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PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF Ia+ AND Ia- SUBLINES OF THE HUMAN MONOCYTE-LIKE CELL LINE, U937

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This is to certify that the dissertation prepared by Coles Meredith Squire entitled Phenotypic and Functional Characterization of Ia+ and Ia- Sublines of the Human Monocyte-like Cell Line, U937 has been approved by her committee as satisfactory completion of the dissertation requirement for the degree of Doctor of Philosophy.

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PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF Ia+ AND Ia- SUBLINES OF THE HUMAN MONOCYTE-LIKE CELL LINE, U937

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology at the Medical College of Virginia, Virginia Commonwealth University.

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DEDICATION

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell cytotoxicity
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ß2M	Beta-2 microglobulin
CML	Cell mediated lympholysis
Con A	Concanavallin A
FcR	Receptor for Fc portion of immunoglobulin
G-IFN	Gamma interferon (immune interferon)
H-2	Murine MHC
HIV-1	Human Immunodeficiency virus
HLA	Human lymphocyte antigens-Human MHC
Ia	MHC class II molecules
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IM	Indomethacin
K562	Human erythroleukemia cell line
LPS	Bacterial lipopolysaccharide
mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
MLR	Mixed lymphocyte reaction

MØ	Monocyte/macrophage
NK	Natural killer cell
PDBu	Phorbol dibutyrate
PHA	Phytohemagglutinin
PMA	Phorbol myristate acetate
REH	Human null cell leukemia cell line

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF Ia+ AND Ia- SUBLINES OF THE HUMAN MONOCYTE-LIKE CELL LINE, U937

Abstract

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology at the Medical College of Virginia, Virginia Commonwealth University.

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Sublines were derived from the U937 human monocytic cell line by limiting dilution techniques. Several of the sublines derived in this manner were found to constituitively express Ia, unlike most U937 cell lines previously examined. Thus the Ia+ and Ia- sublines were examined and characterized phenotypically and functionally. Morphologically the sublines, both Ia+ and Ia-, were found to be similar to the originally described U937 parent cell line. They were positive for production of non-specific esterase and expressed the HLA phenotype A(3,X), B(51,18), DR(2,X). The sublines were examined by immunofluorescence techniques with a large number of antibodies specific for cell surface structures and found to express MHC class I, MHC class II the major type being HLA-DR and perhaps HLA-DQ in small amounts, Fc receptor, TA-1 structure typical of monocytes and T cells, endothelial antigens and CD4. The sublines and parent U937 cell line were found to express few cell surface antigens typical of mature monocytes/macrophages; however, they expressed myeloid antigens typical of early monocytic or promonocytic lineage. Expression of MHC class II

by the sublines was confirmed by immunoprecipitation with monoclonal antibody to a framework determinant of human Ia and SDS-PAGE autoradiography which gave bands at p29:34 for Ia+ sublines but not for Ia- parent U937 cell lines. Cell surface expression of Ia was increased to a significant degree by treatment with gamma interferon which peaked at 24-48 hours of treatment.

Functionally the parent U937 cell line and several Ia+ sublines were examined in several assays. The Ia- and Ia+ U937 cells were found to stimulate the generation of specific CTLs in CML assays to approximately the same degree. The Ia+ sublines were found to stimulate in MLR assay, although variable results were obtained and indicated that the U937 parent and subline cells produced factors which were found to be inherently immunosuppressive. The sublines were able to substitute for monocytes by reconstituting the CD3 mediated T cell mitogenic response. The Ia+ sublines and the parent U937 cell line (also weakly Ia+) were found to present tetanus toxoid antigen to nylon-wool purified T cells following an overnight pulse with antigen, and this response was found to be significantly abrogated by addition of antibody specific for CD4 and MHC class II, but not MHC class I. Preliminary characterization of the immunosuppressive factor produced by the U937 cell line and the sublines revealed that it was strongly antiproliferative and affected lymphoid cells somewhat more than non-lymphoid cells; that its probable molecular weight was approximately 90,000, it was not inactivated by treatment with trypsin or chymotrypsin, was not immediately inactivated by freezing although dialysis and long term storage diminished its activity, and was partially inactivated by heat treatment at both 56°C and 80°C. Clear indication of soluble IL-1 production by the U937 parent cell line and the sublines was problematic due to the strong inhibition of proliferative assays by supernatants; however, partial inactivation of inhibitory activity by heat treatment as well as partial removal of the inhibitor fraction by gel filtration indicated that IL-1 or a cytokine with IL-1 activity was constituitively produced by the cells. Membrane IL-1 was

detected in very low amounts in unstimulated cells and was significantly increased by treatment with phorbol esters but not other immunomodulators. Preliminary examination of Northern blots of HLA-DR alpha and HLA-DQ alpha mRNA production by the parent U937 cells and the sublines revealed that both HLA-DR alpha and HLA-DQ alpha mRNA were detectable for Ia+ sublines E11, G4 and G11, that only trace amounts were detectable for relatively Ia- subline E9 and that surprisingly, HLA-DQ alpha was detectable for the 2-1 parent cell line but no HLA-DR alpha mRNA was detectable. The results of mRNA analysis following gamma interferon treatment, as well as treatment with LPS and phorbol esters, were variable for the different sublines and indicate that in some sublines gamma interferon treatment appears to augment Ia specific mRNA and in others the levels are decreased indicating that the expression of MHC class II specific mRNA may be differentially regulated in the different sublines.

INTRODUCTION

Immunity and the role of monocytes and macrophages in competent immune function. Maintenance of the integrity of the organism in the face of hordes of invaders is dependent on both innate relatively non-specific mechanisms and adaptive specific mechanisms.

Innate, non-specific, non-adaptive mechanisms of immune function. Innate mechanisms include the presence of healthy intact skin and mucous membranes, phagocytic cells present in a wide variety of tissues and organs and the enzymes and secreted substances such as lysozyme and reactive oxygen intermediates which are products of phagocytic cells once invaders have been encountered. Innate mechanisms of immune function should not be underemphasized in the protection of the individual. Maintenance of intact innate mechanisms protects the individual from all of the invaders except those which have developed mechanisms for penetration. The importance of innate immune function becomes obvious in the overwhelming infections common to patients with severe burns and exfoliative diseases, the current finding that the small skin and mucous membrane lesions produced by infection with Herpes virus or syphilis predisposes individuals to human immunodeficiency virus (HIV) infection (1) and the infections, especially opportunistic bacterial and fungal infections, common in chronic granulomatous disease (2, 3, 4). Although these are merely a few examples, they point to the severity and life-threatening nature of a compromised host.

Specific, adaptive immune function. Specific mechanisms of immune function

are characterized by two features: they are anamnestic, i.e. they have intrinsic memory; and they are exquisitely specific. Both specificity and the information required for memory are encoded by members of the immunoglobulin supergene family, so named because the genes and gene products share a battery of characteristics which allow for the production of vast numbers of different, multichain receptors having domain structure from a finite number of encoded genes.

The specific arm of the immune response is characterized by interactions between several types of cells, the most common of which are T lymphocytes, B lymphocytes, monocytes, macrophages and dendritic cells. It is now generally accepted that the production of a specific response necessitates the production of a termolecular complex between an antigen in a presentable form, a major histocompatibility complex (MHC) encoded class I or class II receptor on an appropriate antigen presenting cell, and a specific T cell receptor capable of recognizing the antigen bound to a particular MHC. This interaction is capable of initiating a specific response to an almost infinite number of antigenic determinants engendering a level of complexity peculiar to the immune system. Several complex processes are required for the production of each component of the termolecular complex which is followed by signal transduction leading to T cell activation, clonal expansion of helper and effector cells, and expression of either a humoral or cellular response to an antigen. Production of an immune response is, therefore, dependent on successful accomplishment of a series of steps: expression of MHC class I or class II receptor, antigen processing and presentation by an appropriate antigen presenting cell (APC), expression of a specific T cell receptor on a T lymphocyte which necessitates the prior elimination of self-reactivity and concomitant preservation of a diverse T cell repertoire, and T cell activation involving transduction of signals across the cell membrane to the nuclear compartment for the induction of secretory products and initiation of cellular proliferation.

The T cell receptor. The enigmatic T cell receptor, variously postulated to be either a single receptor with dual recognition properties or two separate receptors, was finally caught and extensively investigated using the development of monoclonal antibodies to T cell hybridomas and clonally expanded T cell poplulations (5, 6, 7) in 1983. A single receptor was found to convey both antigen and MHC specificity and has convinced most investigators of the correctness of the single receptor-dual recognition model (8, 9, 10). The receptor was found to be a disulphide-linked heterodimer having a combined molecular weight of 80-90 kd composed of an acidic glycoprotein alpha chain with a molecular weight of 39-49 kd and a second, but more basic, 38-44 kd beta chain which is also glycosylated. (There is still disagreement in the literature on the characteristic size of each chain.) (Reviewed in 11, 12, 13, 14, 15) The two polypeptides were found to be encoded by separate genes but shared several features including allelic exclusion, domain structure and features of gene arrangement which were also very similar to the genetic arrangement of immunoglobulin genes (16, 17, 18). The alpha chain is located on chromosome 14 in both mice and humans (19, 20, 21), and the beta chain is located on chromosome 6 in mice and chromosome 7 in humans (22, 23, 24); therefore, as with immunoglobulins, a multichain product is constructed from the products of separate chromosomes. Structurally the alpha chain gene is composed of three types of elements in addition to a leader sequence: approximately 50-100 variable (V) genes, 20-50 J region genes, and a single constant (C_a) region gene. The beta gene of humans is composed of approximately 50 $V\beta$ genes, 2 $D\beta$ genes, 13 $J\beta$ genes, and 2 $C\beta$ genes. The mouse beta chain genes are somewhat more limited and consist of 21-30 variable ($V\beta$) genes and only 12 $J\beta$ genes. The beta genes are also arranged such that the variable genes are followed by two clusters of DJC genes as follows (13,15):

$$[D\beta1...J\beta(1\text{-}7)...C\beta1] \ \& \ [D\beta2...J\beta(7\text{-}12)...C\beta2].$$

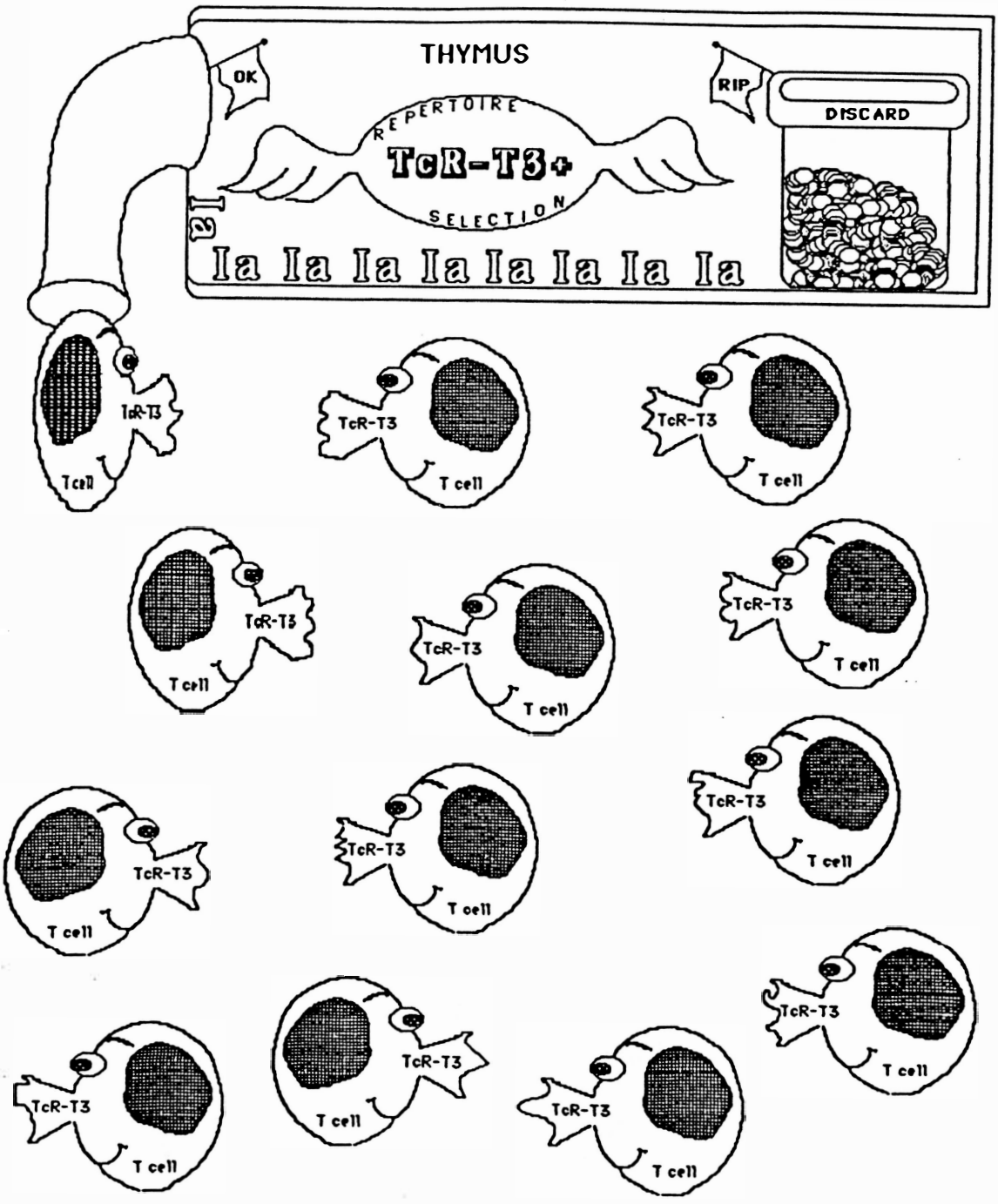
The generation of specificity and diversity is accomplished by the arrangement of

all the possible combinations of VDJ for alpha and beta chain genes with the added measure of diversity conveyed by the possibility of using the Dβ genes in either orientation and by the addition of random nucleotides at N regions. The total number of postulated combinations (T cell receptors) is greater than 1 X 10^7 (25, 26). This number of possible T cell receptors is more than sufficient to recognize the antigens available to the animal, but the repertoire is selected such that far fewer clonally distributed specificities are present.

T cell repertoire selection. Selection of a clonally distributed repertoire of T cell receptors, illustrated schematically in Figure 1, is a complex process which seems to take place in the thymus of the developing animal and results in the production of sufficient diversity of T cell receptors to recognize all invading foreign antigens in the context of self-MHC while at the same time insuring tolerance to self. Production of both immune capability and self tolerance requires the selection and clonal deletion or suppression of all clones which have high affinity for interaction with either [self-MHC] alone or with [self-MHC+self-products]. This necessarily requires the selection and death of a large number of possible clones. It is known that of the lymphocyte precursor cells which enter the thymus, very few, perhaps 1%, remain alive to leave (27). There must, however, be a simultaneous selection of clones having receptors which will recognize nonself products in the context of self-MHC. The widely accepted view that the mechanisms involved in repertoire selection must be poised to eliminate clones having high affinity receptors while at the same time selecting clones which have weak affinity receptors for the same molecular structures, self-MHC (25, 28, 29), eliminating in the process receptors for nominal antigen, appear to be mediated through processes of both positive and negative selection (30). Although the mechanisms involved in repertoire selection have been difficult to investigate, it is currently an area of active investigation, and a coherent picture is beginning to emerge.

FIGURE 1. Selection of the T cell repertoire.

The thymus is the site for both selection of (antigen+self)-MHC specific T cell receptors (TcR's) and elimination of clones bearing (self-MHC) or (self-protein+self-MHC) specific TcR's. Selection is made from TcR bearing lymphocytes which express both CD4 and CD8; therefore, if a TcR reacts with self-MHC of class I and interacts with CD8 on a T cell, that clone will be eliminated and that T cell receptor will not appear on either $CD4^+$ or $CD8^+$ T cells in the selected repertoire (32, 33, 34).


THYMUS
OK
RIP
DISCARD
REPERTOIRE
TcR-T3+
SELECTION
Ia Ia Ia Ia Ia Ia Ia Ia
TcR-T3
T cell

During the gestational development of the mouse, populations of lymphocyte precursors appear in the thymus at about day 12; by day 17 T cell receptors are rearranged and expressed on thymocytes positive for both CD4 and CD8, and by day 18, $CD4^+$ ($L3T4^+$) T cell receptor positive functional helper T cells are present (31). Recent evidence from several laboratories, notably von Boehmer et al. and Sha et al. (32, 33, 34), using transgenic mice in which the rearranged T cell receptor (TcR) genes for the male-specific minor histocompatibility antigen H-Y were expressed only on $CD8^+$ cells and only if the original MHC ($H\text{-}2D^b$) was present supports the occurence of positive selection in the thymus. The findings indicate that the double positive $CD4^+8^+$ immature thymocytes are the precursor population from which both $CD4^+8^-$ and $CD4^-8^+$ cells develop and that the development of the mature TcR-bearing single positive cell is dependent on the presence of thymic MHC, either class I or class II, and the presence of heterodimeric TcR. They postulate that the selection of permitted TcR's is mediated by interaction of MHC with TcR in the absence of nominal antigen, in this case H-Y, probably by receptor crosslinking strongly augmented by cointeraction with CD4 or CD8 (33).

Both elimination of self reactivity and generation of self-MHC restriction are dependent on the presence of self-MHC in the thymus; however, this is apparently not all that is required (25, 28, 29, 31). Kappler and Marrick et al. have shown that there is a correlation between the use of the T cell receptor variable gene, $V\beta17a$, and the presence of the Ia gene products for any haplotype of I-E. Some strains of mice do not express I-E, ($H\text{-}2^{b,s,f,q}$) (35) and these mice were found to make use of the $V\beta17a$ variable gene in approximately 10% of the T cell repertoire. Strains which do express I-E were found to have very few T cell receptors utilizing this variable gene and those which were present did not recognize I-E. Moreover, $I\text{-}E^+$ animals reconstituted with $I\text{-}E^-$ cells after lethal irradiation were found to *be repopulated* with $V\beta17a^+$ cells leading to the conclusion that the presence of I-E on the remaining

endothelial and stromal cells of the thymus was insufficient for the self tolerance inducing deletion of cells bearing the Vβ17a variable region gene product (25). Von Boehmer et al., from experiments using deoxyguanosine treated mice, postulate that induction of tolerance to self-MHC is dependent on MHC$^+$ thymic epithelial tissue, but development of self-MHC restriction is dependent on bone marrow derived cells in the thymus (29). Other recent work from Kappler and Marrick (36) and others (37, 38) in the Mls system may begin to shed some light on the selection against self reactive clones. As in the Vβ17a experiments, it was noted that mice having an Mls^a genotype expressed few if any T cell receptors using the variable gene, Vβ8.1. MacDonald et al. noted that these individuals also did not express T cell receptors using the Vβ6 variable gene (37). Mls^a (mixed lymphocyte stimulatory) is a nonMHC antigen which in MHC matched but Mls disparate mice will cause the generation of a very strong mixed lymphocyte reaction. A human analogue has been postulated but has not been identified. Both Vβ8.1 and Vβ6 variable genes used in T cell receptors confered strong Mls^a reactivity in spite of MHC haplotype and also inspite of the J region gene used, although Mls^a is recognized in conjunction with MHC. Mature thymocytes bearing the Vβ8.1 and Vβ6 gene products in mice expressing Mls^a were not found indicating that there had been clonal deletion of the Vβ8.1 and Vβ6 expressing clones. It is suggested that Mls^a may be an MHC binding protein which binds to a possible groove in the MHC molecule in the thymus thereby producing a broadly reactive selection receptor for elimination of certain self-MHC reactive clones and thus retention of useful T cell clones (36, 37, 38). Based on their finding that normal percentages of these receptor bearing cells (Vβ17a and Vβ8.1) are present in early thymocytes (double positive or CD4$^+$8$^+$ cells), Kappler and Marrack et al. have proposed that there is a developmentally regulated "abortion" of any cells having reactivity to self such that both mature thymocytes and peripheral blood T cells have been selected and no longer contain the offending receptor bearing populations. The

details of this negative selective mechanism are in agreement with several groups of investigators (28, 29) and not with others (33).

The sequence of events during thymic selection of permitted clones is not known; however, positive selection mechanisms have been thought to occur before negative selection events based on the finding that MHC expressed on thymic cortical epithelial cells appears to interact with TcR on double positive cells which are found primarily in the thymic cortex (29, 39) and negative selection requires the bone marrow derived cells found in the medullary region (29, 40). The proposed mechanisms for positive and negative selection in the thymus remain somewhat contradictory, and the spatial and temporal relationships are not entirely understood. In negative selective processes engagement of the TcR is a signal for death in immature thymocytes, and in positive selective processes it is the means for preservation and permitted maturation. Presumably the same type of TcR interaction in the periphery has the more positive effect, i.e. causing IL-2 production and clonal expansion.

Ramarli et al. (41) postulate that the CD2 (T11) receptor plays a pivotal role in the thymus in that it is the first observable T cell antigen which is expressed and their finding that engagement of T11 causes an alternative pathway of T cell activation. If the TcR's on $T11^+$ thymocytes are subsequently crosslinked, T11 mediated IL-2 production ceases, although IL-2 receptor and TcR production are not affected. They contend that IL-2 is required for growth and maintenance of these clones and its absence causes them to die, again making engagement of the TcR in the thymus a signal for clonal death (28, 29, 41). Saito and Germain (8) and Ashwell et al. (42) report that TcR interaction with antigen+MHC causes growth inhibition, i.e. cessation of lymphokine production, unless further signals are delivered. Particular variable region genes, either V_a or V_β, may predispose a T cell receptor to recognize a particular MHC; however, evidence currently indicates that the *total specificity* of the receptor is dependent on contributions made by both alpha and beta chains. Rather than

selection by high and low affinity, selection by modified self available only in the thymus is favored, supported by evidence that Vβ17a recognizes an allogeneic MLR-stimulating determinant only on I-E$^+$ B cells and peritoneal macrophages and not on I-E$^+$ macrophage cell lines and transfected fibroblasts, suggesting that a self product binds with the I-E molecule to produce the I-E allogeneic ligand and that this product is not available in the macrophage cell line or the transfected fibroblast (31). The effect is not related to density of expression of I-E on the cell surface (43). This finding in combination with the recent advances in the understanding of Ag processing and presentation to be discussed below (44, 45, 46) would seem to lend weight to the idea that allogeneic responses are polyclonal responses to foreign MHC complexed to self peptides (47). The large number of self peptides, which in combination with self MHC are not recognized, would, in combination with foreign MHC, be recognized by large numbers of clones.

Thus it is obvious from the brief mention of work from several groups that progress is being made in determining mechanisms for repertoire selection and tolerance induction; however, the picture is far from clear and there is currently no consensus on the details. There is growing agreement, however, that selection of the T cell repertoire is dependent on thymic MHC, both class I and class II, and interactions between MHC and TcR's expressed on immature T cells which are double positive CD4$^+$8$^+$ thymocytes; and that this selection process works in negative ways for the elimination of autoreactive clones and in positive ways for the selection of TcR's which can interact with self-MHC for the production of a TcR-bearing, single positive repertoire sufficiently diverse to offer induction of immune function to all potentially harmful foreign antigens. The growing body of knowledge of the mechanisms of allelic exclusion and expression of the alpha and beta chains of the T cell receptor in conjunction with the several constant chains of the CD3 complex, *however*, *has* been more clearly shown and has contributed directly to investigation of repertoire

selection.

The clonotypic T cell receptor and the monomorphic CD3 complex. The T cell receptor is found as an integral membrane protein in close association with the polypeptide chains of the CD3 complex. The CD3 complex in humans is composed of a gamma chain (25-28 kd glycoprotein); a delta chain, the originally detected ligand for OKT3 monoclonal antibody, (20 kd glycoprotein); and an epsilon chain (20 kd non-glycosylated protein) (15). The murine CD3 equivalent was found (48) and upon analysis was found to be composed of several additional chains. The N-glycosylated 28 kd delta-equivalent, 21 kd N-glycosylated gamma, and 25 kd non-glycosylated epsilon were present; however, an additional 34 kd non-glycosylated homodimer designated zeta was found as was a 21 kd non-glycosylated chain (p21) disulphide linked in some instances to zeta (49). Zeta was subsequently identified in humans but did not comodulate or coprecipitate with either T cell receptor or antibody to the CD3 complex (15). The components of the CD3-T cell receptor complex apparently are produced in excess quantity by T cells, the various chains are glycosylated and the complex is assembled prior to expression on the cell surface, the excess chains being rapidly degraded intracellularly (50, 51). Production of components, glycosylation, assembly and final processing were found to be very rapid events as shown by pulse-chase experiments in which intracellular components were evident in less than 10 minutes; however, membrane expression required a minimum of 90 minutes (51) and the presence of the zeta chain was found to be a limiting factor in the murine system (50). The gamma and delta genes of the CD3 complex are very similar and are believed to have arisen by gene duplication prior to the divergence of mice and humans. The epsilon gene is also related to the gamma and delta genes but diverged somewhat earlier (52). Functionally the CD3 complex appears to be central to the transmission of signals from the engaged T cell receptor, probably via both TcR alpha and beta chains (51), to the

CD3 complex into the cell by second messengers. The gamma chain of the CD3 complex is a substrate for protein kinase C mediated phosphorylation during T cell activation (15, 53).

A second type of clonally distributed T cell receptor has been isolated. When originally isolated the gamma chain of this second type of receptor was mistakenly thought to be the TcR alpha chain. Although it had immunoglobulin-like variable and constant regions, its apparent lack of N-glycosylation sites made it suspect (17). The gamma chain has been more completely described and found to be composed of variable, joining, and constant regions which are rearranged early in thymocyte development (54, 55, 56). The delta gene has also been isolated and sequenced and found to be located next to the components of the TcR alpha chain genes. This chain is composed of variable, joining, diversity and constant regions (57, 58). The gamma and delta chains are both glycosylated, contrary to original findings, and have molecular weights of 35 kd (mice)-55 kd (humans) and 40-45 kd, respectively. The receptor is expressed as a disulphide linked CD3 associated heterodimer on $CD4^{-}CD8^{-}$ thymocytes and a small number of peripheral blood lymphocytes and appears earlier in thymocyte development than the alpha:beta heterodimer (54, 55, 59, 60). It is postulated that gene rearrangements for chains of both types of TcR begin early in thymocyte development and productive rearrangement of gamma:delta prevents further rearrangement of alpha:beta. If on the other hand, the gamma:delta does not productively rearrange, the alpha:beta genes continue, giving rise to populations of cells which express either receptor, but not both. The thymocytes expressing gamma:delta and those expressing alpha:beta are, therefore, separate T cell lineages which have common progenitors. There has been extensive speculation about the possible roles played by this second T cell receptor (54, 55). There continues to be controversy as to the level of diversity expressed within the population of cells which

express this type of TcR and whether the CD3 complex expressed with it is identical to that expressed with alpha:beta expressing cells (61, 62). Cells bearing the gamma:delta receptor can be stimulated to produce IL-2, IL-4 and gamma interferon (54, 62), have been reported to be cytolytic in both MHC specific and non-specific ways (54, 55, 63, 64). It is proposed by some that they may represent both an ontologically and phylogenetically earlier form of T cell receptor which may represent a functional defense against development of infections and neoplasms through recognition of non-polymorphic MHC class I molecules (55) by gamma:delta bearing epithelial dendritic cells (65) present in skin. Gamma:delta T cell receptors have been found on a wide assortment of T cell hybridomas, natural killer-like clones and responding cells in mixed lymphocyte reactions (62, 66, 67). It is apparent that the functional relevance of this type of receptor expression is currently a topic of extensive research; however, at present very little is known with certainty.

T cell activation. T cell activation involves a complex series of interactions leading eventually to clonal expansion. The elucidation of the structure of the T cell receptor complex in both the murine and human systems as the products of a gene family similar to that of immunoglobulin has opened up avenues for disclosure of how the three necessary components-T cell receptor, antigen in presentable form and MHC molecules-come together for T cell activation which is necessary for the production of specific immune effector function. The series of interactions is initiated by contact and binding of the clonotypic antigen-specific T cell receptor to its ligand which is an MHC molecule, either MHC class I or class II, which probably has a presentable fragment of antigen already attached (44, 45). T cell activation leads to modulation or internalization of the T cell receptor and its associated CD3 complex, production of IL-2, expression of IL-2 receptors and DNA synthesis leading finally to clonal expansion. The series of interactions is not completely understood; however major strides have

been made in characterizing some of the early signals and second messengers involved.

Signal transduction mechanisms. Signals are carried from the T cell receptor interactions at the cell surface into the interior to initiate the events characteristic of T cell activation. Following receptor-ligand interaction on the external surface of the T cell, a signal is transduced into the interior of the cell perhaps mediated by a conformational change due to interfacial receptor binding, i.e. binding by two receptors which are integral membrane glycoproteins constrained by their anchoring in two lipid bylayers in close apposition, and crosslinking by accessory molecules such as CD4, CD8 or LFA-1. This signal may be propagated by way of both alpha and beta chains interacting with the closely, but noncovalently, expressed chains of the CD3 complex (14,15). Although the complete details of activation are unknown, there is evidence that this signal initiates phospholipid metabolism and a rise in intracellular [Ca^{++}] (68) presumably by first interacting with a GTP binding protein (G protein) which is pertussis toxin insensitive (69) and cholera toxin sensitive and seems to have some Gs character, but is not Gs, and has not been identified (70, 71). The G protein activates a phosphodiesterase, phospholipase C, which hydrolyzes a diphosphorylated phosphatidylinositol present in the membrane, PIP_2 [phosphatidylinositol bisphosphate], into two second messengers: IP_3 and diacylglycerol, thus giving rise to a "bifurcating signal transduction system" described by Berridge (72). IP_3 [inositol 1,4,5 triphosphate] which is soluble in the cytosol is responsible for mediating a calcium (Ca^{++}) flux which characteristically has two phases (72). Phase I involves Ca^{++} release from intracellular stores in the endoplasmic reticulum giving rise to an initial spike in intracellular Ca^{++} concentration [Ca^{++}] seen in studies using the Ca^{++} indicators Quin 2 and more recently Indo 1 (73, 74). Current evidence using excised patches from Jurkat cells and extracellular patch clamp techniques indicate that the second phase of the calcium response is mediated by activation of a voltage

insensitive Ca^{++} channel by IP_3 (75) causing an influx of extracellular Ca^{++} thus giving rise to the second Ca^{++} spike. The biphasic Ca^{++} response is necessary for production of IL-2, although expression of IL-2 receptor is insensitive to intracellular [Ca^{++}] (69, 76). Chelation of extracellular Ca^{++} prevents cells from dividing after T3-Ti stimulation (69) probably due to inhibition of IL-2 induction. The second second-messenger formed after PIP_2 hydrolysis is diacylglycerol which activates protein kinase C (77) and mediates its translocation from the cytoplasm to the plasma membrane (78, 79). There in concert with phosphatidylserine, diacylglycerol activates protein kinase C to phosphorylate a number of receptor molecules including the gamma chain of CD3 and the CD4 and CD8 receptors (53, 79, 80). T cell activation is also accompanied by an increase in intracellular pH by activation of the Na^+/H^+ antiporter; however, this cellular alkalinization has not been shown to be critical to other events leaving its role in question (69, 76). The signal transduction mechanisms so far described are very rapidly and transiently active (72). IP_3 is subsequently metabolized or further phosphorylated to IP_4 which may also have regulatory function. Diacylglycerol is either reconstituted to form phosphatidic acid or further degraded releasing arachidonate for production of prostaglandins and leukotrienes (72, 81). The activated protein kinase C, which is auto-phosphorylating, is inactivated or down regulated, perhaps by dephosphorylation by a phosphodiesterase or by interaction with cAMP dependent mechanisms (77). Thus the early events of signal transduction take place in the cytoplasm, and though the signals are transient they lead to profound changes in T cell behavior over several days. The events following initial signal transduction are not understood and neither the messengers nor the messages conveyed to the nucleus are known (70, 72, 77). In publications containing figures describing the events of T cell activation, this area of the diagram is generally labeled THE FINAL EVENT-CELL DIVISION.

Cellular proto-oncogenes. Following T cell activation, it has been noted that a series of identified cellular proto-oncogenes are temporally expressed in proliferating cells giving rise to the hypothesis that these genetic elements may code for growth or "progression" factors necessary for DNA replication which either turn on genes for DNA binding proteins or are DNA binding proteins themselves (82, 83, 84). Several of these proto-oncogenes code for proteins which are found exclusively in the nucleus. Among these genes, one of the first expressed is the c-fos gene which appears within 5-10 minutes of cellular activation and has been postulated to be a "master switch" for the triggering of an assortment of other genes which lead to DNA replication (85). Other proto-oncogenes which code for nuclear binding proteins, thus apparently involved in DNA replication, which have also been examined relative to T cell activation are the c-myc gene and the c-myb gene. As an isolated event, stimulation through interaction with the T cell receptor in the absence of IL-2 is not mitogenic but appears to induce the expression of c-fos very early and c-myc mRNA slightly later. Using several systems derived from human peripheral blood mononuclear cells, the expression of these oncogenes has been found to be associated with a change in the status of the T cells which may be a change from the resting G_0 state to the still resting but "competent" state of G_1 (86, 87, 88). The expression of these genes has also been found to take place in spite of chelation of extracellular Ca^{++}, although expression was suboptimal (88). The addition of IL-2 to cells given an incomplete activation signal, i.e. stimulation which was insufficient to lead to a mitogenic response (87), or delivery of a complete mitogenic signal (86, 88) caused a post-IL-2 late expression of c-myb and a second induction of c-myc expression, both of which required extracellular Ca^{++} and interaction of IL-2 with its receptor. Although the specific effects mediated by the products of these oncogenes are still unknown, both their spatial and temporal expression relative to T cell activation is *suggestive of intimate* involvement. If there is any analogy to the finding that more that 70 mRNAs were

induced by NIH 3T3 cell interaction with growth factors in serum (89), there may be many other gene products involved in T cell activation which have not been investigated.

Expression of proto-oncogenes has also been examined in myelomonocytic cell lines in an attempt to determine the relationships between proto-oncogene expression and differentiation in normal macrophages (90, 91, 92, 93). Differentiation is frequently equated to phorbol ester treatment of cells to produce adherent cells having characteristics of mature macrophages. Findings indicate that during the course of differentiation, the c-myc and the c-myb gene expression is decreased and c-fos and c-fms gene expression is increased. As indicated by Mitchell et al. (94), proto-oncogene expression, the kinetics of its transcription and the duration of availability of its gene product are dependent on the cell type being examined, i.e. lymphoid cells are quite different from monocytic cells which are in turn different from fibroblasts. A question raised frequently in the literature is whether the appearance of a proto-oncogene transcript or gene product has any causative relationship in mediation of events leading to differentiation and the answer, when causation can be examined, frequently is no (94). As with the c-fos gene examined in the U937 monocyte-like cell line (95), the expression of the c-fos gene occurs but can not be shown to be sufficient nor even necessary for differentiation (94).

Alternative methods for T cell activation. T cells can be activated in more than a single way. Although activation by engagement of the antigen specific T cell receptor (T3-Ti) by a membrane bound, antigen bearing MHC molecule is the most immunologically relevent form of activation, T cells can be activated by a number of other paths, many of which evoke a polyclonal T cell activation or mitogenic response. The features of a polyclonal response are the same as an antigen specific response except that more cells are capable of responding. Much of the seminal work on T cell

activation has been dependent on activation mediated by monoclonal antibodies to the clonotypic T cell receptor or monoclonal antibodies to the CD3 complex (70, 96, 97). Soluble monoclonal antibodies to the TcR and to CD3 were found to be unable to activate purified populations of resting T cells without combinations of additional signal compounds such as phorbol esters, calcium ionophores, interleukin 2 (IL-2), interleukin 1 (IL-1) or crosslinking of antibodies by immobilization on substrates such as sepharose beads (69, 70, 76). The responses of purified truly resting T cells were found to be different from responses of previously stimulated T cell clones and hybridomas even though rested for long periods (70), thus the picture, though still unclear, seems to indicate that in unprimed T cells there is an absolute requirement for IL-2 and probably a requirement for IL-1 for the production of a proliferative response. Autocrine production of IL-2 may be sufficient for activation; however, macrophages or other cells are required to supply IL-1. The membrane form of IL-1 may be a necessary additional signal in initiation of T cell clonal expansion (98). Whether there is an absolute necessity for accessory cells or some unidentified product of accessory cells is still controversial (69, 70, 76). The monoclonal antibody OKT3 was found to be highly mitogenic for T cells in the presence of Fc receptor (FcR) bearing accessory cells (99). Other antibodies to CD3 such as UCHT1 were found to be nonmitogenic for a substantial portion of the population and the defect was traced to an inability of their monocyte-FcR to interact with the particular antibody isotype (100, 101). There is, however, continuing controversy as to whether accessory cells or their soluble products are required in CD3 mediated mitogenesis (102, 103, 104). Plant lectins such as phytohemagglutinin (PHA) and concanavalin A (Con A) which seem to adhere to the T cell receptor glycoproteins as well as other membrane glycoproteins (105, 106) cause a polyclonal T cell response. These mitogenic plant lectins may also mediate other effects by their ability to crosslink receptors to which they bind and to stick cells together. The mRNAs expressed and the kinetics of their

expression for oncogenes c-fos, c-myc and c-myb induced by PHA stimulation of human peripheral blood mononuclear cells is the same as that seen with other modes of T cell activation. There is also evidence that they may activate additional mechanisms for causing Ca^{++} influx (69).

Other means for T cell activation involve interaction of membrane receptors other than the TcR with appropriate ligands. The CD2 (T11) receptor, the sheep erythrocyte receptor on T cells, is expressed early in T cell ontogeny and is capable of transmitting a mitogenic signal when stimulated with a combination of monoclonal antibodies (107, 108). A natural ligand for T11 is the lymphocyte function associated antigen (LFA-3) glycoprotein found widely distributed on both hematopoietic and nonhematopoietic cells (109). Although fully mitogenic T cell activation is not stimulated by the binding of sheep erythrocytes or a monoclonal antibody specific for the epitope on T11 to which SRBCs bind (anti-$T11_1$) (76, 110), T cell activation by binding of monoclonal antibodies to two other epitopes is very effective. The monoclonal antibodies have been designated anti-$T11_2$ and anti- $T11_3$ (107). Binding of anti-$T11_2$ induces the expression of the epitope recognized by anti-$T11_3$ which is only available to be bound on activated T cells. Cellular proliferation results only from a combination of the antibodies, and the mechanisms involved in induction of cell division are similar to those seen in TcR (T3-Ti) mediated T cell activation, although the kinetics appear to be slightly slower. Reinherz et al. postulate that T cell activation by way of the T11 receptor is important in selection of the T cell repertoire in the thymus, and, in the periphery because binding the TcR causes induction of the $T11_3$ epitope, subsequent activating signals leading to IL-2 production are transmitted by the T11 receptor rather that the TcR-CD3 complex (76); however this remains somewhat controversial (70, 108).

Accessory molecules: CD4, CD8 and LFA. Other receptors including CD4, CD8

and lymphocyte function-associated antigens (LFA's) are important accessory molecules in T cell activation. Interaction of T3-Ti with antigen and MHC is not sufficient for fully mitogenic T cell activation except perhaps when the T cell receptor is of extremely high affinity. Other interactions are required. Two such membrane receptors are CD4 and CD8. Both the CD4 and CD8 receptors have been isolated and their genes have been cloned (111). Because of their homology to immunoglobulin light chain variable regions and domain structures, they have been included in the growing list of members of the immunoglobulin supergene family (112, 113). CD4 (called T4 or Leu 3 in humans, L3T4 in mice) is a nonpolymorphic 55 kd integral membrane glycoprotein characterized by five external domains, a hydrophobic transmembrane segment and a highly conserved cytoplasmic tail (114, 115). CD8 (called T8 or Leu 2 in humans, Lyt 2 and Lyt 3 in mice) is apparently different in humans and mice in that the human 34 kd CD8 is expressed as a disulfide-linked homodimer (or multimer) on the surface of cytolytic/suppressor T cells (116) and the murine CD8 is expressed as a heterodimer composed of a 34-38 kd Lyt 2 chain disulfide linked to a 30 kd Lyt 3 chain (117). The general structure of the CD8 molecule is a single external domain, hinge region, transmembrane region and highly conserved cytoplasmic tail (111). Some early thymocytes express both CD4 and CD8, some express neither CD4 nor CD8; however, shortly after expression of functional TcR, the majority of thymocytes express either CD4 or CD8 but not both. There is currently strong evidence that these cells develop from a double positive precursor population found in the cortical region of the thymus (29, 33, 118). In general, T cells which fit the helper inducer phenotype are $CD4^+$, and T cells which are phenotypically cytolytic are $CD8^+$; however a few $CD4^+$ cytolytic cells and a few $CD8^+$ helper cells have been found. $CD4^+$ cells are restricted by MHC class II molecules whether they are helper cells or cytolytic cells, and $CD8^+$ cells are restricted by MHC class I molecules (119). $CD4^+$ helper or inducer cells mediate B

cell activation for antibody production and interact in allogeneic mixed lymphocyte reactions and delayed type hyper-sensitivity. $CD8^+$ T cells are specific cytolytic T cells for virally infected cells and tumor cells and may also contain the elusive suppressor T cells. Both membrane glycoproteins have been postulated to be focusing receptors which could bind to a monomorphic ligand on accessory cells to stabilize the binding of the TcR, antigen and MHC molecules which were characterized as a loose confederation ready to dissociate unless cemented together by other interactions. Current understanding of the relatively stable interaction of MHC molecules with presentable antigen prior to interaction with the TcR has necessitated a change in view (44, 45). The CD4 and CD8 receptors are now thought to bind to monomorphic determinants on the MHC molecules themselves and serve as both enhancers of binding avidity and as functional receptor crosslinkers capable of aiding in the delivery of a transmitted activation signal across the membrane (120, 121, 122, 123). Evolutionary conservation of the transmembrane and cytoplasmic tail regions of both genes is postulated to be related to an important but not yet clearly understood function of these receptors in signal transduction (111, 124). CD4 and CD8 molecules are both phosphorylated during T cell activation of their respective types of T cells and are comodulated from the cell surface with the TcR-CD3 molecules after TcR interaction (80, 125, 127). There is evidence that although approximately 5% of the CD4 molecules on the surface of a resting lymphocyte are associated with the TcR-CD3 complex, TcR interaction causes a rapid redistribution of the CD4 molecules to the TcR-CD3 complex (127, 128). CD4 is found primarily on T lymphocytes but is also found on monocytes at lower concentration (129, 130). The CD4 molecule has been identified as the cellular receptor for human immunodeficiency virus (HIV) and $CD4^+$ lymphocytes are selectively eliminated in active AIDS patients leading to profound fatal immunodeficiency (131). The presence of the CD4 receptor on monocytes is also the means by which HIV gain entry into monocytes where the virus is protected from

immune surveillance. Within this safe haven, HIV appears to be able to evade the cellular degradative machinery and instead causes the macrophages to cease to function effectively against opportunistic invaders (132, 133).

The lymphocyte function associated antigens (LFA's) have been shown to be important in cellular adhesion and aggregation (134). A family of cellular adhesion molecules has been identified which are composed of heterodimers and which share the expression of one chain. The LFA-1, Mac-1, p150,95 family shares expression of beta chains but have different alpha chains and have been shown to be immunologically very important largely through the identification of the life-threatening bacterial and fungal infections charactistic of individuals with a severe deficiency of these membrane adhesion molecules (135). LFA-1 is present on hematopoietic cells including lymphocytes, granulocytes and most monocytes. It is not expressed on nonhematopoietic cells, erythroid and myeloid precursors and resident murine macrophages (134). LFA-1 is important in adhesion of specific TcR bearing lymphocytes with antigen presenting cells or with cells bearing allogeneic MHC class II antigens in mixed lymphocyte reaction, adhesion of CTL's to specific target cells and natural killer cells to NK targets. Although T cell activation is postulated to be the trigger for inducing adhesiveness, adhesion by LFA-1 is relatively antigen independent; however, stable aggregation of T effector cells with targets or antigen presenters facilitates the specific interactions. The natural ligand for LFA-1 is currently believed to be ICAM-1 (intercellular adhesion molecule-1) which is a heavily glycosylated 90-114 kd glycoprotein widely distributed on lymphoid cells, vascular endothelial cells, tissue macrophages, dendritic cells, and epithelial cells in the thymus (136). Expression of ICAM-1 is greatly increased in inflammation and is positively regulated by IL-1, gamma interferon and tumor necrosis factor (134, 136). LFA-2 is another name for CD2, the human sheep erythrocyte receptor, which has been described. The rat and mouse homologues have also recently been identified and cloned and appear to be

similar to human CD2 (137, 138). The natural ligand for CD2 appears to be LFA-3 which is a 55-70 kd glycoprotein widely distributed on endothelial, epithelial, connective and hematopoietic cells (109, 139).

Soluble mediators. Immune function is regulated in part by signals conveyed by soluble mediators. Cellular interactions transmitted by membrane receptors serve to initiate cellular changes which ultimately lead to development of effector function. Initiation, however, must be closely followed both temporally and spatially by propagation of those signals by cytokines. Although the list of important cytokines, lymphokines and interleukins grows longer and more complex, a few soluble mediators have been chosen for mention because they have been shown to be of particular importance in T cell activation, antigen processing and presentation or modulation of macrophage function.

Interleukin 2 (IL-2). Originally called T cell growth factor, IL-2 has been shown to be an integral component of the development of T cell effector function capable of stimulating the progression from G1 in the cell cycle through S->G2->M (140). IL-2 is a 15,500 dalton glycoprotein (141) encoded on chromosome 4 in humans (142) which is producted by T cells upon stimulation by presented antigen+MHC, mitogens and combinations of phorbol esters and calcium ionophores. The subpopulation of T cells producing IL-2 has been designated TH1 by Mosmann et al. who noted that T helper clones could be shown to produce either IL-2 and gamma interferon (TH1) or IL-4 (TH2) (143). Investigation of the IL-2 receptor has been greatly facilitated by development of the anti-Tac monoclonal antibody by Uchiyama et al. (144) leading to the finding that two types of receptors are expressed, the majority of which are of low affinity (145, 146). High affinity receptors for IL-2 must be expressed for the proliferative response by T cells, as it is the interaction of IL-2

with the IL-2 receptor which apprears to convey the message for DNA replication and clonal expansion. As an initiating signal the stable interaction of the TcR-CD3 complex with antigen-MHC confers specificity, and the signal transduction emanating from that interaction is the trigger which activates the autocrine production of IL-2. The nature of that signal is not known at present; however, transcription of messenger RNA is rapidly apparent within 1-2 hours and peaks from 5-10 hours after stimulation, depending on the cells or cell line used (147, 148, 149, 150). IL-2 mRNA is also short lived and quickly begins to disappear, returning to near basal levels in from 10 to 24 hours, again depending on whether Jurkat cells, EL4.E1 cells, peripheral blood mononuclear cells or human tonsil cells are examined (147, 148, 149, 150). Although there is some disagreement among investigators, it is probable that as soon as the stimulus for activation is no longer present, there is an increased rate of IL-2 degradation or repression which can be blocked by cyclohexamide treatment, indicating that protein synthesis is required for the repressor, in spite of continued transcription of mRNA (148, 149, 150). This constitutes a negative feedback control to limit the continuation of activation beyond its antigen specific initiation (147). Expression of gamma interferon mRNA appears at the same time as IL-2 mRNA (147) indicating the possibility of coordinate regulation. Although gamma interferon production is induced by IL-2, initially they are probably regulated by a series of overlapping and non-overlapping signals (151). Expression of proto-oncogenes c-fos and c-myc preceed IL-2 gene expression (88, 152, 153, 154); however, their relationship to IL-2 expression or repression is not known. Expression of functional proteins for both IL-2 and IL-2 receptor do not peak until 24-48 hours, and IL-2 receptor preceeds IL-2 (153). Because resting T cells express neither IL-2 nor IL-2 receptor, the period of time when a cell is primed by activation through the TcR, producing IL-2 and receptive to its interaction through the IL-2 receptor is of relatively short duration. If the activation sequence is not completed during this period, the cell enters a refractory

period during which it is impervious to further stimulation (145, 147).

Although the primary function noted for IL-2 is in T cell proliferation, IL-2 is functionally pleomorphic having been shown to cause B cell proliferation and activation of macrophages when used at high concentration (140, 145). IL-2 receptors have been found on roughly 5% of T cells in the peripheral circulation and 20% of the T cells in lymph nodes probably indicating transient activation. IL-2 receptors at low density have also been found on some B cell lines and on monocytes, macrophages, Kupffer cells and skin Langerhan's cells (145). Unlike IL-2 receptor expression, IL-2 production is sensitive to Ca^{++} blockade, the effects of glucocorticoids such as dexamethasone (155) and cyclosporin A which inhibits IL-2 at the level of transcription (150), has Ca^{++} channel blocking character and also appears to block the interaction of IL-2 and the IL-2 receptor (156).

Interleukin 4 (IL-4). Interleukin 4, called B-cell stimulating factor (BSF-1) prior to renaming as an interleukin, is the product of activated T cells, the TH2 type of lymphocyte (143). IL-4 is a 20,000 dalton glycoprotein found to costimulate resting B cells when treated with anti- immunoglobulin, stimulate resting B cells to increase MHC class II expression, and LPS-activated B cells to increase production of IgG_1 and IgE (157). Although characterized as a B cell specific mediator, IL-4 has been found to have effects on many other cell types including T cells, macrophages, erythroid precursors and granulocytes (157). A high affinity receptor shown to bind purified IL-4 has been found, widely distributed, on both hematopoietic and nonhematopoietic cells (158). Recombinant IL-4 has been found to increase B cell antigen presentation at least partially by increasing MHC class II expression (157). More recently IL-4 has been shown to increase monocyte/macrophage antigen presentation in a bone marrow derived murine macrophage cell line but not in several macrophage tumor cell lines (159). In another study using centrifugally elutriated human monocytes, IL-4

caused an increase in MHC class II expression, receptor for C3bi (CR3) and p150,95 adhesion molecules and concomitant decrease in cytostatic factor production, IL-1 production and morphological changes purported to indicate stimulation of differentiation (160). Thus IL-4, like many other interleukins, appears to possess multiple reactivities which at present are not clearly defined.

Interleukin 1. Interleukin 1 (IL-1), which has been referred to by many function-related names and is produced by a wide range of different cell types, is a polypeptide cytokine having diverse biological activities including production of fever, costimulation of thymocytes, stimulation of acute phase reactants, resorption of cartilage and wasting of muscle (161, 162). Some of these effects have been attributed more recently to tumor necrosis factor (TNF_a) which is induced by the same stimuli as IL-1 (163). Immunologically, IL-1 was shown to be a product of monocytes and referred to as lymphocyte activating factor (LAF) because it was shown to costimulate murine thymocytes when combined with submitogenic doses of phytohemagglutinin (164). Although IL-1 is characterized as a monocyte product, it has been shown to be produced by a vast array of cells throughout the body including endothelial cells, hepatocytes, chondrocytes, synovial cells and virtually every type of cell examined (165). Of particular importance in the immune system, IL-1 has been shown to be produced by dendritic cells (166), B lymphocytes (167) and also has been reported to be made by some T cell leukemia cell lines (168) in addition to the originally identified producer, the monocyte.

Characterization of IL-1 has been hindered by a scarcity of cell lines producing large quantities; however, the murine P388.D1 macrophage cell line (169) and human cell lines THP-1 (170) and a subline of the monocytic cell line U937 (U937.1) (171) have been found to produce IL-1 under appropriate stimulation. IL-1 has recently been characterized at the molecular level by several groups (172, 173,

174). The consensus has been that there are two forms in both mice and humans. They are referred to as IL-1a and IL-1ß and have isoelectric points of approximately 5.0 and 7.0, respectively (172, 175). Although there is agreement on the existence of these two types of IL-1, there are also reports of other IL-1's having different isoelectric points, particularly a pI 6 form which is similar to IL-1a (174); therefore, there may be a whole family of interleukin 1 molecules perhaps produced by different cells (162). The genes for both IL-1a and IL-1ß are tightly linked and found on chromosome 2 in both mice and humans (176, 177). Although in mice the interleukin 1 genes are in close proximity to beta-2 microglobulin (176), in humans beta-2 microglobulin is on a separate chromosome. The amino acid sequences for IL-1a and IL-1ß are very dissimilar showing only 22% and 26% homology to each other in mice and humans, respectively; however, the homology of each type of IL-1 across species is fairly high, shown to be 62% for IL-1a and 67% for IL-1ß (178). The functional activity resides in the carboxyterminal 140-150 amino acids of the gene products which are translated as 33 kd polypeptide precursors (179). The IL-1a precursor appears to have IL-1 activity; however, the precursor IL-1ß appears to be inactive, although trypsin treatment causes activation (178, 180). Interleukin 1 was originally characterized as a soluble product; however, more recently it has been found that a far greater proportion of IL-1 remains intracellular and a large part of that pool is in the precursor form (174). IL-1ß is approximately 10 times more abundant that IL-1a, although IL-1a has been found to have considerably higher specific activity (178, 181). Upon stimulation, a membrane IL-1 is expressed, which has been found to be IL-1a, prior to appearance of the secreted form (98, 182). The finding that most IL-1 is intracellular correlates well with the additional finding that neither form of IL-1 has a competent signal peptide (178, 180). Explanation of a means by which it might be excreted have been problematic and it has been suggested that it is only present extracellularly when cells are ruptured (178). IL-1 does however have

highly conserved COOH terminal hydrophobic amino acids and is clearly present on membranes apparently as an integral membrane protein; therefore, it has been suggested that it is possibly attached by a glycosyl phosphatidyl inositol membrane anchor (180) which has been found to be an important membrane attachment mechanism for a number of immunologically relevant molecules including LFA-3 and Thy 1 (183). Although little is currently known about this type of attachment, the actual site of attachment and subsequent transport to the membrane, it has been studied in *Trypanosoma brucei* (183). Attachment is postulated to occur in the endoplasmic reticulum and appears to occur very rapidly, within one minute of the end of translation for the variant surface glycoprotein (VSG) protein of *Trypanosoma brucei.* This type of attachment appears to allow for rapid expression, extremely high receptor motility allowing for rapid redistribution after stimulation and expression of an active receptor which can be easily cleaved, perhaps by extracellular elastases, to give a fully functional hydrophillic soluble moiety (174). This suggestion may in part explain the controversial finding of 23 kd membrane IL-1 and a 17 kd soluble IL-1. Membrane IL-1 has been characterized on both macrophages and B cells (98, 184). Although IL-1a and IL-1ß are very different in amino acid sequence, they appear to mediate the same diverse functions and also interact with the same high affinity receptor present in small numbers on T cells and other cells (185, 186). The EL4 cell line has been beneficial in investigation of the IL-1 receptor because it appears to express a relatively large number of receptors, unlike normal T cells (185, 187). Receptor ligand interaction has been shown to down regulate receptor expression, and internalized IL-1 has been visualized by immunofluorescent techniques to collect in the nucleus. The significance of this finding is not known at present; however, it may have relevance in the interaction of IL-1 with T cells in T cell activation via the T cell receptor (185, 187).

Many of the effects of IL-1 on macrophage activation and on T cell activation

remain controversial (162, 188). Tumor necrosis factor (TNF) interaction with IL-1 has been a further complication, as both IL-1 and TNF are induced by the same stimuli (163), TNF is induced by IL-1 (189) and they apparently synergize in some systems and antagonize in others (189, 190). Interleukin 1 is strongly inhibited by prostaglandin and also stimulates the production of PGE_2 which may act as a negative feedback mechanism (191). Prostaglandins have also been shown to down regulate the expression of MHC class II molecules (192). By suppression of both IL-1 production and MHC class II expression, the cell may curtail the immune response by preventing T cell activation shown to be dependent on the levels of expression of both membrane IL-1 and MHC class II (193). IL-1 production is also inhibited by glucocorticoid hormones which suppress at the level of mRNA transcription but also increase the rate of mRNA degradation (194). Interleukin 1 transcription has been shown to be regulated by DNA methylation in the U937 monocytic cell line in which IL-1 is not normally detected even after treatment with lipopolysaccharide (LPS) (171). Treatment with 5-azacytidine induced expression of both IL-1 mRNA and soluble IL-1 in U937.1, a subline of the U937 monocytic cell line and vastly increased expression of IL-1 in the THP-1 cell line after LPS stimulation (171). Bacterial LPS is the inducing agent used most frequently for IL-1 production. Although *E. coli* LPS is typically employed, *Salmonella* LPS has been found to be significantly more potent in human monocytes (195). Monocytes isolated and grown in endotoxin free culture express little detectable IL-1 or IL-1 mRNA. There is a transient expression at 3-6 hours of intracellular IL-1 presumably due to adherence which has been shown to be a fairly weak inducer of IL-1 (195). *Salmonella* LPS causes a rapid expression of intracellular IL-1 within 2-3 hours which declines at about 20 hours. Secreted IL-1 appears somewhat later and continues to rise after 24 hours. Fuhlbrigge et al. (196) found that adherence of peritoneal macrophages caused significant IL-1 production in the absence of endotoxin and that intracellular and membrane IL-1 were expressed

transiently and early, peaking within 4 hours, but that secreted IL-1 continued for more that 24 hours and suggested that monocytes which are isolated by adherence are not resting cells. LPS stimulates a transient but significantly higher level of expression. Phorbol esters are potent inducers of IL-1 and synergize with LPS, apparently by activating somewhat different induction pathway (197). The transient nature of LPS-induced IL-1 production has been attributed to the coinduction of a cyclohexamide inhibitable repressor, similar to what is seen with IL-2 (198). Many other signals are capable of inducing IL-1 production by monocytes including muramyl peptides which have adjuvant activity (199), PPD (200), immunoglobulin Fc (201), C5a as well as C5a des arg, inactive C5a lacking a terminal arginine residue (202) and heat killed *Listeria monocytogenes* (98). Weaver and Unanue (203) have reported that IL-1 induction by bacterial products is a direct effect and does not require other cellular interactions; however, stimulation by soluble antigens require interaction with T cells or with a T cell product which they have found is not IL-2, gamma interferon, IL-4 or colony stimulating factor-1 (CSF-1). This interaction appears to be necessary to get macrophages ready to present antigen. The capacity to produce IL-1 may also be related to the maturity of the macrophage. Kurt-Jones et al. (98) found that macrophages which had been stimulated to produce both secreted IL-1 and membrane IL-1 were capable of producing only membrane IL-1 when restimulated. Also when human alveolar macrophages are compared to peripheral blood monocytes, monocytes were found to be significantly more capable of stimulation to produce IL-1 and within both populations there was a correlation between IL-1 production and mature phenotype measured by morphology and cellular density (204). Because IL-1 is a potent mediator in inflammation, a down regulation of production may be a mechanism for tissue protection.

IL-1 has been shown to be necessary in the induction of T cell activation in naive or resting T cells. Lowenthal and MacDonald have reported that $CD4^+$ cells, but not

$CD8^+$ cells, show an absolute requirement for accessory cells for activation and that this requirement is for membrane IL-1 (205). Similarly, Kurt-Jones et al. (98) have found that macrophages can present antigen only if both MHC class II and membrane IL-1 are expressed. The finding that many T cell clones and hybridomas have no requirement for IL-1 has been attributed to their being in a primed state rather than in a truly resting G_0 state (70). Thus the investigation of the role of IL-1 in immune function is actively being pursued and many areas remain controversial. IL-1, perhaps more than any other interleukin, has been shown from early studies to be extremely pleomorphic in both its molecular structure and in its functional capabilities leading to questions of its true identity. The role of IL-1 in immune functions is clearly a minute portion of its overall function in the whole body.

Gamma interferon. Gamma interferon (G-IFN), also called Type II or immune interferon, is a non-virally induced lymphokine produced by $CD3^+$ T cells, the TH1 cells which also produce IL-2, IL-3 and GM-CSF, in response to antigen presentation, PHA, Con A, IL-2 and antibodies capable of inducing T cell activation (143). Gamma interferon is also produced by $CD3^+$-$CD16^+$ and $CD3^-$-$CD16^-$ lymphocytes having characteristics of natural killer cells (206) Interferon gamma, like interferons alpha and beta, interferes with viral infection, thus, the name. One manner in which G-IFN continues to be quantitated is in terms of its antiviral specific activity, measured as the ability to inhibit the virally produced cytopathic effect on cells in culture. Gamma interferon is produced only in activated cells and then in minute quantities making its collection and characterization difficult (207); therefore, the production of recombinant G-IFN has facilitated many more studies. Gamma interferon is the product of a single gene on chromosome 12 in humans (208), and three different size glycoproteins have been isolated: 15-17 kD, 20 kD and 25 kD glycoproteins (209, 210). The differences in size are apparently due to micro-heterogeneity in

glycosolation and differentially processed COOH terminal forms but are functionally very similar and are not homologous to interferons alpha and beta (211). There is evidence that G-IFN self associates and may exert its effects as a homodimer or tetramer (207). It is a basic protein having a pI of 8.5 and is extremely acid labile being, rapidly destroyed by treatment at pH 2 (210). Natural G-IFN and recombinant G-IFN produced in eukaryotic cells are 5-10 times more active than the unglycosylated recombinant G-IFN produced in *E. coli* (207). Glycosylation appears to be the explanation for this difference; however, glycosylation does not affect its ability to bind to its receptor (210). The G-IFN receptor is expressed on many cell types and is coded for by a gene on the long arm of human chromosome 6 (212). There is controversy as to whether the receptors on all cell types are identical; however, there is apparently a single type of high affinity receptor on particular cell types (213). There are as few as 500 receptors on resting T cells but 13,000 on monocytes and a very small proportion of the available receptors need to be occupied in order to stimulate a response (214). As few as 40-60 molecules of G-IFN per cell produces maximal MHC class II induction and anti viral effects in monocytes (210). The original finding of receptors having multiple affinities on populations of cells apparently was due to the fairly high degree of nonspecific binding of G-IFN to cells (210). Once G-IFN interacts with its specific receptor, it is very rapidly endocytosed in clathrin coated pits facilitating down regulation of the receptor on the surface; however, the endocytosed receptor separates from the bound ligand in the acidic endosome and is recycled to the surface (210). The G-IFN present in the endosome has variously been found to be degraded after endosomal fusion with enzyme-laden lysosomes or to be transported to the nucleus (210, 215). Mediation of G-IFN effects requires both the interaction of G-IFN with its receptor and a gene product coded for on chromosome 21 which has not been identified (207).

Induction of G-IFN production in activated T cells may be biphasic with an early

peak seen concomitant with the appearance of IL-2 and a later larger peak which is possibly induced by the endogenous production of IL-2 (216). Gene regulation of G-IFN has been shown to be coordinately regulated with IL-2; however, G-IFN is also induced by IL-2 (206). The finding of a cis-acting consensus sequence in the 5' flanking region of the G-IFN gene which is 83% homologous to a sequence in the IL-2 gene has led to speculation that both genes may be regulated by the same DNA binding protein (217). When the human G-IFN gene was transfected into murine lymphoblasts and fibroblasts, expression was seen only in the lymphoblast cells leading to the conclusion that regulatory factors are present only in lymphoid cells. Thus like other cytokines, the regulation of expression of G-IFN is intimately connected with that of other cytokines and is confined to particular cell types (218). Gamma interferon production is suppressed by treatment with glucocorticoids (219) and cyclosporin (150) which may or may not be secondary to the effects of those compounds on IL-2 expression.

Gamma interferon has a variety of effects on immune function. The best characterized effects are the induction of MHC class I and class II molecules on the surface of monocytes and other tissue cells, the antiviral effects which it induces and delivery of the priming signal in macrophage activation (207, 220, 221). Gamma interferon is considered to be the T cell product having the greatest influence on macrophage function and differentiation. Gamma interferon causes a significant increase in Fc receptor expression and a concomitant increase in phagocytic activity and antibody dependent cell cytotxicity mediated by this receptor (222); however, in contrast to the Fc receptor, both C3b receptor (CR1) and mannosyl-fucosyl receptor expression are down regulated and phagocytosis mediated by these receptors is decreased (223). Gamma interferon induces increased MHC class II expression of all types, HLA-DR, DQ, and DP and also invariant chain (220, 225, 226). The marked increase in monocyte/ macrophage mediated accessory function is probably directly

related to this increased MHC class II expression and perhaps also to the increased expression of IL-1 stimulated by G-IFN (220). Gamma interferon increases the transcription of TNF and urokinase mRNA (227), increases expression of complement components C2 and factor B (207, 228), and has been found to induce the expression of IL-2 receptors on monocytes (229). Gamma interferon activation of macrophages increases the ability of these cells to kill intracellular parasites in both mice and humans (221), increases the respiratory burst and release of reactive oxygen compounds (221) and increases macrophage mediated tumor cell cytotoxicity (221). Induction of the 2',5'-oligoadenylate synthetase, endoribonuclease, phosphodiesterase system of enzymes occurs after addition of G-IFN (207); however, its significance in the functional activation remains a mystery. Gamma interferon interaction with its specific receptor apparently induces activation of a protein kinase C and a slow increase in intracellular calcium concentration and may induce a cascade similar to the signal transduction seen in T cell activation (221). In non-macrophage cells, G-IFN also has marked effects on cytolytic function including generation of cytolytic T lymphocytes and activation of natural killer cells (207). Gamma interferon induces the expression of both MHC class I and class II antigens, beta-2 microglobulin and invariant chain on tissue cells (220, 225, 226, 230, 231). Gamma interferon does not induce increased MHC class II expression on B cells (232); however, it has been shown to modulate antibody isotypes expressed by B cells (233). Gamma interferon stimulates the production of IgG_{2a} and inhibits the production of IgG_1 and IgE which are stimulated by IL-4.

Thus it is apparent that G-IFN is similar to other cytokines in that it has hormone-like capacity to interact with a specific receptor to mediate the expression of a complex array of functions, that it is transiently expressed, under strong regulatory control and its mRNA has a relatively short half-life, and that it acts in conjunction with a large number of other regulatory molecules to induce and modulate an immune

response.

Antigen processing and presentation.

Antigen must be processed before it can be productively presented to T cells. The puzzle afforded by the apparent difference between the recognition of antigen by B cells and by T cells is beginning to yield some clues to its intricasies. B cells were found to recognize and bind antigen in its native form without any modification and indeed were found to cease to recognize antigens if they were sufficiently denatured (234). When binding sites were analyzed, B cell recognition was found to involve conformational epitopes on antigen molecules (235). The B cell receptor for antigen was also found to be a membrane anchored form of antibody which had previously been found to be the mediator of humoral immune function (236). Analysis of antibody structure and immunoglobulin genes has revealed the extremely complex nature of the generation of the exquisite specificity exhibited by the interaction of antibody with antigen (237). Analysis of the interaction of T cells with antigen proved to be more difficult, largely because there was no soluble product produced in large quantity which could undergo biochemical characterization and also be used as an antigen for the production of antibody with which to fish out the elucive receptor. And because the T cell receptor is clonally distributed, a single T cell receptor is present in vanishing small quantity within the available cells in the T cell repertoire. The advent of monoclonal antibody production and collateral techniques for T cell hybridoma formation, T cell cloning procedures and techniques for molecular genetic analysis made isolation and characterization of the T cell receptor complex possible. The explosion of findings led to inclusion of the T cell receptor in the immunoglobulin supergene family because of its genetic organization (26). The antigen specificity exhibited by T cells using a multichain receptor is similar in many respects to that of B cells; however, the manner in which antigen is recognized appears to be very

different. T cells recognize epitopes dependent on the primary sequence of antigens rather than conformational determinants (234), and that recognition of antigen is strictly dependent on the presence of accessory cells (238). The requirement for accessory cells has been shown to involve the presence of molecules encoded by the major histocompatibility complex (MHC) and the necessity for recognition of antigen by the T cell receptor only in association with MHC (239, 240).

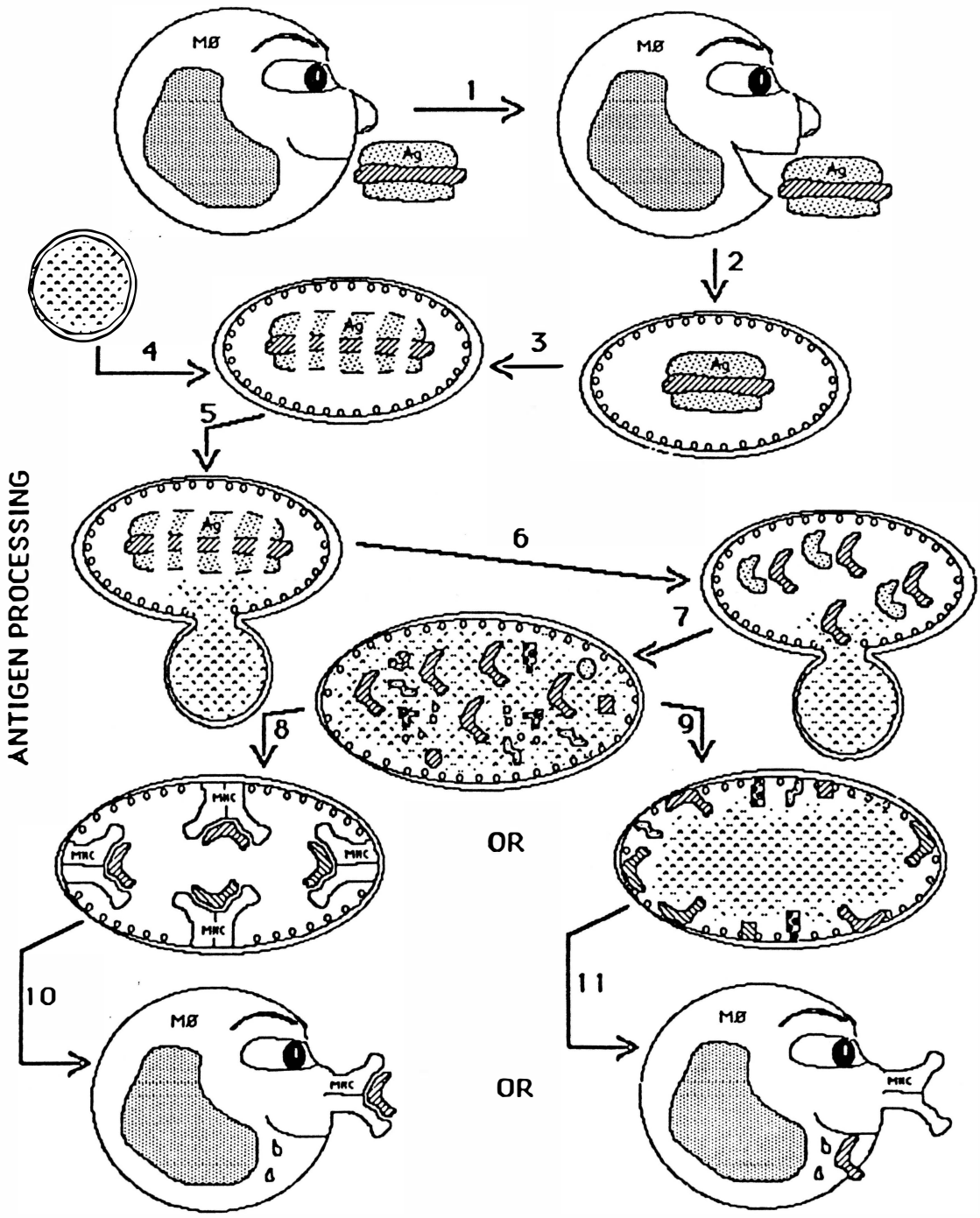
Several cell types can serve as accessory cells, principally including monocyte/macrophages, B cells and dendritic cells (241, 242). The ability of these cells to act as accessory cells is dependent on their capacity to express MHC class II molecules (243). Although macrophages were vehemently defended as the archetypical antigen presenting cell, Chestnut and Grey (244) clearly showed the competence of B cells to present antigen to T cells. B cells have also been shown to be able to process antigen for presentation (245). Lanzavecchia (246) elucidated the possible mechanisms for a direct role of B cells in the mediation of T cell help which seeks to explain the manner in which a signal from an antigen specific T cell receptor can mediate activation of B cells to produce vast quantities of an antigen receptor, antibody, specific for totally different epitopes on the antigen molecule. B cells have the advantage of possession of a specific membrane receptor which can recognize antigen in its native form, bind it, internalize it, process it and reexpress fragments of it in association with MHC class II molecules to T cells with specific receptors which can then stimilate the presenting B cell to proliferate and differentiate into an antibody factory cell, a plasma cell, and in the process generate memory B cells. Dendritic cells are morphologically complex cells found intimately associated with T and B cells in organs of the immune system where they may serve as repositories of antigenic competence for extremely long periods of time (247). There are several different types of dendritic cells which appear to differ in at least some of their functional capacities; however, there is agreement that dendritic cells express large quantities of

MHC class II on their surface, are extremely potent stimulators of the mixed lymphocyte response to foreign MHC class II antigens and are also very potent antigen presenting cells (247, 248, 249). Although B cells and dendritic cells have been shown to present antigen very efficiently, accessory cell function by monocyte/macrophages will be emphasized chiefly for two reasons: first because that is the cell in which the largest body of work has been done and second, because this project involved using the U937 cell line which is derived from a histiocytic lymphoma and has monocytic characteristics (95).

The interaction of monocytes or macrophages (MØ) with antigen, illustrated schematically in Figure 2, stimulates endocytosis which may be pinocytosis or phagocytosis. The process of phagocytosis requires an initial interaction with some type of membrane receptor on the MØ, primarily receptors for immunoglobulin Fc, complement receptors and mannosyl/fucosyl receptors, although there are probably many other MØ receptors which are capable of receptor mediated endocytosis (250, 251). Particulate antigens, opsonized by antibody or free, adhere to an appropriate receptor and are engulfed by the MØ by receptor mediated endocytosis. The events which follow endocytosis leading finally to interaction of fragmented antigen with the MHC molecules on the surface of the cell remain one of the most active areas of current research in immunology. It is postulated that after antigen is endocytosed it is digested partially and somehow is reexpressed on the surface of the MØ where it is presented to a specific T cell in conjunction with an MHC molecule (252, 253). Although there is still controversy over the fate of antigen in the endosome, its trafficking through the interior of the cell and the location of its final interaction with MHC, information on the cell biology of intracellular proteolysis, acidic compartments and mediation of bulk and directed flow of intracellular vesicles is relevant to the events taking place in antigen processing. Antigen attached to specific receptors appears to have two paths for degradation (252, 253, 254). Once internalized, the endosome is acidified probably

FIGURE 2. A schematic view of antigen processing.

Antigen must first be recognized by a macrophage by interaction with a receptor (1) followed by engulfment by receptor mediated endocytosis (2). Following this phagocytic function, the endocytosed antigen is contained within a phagosome (3) which may fuse with a primary granule (4) which contains proteolytic enzymes to form the acidic compartment (5) where the antigen is successively digested into smaller fragments (6-7). Two paths are available according to two proposed mechanisms for antigen fragment reexpression on the surface of the cell. Pathway (8) (44) requires that MHC class II antigens present on the internal surface of the phagosome salvage antigenic peptide fragments from the digestive environment of the endosome and reexpress these fragments on the surface of the cell (10). Pathway (9) illustrates the antigenic peptide fragments adhering directly to the lipid bilayer by means of the hydrophobic face of the amphipathic and probably alpha helical fragment configuration leading to reexpression directly on the surface of the cell (11) where interaction with MHC class II takes place by some unspecified mechanism (254).


ANTIGEN PROCESSING
MØ
Ag
1
2
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MHC
OR
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b
a

changing the binding affinity of the receptor and releasing the antigen. The receptor may be shuttled back to the cell surface and reexpressed or may accompany the antigen as the endosome fuses with lysosomes which release their store of proteolytic enzymes into the lumen of the phagolysosome. There are also enzymatic reactions of limited proteolysis within the endosome which are potentiated by decreased pH (255, 256, 257). These enzymes may cleave antigen into peptide fragments of varying lengths. Another site proposed for limited proteolysis is the cell membrane itself where a serine protease which is glutaraldehyde insensitive have been identified (258). Although this site for processing has not been widely accepted, its possible role in experiments using glutaraldehyde fixed antigen presenting cells is open to speculation. Treatment of cells with lysomotrophic agents, such as chloroquine which raises the pH of lysosomes, effectively eliminates the ability of the MØ to present antigen (259). Although not generally accepted because of the large body of research which supports a fairly specific type of action for lysomotrophic agents, it has been argued that chloroquine may have other effects on cell physiology that are unrelated to antigen processing but that can inhibit cell function thereby causing decreased antigen processing or presentation by indirect pathways (254). There is evidence that chloroquine does inhibit the dissociation of invariant chain from MHC class II molecules and decreases the expression of MHC class II molecules on the cell surface (260). These effects would certainly be expected to influence antigen presentation. The findings of Gruenberg and Howell (261) that a transmembrane protein, in this case the G protein of vesicular stomatitus virus (VSV-G), once internalized by endocytosis, spends a very short time, less than 5 minutes, in an endosome which is competent to fuse with a lysosome probably has relevance. The internalized VSV-G takes one of two possible paths: it is reshuttled to the cell surface or it remains in the portion of the endosome which fuses with a lysosome and is degraded. If the concentration of VSV-G in the cell membrane is increased or if the VSV-G is coupled to antibody, the proportion of

membrane protein which takes the lysosomal fusion path is markedly increased. Indeed it has been shown that if antigen to be endocytosed is opsonized by antibody, phagocytic activity is markedly enhanced and the rate of Fc receptor expression is increased (224). Macrophages then utilize Fc mediated endocytosis of antigen almost exclusively and down regulate mannosyl-fucosyl receptors.

The findings by Rothman et al. (262, 263) and others that organelle specific retention and vesicle trafficking are dependent on the presence and/or absence of specific amino acid sequences may also have relevance for the processing of some antigens for presentation rather than complete catalysis. Cytoskeletal elements appear to have an important role in receptor mediated endocytosis and endosome traffic also (264). The organized network of microtubules originating from the centrioles in the Golgi region are important pathways for shuttling cellular vesicles within the cytoplasm in unidirectional fashion propelled by so called motility motors such as kinesin reminiscent of actin-myosin interaction in muscle (265). Although control of their function and the concerted interplay of the whole array of cellular machinery for engulfing, degrading and expelling as well as the integration with biosynthesis is not at all clear, one looks forward to rapidly advancing knowledge on many fronts.

Processing requires that most antigens be cleaved into fragments; however, a minimum size requirement for presentation appears to be approximately 7-10 amino acids and for many peptide fragments studied a longer peptide is more antigenic (266). This would seem to indicate that enzyme digestion must be limited because degradation to individual amino acids would obviate presentation; therefore, the fragments made available to the T cell during presentation must be derived from the endosome or retained and somehow rescued from proteolysis in the phagolysosome (267). This led to the suggestion that the MHC molecules fulfilled the role of rescuing bindable fragments from the enzyme soup within the phagolysosome. This idea has been discounted by several groups because it is felt that if the receptor MHC molecules are

present in the phaglolysosome, they too would be degraded rather than shuttled back to the surface (254, 268). Interaction of antigenic fragments with intracellular MHC molecules, however, remains a major requirement in the generally accepted scheme for antigen presentation. There are also several antigens which have been described which do not require processing for presentation. Allen et al. (269) found that fibrinogen A alpha chain can be presented without proteolytic cleavage or modification. Previously Allen et al. (253) had shown that proteolytic fragmentation was required for presentation to some but not all available HEL specific T cells as clone 3A9 was capable of reacting with carboxymethylated HEL. Ziegler et al. (270) also found that particulate antigens (*Listeria monocytogenes*) required processing; however, some bacterial products could be presented by paraformaldehye treated antigen presenting cells without additional modification. Berzofsky et al. (254) found that simply denaturing sperm whale myoglobin was sufficient to allow for antigen presentation to T cells. The antigens which can be presented without fragmentation are soluble and probably adopt a random coil configuration in solution (269, 270). This is apparently true for the portion of the fibrinogen A alpha chain which binds directly to the I-E^k molecule. T cell antigen epitopes have begun to be characterized and, although there is still disagreement, these epitopes appear to be composed of segments of amino acids in random coil or having high segmental mobility which can, under appropriate conditions, adopt an alpha helical configuration (254). Adoption of the alpha helical configuration may be dependent on the interaction with the binding groove of the MHC molecules which may cause the alignment of the hydophobic amino acids to one face and the hydrophilic residues to the opposing face thereby producing an amphipathic helical structure having two thirds of its surface relatively hydrophobic and one third hydrophilic. Indeed the primary structure of presentable T cell epitopes has been

postulated to be composed of the following general structure:

NH4--GLY (or charged a.a.)-hydrophobic a.a.-(hydrophobic a.a.)1-2-hydrophilic a.a.-COOH

which would generate an amphipathic alpha helix (254, 269). Although this view is championed by several groups, there is not complete agreement and other T cell epitopes may prove to be very different. Using HEL peptide fragments having known amino acid sequence and defined length, Allen et al. (266) were able to determine a minimum peptide length of 7-10 amino acids for presentation. A peptide fragment of this size allows for at least two and probably three polar residues on its hydrophilic face if it assumes an alpha helical configuration. This would appear to provide a T cell receptor interaction site to which the antigen contributes three to four residues protruding from the MHC binding groove. The geometry of this face of the antigenic peptide is necessarily governed by several parameters, two of which are the periodicity of alpha helices and the interaction of the opposite face of the alpha helical fragment with the binding site on the MHC receptor. Periodicity of peptide alpha helix is 3.6 residues which affords a staggerred array of polar residues if the general amino acid configuration of antigenic peptides holds true (254). Thus the first and seventh residue would fall in phase with each other to a greater degree than the first and third or fourth. The interaction of the opposite face of the alpha helical fragment imposes constraints both by hydrophobic interactions and by the fractal nature, i.e. the bumpiness, of the peptide fragment and the MHC molecule with which it is interacting which will tend to induce bending or eversion of the residues on the hydrophilic face. The finding that the signal sequence for membrane protein insertion adopts an alpha helix if successfully inserted into the membrane but adopts a beta pleated sheet configuration atop the membrane if insertion is frustrated (254) illustrates the pleomorphic nature of particular amino acid sequences and their dependence on environmental constraints for secondary structure.

The requirement for antigen processing became apparent from the seminal work

on antigen presentation by a number of groups using fragments isolated from enzyme cleavage of protein antigens notably hen egg lysozyme (HEL) (44), chicken ovalbumin (OVA) (45), pigeon cytochrome c (271) and lambda repressor (272). Ziegler and Unanue (273) showed that if accessory cells were incubated with antigen and subsequently gently fixed with paraformaldehye, they were capable of presenting antigen to T cells just as unfixed cells were; however, if the antigen was added after fixation, no presentation was achieved. This finding seemed to clearly indicate that the antigen must be altered in some way by the macrophage to mold or process it into a recognizable form. To take this type of study one step further, Shimonkevitz et al. (274) determined that a peptide fragment of antigen added directly to fixed cells could substitute for the processing events and be presented; and indeed Watts et al. and others (275) showed that peptide fragment could be presented by MHC class II molecules anchored in synthetic membranes. Thus the requirement for "processing" seems to hold true for most antigens examined. The findings of Walden et al. (276) that whole hen egg lysozyme chemically crosslinked to Ia containing liposomes would also stimulate specific T cell activation has been cited as evidence to counter the claim for a processing requirement; however, there is a strong possibility that either there was denaturation of the HEL during cross linking or less likely that there was very slight contamination of the original preparation with peptide framents (277). Identification of naturally processed peptide fragments on the surfaces of antigen presenting cells has not been deemed to have been successful. Although recent studies by Berzovsky and Delovitch et al. (278) have purported to show the intracellular route traversed by antigen in the process of being "processed," the major criticism continues to be questions of whether the label being followed remains associated with any part of the antigen, much less any relevent epitope.

Antigen processing discussed thus far applies to soluble proteins and particulate matter which enter the antigen presenting cell most commonly by receptor mediated

endocytosis and follow the endocytic pathway to presentation on the cell surface, whatever that actually involves.

The pathway is considerably more complex when the particulate matter is bacterial, viral or parasitic and has mechanisms of its own to evade degradation or to stifle the ability of the macrophage to kill and degrade the antigen for presentation. Although vast numbers of examples of evasion mechanisms of various organisms have been demonstrated, the findings of Cluff and Ziegler (279) using *Listeria monocytogenes* demonstrate anew that investigations using killed or avirulent organisms, while important in a mechanistic sense, are not strictly relevent to an animal's immune response to that organism under environmental conditions. In studying hemolysin negative and hemolysin positive *Listeria*, Cluff and Ziegler found that mice were capable of processing and presenting hemolysin negative bacteria with great facility; however, hemolysin positive bacteria even in small numbers were not processed and presented, resulting in virulence increased by a factor of 10^3 as measured by LD_{50} values. Increased virulence was also not attributable to either decreased Ia expression or direct killing of the macrophages after bacterial ingestion. Viruses poses problems which are more complex than most other infectious agents. Their routes of entry and escape from membrane compartments and their evasion mechanisms which make use of the cells own resources make presentation of viral antigens a different process. Viral proteins are produced by the cell once infected and these proteins may be formed largely on the free ribosomes in the cytosol which in uninfected cells are responsible for the production of proteins involved in the intermediary metabolic functions of the cell (280, 281). Although these proteins may be transported to membrane bound vesicles and cycled to the surface of the cell, the fate of viral proteins produced in this manner is not well understood and the problem of inserting material from the hydrophilic environment of the cytosol through a hydrophobic membrane into a compartment containing MHC class I molecules is

unexplained (282). The very fact that virally infected cells and perhaps tumor cells are usually recognized and eliminated by a different system of MHC molecules, MHC class I, and T cells, $CD8^+$ rather than $CD4^+$, indicates that the processes involved in what Braciale et al. (283) refer to as the "endogenous presentation pathway" are very different from those employed for the processing and presentation of exogenous antigen. The requirement for processing of endogenous viral antigen is favored but has not been clearly demonstrated; however, viral proteins may be subject to various degradative processes while in the cytosol including ubiquitin dependent degradation (284), PEST sequence mediated degradation (285) and Ca^{++} dependent proteases (286). Although the most commonly found cytolytic T cell is a $CD8^+$ cell which recognizes viral antigen in association with MHC class I molecules, Braciale et al. (283, 287) compared the cytolytic T cells specific for influenza hemagglutinin which were $CD8^+$ with those which were $CD4^+$. Morphologically $CD4^+$ CTL's were indistinguishable from $CD4^+$ helper cells and the "lethal hit" delivered by both $CD4^+$ and $CD8^+$ CTL's was identical. $CD4^+$ CTL's recognized cells treated with antigen from both infectious and non-infectious virus and presentation was inhibited by the lysomotrophic agent, chloroquine; however, $CD8^+$ cells recognized antigen only from infectious virus and recognition required de novo protein synthesis and was not inhibitable by chloroquine. Thus processing and presentation of viral antigen to $CD4^+$ T cells probably followed the typical exogenous pathway through endosome-lysosome degradation and reexpression prior to recognition in conjunction with MHC class II molecules. $CD8^+$ cells were able to recognize antigen in conjunction with MHC class I molecules only if the virus had successfully infected the target cell and initiated production of its viral proteins by the cell. These findings have significant implications for the induction of viral immunity by immunization using killed versus attenuated virus in terms of the type of response generated.

Thus processing of antigens proceeds probably by all the degradative routes

available within the cell and runs the gamut between no degradation to fragmentation into short peptides. It remains difficult to reconcile the need for an intact peptide of seven or more residues with progression of ingested material through the lysosome where it is subject to the effects of 40 or more enzymes, the products of which would appear to be too small to interact with MHC molecules to be presented to T cells. It is also apparent that antigen processed by different compartments, i.e. within endosomes or within the cytosol, is presented by different MHC molecules primarily to different subsets of T cells; however, like other systems encountered in immune function, the systems are not totally exclusive and in fact are overlapping.

Once processed, antigen is presented in recognizable form to T cells in association with MHC molecules. Zinkernagel and Doherty in 1974 (240) showed that response to antigen required that the MHC haplotype of the antigen presenting cell be the same as that of the T cell being asked to respond. The response was, therefore, MHC "restricted;" however, the manner by which this restriction was imposed was unknown. Restriction proved to reside in a portion of the MHC which was separate from the transplantation antigens and was designated as the I region. Questions relating to MHC restriction led to intense debate over the nature of the T cell receptor interaction with antigen and the concomitant but incongruous necessity for recognition of MHC I-region gene products on accessory cells. A second important aspect of immune function concerned the finding that in guinea pigs and in mice when antigens of limited epitope heterogeneity were administered, some strains of animals made a strong response and other strains made little if any response (288). Thus arose the problem of responder and non-responder strains which when further examined also was dependent on the haplotype of the MHC region immune response genes (IR genes). The nature of both restriction and response/non-response remained a topic of investigation and conjecture, clarified somewhat by the finding that the serologically defined Ia

antigens on accessory cells were the products of IR genes. Identification of the clontotypic T cell receptor as the single recognition structure for both antigen and MHC dispelled the problem of dual receptors but did not indicate how polymorphic but non-clonotypic structures, the MHC molecules, could bind to all the different antigens for which the T cell repertoire was selected. Instead a loose association of antigen stabilized by T cell receptor interaction was postulated to account for the MHC lack of specificity. Recent findings indicate quite clearly that MHC molecules are antigen binding structures in the absence of T cell receptors (289) and further that antigen remains tightly associated with the MHC molecules for relatively long periods of time (253). Babbitt et al. (44) and Buus et al. (45) demonstrated that MHC class II molecules bound free antigenic peptides and their propensity to bind peptide fragments correlated with the ability of cells with that haplotype to respond to the particular antigenic fragments used (252, 253). Thus MHC class II molecules are direct binding structures which select determinants which will be available to T cells in conjunction with MHC (290, 291). Moreover, the antigenic fragments of several antigens were found to compete for association with MHC in a haplotype restricted manner indicating that there is a single antigen binding site on MHC molecules which serves all antigen interactions. Other recent studies utilizing photoaffinity labeling and fluorescent photobleaching have supported the finding that MHC directly binds peptide fragments (292), although findings of Delovitch et al. (289) using beef insulin indicated that all MHC molecules bound antigen but to different degrees. Experimental evaluation of MHC binding affinity using equilibrium dialysis has confirmed a dissociation constant of 10^{-5}-2 X 10^{-6}M/sec^{-1} for antigenic peptides tested (45, 253). The reaction has been shown by Buus et al. (45) to be very temperature dependent and stable requiring fairly long times for dissociation of the complexed antigen giving a $t_{1/2}$ of 30 hours at room temperature and 5-10 hours at 37°C. Further evidence for direct MHC binding of peptide was afforded by the crystallographic data obtained for the structure of the

human HLA-A2 molecule, an MHC class I molecule, which indicates that MHC molecules may seldom be devoid of antigen (293, 294). Recent structural information derived from amino acid sequence data indicate that MHC class II structure is similar to that of MHC class I molecules and may involve the same type of antigen groove (295). Thus the concept of MHC restriction and responders and non-responders is now clearly seen as a direct capacity of the MHC molecules to directly bind to fragments or conformationally permissive segments of antigen. Different haplotypes of MHC molecules allow interaction with different peptide fragments. In globular proteins, a heterogeneous array of peptide fragments can be released by limited proteolysis. Within a given haplotype, the probability is great that there will be one or more epitopes with which the MHC molecules can interact. For antigens with limited epitope heterogeneity such as the random terpolymers of glutamic acid, alanine, tyrosine (GAT) or glutamic acid, lysine, phenylalanine (GLØ), the MHC molecules expressed by some haplotypes do not bind to the one or two epitopes available and thus do not respond. MHC restriction, therefore, is governed by the determinant selectivity of the MHC molecules which are the structures required for interaction with antigen-specific T cell receptor (253, 291). MHC interaction is necessary for immune response; however, it does not insure that a corresponding T cell receptor has been selected with which this complex can interact. T cell repertoire selection appears to clonally delete or markedly diminish T cells which interact with unbound self-MHC molecules or self-MHC molecules bound to self peptides but selects for self-MHC molecules bound to foreign epitopes. For this reason, the T cell repertoire recognizes only cells which have the same MHC molecules which were present during the selection process in the thymus. Foreign MHC may be recognized as analogous to self-MHC bound to foreign antigen or self-MHC bound to self antigen, although there is conflicting evidence concerning any requirement that foreign MHC antigen be processed prior to recognition (296, 297).

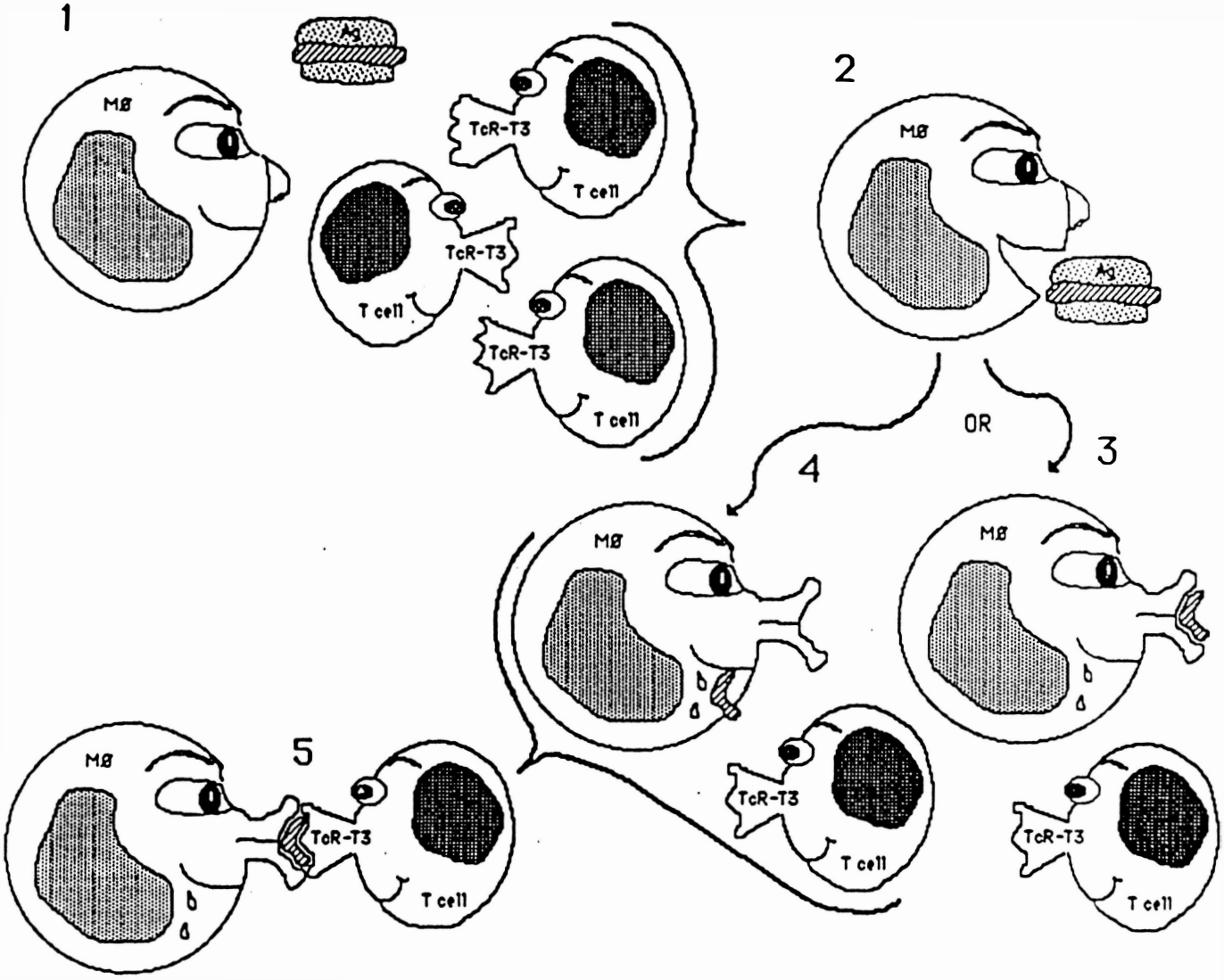
The site of interaction of MHC molecules with antigen to be presented remains controversial. Two primary hypotheses have been postulated to explain the interaction: first, that the antigen and MHC bind while in the endosome (or phagolysosome) or in the trans-Golgi where newly synthesized MHC molecules are available and are reexpressed on the surface of the cell together (241, 297); and second that the antigen fragments which, as previously stated may have a hydrophobic face, are attached directly to the phospholipid of the endosomal membrane and reexpressed on the surface of the cell still attached to the membrane with MHC interaction occurring on the extracellular surface (254). The first hypothesis is more generally accepted at least partially due to the problem of retention of antigen after processing, its reexpression and subsequent disengagement from the cell surface and interaction with MHC. There is evidence from Cresswell (298) that MHC has access to antigen in an intracellular compartment. Berzofsky et al. (254) champion the second hypothesis and explain antigen retention by hydrophobic interactions, although MHC interaction remains unclear. Findings of Falo et al. (299) that treatment of antigen pulsed cells with phospholipase prevents antigen presentation of soluble antigen but not allogeneic MHC tends to support the second hypothesis since it is their contention that the phospholipase is removing antigen or antigenic fragments which are interacting directly with phospholipids of the cell membrane. Allogeneic MHC is not affected because it is an integral membrane protein.

It is apparent that recent developments have clarified the role of MHC molecules with respect to restriction phenomena and T cell activation by antigen; however, the cell biology of antigen processing and MHC interaction remains to be fully explained. As shown in Figure 3 which is a cartoon representation of antigen presentation, the components necessary for transmission of antigen specific signals to T cells to initiate clonal expansion and production of efferent immune function are macrophages, antigen in presentable form in association with MHC class II molecules and appropriate T cells

FIGURE 3. Antigen presentation.

As shown in cartoon form, the components required for successful presentation of antigen (1) are macrophages with appropriate receptors for mediation of endocytosis of antigens, antigen and a T cell with the appropriate T cell receptor previously clonally selected by MHC interaction in the thymus. Antigen is processed by the macrophage (2) and reexpressed on the cell surface in association with MHC class II through either association of antigen fragment with MHC class II intracellularly (3) or extracellularly (4), followed by interaction with the T cell bearing the appropriate receptor (5) leading to conveyance of activation signals across the T cell membrane.

ANTIGEN PRESENTATION



present in the clonally selected repertoire. As discussed in previous sections but not indicated in the drawing, there are other important interactions which take place in antigen presentation and T cell activation involving cell surface receptors including CD4 and LFA and soluble products present in the microenvironment produced by both T cells and macrophages.

Major histocompatibility complex (MHC). The molecules of the major histocompatibility complex are also members of the immunoglobulin supergene family (26). More than fifty years ago, Peter Gorer proposed that the mouse H-2 complex was the major determinant of graft acceptance in mouse transplantation (300, 301). Since that time the genes for the mouse H-2 and the analogous human HLA have been examined at length and the genetic map has been drawn and redrawn many times. The mouse H-2 was identified on chromosome 17 (302) and the human HLA was found on the short arm of chromosome 6 (303). Although the genes encoded are very similar, the genetic organization of the murine H-2 underwent a major change at a point in evolution after speciation estimated to be approximately 75 million years ago (304). The murine type of MHC having a split class I region is apparently anomalous and found to occur only in mice and rats. The human MHC gene arrangement has been found to be common to other species, predicted even for the molerat cousin of mice, making it the more ancient form (304). Because mice have been the archetypical immunological beast, the murine MHC has been the most extensively examined and sequenced; and although the gene arrangement proved to be different, the major features of the murine MHC have also applied to the human MHC. Figure 4 shows a compilation of the genes of the murine and human major histocompatibility complexes. Four types of genes are found in both mice and humans, as well as all other animals which have been investigated: (1) class I, (2) class II, (3) class III and (4) other genes.

Class I genes encode the classical transplantation antigens which exhibit an

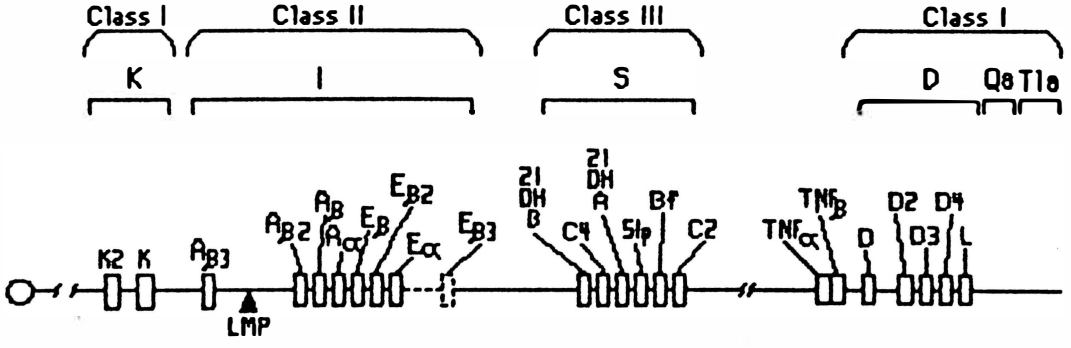
FIGURE 4. Major Histocompatibility Complex.

The murine MHC, found on chromosome 17, is composed of split class I, class II, class III and unclassified genes. Not all haplotypes have been shown to have all genes illustrated. Two class I MHC K genes (K and K_2) have been demonstrated as well as four D genes (D, D_2, D_3, D_4) and a single L gene. The I region contains alpha and beta chains for I-A (A_β, $A_{\beta 2}$,A_a) and I-E (E_β, $E_{\beta 2}$, E_a and postulated $E_{\beta 3}$). The class III genes present in the murine MHC include C2, C4, Bf, and Slp. Unclassified genes found to be present include 21-hydroxylase A and B (21 OH-A & 21 OH-B), TNF_a and TNF_β and LMP (304, 305).

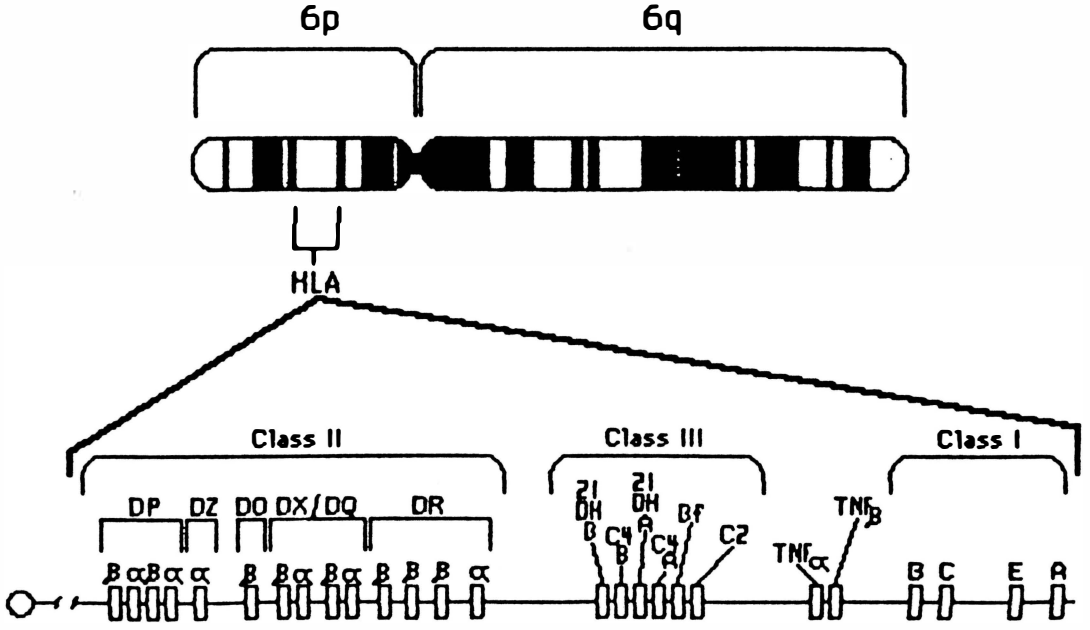
Human HLA, located on chromosome 6 in the center of 6p, is composed of non-split class I, class II, class III and unclassified genes. The class I genes include HLA-A, B, C & E. Class II genes include the alpha and beta benes of DR (a and β_1, β_2, β_3), DQ (a and ß), DX (a and ß), DO (ß), DZ (a) and DP (a_1, β_1, a_2, β_2). Gene order for DQ and DX is not clearly known. Class III genes present are C2, C4-A and C4-B and Bf. Unclassified genes include 21-hydroxylase A & B (21 OH-A & 21 OH-B) and TNF_a and TNF_β (306, 307).

MAJOR HISTOCOMPATIBILITY COMPLEX

H-2 MOUSE CHROMOSOME 17



HLA HUMAN CHROMOSOME 6



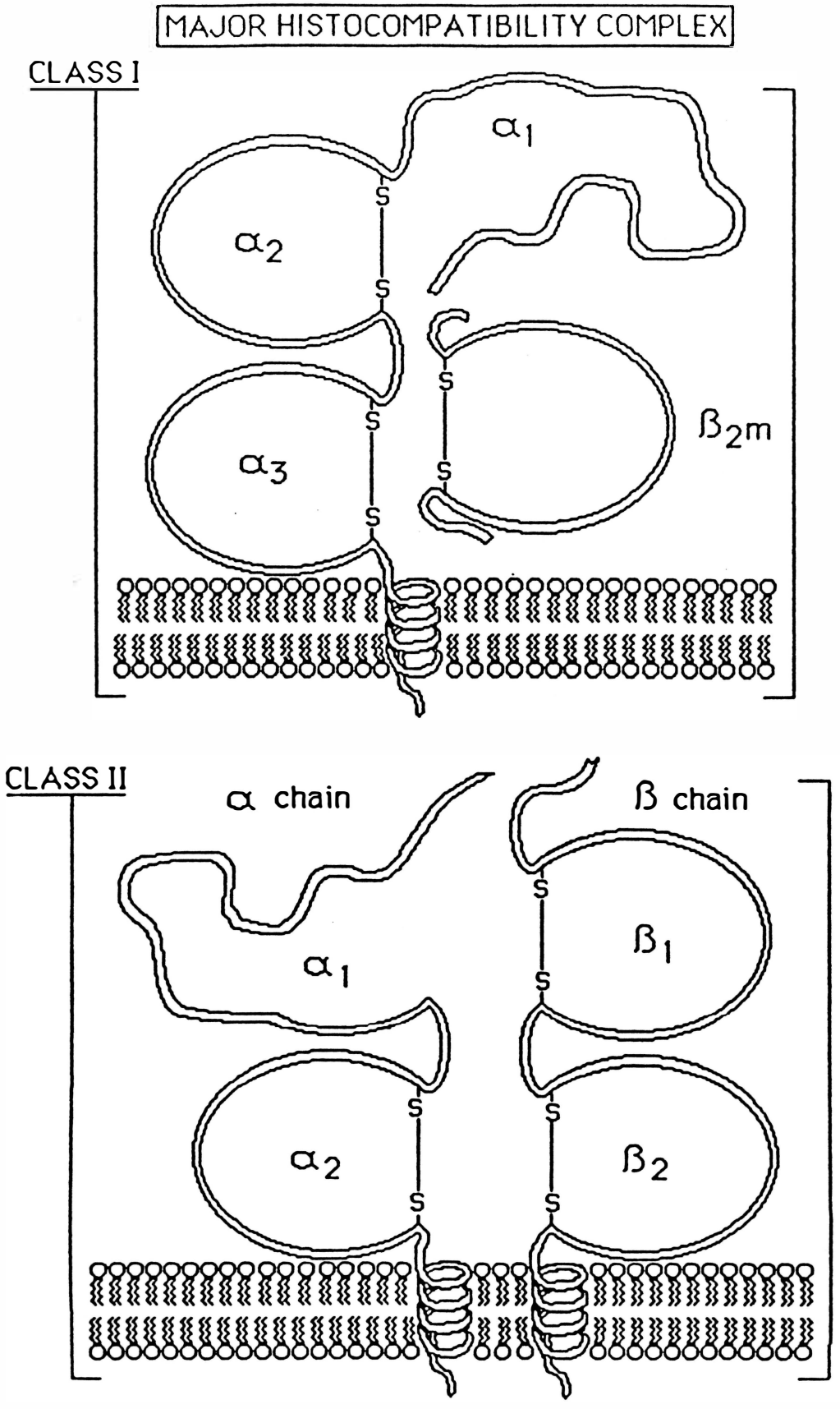
extremely high degree of polymorphism within populations and encode a 44-45 kD membrane bound glycoprotein which is found on nearly all cells of the body and which is expressed in conjunction with beta-2 microglobulin, a 12 kD protein encoded on a separate chromosome (308). The class I molecule illustrated in Figure 5 is composed of three extracellular domains each comprised of approximately 100 amino acids, a transmembrane segment and an intracellular tail. The external domains, a1 and a2 at the N terminal are extremely polymorphic and associate to form the antigen binding site. The a3 domain which is disulfide linked and shows sequence homology to immunoglobulin constant domains is non polymorphic and lies close to the membrane. This domain shows structural homology to beta-2 microglobulin with which it is closely associated to form the scaffolding for the antigen binding structure (293, 294). Polymorphic class I MHC molecules are important in recognition of virally infected cells and tumor cells and their elimination by specific cytolytic T cells. The class I MHC genes of the mouse are the K and D (L) genes and the human genes are HLA-A, B, C and newly described E (306, 308). Within the human HLA, the best characterized genes are the A and B loci which have been found to be extensively polymorphic, especially the B locus (309). There are also large numbers of non-polymorphic class I genes found in the Qa and Tla regions (308). These genes are similar in structure to polymorphic class I genes and may have served as repositories for genetic information for recombination or gene conversion with polymorphic class I genes; however, they have not been found to have a role in T cell activation, and although they are currently the subject of intense investigation, will not be treated further.

Class II genes in the mouse have been investigated successfully due to the development of inbred and congenic strains of mice which differ only at specific segments of the MHC. Findings that the Ia antigens expressed on accessory cell membranes were, as suspected, synonymous with the IR gene products (305) was

FIGURE 5. MHC class I and class II molecules-protein structure.

MHC class I molecules are composed of a heavy chain of 44-45 kD comprised of two highly polymorphic external domains (a1 and a2), a non-polymorphic domain (a3) in close proximity to the cell membrane, a transmembrane segment and cytoplasmic tail. The heavy chain is associated with beta-2 microglobulin which also has domain structure. Each domain contains approximately 100 amino acids and the a2 and a3 domains and beta-2 microglobulin domains are disulfide bonded. The antigen ginding structure is formed by the configuration of the a1 and a2 domains supported by the scaffolding structures of the a3 and beta-2 microglobulin (ß2m) (293, 294).

MHC class II molecules are composed of two chains which are closely, but non-covalently associated. The alpha heavy chain has a molecular weight of 34 kD and is composed of two domains of approximately 100 amino acids each. The beta light chain has a molecular weight of 29 kD and is also composed of two domains. The external domains (a1 and ß1) are highly polymorphic, whereas the membrane proximal domains (a2 and ß2) are non-polymorphic. The a1 domain, like the class I heavy chain a1 domain, is not disulfide bonded; however, all other domains do contain a disulfide bond. Both chains have a transmembrane region and a cytoplasmic tail (295). The configurations of both class I and class II molecules are similar in that they provide highly polymorphic binding grooves composed of two domains, one of which contains a disulfide bond and one which does not. It is proposed that this groove constitutes the antigen binding site (293, 294, 295).


MAJOR HISTOCOMPATIBILITY COMPLEX
CLASS I
α1
α2
α3
ß2m
CLASS II
α chain
ß chain
α1
α2
ß1
ß2

followed by the use of molecular genetics techniques to examine the MHC region genes at the molecular level for large segments of the genome causing major redrawing of the murine MHC, eliminating several postulated immune response genes and identifying "hot spots" of recombination which had caused the MHC to falsely appear to be larger because of increased recombinatorial frequency (310, 311). When the dust settled, the murine class II region consisted of genes which encoded membrane bound glycoproteins of only two types, I-A and I-E (305). The investigation of the human MHC class II genes did not have the benefit of congenic strains and has necessarily been more problematic. It has also been found to contain more than two types of genes. The human equivalent of I-A and I-E have been found to be DQ and DR, respectively; however, several other class II genes, specifically DP, DO, DX, DZ, have also been found which appear to be less polymorphic than DR but complicate assessment of class II molecular function (306, 312, 313, 314). Sequence analysis of murine and human loci has indicated that several murine genes located in the I-A or I-E regions may actually be analogous to the additional human class II genes, e.g. murine $A_{\beta 3}$ = human DP_{β}, murine $A_{\beta 2}$ = human DO_{β} (313). Each type of class II gene encodes a single alpha chain and one or more beta chains. The MHC as currently drawn is certainly not the final picture and as new genes are found, both evolutionary and functional questions may be answerable. Class II molecules are membrane bound heterodimers composed of a 34-35 kD glycoprotein heavy chain encoded by the alpha chain loci and a 28-29 kD glycoprotein light chain encoded by the beta chain loci (313, 314). As a general rule combinations of mismatched class II molecules are not expressed; however, mismatched pairs, i.e. combinations of A alpha + E beta or E alpha + A beta chains, have been observed under certain conditions and their ability to pair may reside in haplotype specific inherent conformational constraints on juxtaposition, i.e. some of the chains of different haplotypes may just not fit together no matter how hard they try. MHC class II polypeptides have a characteristic disulfide linked domain structure shown in Figure

5. The alpha chain contains an N-terminal polymorphic non-disulfide linked domain (α1), followed by a disulfide linked relatively non-polymorphic domain (α2), a transmembrane anchor and a short cytoplasmic segment (313). The beta chain is composed of an N-terminal polymorphic disulfide linked domain (ß1), followed by a second disulfide linked domain which is also relatively non-polymorphic (ß2), a transmembrane anchor and a short cytoplasmic segment (313). Each domain consists of approximately 100 amino acids. Both alpha and beta chains are glycosylated at asparagine residues (N-linked), the alpha chain at two sites and the beta chain at one. Further modifications of the chains include O-linked glycosylation of the alpha chain, addition of palmitic acid and phosphorylation of the cytoplasmic tail (317). The alpha and beta chains are non-covalently linked but are tightly held together. Like the T cell receptor heterodimers, single chains are apparently not expressed (305). Invariant chain, a 31 kD basic glycoprotein, may have a role in MHC class II gene expression, although it is encoded on a separate chromosome (318). Evidence has indicated that invariant chain is necessary for assembly of the heterodimer, for transport to the cell surface or for membrane insertion (317); however, all of these events have been noted to take place in the absence of invariant chain (319) making it unclear whether invariant chain has a role in causation or simply appears as a result of these events. Invariant chain which has a protein core is largely composed of a glycophorin structure which binds to class II molecules intracellularly and disengages generally before surface expression (320). The function of invariant chain is not fully understood; however, Cresswell (317) suggests that attached invariant chain has a role in the intracellular binding of antigenic fragments to MHC molecules in vesicles and that once bound, the invariant chain is dissociated from the complex probably by acidification.

Class III genes found within the murine and human MHC encode complement components: C2, C4 and factor B (Bf). These are apparently highly conserved as is their genetic arrangement (307).

The fourth group of genes encoded by the MHC is the group of all other gene products which have over the years been mapped to the MHC but which defy classification into one of the classes of molecules (306, 321, 322). These include the recently discovered genes for tumor necrosis factors alpha and beta (322), genes for 21 hydoxylase (322), neuraminidase (304) and low molecular weight proteins (LMP) (323). Whether these genes are intimately associated with the transcription, translation or functions of the class I, II, or III genes in the MHC is not known. Their genetic material and gene order are highly conserved and there are probably more of them present in the uncharted regions of the genome both between and within the classes (324). Although MHC disease associations may be primarily attributable to some inherent characteristic of class I or class II mediated antigen presenting function, it is also possible that there are some MHC disease associations which are actually due to closely linked genes.

The MHC genes of particular interest in antigen presentation and T cell activation are the class I and class II genes and their products. Recent structural analysis of HLA-A2 molecules by x-ray crystallography (293, 294) and comparison of these structural revelations with sequence data for class II molecules (295) has clarified the nature of the antigen binding site, its existence as a single site rather than multiple sites and its structure composed of a groove formed by alpha helical segments and floored by ß-pleated sheet. The structural interaction of the non-polymorphic domains clearly analogous to immunoglobulin domains has been revealed to stabilize the antigen binding structure and the polymorphic portions of the molecules have been shown to play the major role in antigen binding. Thus we begin to know what MHC molecules look like and perhaps where and how in the molecular sense they interact with antigen.

MHC class II antigen (Ia) is constituitively expressed on relatively few types of cells in the unstimulated host unlike MHC class I molecules. MHC class II molecules are constituitively expressed on monocytes, macrophages, B cells and dendritic cells in humans (312). In mice, Ia is constituitively expressed on B cells and dendritic cells but must be induced on macrophages (241). Expression of MHC class II molecules on murine and human macrophages is subject to regulation by cytokines, particularly gamma interferon (220). Gamma interferon will also induce expression on endothelial cells and other tissue cells (207). Macrophages are very sensitive to gamma interferon and their ability to present antigen to T cells is markedly enhanced by gamma interferon treatment because of the increased level of MHC class II expressed on the cell surface (220, 225, 226). Initial expression of MHC class II molecules appears to require demethylation of the MHC class II genes (325), although exactly opposite results have also been noted (325, 326) and subsequent cycles of expression and loss of expression may not require further demethylation (327). Consensus sequences referred to as X and Y boxes (or A and B boxes) containing typical CCAAT box sequences (328) and W sequences (329) have been identified in the 5' flanking region of the MHC class II genes which appear to be sites for regulatory DNA binding proteins. Several groups have noted that one or more of these sites is regulated by gamma interferon (329, 330). Evidence from MHC class II nonexpressing severe combined immunodeficiency indicates that other non-gamma interferon trans-acting factors also regulate class II expression in all types of cells (331). B cells are stimulated to express class II by IL-4 rather than gamma interferon (157) and have been shown to be regulated by products of a gene locus which does not affect MØ Ia expression (332). Other regulatory sequences which have been identified in the 5' flanking region of MHC class II genes appear to confer tissue specificity (333). Thus the expression of MHC class II gene products on macrophages as well as B cells is a closely regulated event.

Macrophage differentiation.

Macrophages arise from pluripotent stem cells in the bone marrow. Experimental evidence obtained by Till and McCulloch (334) in the early 1960's bolstered the position of those whose contention it was that all of the cellular elements of the hematopoietic system were derived from single pluripotent stem cells present in the bone marrow. Using radiation depletion of hemopoietic progenitors followed by reconstitution with syngeneic bone marrow stem cells, they investigated the populations of cells in resultant progenitor colonies formed in the spleen. Their findings indicated that colonies contained a mixture of cell types and that the reconstituting bone marrow cells were capable of maintaining stability within the peripheral blood under varied physiological states. Although the monocyte was not one of the cells originally ascribed to the pluropotent lineage, it was added a short time later (335). Since that time the pluripotent stem cell has been firmly established, although its identification is still problematic and its presence can only be proved retrospectively by the progeny it produces (336). More recent findings seem to indicate that the pluripotent or totipotent stem cell population is indeed present in the bone marrow; however, it may remain in a somewhat dormant state unless extreme conditions develop. The requirements for routine maintainance of hematopoietic elements resides with the population of multipotent stem cells which are capable of rapid proliferation into functional cells of more than a single phenotype (336). The signals for induction of proliferation and designation of selected phenotype appear to be regulated by a battery of colony stimulating factors (CSF), synergistic factors and interaction with the bone marrow stromal cells (337). The progenitor populations of monocytes arise from multipotent stem cells which, depending on the type of CSF available, can be pushed to differentiate into either monocyte or granulocyte lineage (336, 337). Several CSF's have been identified and characterized; however their functions in vivo are not clearly known, although their continuous presence during

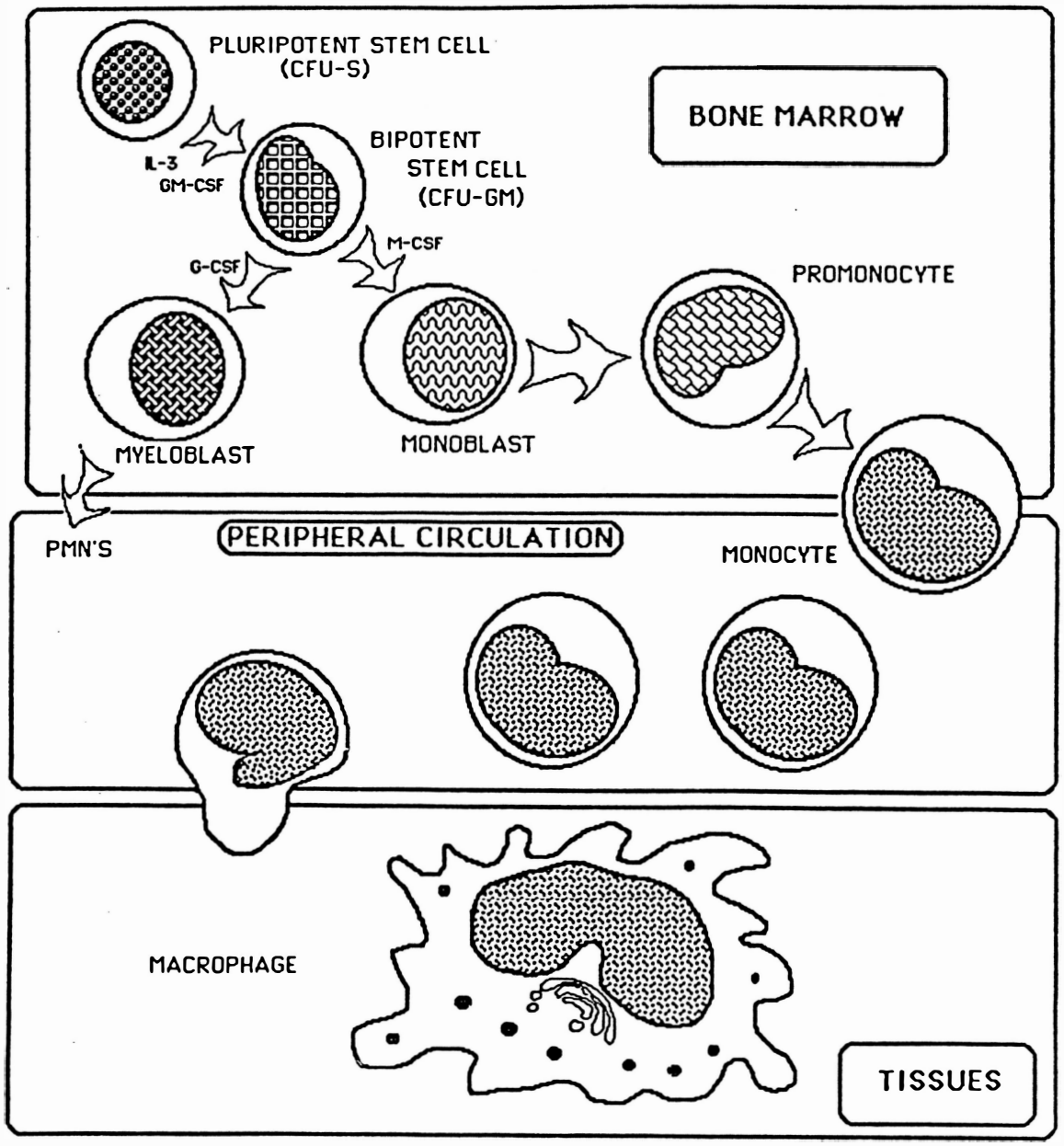
maturation and differentiation of hemopoietic progenitors is absolutely required to sustain cellular viability (337). Of these interleukin 3 (IL-3), which was named as an interleukin when it was characterized as a lymphocyte product having lymphocyte modulating activity in addition to induction of 21-hydroxylase activity, has since been characterized as a broadly active colony stimulating factor having specificity for multiple lineage stimulation which seems to be quite active on early progenitor cells, possibly the pluripotent stem cells (338). Granulocyte monocyte CSF (GM-CSF) stimulates multipotent monocytic-granulocytic differentiation causing progression of the multipotent stem cells without selection of final lineage. Terminal lineage selection appears to be dependent on monocyte CSF (M-CSF) or granulocyte CSF (G-CSF) to drive the immature cells to differentiate into fully functional and identifiable monocytic and granulocytic phenotypes (337, 339). The whole scheme is complicated by the findings that hemopoietin I and perhaps several other factors have synergistic effects with colony stimulating factors and while insufficient to sustain life or induce differentiation on their own clearly modulate the pathways taken by progressing cells (337). The network of in vivo effects of both stimulatory factors and inhibitory factors in conjunction with the effects mediated by stromal cell interaction produce a finely regulated self-renewing hemopoietic system which has been only partially dissected.

The development of circulating monocytes is similar to that of other hemopoietic cells. Pluripotent stem cells give rise to multipotent stem cells, as shown in Figure 6, having more limited differentiative capacity (335, 336, 340). Pluripotent stem cells, also refered to as colony forming units-spleen (CFU-S), are probably small mononuclear cells resembling lymphocytes. These cells have been found to express MHC class I and class II as well as theta antigens. Multipotent, or bipotent, stem cells give rise to monoblasts which are identified as having fine chromatin structure, visible

FIGURE 6. Macrophage Differentiation.

Pluripotent stem cells reside in the bone marrow where they may remain in a dormant state while the normal requirements for cellular proliferation are met by bipotent cells which are driven to differentiate along either a granulocytic pathway or a monocytic pathway dependent on the colony stimulating and progression factors present. The promonocyte is the first clearly identifiable monocytic cell. After several cell divisions the mature monocytes enter the peripheral circulation where they remain until they are attracted into the tissues where they may reside for long periods of time as tissue macrophages or where they become activated by inflammatory processes. Signals for macrophage movement into tissues are not well understood except for several well characterized chemotactic factors.

MACROPHAGE DIFFERENTIATION



nucleoli and positive reactions for non-specific esterase. Their morphological identification is based solely on their proximity to identifiable monocytes rather than any intrinsic character which they possess, and they have been found to be morphologically synonymous to myeloblasts which are identified in close proximity to granulocytes. Monoblasts apparently undergo a single cell division to produce promonocytes which are the first clearly recognizable cells in the monocytic lineage and contain a large indented nucleus and azurophilic granules. Promonocytes are weakly phagocytic, adhere to glass, contain non-specific esterases and peroxidases including acid phosphatase and arylsulfatase, and remain in the bone marrow where they probably undergo at least three divisions before giving rise to monocytes which are extruded into the circulation, where as a rule they no longer proliferate (335, 336). Few mature monocytes remain in the bone marrow unlike polymorphonuclear leukocytes, thus an increased number of monocytes in circulation is due to de novo produced monocytes rather than release of a sequestered pool (336). Mature monocytes constitute 1-9% of the circulating leukocytes and average approximately 400 monocytes/μl blood in adults, slightly more than 1000 monocytes/μl blood in infants (336). Monocytes remain in circulation an average of 2-5 days before migrating into tissues to become tissue macrophages. Throughout the progression from pluripotent stem cell to terminally differentiated tissue macrophage, the cells successively lose differentiative plasticity as well as proliferative capacity and in turn acquire more specialized functional capacities many of which can be modulated by environmental factors.

Circulating monocytes are large cells measuring 12-15 μm in diameter. When examined in stained blood smears they are characterized by a large, reniform, eccentrically placed nucleus (336, 340). The cytoplasm of monocytes contains a variety of granules and vacuoles. Monocytes contain relatively little endoplasmic

reticulum but contain a well developed golgi region, and the centrosome located in close proximity to the nuclear cleft serves as the microtubule organizing center for the extensive array of microtubules and microfilaments which are involved in movement of cellular organelles and movement and attachment of the cells to substrata. The propensity of monocytes to adhere to plastic and glass surfaces, indeed to almost any surface, is very characteristic and has been employed as a primary means of isolation. Monocytes adhere as circular cells and exhibit marked membrane ruffling or veils on the leading edge toward which the cell is moving. Unlike dendritic cells, monocytes remain firmly attached to surfaces and in general maintain a rounded morphology without the formation of extended processes (247, 336).

Monocytes develop into macrophages and multinucleated giant cells. These cells are larger and more amorphous in shape, contain large quantities of lysosomal enzymes, have increased metabolic activity indicated by the increase in size and complexity of the Golgi and increased numbers of larger mitochondria and show increased phagocytic and pinocytic activity (336, 341). Monocytes in circulation are considered to be immature macrophages because their sojourn in the circulatory system takes them to all parts of the body where they are able to respond to chemotactic inflammatory products which cause them to marginate and leave the circulation by diapedesis (342). Once out of the circulation, monocytes migrate to sites of inflammation. There are other homing mechanisms probably involving adhesion molecules such as fibronectin-laminin (341) and LFA-1-ICAM-1 interactions (343) for localization of monocytes in tissues under normal cellular renewal conditions. Once localized, the monocytes take on morphology and functional capability characteristic of the histiocytes of the particular tissue in which they are embedded.

Macrophages are found in nearly all tissues where their specific functions as

mononuclear phagocytes serve as an important means for removing foreign invaders as well as effete autologous particulate matter. Phagocytosis (and endocytosis) by macrophages is probably mediated by an extremely diverse array of receptors including hormone receptors, mannosyl-fucosyl receptors, alpha-2 macroglobulin, iron-binding protein receptors for lactoferrin and transferrin, fibrin and fibrinogen specific receptors, extracellular matrix protein receptors for fibronectin and laminin, receptors for lipoproteins, as well as receptors for opsonized materials, Fc receptors for IgG and IgE and complement receptors (341). Monocytes treated with gamma interferon have also been shown to express receptors for IL-2 in addition to increased Ia expression (220, 344). Elie Metchnikoff noted the ability to engulf foreign particles by cells of the starfish larvae in 1882 (345). He called these cells phagocytes and noted that they actively "ate" (from the Greek phagein, to eat or digest) as opposed to being entered by an invader as previoulsy believed. In 1884 he extended his observations to the leukocytes of humans and rabbits and noted that these cells were also phagocytic and would actively remove bacteria from their surroundings (346). Metchnikoff subsequently left Russia to work at the Pasteur Institute in 1888 and went further to study inflammation and the role of the phagocytic cell in the inflammatory response. He is credited with being the founder of the theory of cellular immunity; however, he and his followers were embattled for most of the early years of the twentieth century by the proponents of humoral immunity, including Jules Bordet who discovered complement mediated lysis while working in Metchnikoff's laboratory. As an indication of the inability to resolve the issue, Metchnikoff was awarded the Nobel Prize in 1908 along with Paul Ehrlich, the founder of the theory of humoral immunity (347).

Monocytes, primarily as more highly differentiated macrophages, play a major role in host immunity due to their ability to engulf foreign matter and the body's obselete cellular products and subject them to proteolytic breakdown and disposal.

Invasive organisms which have developed methods for evasion of phagocytosis, or once engulfed evade the effects of the vast array of proteolytic digestive machinery in the acidified vesicles of the macrophage, tend to have profound effects on the host (341). Mycobacterial infections are an example of bacterial escape of macrophage defense mechanisms. Although they may be phagocytosed, they remain intact within the macrophage because their waxy coats provide protection from digestion. Another organism which appears to enter macrophages but does not undergo degradation is the human immunodeficiency virus (HIV-1) which enters macrophages and monocytes presumably by attachment to the CD4 receptor on the surface which is then taken in by receptor mediated endocytosis (132, 133). The virus however, continues to live within the monocyte and indeed the monocyte may serve as a viral repository for subsequent infection of T cells at distant sites (132, 348). Phagocytosis of particulate matter is intimately associated in free macrophages with stimulation of the respiratory burst and release of reactive oxygen intermediates and other bioactive secretory products (341, 349).

Monocytes and tissue macrophages also serve as secretory cells, as antigen presenting cells and as directly cytotoxic cells for elimination of tumor cells. The antigen presenting capacity of monocytes and macrophages has been previously discussed. Monocytes and macrophages secrete more than 100 different products, most of which have profound effects on their microenvironment and its cellular inhabitants (350). Among the products secreted are a variety of hormone-like substances including interleukin 1, tumor necrosis factor-alpha, alpha interferons, platelet derived growth factor, transforming growth factor-ß and other fibroblast growth and activating factors, and colony stimulating and hemopoietic modulating factors. Many of the complement components and coagulation factors are produced by macrophages. A wide variety of enzymes including neutral proteases such as plasminogen activator and

elastase, lipases, lysozyme, lysosomal enzymes as well as enzyme inhibitors are also produced. Extracellular matrix proteins such as fibronectin and binding proteins such as transferrin and apolipoprotein E are other polypeptide products. Smaller molecular weight products include highly active cyclooxygenase and lipoxygenase products such as prostaglandins E_2 and F_{2a}, leukotrienes and mono- and di-hydroxyeicosatetranoic acids; platelet activating factor; neopterin; purine and pyrimidine salvage pathway products; and reactive oxygen intermediates, superoxide and hydrogen peroxide (350). This is an abbreviated listing of macrophage secretory products and in no way claims to be comprehensive; however, it is apparent that macrophages secrete a vast array of products most of which are very active in mediating significant chemical reactions or in inducing receptor mediated cellular modulation. It is also apparent that among the products are many which have overlapping capacities and also many which have clearly antagonistic capacities. The modulation of secretory function, therefore, must be under stringent control to insure that a particular effect can be elicited (350). Macrophage secretory function is modulated by the state of activation and by the site of tissue localization (241). The concept of macrophage activation is most clearly delineated in the murine system in which peritoneal macrophages can be described as resident, elicited or activated depending on the stimulus used (351). In studying human macrophages and monocytes, the designations are less clearly defined and within a population taken from a normal individual, multiple stages of activation may be represented (352). In the murine system, activated macrophages are characterized by a respiratory burst involving the secretion of both proteolytic enzymes and reactive oxygen intermediates which arm the macrophage for tumoricidal activity and cause the macrophage to express MHC class II antigens in a transient manner (349). Human macrophages are also capable of exhibiting a respiratory burst but express MHC class II molecules constituitively, albeit at a relatively low level compared to dendritic cells and B cells. The expression of MHC class II molecules is increased upon activation

probably as a result of transcriptional regulation by gamma interferon produced primarily by T cells (312). Fully differentiated tissue macrophages such as alveolar macrophages and Kuppfer cells in the liver display a reduced abilty to secrete reactive oxygen intermediates and inflammatory products unless stimulated over a period of several days (353). This characteristic is postulated to reduce the problem of bystander tissue damage by cells which are constantly in contact with noxious substances, i.e. antigens. Circulating monocytes which are attracted to sites of inflammation are more readily activated to produce highly damaging reactive oxygen compounds; however, they would not be activated to risk destruction of normal tissues along with destruction of treatening invaders until the development of the initial inflammatory lesion attracted them to marginate and diapedese (341). Therefore there are probably large differences in the capacities of tissue macrophages depending on the receptors which were utilized to attract them to their sites of localization.

The relationship between macrophages and dendritic cells is not entirely clear. Both types of cells may be derived from a common progenitor which arises within 4-5 weeks following fertilization in the human fetus and develops in the yolk sac and mesenchyme prior to the development of either the thymus or the bone marrow (336). The finding that dendritic cells express large quantities of MHC class II molecules, are very effective antigen presenting cells and stimulators of mixed lymphocyte reaction makes them very important in immune function (354) and cells previously identified as tissue macrophages such as Langerhans cells of skin have more recently been identified as dendritic cells (355). Efforts to dismiss the macrophage as an antigen presenting cell have not been entirely successful if only because synthetic membranes which contain MHC class II molecules in a proper orientation have been shown to competently present antigen (275). One can not escape the intimate relationship between macrophages and antigen and their capacity to endocytose, degrade and disgorge processed antigen. Macrophages, therefore, have a vital role to play in immune

function through their capacities to phagocytose material, secrete bioactive products and present fragments of antigenic matter in conjunction with MHC class II molecules to T cells.

The necessity of isolation of monocytes and macrophages constitutes a problem for their study. The elucidation of the central role played by the macrophage in the maintenance of competent immune function begun by Metchnikoff has been extended in numerous species, particularly mice and humans, and the morphological and functional characteristics of this cell type have been reported in volumes of reports. Finding sources of large numbers of unperturbed monocytes and macrophages for study has been a continuing problem. The cells studied in the murine system are generally isolated as peritoneal exudate cells, elicited with sterile inflammatory agents such as proteose peptone or thioglycolate or activated macrophages stimulated prior to removal from the animal by injection of bacterial extracts such as *E. coli* LPS. Alternatively macrophages are isolated as a portion of the population of cells in the spleen, lymph nodes, or bone marrow (349). In the human system the cell source is generally peripheral blood monocytes, occasionally alveolar macrophages isolated by lavage and even less frequently macrophages from tissues excised as a result of medical treatment or autopsy such as spleen, lymph nodes, liver, bone marrow or cells from peritoneal dialysis. Although in all cases the cells being examined are of macrophage lineage, it is unlikely that they are isolated at the same stage of differentiation; therefore, the results found in a given species may not necessarily be comparable to another species. Isolation of human monocytes/macrophages involves multiple step separations and has been plagued by the necessity of removing this cell type from a mixture of cells of which they are a minor component using isolation procedures which frequently are dependent on the propensity of the monocyte to adhere to glass or plastic. This step must invariably be followed by removal of adhered cells by enzyme treatment or

scraping which frequently changes functional capability or decreases the number of viable cells recovered. Other procedures make use of counter flow elutriation (356) or density gradient centrifugation on percoll gradients (357). These procedures require large volumes of blood from donors (who are relatively scarce) if one is to recover working numbers of monocytes. There have been indications that these methods are not innocuous to monocytes and with Percoll separations the monocytes may phagocytose the colloidal silica (358). The problem is further compounded in the human system by the lack of constant sources of MHC identical cells necessitating complex screening and matching of cells from donors. There are in the literature many reports of methods for isolation of monocytes/macrophages and each method yields a product which is slightly different from every other.

Stable murine macrophage cell lines have been developed. In order to circumvent the difficulty of macrophage isolation, investigators have sought to develop macrophage cell lines which can be grown using standard tissue culture techniques. In the murine system, this has been relatively successful, and there are numerous cell lines available for study of which P388D1, J774, WEHI-3 and RAW264 (359, 360, 361, 362) represent a few of the available cell lines derived from murine macrophages. Other strategies for establishment of cell lines have relied on transfection of murine macrophages with an origin of replication deficient (ORI^-) SV40 DNA construct developed by Sambrook (363).

The development of monocyte or macrophage-like cell lines in the human has been less successful, although there are now several cell lines which have been successfully used. Early attempts to derive cell lines from cultured human monocytes have met with little long term success (364, 365, 366). More successful recent developments have yielded cell ines which are stable for long periods of time and which

retain major monocytic charateristics especially expression of MHC class II antigens, HLA DR and DQ. This has been achieved by using techniques such as somatic cell hybridization of human monocytes with mouse myeloma cells (367) and transfection of human monocytes with the aforementioned ORI$^-$ SV40 construct (368). Immortalized cell lines produced by these methods are still being evaluated and may prove useful; however, in the study of the effects of expression of MHC class II antigens, the addtional expression of either mouse derived glycoproteins or viral T antigens on these cells may complicate interpretation of any results. Attempts to isolate cell lines having monocyte characteristics from tumor tissues or from naturally transformed cells has met with limited success; however, in recent years several cell lines have been isolated from various leukemias which have monocyte/macrophage-like characteristics or can be induced to have those characteristics, are stable over a long period of time, and can be propagated under normal tissue culture conditions. Among these are U937 (95), HL60 (369), KG-1 (430), and THP-1 (371), of which HL-60 and KG-1 are more typically of the granulocytic lineage than monocytic lineage. These cell lines have all been used for the study of human monocyte/macrophage function in the last several years. HL60 cell line has been used extensively because it can be induced to differentiate along either the monocytic or granulocytic pathways. KG-1 and THP-1 have been more recently developed. THP-1 has been especially useful in investigation of IL-1 induction (372).

The human monocytic cell line, U937. The U937 monocytic cell line was isolated from the pleural effusion of a 37 year old male diagnosed with diffuse histiocytic lymphoma (95). The cells isolated by Sundstrom and Nilsson in 1976 by utilizing organ culture techniques and fibroblast or glial cell feeder layers retained the characteristics of the tumor cells found in the pleural effusion. The cells were isolated following both radiation and chemotherapy; however, the tumor was rapidly fatal to the

patient and continued to grow vigorously in culture in rich tissue culture media.

The cells were characterized as typical of monocyte-precursors. By light microscopy they appeared to be morphologically heterogeneous in that they ranged in shape from round to polygonal with frequent cytoplasmic projections which on scanning electron microscopic examination appeared mainly as blebs and occassional lamellar projections, sometimes sharp barbs. The cells were found to be 12.5 ± 4.4 μ in diameter with moderate amounts of cytoplasm containing numerous acidic granules, well developed Golgi apparatus, many polyribosomes, well developed mitochondria and little endoplasmic reticulum. The single nucleus was found to be variable in shape although frequently lobulated and contained at least one large nucleolus. The cells grew as single cells in suspension culture. Although in the first years of growth the cells were maintained on feeder layers of glial cells, the U937 cells did not strongly adhere to the feeder layer. Cytochemically U937 cells were found to be strongly positive for nonspecific esterase and exhibited NaF inhibitable naphthol AS-D esterase activity which has been described as very characteristic of human monocytes (95). The cells were found to have cell surface receptors for immunoglobulin Fc and complement component C3. They produced no immunoglobulin, either intracellularly or on the cell surface, and no EBV genome or viral proteins were detected. The U937 cells were typed for HLA by microcytotoxicity and found to be HLA-A 3,w19; B 5,18; Cw1,w3. No MHC class II antigen profile was reported. As further evidence for the monocytic lineage of these cells they were found to produce and secrete lysozyme at fairly high concentration; but unlike monocytic cells, only a small percentage of the U937 cells were found to be actively phagocytic and they were negative for acid phosphatase and peroxidase (95). Chromosomally they exhibited aneuploidy but were characterized as arising from a monoclonal lineage. A recent karyotypic analysis of three U937 cultures (373) obtained from geographically dispersed sites including cells obtained from the laboratory of origin found that there was great diversity in chromosomal

makeup of the three cultures and at first it was believed that the cells must be different cell lines; however, changes in four chromosomes were constant for all three U937 cell lines tested. These changes were $3q^-$,$11q^-$, $16p^+$, and $17p^-$. Deletion in chromosome 11 has been found to correlate with deletions characteristic of monocytic and myelomonocytic malignancies. Although the genes involved have not been determined, this deletion is probably related to the original tumor. The significance of the other consistent abnormalities is not known (373).

The U937 cell line has been extensively utilized as a tool for study since its development. It is variously characterized as histiocytic, monocytic and monoblastic, although the distinctions are not clear. Harris and Ralph (374) reviewed the explosion of information available for both HL60 and U937 cell lines in 1985. Since that time, the U937 cell line-related literature has continued to expand at an accelerated rate. It was felt that a detailed comprehensive review of that literature, both the new information and the many redundancies, was beyond the scope of this project. Therefore a brief overview of selected illustrative citations was employed to indicate the major areas of endeavor in utilization of the U937 monocytic cell line. In addition to the characteristics of the U937 cell line originally reported, the cell line has been found to secrete a large number of bioactive products such as elastase (375) and glycosidases and acid and alkaline phosphatases (376), cathepsin G (377, 378), cholesteryl esters (379, 380), to express cell surface receptors for IgE Fc (381), insulin receptor (382, 383), gamma interferon receptor (384, 385), histamine H2 receptors (386), CD4 cell surface antigen (387, 388), and to produce an array of complement components (C1-INA, C1r, C8, Factor H, Factor D, and small amounts of C3 (389, 390, 391, 392), and factors having suppressive activity (393, 394, 395). The constituitive production of interleukin 1 has been controversial (396, 397). The cells can be activated by gamma interferon (398), phorbol esters (399), vitamin D metabolites (400), retinoic acid (401) and a number of other agents and combinations

of agents to become more differentiated concomitantly expressing more macrophage characteristics and performing macrophage functions such as inhibition of intracellular parasites (402), tumor cell killing (403), antibody dependent cell cytoxicity of chicken erythrocytes (404), phagocytosis and stimulated production of reactive oxygen compounds (405), chemotaxis (406), adherence to glass and plastic surfaces and cessation of proliferation (407), expression of IL-2R (408), I-CAM 1 (409) and p150,95 (410) adhesion related structures, release of eicosonoids, PGE_2 and thromboxane (411, 412), and release of plasminogen activator inhibitor-2 (413, 414). Although all agents listed affect the cells in different ways and none are able to mediate all of the changes described, they all tend to push the U937 cell line toward a more activated and terminally differentiated state, except gamma interferon which merely activates in a reversible manner. A major portion of the literature concerning the U937 cell line involves its use in differentiation studies, both for assessment of the effects of the agents used to induce differentiation and for the information gained about progression through monocytopoiesis.

In more clinically relevant research, the U937 cell line has also been useful in investigating the effects of chemotherapeutic agents on tumor cells. Although the true histiocytic malignancies are relatively rare, the U937 cell line serves as a ready source of tumor cells for testing to determine effects of new combinations of treatment modalities (415, 416). In addition the finding that the U937 cell line can be chronically infected with HIV-1 (417, 418, 419) makes it a potentially valuable adjunct to AIDS research.

A third area in which U937 cells have been useful is in the realm of immune function and gene regulation. This area overlaps with the area of differentiation especially with regard to investigations into the expression and the role of oncogenes in the differentiation process (420, 421) and in the altered functional state following terminal differentiation (398-414). A second area of investigation into gene

regulation concerns the expression of MHC class II molecules. Several groups have reported induction, or lack of induction, of MHC class II expression on U937 using either gamma interferon (422) or phorbol esters (423) or gamma interferon following treatment with demethylating agent, 5-azacytidine (424). There is no explanation for the disparity seen between the U937 cell lines in different laboratories which apparently behave in different ways. There are also reports of constituitive expression of MHC class II molecules from a number of laboratories including our own. The Ia^+ I937 cell line derived by cell sorting from an Ia^+ U937 cell line by Gitter et al. (425) has been investigated and appears to constituitively express a high concentration of surface MHC class II and also has been shown to stimulate a mixed lymphocyte reaction. This is one of the only reports of immune function requiring interaction between T cells and accessory cells in which the relevant accessory function was mediated by the U937 cell line.

Rationale for pursuit of this project. At the time that this project was begun, the U937 was one of only two human monocytic cell lines, U937 and HL60, which had been fairly well characterized. Neither standard cell line had been found to express MHC class II antigens and HL60 was used to investigate granulocyte differentiation to a larger degree than monocytic differentiation. Although the U937 cell line had been extensively investigated for macrophage characteristics and capacities, its lack of MHC class II molecules and its inability to be induced to express MHC class II prevented the cell line from serving as a macrophage analog in immune function in which MHC class II molecules were thought to be intimately involved, i.e. antigen presentation and stimulation of mixed lymphocyte reaction. One of the U937 cell lines available in the laboratory had been found to express MHC class II molecules constituitively. It was felt that the availability of a well characterized, easily propagated human monocytic cell line constituitively expressing known MHC class II molecules would be of great value in

the dissection of antigen presentation and the role of MHC class II molecules. In spite of the obvious questions raised by the use of a tumor cell line for the extrapolation of normal macrophage function, the potential of this cell line for elucidation of human macrophage function in antigen presentation, mixed lymphocyte reaction, generation of specific cytolytic T cells, production of monokines, antigen endocytosis and processing, presentation of antigen fragments to antigen specific T cells, macrophage secretory capacity, and regulation of expression of MHC class II molecules and other cell surface receptors continues to be apparent. The phenotypic and functional characterization of this cell line is by no means complete, and on many occasions more questions arose from experiments than answers; however, the MHC class II positive U937 cell lines offer a means for dissecting relationships in a cell type of exquisite complexity.

MATERIALS AND METHODS

Media. Cells were grown and assays were conducted in RPMI 1640 tissue culture medium which was obtained in powdered form from GIBCO, Grand Island, NY, or from Hazelton Laboratories, Denver, PA. Packets of powdered media were reconstituted using ultra-pure deionized water (Millipore Corp., Bedford, MA) and sterilized by filtration through Millipore (Millipak) 0.22μ filters into sterile bottles. Media was supplemented with 100 units of penicillin/ml. and 100 micrograms of streptomycin/ml. (GIBCO, Grand Island, NY). Media was also supplemented with 2 mM L-glutamine (GIBCO, Grand Island, NY) during early experiments but additional glutamine was found to be unnecessary for the routine growth of the U937 cell line.

Sera. Fetal bovine serum (FBS) was obtained from several suppliers. Prior to purchase, serum was screened in the laboratory to ascertain which lots would satisfactorily support the growth of the cell lines carried in the laboratory. During the period of this project, fetal bovine serum used for growth of U937 cells was obtained primarily from Sterile Systems (Logan, UT) and from Hazelton Laboratories (Denver, PA). Standard tissue culture media used for growth of the U937 cell line was RPMI 1640 supplemented with penicillin, streptomycin and 10% FBS.

Normal human serum (NHS) was obtained by pooling serum drawn by venipuncture from approximately 20 healthy donors. Parous women and individuals on medications other than birth control pills were excluded from the donor pool. Fasting (preferred but not always available) samples of 200 ml. were drawn using all necessary precautions after informed consent was obtained from each donor. Blood was

allowed to clot and serum was removed after centrifugation. When all cells had been removed, the serum samples were combined, heat inactivated at 56°C for 30 minutes, centrifuged at 15,000 x g for 60 minutes, filtered through a 0.45µ sterile filter (Millipore Corp., Bedford, MA), aliquoted into sterile tubes and frozen at -20°C. Samples of each batch were tested for sterility by addition to media and incubation at 37°C for several days. RPMI 1640 (penicillin and streptomycin added) containing 15% normal human serum was used in mixed lymphocyte cultures (MLC), cell mediated lympholysis assays (CML) and for antigen presentation assays.

Tissue culture. U937 cells were maintained in suspension culture in RPMI 1640 containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml. penicillin and 100 µg/ml. streptomycin in 25 cm^2 or 75 cm^2 tissue culture flasks (Costar, Cambridge, MA) at 37°C, in 5% CO_2 in an incubator with humidified atmosphere. L-glutamine (extra 2 mM) was occasionally added to the culture media; however, this addition was found to be unnecessary. These cells are very metabolically active and grow rapidly causing the pH of the media to become very acidic. Although they have been found to grow optimally in a moderately acidic medium (pH 6.8-7.0), the cultures rapidly lost viability after six days unless divided. For this reason adherence to a maintenance schedule was important and cells were routinely propagated by splitting cultures at a 1:8 dilution every fourth day. U937 cultures grew poorly in the presence of HEPES buffer. Periodically samples of the cell line and isolated clones were frozen in liquid nitrogen for future retrieval. Cells to be frozen were removed from culture, centrifuged to concentrate the cells in a loose pellet. The supernatant was decanted or pipetted off of the pellet and the cells were resuspended in cold 10% DMSO/40% FBS and subjected to control rate freezing. These samples were maintained in either liquid nitrogen or nitrogen vapor phase storage.

Tissue culture techniques and all cellular assays requiring sterile preparations

were performed in laminar flow tissue culture hoods.

Isolation of clones of U937 by limiting dilution techniques. Both Ia positive and Ia negative parent lines of U937 were plated in 96 well flat bottom tissue culture plates (Costar, Cambridge, MA) at cellular concentrations of 0.5, 1, 2, 5, 10 and 100 cells/well in 200 µl. (426) to isolate clones by limiting dilution techniques for further study. Twenty-four or forty-eight wells were plated for each of the concentrations, except 100 cells/well which was plated in twelve wells only as a control that living cells were plated. Plates were maintained in 5% CO_2 for several weeks and were scanned 2-3 times each week for cell growth. Growth was slow in wells at low cell doses. After growth was well established in the wells of individual dilutions, half of the old media was removed by pipet and an equal amount of fresh media was added until the growth in the well was confluent at which time the cells were pipetted into wells in a 24 well plate to which 2 ml. of medium was added. These cells were fed by removal of spent media and addition of fresh media as gauged by pH and cell concentration. When the cell concentration became confluent in the 24 well plates, the cells were transfered to 25 cm^2 tissue flasks. When growth was stable, aliquots were tested for membrane Ia and samples of each clone were frozen. Cells plated at each concentration were not considered to be clonally derived unless no growth occurred in at least 37% of the wells (426, 427, 428).

Phenotypic analysis of U937 clones and parent line by fluorescent antibody staining and flow cytometry. Both direct and indirect immunofluorescence assays were performed. Cells, 5 X 10^5 - 1 X 10^6 cells per assay, were washed three times with cold phosphate buffered saline (PBS) containing 1% FBS and 0.02% sodium azide (wash buffer) by centrifuging at 400 x g, decanting, resuspension of the pellet by gentle agitation and addition of fresh wash buffer. Assays were performed in disposable

glass tubes (12 x 75 mm). For direct assays appropriate dilutions of each of the antibodies to be tested were added to the washed pellet of cells, mixed gently by finger tapping and incubated on ice for 30 minutes. The antibodies used were directly conjugated with fluorescein isothiocyanate (FITC) and intended for use on human blood cell samples. These antibodies were used according to the manufacturer's instructions as direct reagents. In most cases these reagents were added to cells after the cells were incubated with a non-specific isotype matched antibody in the form of a hybridoma supernatant or normal human serum for 30 minutes on ice followed by three washes with wash buffer. Indirect immuno-fluorescence assays were performed in much the same fashion; however, the unlabeled first antibody was added to the cells either after non-specific antibody or normal human serum treatment or diluted with PBS in the presence of non-specific antibody or normal human serum. After an incubation period of 30 minutes on ice, the cells were washed 3 times with cold wash buffer at 400 x g and an aliquot of FITC labeled second antibody was added. The second antibodies most commonly used were $F(ab')_2$ goat anti-mouse IgG or $F(ab')_2$ sheep anti-mouse IgG (Organon Teknika-Cappel, Malvern, PA). FITC labeled antibodies were titered with known positive antisera to obtain an appropriate dilution of antibody to give the highest percentage of staining without giving an increase in background staining. Controls for immunofluorescence consisted of unstained cells for direct assays and cells pretreated with isotype matched non-specific antibody or normal human serum and stained with the second antibody alone for indirect assays. Normal human mononuclear cells were also examined and particular cell populations were gated for analysis in some assays. Capping was prevented by the presence of sodium azide in the wash buffer and by keeping the cells in the cold at all times.

Cells were examined using the Ortho Spectrum III laser activated flow cytometer equipped with an argon-ion laser with 488 nm wavelength emission. The trigger region was set to include the entire viable population, and gain settings were set

such that the whole population was evenly dispersed within the gated field. Forward and right angle scatter and green fluorescence were measured for each sample and histograms were recorded.

If a large number of samples were to be read at one time, the samples were centrifuged at 400 x g, decanted and the cells were resuspended in PBS containing 1% paraformaldehyde and kept on ice. Prior to examination, cells were further diluted in PBS + 1% FBS + 0.02% sodium azide.

Isolation of human mononuclear cells for microcytotoxicity assay and cellular proliferation assays. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples drawn from normal volunteers after informed consent was obtained. Standard separation techniques developed by Boyum (429) were used. Blood samples were heparinized with preservative free heparin (O'Neil, Feldman & Jones, St. Louis, MO), mixed gently, diluted with RPMI 1640 1:2-1:3 and layered onto tubes containing approximately an inch depth of ficoll-hypaque separation medium (specific gravity 1.077-1.079), i.e. 4 ml./17 x 100 mm tube or 15 ml./50 ml. tube. Diluted blood was carefully pipeted or poured onto the ficoll-hypaque cushion. Tubes were centrifuged at 400 x g for 20-30 minutes. Mononuclear cells were removed from the interface between media and separation medium carefully with a pipet. These cells were washed 3-4 times with media by centrifuging at 400 x g and decanting. Cells to be used for microcytotoxicity assay for HLA phenotype were treated with Tris NH_4Cl to remove erythrocytes by adding the solution to the cell pellet, incubating at 37°C. for 5 minutes followed by washing.

Isolation of plastic adherent cells. The mononuclear cells isolated by ficoll hypaque density centrifugation were diluted to a concentration of 2 X 10^6/ml. and plated into 100 mm petri dishes (Falcon, Oxnard, CA) and incubated for 60 minutes at 37°C in the CO_2 incubator. After incubation, the dishes were swirled and the non-

adherent cells were removed by gently washing the dishes with media. The plates were additionally washed with more vigor using a pipet to remove any stubborn lymphocytes which were discarded. Adherent cells were removed by scraping with a rubber policeman or with disposable plastic policemen (Costar, Cambridge, MA) and transfered to a polypropylene tube on ice, to minimize sticking, until further use.

Isolation of nylon wool non-adherent cells. Nylon wool columns were prepared by addition of 1.0 gram of nylon wool (Fenwall Laboratories, Deerfield, IL) to a disposable 10 ml. plastic syringe. The nylon wool was manually fluffed before packing loosely into the syringe. The columns prepared in this manner were autoclaved with vacutainer stoppers containing a blunt 18 gauge needle in paper bags to sterilize. To prepare columns for use, they were assembled and attached to a ring stand in a tissue culture hood where they were connected to a 50 ml. plastic syringe reservoir. Columns were washed with 100 ml. of warm RPMI 1640 followed by addition of the non-adherent fraction of mononuclear cells removed from plastic petri dishes in a volume of 1-3 ml. Cells were slowly run into the column and incubated at 37°C for 45 minutes. During the incubation period media was added and carefully run into the column twice at 15 minute intervals. After incubation, the column was again attached to the reservoir and the non-adherent cells were eluted with 50 ml. of warmed (37°C) RPMI 1640. These cells were collected, concentrated by centrifuging at 400 x g for 10 minutes, counted and resuspended in RPMI 1640 + 15% NHS.

Isolation of T and B cells for microcytotoxicity assay. Blood samples (30 ml.) for microcytotoxicity assay were depleted of monocytes by addition of approximately 2 g. carbonyl iron particles (GAF Corp, New York, NY). After incubation at 37°C for 10 minutes, tubes were centrifuged and the particles and phagocytic cells which had ingested them were removed by passing the blood over a strong magnet while decanting into a second tube. The mononuclear cells were isolated from this blood by standard ficoll-hypaque density gradient centrifugation previously

described. The lymphocyte population isolated in this manner was used for HLA class I antigen determination by microcytotoxicity. For HLA class II antigen determination, B cells were isolated. Lymphocytes were adjusted to a concentration of 1 x 10^7 cells/ml. and added to a nylon wool (0.1 gram) containing sealed plastic drinking straw. The straw was incubated for 30 minutes at 37°C and the non-adherent cells were washed out with a syringe containing 20 ml. of McCoy's media + 10% FBS (GIBCO, Grand Island, NY). The adherent cells were then dislodged from the nylon wool by manual manipulation (squeezing and squishing with the fingers) and eluted from the nylon wool by addition of 20 ml of McCoy's + 10% FBS. These B cells were used for HLA-DR & DQ determination by microcytotoxicity.

Determination of HLA phenotype by microcytotoxicity assay. U937 parent lines, clones and cells from normal donors were tested for HLA phenotype by the Tissue Typing Laboratory at MCV using microcytotoxicity techniques employing a panel of specific antisera and low cytotoxicity, pretitered complement (430). Mononuclear cells were isolated by ficoll-hypaque density centrifugation, washed and erythrocytes were removed by treatment with Tris-NH_4Cl treatment. Cells were adjusted to 2 X 10^6 cells/ml. in RPMI 1640 and plated into microwells (1 µl/well) of Terasaki tissue typing plates (One Lambda, Los Angeles, CA) with a Hamilton repeating syringe (Hamilton Co., Reno, NV). Plates were incubated for 30 minutes at room temperature. Barbital buffer was added to each well to wash, the plates were emptied (flicked) and 5 µl. of pretitered rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada) was added to each well. Plates were again incubated followed by another wash and flick and addition of a tiny drop of trypan blue. Percent cytotoxicity based on incorporation of trypan blue by dead cells in each well was assessed by reading the plates on an inverted microscope. Wells containing greater than 20% dead cells were considered to be positive. Negative controls consisted of wells containing buffer alone. For typing

HLA class I antigens, total lymphocyte population was used and positive controls consisted of wells containing anti-thymocyte globulin (ATG). For typing HLA class II antigens, nylon wool purified B cells were plated in typing trays containing heterologous antisera of known HLA-DR specificity (One Lambda, Los Angeles, CA) and microcytotoxicity assays were performed as for determination of HLA-A and B phenotype. In HLA-DR determinations positive controls consisted of wells containing anti-B cell antibody.

Non-specific esterase determination. Non-specific esterase as alpha naphthol acetate esterase activity was determined on U937 cells using the reagents and procedure provided in a kit purchased from Sigma Chemical Co., St. Louis, MO. Cells were smeared on slides or cytocentrifuged onto microscope slides using a Shandon Cytospin (Shandon Southern Instruments, Sewickly, PA), air dried, fixed in citrate-acetone-methanol fixative for 30 seconds, washed with deionized water, stained in tris-alpha naphthol acetate-Fast Blue RR stain for 30 minutes, washed in tap water and examined microscopically for the inclusion of black granulation in the cell cytoplasm.

Immunoprecipitation of HLA-DR and SDS-PAGE analysis. U937 parent cell line and cloned cells (3 X 10^7 cells of each type) were washed three times with PBS to remove serum proteins and soluble products from tissue culture media. If cell viability was less than 90% by trypan blue exclusion, non-viable cells were removed by ficoll-hypaque density gradient centrifugation. Cell surface proteins were radioiodinated by lactoperoxidase-catalyzed addition of ^{125}I as sodium iodide (Amersham, Arlington Heights, IL) to tyrosine residues of the exposed integral membrane proteins. Cells remained intact. The procedure used is a *modification of* several published methods (431, 432, 433). Using a micropipettor, sodium iodide

(300-500 μCi in 3-5 μl.) was carefully added to each sample, followed by the addition of lactoperoxidase (10 μl. of 166 IU/ml. solution) (Worthington Biochemical Corp., Freehold, NJ). The reaction was initiated by quickly adding hydrogen peroxide (15 μl. of 0.03% solution). Tubes were mixed continually and addition of lactoperoxidase and hydrogen peroxide was repeated twice more at one minute intervals. The reaction was stopped by addition of the reaction mixture to RPMI 1640 containing 10% FBS (10 ml.). The cells were centrifuged at 500 x g for 10 minutes, decanted and resuspended in 1 ml. media followed by incubation for 1 hour at 37°C. To remove unbound ^{125}I, the cell suspension was layered onto a FBS cushion and centrifuged at 700 x g for 4 minutes. The cells were washed once with PBS prior to addition of 1 ml. of 0.5% NP-40 in PBS and 10 μl. 100mM phenyl-methyl-sulfonyl-fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) to solublize the cells and prevent enzymatic degradation of the liberated cellular proteins, respectively. After incubation at 4°C. for 30 minutes and centrifugation to sediment insoluble cellular components, the supernatant was removed and precleared with an irrelevant monoclonal antibody prior to specific immunoprecipitation (434). This was accomplished by adding 50 μl of antibody (usually anti-LETS which is a monoclonal antibody specific for fibronectin) to 0.5 ml. of cell extract and incubating the mixture at room temperature for 30-60 minutes followed by addition of 0.5 ml. *Staphylococcus aureus* Cowan strain (heat killed and kept frozen) previously treated with rabbit anti-mouse IgG (SAC). After incubation of this mixture for 1 hour at 37°C. and centrifugation, the supernatant was removed and 30 μl. Monoclonal antibody to a framework determinant of human HLA-DR, Kula 2 ascites, was added to 0.5 ml. of supernatant. Following an overnight incubation at 4°C., SAC was added to insolublize the immunoprecipitated proteins which were retrieved by centrifugation. The pellet was washed several times, electrophoresis buffer was added and the sample was boiled for 5 minutes. The liquid was removed, cleared by quickly centrifuging at 10,000 x g, and an aliquot was counted in a gamma

counter to gauge the amount of labelled material being loaded on the gel. Bromophenol blue tracking dye was added to the sample which was then loaded onto a 10-16% gradient SDS-polyacrylamide gel and electrophoresed at 60 V for several hours in a vertical slab gel electrophoresis apparatus (Hoefer, San Francisco, CA). Following electrophoresis, gels were stained with 0.5% Coomassie Blue in 25% 2-propanol/10% acetic acid and destained with 10% acetic acid. Once gels were sufficiently destained to visualize molecular weight standards, gels were soaked in 10% acetic acid-1% glycerol for 30 minutes. After destaining, gels were dried for 3 hours on a vacuum gel drier after which they were placed in X-ray cassettes containing intensifier screens with XR5 X-ray film (Kodak, Rochester, N.Y.) and placed in a -70°C freezer for autoradiography (436). Film was exposed for various times gauged by trial and error. Film was developed in automatic developers provided in the Radiology Department of MCV Hospital.

Cellular inactivation procedures for cells to be used as stimulators.

Inactivation of stimulator populations by irradiation. Peripheral blood mononuclear cells to be used as stimulator cells in mixed lymphocyte assays or cell mediated lympholysis were inactivated by exposure to 3000 rads of irradiation by placing cells in 12 X 75 mm plastic tubes in a cesium source gamma irradiator. The dose rate for the irradiator was calculated by the Radiation and Environmental Safety Department to be 2116 rads/minute. Cells treated with this dosage remained intact and viable; however, they did not incorporate ^{3}H-thymidine when pulsed after incubation for 72-96 hours.

Inactivation of stimulator populations by mitomycin C. Irradiation was found to be ineffective for inactivation of U937 cells and mitomycin C inactivation was used as an alternative. Cells were removed from tissue culture flasks and washed one time with RPMI 1640. Cells were adjusted to 2 X 10^{6} cells/ml. Mitomycin C (Sigma

Chemical Co., St. Louis, MO) was made up fresh or aliquoted and kept frozen for not more than 30 days. When possible, fresh mitomycin C was always used. Mitomycin C was diluted to 250 μg./ml. with RPMI and protected from light by wrapping in aluminum foil. Cells were treated by addition of 25-50μg./ml. of cells. The cells were mixed gently and incubated at 37°C. for 30 minutes, washed 5 times with RPMI 1640 to remove residual mitomycin C and resuspended in RPMI + 15% NHS for use in mixed lymphocyte assays, cell mediated lympholysis, antigen presentation and T3 mitogenesis assays.

Mixed lymphocyte reaction. Peripheral blood mononuclear cells from heparinized blood from normal volunteers isolated by ficoll-hypaque density gradient centrifugation were plated in the wells of 96 well flat bottom or round bottom microtiter plates (Costar, Cambridge, MA) at a concentration of 1-2 X 10^5 cells/well in RPMI 1640 + 15% NHS (responder cells). Stimulator cells were added to these wells. Normal mononuclear cells used as stimulator cells were irradiated with 3000 rads using a ^{137}Cs irradiator. These cells were also plated at concentrations of 1-2 X 10^5 cells/well. Because irradiation was found to be ineffective for inactivation of U937 cells, U937 cells used as stimulator cells were pretreated with mitomycin C (Sigma Chemical Co., St. Louis, MO) prior to plating. Concentrations of U937 cells varied and were dependent on the assay being done. Total volume in each well was 200 μl. Plates were incubated at 37°C. in 5% CO_2 for 5 days, pulsed with 1 μCi ^3H-thymidine (ICN Biomedicals, Cambridge, MA), incubated for an additional 16 hours, harvested onto glass fiber filter disks using a cell harvester (PHD Harvester, Cambridge, MA.), transfered to scintillation counting vials to which counting cocktail (RPI, Mt. Prospect, IL) was added and counted in a liquid scintillation counter (Beckman Instruments, Fullerton, CA). Assays were set up in triplicate and assay results were expressed as the mean and standard deviation of these triplicate wells.

Controls included responder cells without added stimulator cells and stimulator cells without added responder cells. As a control when using U937 cells, at least one normal one-way mixed lymphocyte response between a normal responder and a normal HLA-DR mismatched stimulator was set up.

Cell mediated lympholysis assay. Responder cells, stimulator cells and U937 cells were treated and plated as for the mixed lymphocyte reaction except that only 96 well round bottom wells were used. Cells were incubated for a total of 6 days at 37°C in 5% CO_2; however on day 3, 100 µl. of media was removed from all wells and 100 µl. of fresh media was added. For normal stimulators, 1 x 10^7 mononuclear cells originally isolated were added to a 25 cm^2 tissue culture flask in 10 ml. of media to be prepared as PHA blast target cells. On day 2 after isolation, a mitogenic dose of phytohemagglutinin (PHA)(Difco Labs, Detroit, MI) was added to the flask and it was replaced in the incubator. On day 6 of the assay, the PHA targets and aliquots of the U937 cells to be used as targets (4 X 10^6 cells each) were removed from culture, washed with RPMI and labeled with 200 µCi ^{51}chromium as sodium chromate (Amersham, Arlington Heights, IL)for 30-60 minutes at 37°C. Cells were thoroughly washed, counted, resuspended at 5 X 10^4 cells/ml. and 50 µl. of cell suspension was added to the appropriate wells. Plates were incubated for an additional 6 hours at 37°C. Supernatants were harvested by Skatron apparatus (Skatron, Sterling, VA) or plates were centrifuged at 400 x g for 10 minutes and supernatants were removed by micropipettor (100 µl./well) and put into disposable tubes. Samples were counted in a four channel gamma counter (LKB Instruments, Gaithersburg, MD). Control samples consisted of responder cells + targets without stimulator cells (background), stimulator cells + target cells (just to make sure the stimulators weren't doing the killing), target cells + media (spontaneous release) and target cells + lysing buffer (maximum release). Triplicate wells of all test combinations were set up and

calculations were done using the mean of triplicate wells. Percent specific cytotoxicity was calculated using the following formula:

$$\% \text{ Cytotoxicity} = \frac{\text{Experimental cpm-Background cpm}}{\text{Maximun cpm-Spontaneous cpm}} \times 100$$

CD3 mediated T cell proliferation. The capability of U937 clones and parent line to reconstitute the mitogenic response of normal T cells to monoclonal antibody to the T-cell receptor associated T3 cell surface structure (437) was assessed by addition of mitomycin C treated U937 cells to nylon wool-purified non-adherent cells from normal human donors in the presence to a monoclonal antibody to CD3, OKT3, (Ortho Diagnostic Systems, Raritan, N.J.). Peripheral blood mononuclear cells were isolated from normal donors and nylon wool purified non-adherent cells were prepared as previously described. Cells were plated in 96 well flat bottom microtiter plates at a cell concentration of 1 X 10^5 cells/well. U937 cells or peripheral blood adherent cells (isolated and treated as previously described) were added to the wells at varying concentrations of from 2 X 10^3-5 X 10^4 cells/well. Monoclonal anti-T3 antibody (0.1 μg./ml.) was added to the experimental wells. Plates were incubated at 37°C in 5% CO_2 for 72 hours, pulsed with 1 μCi ^{3}H-thymidine for the last 16 hours of incubation and harvested onto glass fiber filters using a cell harvester. Samples were set up in triplicate and counted in a liquid scintillation counter. Controls included nylon wool non-adherent cells (T cells + contaminants) alone ±OKT3 (to assess background OKT3 stimulation) and non-adherent cells + U937 cells without OKT3 (to assess the stimulation of a mixed lymphocyte reaction). Results are expressed as mean ± standard deviation of triplicate wells.

Antigen presentation of tetanus toxoid. The capability of U937 parent line and

sublines to process and present soluble antigen to nylon wool purified peripheral blood mononuclear cells was assessed using a modification of methods described by (438).

Selection of normal donors for testing. Normal donors with specific HLA-DR phenotypes were selected from the MCV Tissue Typing Laboratory cell panel and pretested for cellular response to tetanus toxoid (Massachusetts Department of Health, Jamaica Plain, MA). This was necessitated by the finding that although all individuals on the cell panel had been immunized at some time against tetanus, two individuals made no cellular response until reimmunized. This testing was done by isolation of peripheral blood mononuclear cells as described and plating these cells at 2 X 10^5 cells/well in 96 well round bottom microtiter plates. Tetanus toxoid was added at final dilutions of 1:10, 1:20; 1:50; 1:100; 1:200; 1:400; and 1:1000. Plates were incubated for 96 hours, pulsed for the final 16 hours with ^{3}H-thymidine, harvested and counted as previously described. Control wells contained only cells and media. Wells were set up in triplicate and results were expressed as mean ± standard deviation. A significant cellular response was judged to be any response which exceeded the control response by three standard deviations.

Presentation of tetanus toxoid by U937. Tetanus responders were selected from whom nylon wool non-adherent cells were prepared as previously described. U937 cells were incubated with tetanus toxoid for 1 hour and washed extensively before being treated with mitomycin C. A tetanus toxoid concentration of 1:200 was used because that concentration consistently gave optimal proliferative responses in normal tetanus toxoid assays. Responding cells were plated in round bottom 96 well plates at 2 X 10^5 cells/well in RPMI 1640 + 15% NHS. U937 cells were added to responder cells at concentrations of 2 X 10^2-1 X 10^5 cells/well. Autologous adherent cells isolated as previously described were also added to responder cells and tetanus toxoid. The higher concentrations of U937 were used to assess the effects of the addition of antigen to a mixed lymphocyte reaction. Because of high

background tetanus response in some individuals, tetanus toxoid was not added directly to the assay wells containing U937 cells in most experiments; therefore the only tetanus toxoid present was added either stuck to the U937 cells added or processed and expressed by U937. Plates were incubated for 96 hours, pulsed with 1 μCi ^{3}H-thymidine for the final 16 hours, harvested and counted in a liquid scintillation counter. Tests were set up in triplicate and results are expressed as mean ± standard deviation of triplicate wells. Controls consisted of responder cells alone; responder cells + tetanus toxoid; responder cells + U937; U937 cells alone; U937 cells + tetanus toxoid; and responder cells + U937 at high concentration + tetanus toxoid (MLR control).

Inhibition of proliferative response by addition of antibody. To assess the role of several cell surface proteins in the presentation of tetanus toxoid antigen to normal nylon wool purified mononuclear cells, antibodies to several membrane proteins were added to the antigen presentation assay described above. Antibodies used were as follows: heteroantibodies to specific HLA-DR haplotypes (anti-DR2, anti-DR3, and anti-DR7) which were obtained from the MCV Tissue Typing Laboratory and the Washington University Tissue Typing Laboratory (St. Louis, MO); monoclonal antibody to beta-2 microglobulin (439); monoclonal antibody specific for an epitope on the human CD4 molecule, KT69-7 (440); monoclonal antibody specific for human Fc receptor, KuFc79 (431); monoclonal antibody specific for a framework determinant of human HLA-DR, Kula2; and an irrelevant monoclonal antibody MOPC 11, IgG_{2b} isotype. Antibodies were added to give a final dilution of 1:10. Assays were set up, harvested and counted as described above.

Mitogen stimulation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells were isolated by ficoll-hypaque density gradient centrifugation as previously described. Mitogens added were phytohemagglutinin (PHA) (Difco

Laboratories, Detroit, MI, or Burroughs-Wellcome Laboratories, Research Triangle, NC) at a final dilution of 1:200 or 1:400 and concanavalin A (Calbiochem-Behring, San Diego, CA or Sigma Chemical Co., St. Louis, MO) at a concentration of 50 µg./well. Mitogens were pipetted into microtiter wells in 10 µl. prior to addition of cells. Cells (2 X 10^5 cells/well) were pipetted into sterile 96 well microtiter plates. Media used for mitogen stimulations was RPMI 1640 + 15% NHS. Volumes in wells was adjusted to 200 µl. with additional media when necessary. Plates were incubated for 96 hours, pulsed with 1 µCi ^{3}H-thymidine for the final 16-18 hours of incubation, harvested by cell harvester and counted in a liquid scintillation counter as previously described. Tests were set up in triplicate and results were analyzed as mean ± standard deviation of triplicate wells.

Collection of supernatant from U937 cells. In general, supernatants to be tested were collected from tissue flasks on day 2-3 after splitting. At that time, the cells usually were in optimal condition and the pH of the tissue culture media was still approximately 7.2. Cells were centrifuged for 10 minutes at 500 x g to sediment cells. Supernatants were decanted into disposable syringes and filter sterilized through a 0.22µ Millipak filter (Millipore Corporation, Bedford, MA) and used immediately or stored frozen at -35°C. On two occasions, supernatant samples from the parent line and from clones were collected and divided into equal parts: half of the supernatant was stored in the refrigerator overnight, the second half was dialyzed against RPMI 1640 containing 25 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO) to maintain a neutral pH under atmospheric conditions. The dialysis tubing (American Scientific Products, McGaw Park, IL) which was previously boiled in 1 mM EDTA-2% sodium bicarbonate solution, washed with deionized water and autoclaved before use, was stirred on a magnetic stirrer overnight in the coldroom with two changes of dialysate. Although the dialysis was done under sterile conditions, the samples were refiltered

after removal from their respective bags. Non-dialyzed and dialyzed supernatant samples were aliquotted into tubes and frozen. As a control an equal volume of RPMI 1640 + 10% FBS was subjected to the same treatments as the supernatant samples. In several experiments, supernatants from U937 parent line and clones were pooled and were subjected to further treatments prior to addition to testing assays. Supernatants were treated in the following ways: (a) repeated freezing and thawing through three cycles of freezing for 30 minutes, thawing for 15 minutes; (b) incubation at 56°C. in a water bath for 30 minutes; (c) incubation at 80°C. in a water bath for 10 minutes; (d) incubation at 100°C. in a boiling water bath for 10 minutes; (e) trypsin or chymotrypsin treatment using trypsin and chymotrypsin coated beads (Sigma Chemical Co., St. Louis, Mo.). Beads were resuspended and washed several times in media according to instructions supplied from the manufacturer. One milliliter of supernatant was added to each type of bead and incubated at 37°C. for 30 minutes after which the tubes were centrifuged to sediment the beads. The supernatant was collected by pipet and filter sterilized as described. All treated supernatants were assayed for suppressive activity immediately after treatment.

Supernatant from U937 cells generally contained 10% FBS. In order to lower the FBS concentration to allow for concentration, U937 parent line and sublines were grown up in 100 ml. cultures of normal media for 3 days, 90 ml. of media was removed from each flask and replaced with RPMI 1640 without serum for an additional 24 hours. Culture media from these cells was harvested by centrifuging the cells at 500 x g for 10 minutes, decanting the supernatant and pooling it. A sample of this pooled supernatant were stored in the refrigerator and a second sample was dialyzed overnight against RPMI 1640 + 25 mM HEPES with two additional changes of dialysate. The remaining supernatant was concentrated 50-fold by vacuum dialysis using 1/4 inch dialysis tubing and a vacuum flask at 4°C. The concentration required several days. Concentrated supernatant was finally removed from the dialysis tubing in a volume of

3.2 ml. and dialyzed against PBS.

Assessment of supernatant effects on cell lines. Supernatants and treated supernatants from U937 were added to the culture media of other cell lines to determine how it might affect the viability or ^{3}H-thymidine incorporation of the cell line. Cell lines used for these studies were as follows: (1) REH (human lymphoid cell line, null cell leukemia) (441) and (2) K562 (human erythroleukemia) (442, 443). Cells from continuously maintained cultures of these cell lines were split in the normal manner which yields a cell concentration of 2-3 X 10^5 cells/ml. in normal culture media, RPMI 1640 + 10% FBS. One hundred microliter samples of cells were added to 96 well flat bottom microtiter plates. Dilutions of U937 supernatants were added to plates also in 100 µl. In general, the highest concentration of supernatant tested was 1:4. Supernatant was diluted in RPMI 1640 + 10% FBS and control wells contained cells and media alone. Cells were incubated at 37°C. in 5% CO_2 for 72 hours. Plates to be assessed for ^{3}H-thymidine incorporation were pulsed with 1 µCi for the last 16 hours of incubation, harvested and counted as described. Plates to be assessed for viability were examined microscopically. Cell viability and counts were assessed on a hemocytometer using trypan blue exclusion as an indicator of viability.

Assessment of supernatant effects on cellular assays. Dilutions of supernatants and treated supernatants were added to mixed lymphocyte assays between HLA-DR mismatched donors and to mitogen proliferation assays on normal donor cells. Mixed lymphocyte assays and mitogen stimulations were performed as previously described using peripheral blood mononuclear cells from normal individuals. Control samples consisted of wells without addition of supernatant. Results are presented as % Inhibition according to the following formula:

$$\% \text{ Inhibition} = \frac{(\text{cpm without sup}) - (\text{cpm + sup})}{(\text{cpm without sup})} \times 100$$

Column chromatography of supernatant from cells grown at reduced FBS concentration. An 85 ml. Sephacryl S-200 (Pharmacia, Piscataway, NJ) glass column (1 cm^2 x 85 cm) was poured in PBS, pH 7.2, and charged with a 2.7 ml. sample of concentrated pooled supernatant previously described. The flow rate was 9.2 ml./hour and 1 ml. fractions were collected and monitored by spectrophotometer (Beckman Instruments, Fullerton, CA) at 280nm. Column fractions were pooled based on O.D. 280 readings, filter sterilized and tested in assays described above as well as for IL-1 activity.

Interleukin 1 assay, soluble and membrane bound. Interleukin 1 activity was assayed using the IL-1 dependent indicator cell line, D10.G4.1, provided by Dr. Brian Susskind. The D10.G4.1 originally described by Janeway et al. (444) was maintained and routinely antigen stimulated. Prior to use for IL-1 measurement, cells were rested for a minimum of 8 days; however, longer resting of the cells generally resulted in more sensitivity to IL-1. (The general rule which stated that the worse the cells looked, the better they responded, held true.) For soluble IL-1 assays, supernatants and dilutions to be tested were pipetted into 96 well round bottom microtiter plates. For membrane IL-1 assays, paraformaldehyde-fixed cells at concentrations of 2 X 10^4 or 1 X 10^5 cells/ well were added to 96 well plates (98). Indicator D10.G4.1 cells suspended in RPMI 1640 + 10% FBS containing a submitogenic dose of concanavalin A (Con A), 2 X 10^4 cells/well, were added to give a total volume of 200 µl./well and a final Con A concentration of 2.5 µg./ml. Control wells included D10.G4.1 cells with media + Con A as a negative control and D10.G4.1 cells + Con A + purified IL-1 (0.1, 0.5, or 1 Unit/well) (Genzyme Corp., Boston, MA). Plates were incubated at 37°C. in 5% CO_2 for 72 hours, pulsed for the final 16-18 hours with 1 µCi ^{3}H-thymidine, harvested and counted as described.

Paraformaldehyde fixation of cells. U937 cells were removed from 2-3 day

cultures of cells which had received no additions, added gamma interferon for 24 hours or phorbol ester (as PMA or as PDBu) for 72 hours. Cells were washed three times with RPMI 1640 to remove any residual tissue culture media, serum or immune modulators. Cells were gently resuspended after the last wash in freshly prepared sterile filtered 1% paraformaldehyde in PBS and cells were incubated at room temperature for 15 minutes. Cells were washed with sterile PBS twice, and once with RPMI 1640 and resuspended in RPMI 1640 + 10% FBS in the refrigerator overnight to allow residual paraformaldehyde to diffuse out of the cells. The following day fixed cells were washed an additional 1-2 times with RPMI 1640 + 10% FBS prior to counting with a hemocytometer and plating in 96 well plates for membrane IL-1 determination.

Addition of exogenous immune modulators to cells:

Gamma-Interferon. Purified human gamma interferon (Meloy Laboratories, Springfield, VA and Sigma Chemical Co., St. Louis, MO) was aliquoted after purchase and stored frozen at -20°C until use to avoid repeated freezing and thawing. Gamma interferon was added to U937 cells in culture at a final concentration of 100 units/ml. and incubations other that kinetic studies were 24 hours unless otherwise stated.

LPS. U937 cells in culture were treated with *E. coli* lipopolysaccharide (LPS) (Difco Laboratories, Detroit, MI) at a concentration of 2 or 10 μg./ml. for 24 hours.

Phorbol esters: PMA and PDBu. Phorbol 12-myristate 13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethanol at a concentration of 10^{-2}M and dilutions were made with RPMI 1640. PMA was added to cells at concentrations of 10^{-8}M or below in most assays and cells were incubated for 72 hours at 37°C in 5% CO_2. An equal concentration of ethanol served as a control.

Phorbol 12,13-dibutyrate (PDBu) was obtained from Dr. Carl McCrady at a concentration of 10^{-2}M dissolved in dimethylsulfoxide (DMSO) and kept frozen prior to use. Further dilutions were made using either DMSO or RPMI 1640. PDBu was added to cells at 10^{-7}M and cells were incubated as with PMA. After incubation with either PMA or PDBu, cells were extensively washed prior to use in assays. Cells were treated with an equal amount of DMSO as a control in experiments with PDBu; however, DMSO was found to induce U937 cells to become markedly adherent to plastic tissue culture flasks. To minimize this effect, PDBu obtained in DMSO was freshly diluted in RPMI 1640 prior to use. Control cells were treated with an equal amount of DMSO and did not adhere to the flasks.

Indomethacin. Release of prostaglandins was inhibited by addition of indomethacin (Sigma Chemical Co., St. Louis, MO) to assay media at a concentration of 10^{-5}, 10^{-6} and 10^{-7} M. Indomethacin was dissolved in 95% ethanol at a concentration of 10^{-4} M and was prepared fresh before use.

Transformation of *E. coli* HB101 by plasmid DNA. Competent bacterial cells were prepared by inoculation of L broth with a loopful of recently tested *E. coli* HB101 and growth at 37°C. to a nephelometer reading of 2.0 at O.D. 600 nm. At that time, 7 ml. of bacterial culture was removed and centrifuged to obtain a pellet of cells which were treated with 75 mM $CaCl_2$ followed by centrifugation and addition of ice cold 10 mM $CaCl_2$ and 0.1 µg DNA (T-33 unpublished, HLA-DR alpha probe; obtained from Dr. Jack Strominger) to be incorporated. After incubation on ice for 60 minutes, the cells were heat shocked at 42°C for 2 minutes, L broth was added and cells were added to an ampicillin containing L-agar petri plate. Cells were spread on the plate using a bent glass rod and the plate was incubated at 37°C. When colonies had grown, twelve colonies were picked and a fresh ampicillin plate divided into sectors was streaked with each colony. When these selected colonies had grown up, each colony was picked and a 7

ml. aliquot of L Broth was inoculated. These cultures were grown for 16 hours with rapid shaking at 37°C after which bacteria were sedimented by centrifugation. DNA was isolated by a modification of the mini-prep procedure as described by Maniatis et al. (445) and M. Pucci (personal communication). Bacteria were lysed by addition of lysozyme (1 mg./ml. in Tris-sucrose buffer) (Sigma Chemical Co., St. Louis, MO) followed by addition of 10% sodium dodecyl sulfate (SDS) and 5 M potassium acetate incubated on ice for 30 minutes. Liberated DNA was isolated by centrifugation at 12,000 X g for 15 minutes and phenol extraction and ethanol precipitation. Samples were digested with appropriate restriction endonuclease (Eco R1) (Boehringer Mannheim, Indianapolis, IN) for 30 minutes at 37°C after which each sample was electrophoresed in 1% agarose in the presence of ethidium bromide to make sure that the pUC plasmid containing 1.3 kb of probe insert was present. Bacteria containing the T-33 probe were grown up in L Broth + ampicillin, centrifuged to concentrate, glycerol was added and the suspension was aliquoted and frozen as 50% glycerol stocks at -40°C.

Plasmid preparation and isolation. Investigation of the mRNA produced by U937 cells which was specific for MHC class II alpha chains was facilitated by obtaining DNA probes in the form of plasmids from Dr. Jack Strominger, Harvard University, Boston, MA. Dr. Strominger sent our laboratory a probe for HLA-DR alpha (T-33) and gave his permission for use of a probe for HLA-DQ alpha which was available in Dr. L. B. Schook's laboratory. Another probe which was used for hybridization, albeit relatively unsuccessfully, was a probe for human 28S rRNA which was obtained from Dr. Eric Westin. Thus the following probes were isolated and used for hybridization with RNA on Northern blots:

Probe name	Probe specificity	Plasmid	Type & Size	Reference
T-33	HLA-DR alpha	pUC13	cDNA 1.3kb	unpublished
pDCH1	HLA-DQ alpha	pBR322	cDNA 0.8 kb	(446)
pAD-19.IIa	28S rRNA	pBR322	cDNA 6.2 kb	(447)

The rRNA probe was obtained from Dr. J. Pullen as purified plasmid at sufficiently high concentration that it could be used directly without reintroduction into bacteria and isolation.

Large scale plasmid isolation was accomplished using a high salt/SDS isolation procedure from Dr. F. Macrina and modified by Dr. J. Pullen. Fifty milliliter starter cultures of L-broth containing the appropriate antibiotic (either ampicillin for pUC or tetracycline for pBR322) were inoculated from 2-3 day L-agar + antibiotic plates previously streaked from partially thawed glycerol stocks of probe transformed bacteria (HLA-DR alpha probe or HLA-DQ alpha probe). Starter cultures were grown with vigorous shaking at 37°C for 6-8 hours. One liter cultures divided between four one-liter flasks (4 X 250 ml.) were inoculated with starter culture and grown on a shaker platform vigorously rotated at 37°C overnight. Bacteria were harvested by centrifugation at 6000 x g for 10 minutes, washed and lysed after three cycles of freezing and thawing and lysozyme (1 mg/ml) treatment by addition of SDS (final concentration 1% SDS) (Sigma Chemical Co., St. Louis, MO) and NaCl (final concentration 0.5 M). Bacterial lysate was cleared by centrifugation at 20,000 x g in a 70.2 Ti rotor in a Beckman ultracentrifuge (Beckman Instruments, Fullerton, CA). Cesium chloride (Cabot Chemical Co., Chicago, IL) was added to the lysate (9.12 g CsCl/9.33 ml lysate + ethidium bromide) and dispensed into 15 ml Beckman Quik Seal tubes which were balanced and centrifuged at 41,000 x g for 48 hours. Plasmid band was localized by ultraviolet illumination of ethidium bromide (Sigma Chemical Co., St. Louis, MO) and removed by puncturing the tube with a 23 gauge needle and 3 ml disposable syringe. The isolated plasmid was again added to cesium chloride and

rebanded by centrifuging at 41,000 x g for 48 hours. After subsequent removal of the plasmid band, ethidium bromide was extracted by several cycles of CsCl saturated isopropanol and the plasmid preparation was dialyzed against TE buffer overnight with 4-5 dialysate changes. Plasmid preparations were then ethanol precipitated and resuspended in TE buffer for restriction endonuclease evaluation. DNA was quantitated spectrophotometrically at 260 nm. using a Beckman spectrophotometer and quartz microcuvettes.

Restriction endonuclease digestion and DNA probe isolation by agarose electrophoresis and electroelution. In order to determine whether the plasmid isolated contained the probe required, 1 µl. of the plasmid preparations were digested with the appropriate restriction endonuclease (Eco R1 for plasmids T33 and rRNA and Pst 1 for plasmid pDCH1) by careful addition of enzyme to DNA sample containing the appropriate buffer. Restriction endonucleases and matched cleavage buffers were obtained from either Bethesda Research Labs (Gaithersburg, MD) or Boehringer Mannheim Corp. (Indianapolis, IN). Reaction mixtures were incubated for 1 hour at 37°C and electrophoresed in a 1% agarose gel with bromophenol blue tracking dye in a submarine electrophoresis chamber (IBI, New Haven, CN). Control lanes contained commercially prepared DNA gel markers of known size. For most gels the control lanes contained either Lambda ladder (Hind III fragments of lambda DNA) or a 1 kb ladder (Bethesda Research Labs., Gaithersburg, MD). Gels were run at 100 volts for 1-2 hours, stained with ethidium bromide and photographed on an ultraviolet light source using a Polaroid MP-4 camera and T-55 black and white film (Polaroid Corp., Cambridge, MA). If insert and plasmid DNA were found which matched probe and plasmid size, the preparation was judged to contain the desired probe. The T-33 plasmid specific for HLA-DR alpha was cut with restriction endonuclease (Eco R1) in bulk by overnight digestion, electrophoresed in a preparative slab gel of 1% agarose at

100 volts. When the insert band was clearly separated from the plasmid band, the insert band was cut from the gel, inserted into a dialysis bag and electroeluted from the agarose by continuing to electrophorese the gel inside the bag for 3 hours followed by reversing the polarity of electrophoresis for 3 minutes. Isolated insert was removed from the dialysis bag by Pasteur pipet, precipitated with ethanol and phenol extracted. This purified insert was quantitated by running on another 1% agarose gel (445).

Labeling of DNA probes with by nick translation and random priming techniques. Two types of labeling procedures were used for Northern blot analysis. Initially the HLA-DR alpha T-33 probe was labeled by nick translation using a commercially available kit (Amersham Corp., Arlington Heights, IL) because the insert DNA had been cleanly isolated. Labeling by random priming techniques also using a commercially available kit (Boehringer Mannheim, Indianapolis, IN) was used for all probes for the remainder of the Northern blot analysis. The rationale for use of random priming was the fact that insert DNA need not be isolated prior to labeling and subsequent hybridization. Given the constraints of time and equipment, random priming appeared to provide a short cut to the information sought.

T-33 (HLA-DR alpha probe) DNA was labeled with ^{32}P by nick translation by addition of 20 µl nucleotide buffer + 1 µl H_2O + 22 µl dCTP (Sigma Chemical Co., St. Louis, MO) + 43 µl [alpha ^{32}P]-dCTP (ICN Biomedicals, Inc., Cambridge, MA) + 10 µl DNA polymerase/DNAse to 4 µl (1 µg) T-33 DNA. After incubation for 2 hours at 15°C, unincorporated nucleotide was removed by Elutip column (Schleicher & Schuell, Inc., Keene, NH) and labeled probe was eluted with high salt buffer. An aliquot was counted in a liquid scintillation counter to determine the degree to which the DNA was labeled.

Random primed labeling of DNA probes was carried out as outlined in the product bulletin. Briefly, 1 µg DNA to be labeled in a total of 9 µl was pipetted into a

plastic tube. Sequentially the following additions were made with the enzyme (Klenow fragment) added last: 1 µl each dGTP, dTTP, dATP; 2 µl reaction mixture in 10X buffer (hexanucleotide primers); 5 µl [alpha-^{32}P] dCTP (ICN Biomedicals, Inc., Cambridge, MA); and 1 µl Klenow fragment. Tubes were incubated at 37°C for 30 minutes, the reaction was stopped by the addition of EDTA and unincorporated nucleotide was removed by passing the labeled DNA over 1 ml. Sephadex G-50 (Pharmacia, Piscataway, NJ) spun columns in TE buffer (445). An aliquot of labeled probe was counted in a liquid scintillation counter to determine the degree of labeling.

Isolation of total cellular RNA. Total cellular RNA was isolated from cells using a modification of published procedures (445, 448). Glassware used in RNA extraction procedures was scrupulously cleaned, autoclaved and baked at 180°C overnight. Whenever possible, virgin plasticware items were used instead of glass and reagents used for RNA were handled only with disposable rubber gloves and were not available for uses other than RNA extraction. Cells from which RNA were to be extracted were removed from culture after a variety of treatment protocols and washed with PBS to remove serum proteins. Cells were centrifuged at 400 x g and decanted to give a dry pellet of 1-2 X 10^7 cells. Cells were lysed by the addition of 4M guanidine isothiocyanate (Fluka Chemical Corp., Ronkonkoma, NY) accompanied by vigorous pipetting followed by addition of sodium sarkosyl detergent (Sigma Chemical Co., St. Louis, MO). Complete disruption of the cells was accomplished by repeated expulsion of the liquid through a syringe fitted first with an 18 gauge needle followed by a 23 gauge needle. The liquid was pipetted carefully onto a cushion of ultrapure CsCl (Bethesda Research Labs., Gaithersburg, MD) in a 15 ml Beckman Quik Seal tube (Beckman Instruments, Fullerton, CA), sealed and centrifuged at 41,000 x g in a Beckman 70.2 Ti rotor for 18 hours. The RNA pellet obtained after centrifugation was washed with ethanol, dissolved in water and reprecipitated from sodium acetate with cold ethanol.

The thoroughly dried precipitated RNA was resuspended in 300 µl water and quantitated spectrophotometrically at 260 nm. The protein contamination of the RNA was ascertained by the O.D. 260/O.D. 280 ratio. In general the ratios were between 1.6 and 2.0.

Preparation of RNA blots by electrophoretic transfer from agarose gels. RNA samples were transfered to membranes by a modification of several procedures (445, 449, 450). RNA samples (5, 8 or 10 µg) to be probed were added to wells in 1.3-1.5% horizontal agarose gels. The gel size generally used was 14 X 20 cm containing 100 ml and either 10 (60 µl) or 16 (30 µl) lane combs were used. The gels contained 2.2 M formaldehyde (Fisher Scientific, Pittsburgh, PA) in MOPS (3-[N-morpholino]propane-sulfonic acid) buffer (Sigma Chemical Co., St. Louis, MO). Samples were loaded in loading buffer containing bromophenol blue and electrophoresed in MOPS buffer at 15 volts for 24 hours in an IBI (New Haven, CN) horizontal electrophoresis chamber with Bio Rad Labs (Anaheim, CA) power supply. To maintain even buffering capacity, the chamber was recirculated with a small pump (LKB Instruments, Gaithersburg, MD). Gels were stained after electrophoresis with 30 µl ethidium bromide (10 µg/ml), washed twice with phosphate buffer and photographed on an ultraviolet transilluminator using a Kodak MP-4 camera and T-55 film as previously described. Positions of the obvious ribosomal RNA bands were localized in the photographs by including a ruler in each picture. Gels were washed in 0.025M phosphate buffer, pH 6.5 on a rotary shaker for 60 minutes with two buffer changes. The blotting medium used was Genatran 45 (Plasco, Inc., Woburn, MA) which is a nylon membrane with high binding capacity. Gel-size pieces of blotting membrane were cut and prewet with buffer before being carefully placed on top of the gel using great care to eliminate any air bubbles. The gel and membrane were placed between two sheets of Whatman 3mm filter paper (Whatman International, Ltd., Maidstone,

England) and placed in the gel holders of the transblotting apparatus (Hoefer Scientific Instruments, San Francisco, CA) with the membrane at the anode. Transfer of the RNA to the membrane was accomplished by electrophoresing at 1.5 Amps, 40 volts for 1 hour. The system was cooled by running tap water through an ice bath and then through cooling tubes in the transblotter chamber. Once transfered, the RNA blot was washed twice in 2XSSC, placed between sheets of filter paper and clamped between glass plates, and then dried in a vacuum oven at 80°C for 4 hours. Blots were stored in a vacuum dessicator after drying.

Northern blot hybridization and autoradiography. RNA blots were hybridized by a modification of published procedures (445, 449 451). RNA blots were prehybridized to prevent non-specific binding of labeled probe by incubation in a prehybridization mixture consisting of 100 mM Tris, 6XSSC, 2 mM EDTA, 10X Denhardt's solution, 1% SDS, 200 µg/ml Salmon sperm DNA, and 50% saturated formamide (Aldrich Chemical Co., Milwaukee, WI). Multiple blots were either placed in covered plastic containers containing prehybridization solution and rapidly agitated on a rotating platform in a 42°C incubator or they were sealed in individual plastic bags containing prehybridization solution and plased in a 42°C water bath. Blots were prehybridized for at least 24 hours after which the prehybridization mixture was poured off and ^{32}P labeled probe (1 X 10^7 cpm/blot) was added in fresh prehybridization solution. Both labeled probe and salmon sperm DNA were boiled for 5 minutes prior to addition to the hybridization. Hybridizations of multiple blots were carried out in plastic containers and also in individual sealed bags. Incubations were carried out at 42°C overnight. Blots were removed from hybridization mixture, washed with 3 X 500 ml. high salt wash buffer (2XSSC; 0.1% SDS) for 20 minutes per wash on a shaker platform at room temperature, followed by washes with 2 X 500 ml. low salt wash buffer (0.1XSSC; 0.1% SDS) for 30 minutes per wash at 62°C. After

the final wash, blots were blotted, placed on filter paper cut slightly larger than the blot, wrapped in plastic wrap and placed in X-ray cassettes containing intensifier screens (Dupont Cronex, Du Pont Co., Wilmington, DE) for autoradiography (436). Type XAR-5 X-ray film (Kodak, Rochester, NY) was placed on each blot. Blots were identified by ^{35}S-ink markings. Cassettes were placed in a -70°C freezer for 72 hours to 3 weeks after which film was developed in X-ray film developers in the Radiology Department.

Evaluation of autoradiograms by scanning densitometry. After hybridizing RNA blots with probes for HLA-DR alpha, HLA-DQ alpha and 28S rRNA, quantitative interpretation of autoradiograms was deemed necessary, especially in light of two problems. The first problem arose when it begame apparent that on most blots, the probe for 28S rRNA had given a bubbly, splotchy pattern as though hybridization had been uneven. Photographs of the RNA gels indicated that there was indeed 28S rRNA present on these gels and although the quantities in each well varied to some degree, they did not vary to the degree indicated by the rRNA probe hybridization. The blots had also been checked after transblotting and found to have ethidium bromide stained 28S rRNA bands directly comparable to the bands pictured in the corresponding gel photographs. The rRNA probe had been used as a positive control but more importantly as a means to determine relative quantity of RNA on the blot. The second problem arose from the faintness of most of the autoradiograms and the very messy background found particularly with the HLA-DQ alpha probe. It was decided that for purposes of relative quantitation, readings from a scanning densitometer would suffice. Dr. Guy Cabral made his Hoefer densitometer (Hoefer Scientific Instruments, San Francisco, CA) available for examination of all autoradiograms. Three scans were taken of each band by removing the film after each scan and repositioning it for optimal exposure to the light source. Background readings were taken near the position of the bands but not

including any of the bands for messy blots. In order to have a way of determining relative 28S rRNA on each blot, the Kodak T-55 film negatives of the original gel photographs were scanned, three times each. Background scans were necessary on the photographs because there was a consistent, regular darkening of the negative toward the center. In general the background corresponded to the arc of a circle which could be fitted to the rRNA readings. Because the RNA quantities had been determined by spectrophotometer prior to running of the gels, the quantities of RNA loaded into each well were usually very similar within a single gel. A relative number has been calculated for the mRNA specific for the probes within a single gel by using the following formula:

$$\text{Relative specific mRNA} = \frac{\text{probe dens. reading - probe bkgd. dens. reading}}{\text{rRNA (photo negative) - photo background*}}$$

*Photo background determined in most cases by arc fitting

RESULTS

The U937 cell line, isolated from the pleural effusion of a histiocytic lymphoma patient in 1975-76, was well characterized by Sundstrom and Nilsson both morphologically and biochemically when first reported (95). The cell line was found to exhibit many characteristics of human monocytes and macrophages and thus became the primary and, for a time, the only monocyte-like cell line available in humans. The U937 cell line was made available to our laboratory where the cells were maintained in culture and further characterized. An antibody specific for U937 cells was shown to react in an analogous manner with cell surface antigens only on human monocytes (452), thus strengthening the link between U937 cells and the monocytic lineage. An important functional aspect of U937 cellular capability was the finding that they were able to mediate antibody dependent cell cytotoxicity (ADCC) following overnight activation with lymphokines (404). U937 cells differed in one very important respect from human monocytes, however. Although they clearly expressed MHC class I molecules, there was no expression of MHC class II molecules and thus they were not seen as capable of mediating the immune functions dependent on interaction of monocytes with T cells through interaction between MHC class II and T cell receptor. Of course, at that point in time these interactions were also not well understood; however the requirement for MHC class II had been clearly shown by Zinkernagel and Doherty (239). In 1981, the U937 cell line, which had previously been consistently negative when tested for expression of cell surface Ia, was used as a negative control in an experiment in our laboratory in which the effects of phorbol ester (PMA) on the expresson of cell surface Ia were being investigated. At that time one of the U937

parent cell lines surprisingly exhibited two bands having molecular weights of approximately 29K and 34K on an autoradiograph of cell surface proteins which were iodinated and immunoprecipitated using a monoclonal antibody to framework determinants of human Ia (mAb Kula2). When this parent line was then examined by immunofluorescence using this antibody, approximately 20% of the cells present were positive for cell surface Ia. A second parent line of U937 used at this time remained entirely negative and could not be induced to express Ia by treatment with PMA. The HLA-DR positive parent U937 line became progressively more positive within the succeeding two months during which the percentage of positive cells rose from 20% to approximately 80%.

The HLA phenotype of the HLA-DR positive and HLA-DR negative U937 cell lines was determined by microcytotoxicity assay.

To rule out the possibility that a contaminating cell from another source was responsible for the apparent strong expression of HLA-DR in a cell line characterized as very negative for HLA-DR, both the Ia negative and the Ia positive cell lines were tested by the Tissue Typing Laboratory at MCV using microcytotoxicity techniques employing a panel of specific antisera and low cytotoxicity, pretitered complement and were found to express the reported HLA phenotype for U937: HLA-A(3), B(51,18)(95). The Ia positive line was also found to be positive for HLA-DR2. The concurrence of the HLA phenotype of both the Ia negative and Ia positive U937 cell lines rules out the possibility of overgrowth by a contaminating cell; thus one of the U937 parent cell lines had begun to express MHC class II antigens spontaneously and for unknown reasons.

Sublines of U937 were isolated by cloning at limiting dilution.

Both Ia positive and Ia negative parent lines were plated in 96 well plates at

cellular concentrations of 0.5, 1, 2, 5, and 10 cells/well for limiting dilution techniques (426) to isolate clones of U937 cells for further study. Approximately a hundred clones were isolated and expanded in five separate clonings from wells plated to contain 0.5 or 1 cell/well. Cell surface expression of Ia was measured by immunofluorescence using either a fluorescence microscope or the Ortho Spectrum III flow cytometer with a monoclonal antibody to a framework determinant of human Ia (mAb Kula2). Preliminary evaluation of the cloning data (data not shown) suggests that the plating efficiency of the Ia positive cells was greater than that of the Ia negative cells, i.e. in attempts to isolate clones from either parent, a larger number of clones grew from higher dilutions of the Ia positive parent line than from the Ia negative parent line. A second finding was that of the clones that were successfully expanded from either parent, a disproportionately large number were Ia positive making selection of clearly negative clones difficult. For example in the final cloning of the 2-1 parent line which was approximately 50% Ia^+ at the time of cloning, 15/17 clones isolated were strongly positive, 1 clone was Ia± (21% Ia^+) and 1 clone was negative (5.8% Ia^+). This negative clone was selected for further study and subsequently became Ia positive (clone G11).

Several clones were chosen for further study during the course of the project. When originally isolated, the cells designated as clones were probably derived from single cells as required by the mathematics of limiting dilution analysis (426, 427, 428), i.e. these clonal populations grew from wells originally plated at concentrations of 0.5 or 1 cell/well and were isolated from plates in which greater than 37% of the plated wells were negative for growth. The clones utilized were kept in continuous culture for more than two years during the project and were not subsequently subcloned; therefore, both the possibility of originally plating more than one cell/well and the possibility of subsequent mutation within a clonal population can not be addressed and the clones used in this study will be referred to simply as "sublines."

The cells used in this study, both parent lines and sublines, are listed in Table 1. Two parent cell lines and five sublines were chosen for further study at various times throughout the project. The 2-1 parent line was generally used as the representative parent or original U937 population.

Morphologically the cells of the sublines are similar to the U937 parent line.

Growth characteristics. Unlike monocytes from peripheral blood or macrophages, these cells do not adhere to surfaces unless further treated with DMSO or PMA. They grow rapidly in single cell suspension cultures in RPMI 1640 tissue culture medium if 10% fetal bovine serum is added and require reduction of the population and addition of fresh media approximately every 4-5 days. The U937 cell line grows well in media having a slightly acidic pH ranging from 6.8-7.0, unlike lymphocytes which survive more readily in more alkaline pH of 7.2-7.4. After four days, the cells reach a maximum concentration of 1-1.2 X 10^6 cells/ml. at which time the cells are quite healthy and the medium is usually clearly acidic. Without splitting and feeding, however, the medium, which contains phenol red as a pH indicator, becomes lemon yellow indicating a very low pH within 24 hours and the cells begin to die.

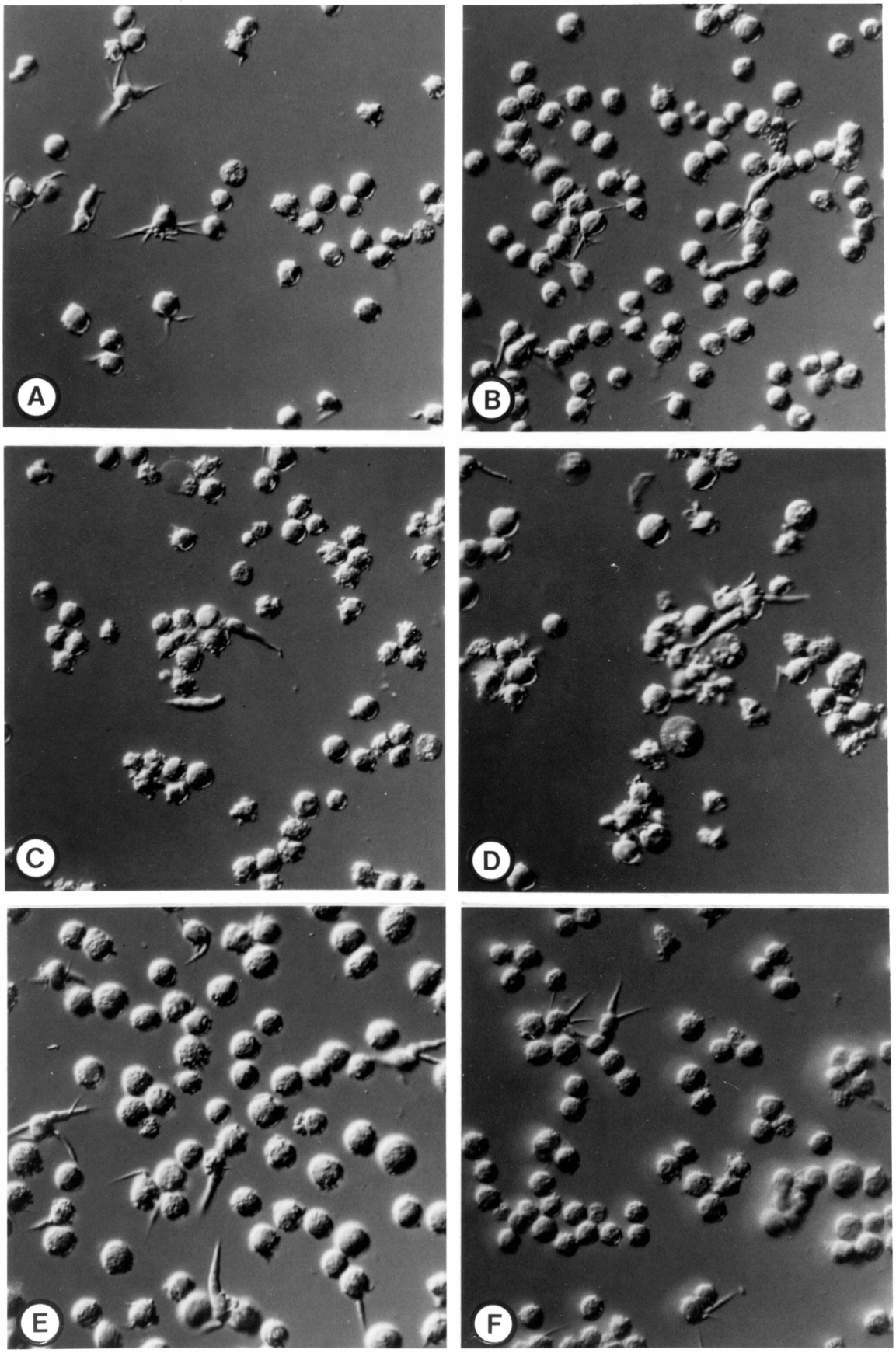
Morphology. The cells have a pleomorphic shape and vary within a population from nearly round to very asymmetrical, as shown in Figure 7. The cells of both parents and sublines are characterized by the appearance of many cell surface blebs and the development of long, sharply pointed or blunt club-like projections attached to the cell by a thinner stalk. All of the sublines examined exhibited the whole range of morphological variations while in culture, usually at the same time; however, when cells in culture flasks were at their peak concentration they tended to appear most homogeneously spherical. The U937 cells are characterized by a nuclear to cytoplasm

TABLE 1. U937 CELLS AND SUBLINES

U937 SUBLINES	ORIGINS
2-1 parent	Parent cell line. Ia negative U937 cells originally frozen 1/5/81 in liquid nitrogen; recovered and recultured 4/5/84.
H-K	Parent cell line. Ia negative U937 cell line obtained from Dr. Hillel Koren, Duke University, Durham, N.C. in May, 1985, to be used as a second Ia negative parent line.
Subline D10	Ia positive subline. Cloned 3/8/84 from an Ia positive parent from which 6 clones were derived out of 24 wells plated at 1 cell/well. Strongly Ia positive.
Sublines G4 & E11	Ia positive sublines. Cloned from an Ia positive parent line referred to as "Grandma" because of the patchy fluorescent pattern apparent when stained for surface Ia using mAb Kula2. Isolated from dilutions of 1.0 and 0.5 cells/well, respectively, both of which yielded growth of cells in 8 of 24 wells plated.
Subline G11	Ia negative subline which converted to Ia positive. Isolated from the 2-1 Ia negative parent line cloned 3/18/85 (at a time when the 2-1 parent exhibited up to 50% Ia positive cells). G11 may not have been clonal when isolated as it was obtained from wells in which growth occurred in18 of 24 wells, thus not satisfying the requirement for at least 37% negative wells.
Subline E9	Ia negative subline. Cloned from an Ia negative parent cell lineby Dr. Carl McCrady prior to April, 1983, and recovered from liquid nitrogen and recultured in April, 1986 (in desperation for an Ia negative subline).

FIGURE 7. Morphology of U937 cell line and sublines in culture.

Parent cell line and sublines were photographed at 20X magnification using Nemarski optics. Panels (A) and (B) illustrate representative populations of the U937 parent 2-1 cell line, panel (C) subline E11, panel (D) subline G4, panel (E) subline G11, and panel (F) subline E9.


A
B
C
D
E
F

ratio approaching 1. The cytoplasm appears to have numerous granules and there is a single, lobed, spherical or kidney shaped nucleus.

The parent lines and sublines are similar in size and within the population there is marked heterogeneity; however, the average cell size in the G11 subline is slightly larger than either the parent or other sublines and the average cell size in the E11 subline is smaller. This became apparent when cells were examined using the Ortho Spectrum III when gating parameters were being set. The increased forward light scatter seen with subline G11 was consistently seen throughout the project. A representative experiment, shown in Figure 8, illustrates that the populations are overlapping both in size as demonstrated by forward scatter and by complexity which is reflected in the right angle scatter.

Nonspecific esterase activity. Nonspecific esterase activity in cytoplasmic granules which is characteristic of monocytic cells was also seen in both parent U937 and the sublines. As seen from the results of alpha-naphthol acetate esterase staining of smears of ficoll-hypaque isolated peripheral blood mononuclear cells and U937 cells shown in Table 2, the black staining granules were apparent in 19% of the PBMC which represents the monocytes present in the preparation and virtually 100% of the U937 cells. Although the percentage of positive cells did not reflect large differences in esterase positivity indicating that nearly all cells contained darkly staining cytoplasmic granules, staining was not uniform in the U937 cells as there was considerable variability in staining intensity within populations. It was noted that the 2-1 parent stained less darkly than the sublines tested, and the G4 subline gave the strongest, most homogeneously positive reaction.

HLA determinations by microcytotoxicity. The five sublines selected for further study were tested by microcytotoxicity and found to express the HLA phenotype,

FIGURE 8. COMPARISON OF CELL SIZE AND INTRACELLULAR COMPLEXITY AMONG U937 PARENT CELL LINE AND SUBLINES.

Size measured as forward scatter and complexity measured as right angle scatter were determined for the U937 parent 2-1 cell line and sublines E11, G4, G11 and E9 using the Ortho Spectrum III laser activated flow cytometer. Standard deviations indicate heterogeneity of all U937 populations examined.

FIGURE 8. COMPARISON OF CELL SIZE AND INTRACELLULAR COMPLEXITY

U937 SUBLINES	CELL SIZE (FORWARD SCATTER)	CELLULAR COMPLEXITY (RIGHT ANGLE SCATTER)
2-1 PARENT	161.0±28.0	78.1±30.2
E11	136.7±28.8	65.0±30.4
G4	157.0±31.9	93.7±35.9
G11	189.1±37.0	122.4±42.0
E9	146.5±29.5	90.0±37.4

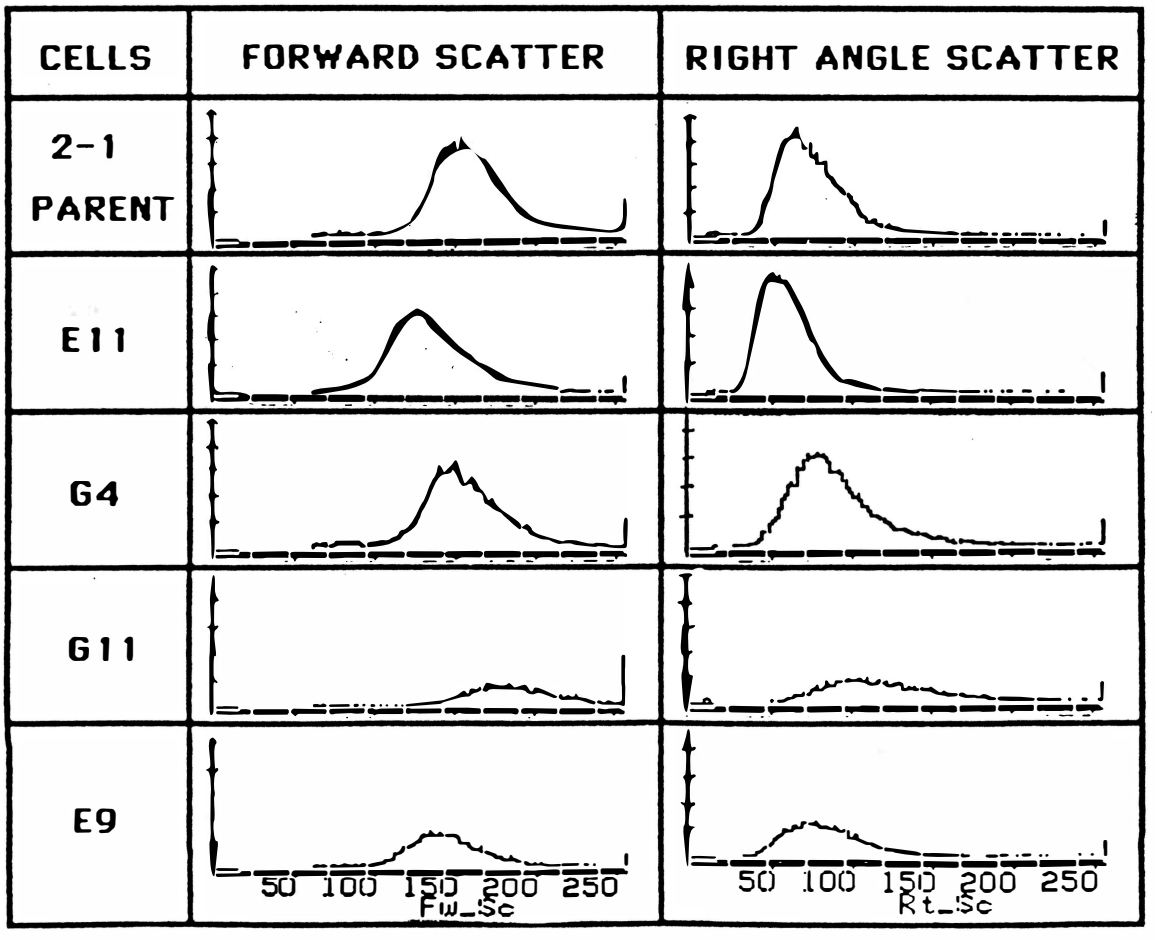


TABLE 2. NONSPECIFIC ESTERASE REACTIVITY OF U937 PARENT LINE AND SUBLINES MEASURED AS ALPHA-NAPHTHOL ACETATE ESTERASE*

CELLS	% ESTERASE POSITIVE
PBMC	19%
2-1 PARENT	96%
SUBLINE E11	98%
SUBLINE G4	100%-STRONGLY POSITIVE
SUBLINE G11	100%
SUBLINE E9	100%

*Alpha-naphthol acetate esterase reactivity was measured on fixed smears of cells using SIGMA kit 90-1A.

HLA-A (3,X), B(51,18) (95). The Ia positive sublines were HLA-DR2. Other MHC class II specificities have not been clearly distinguishable. The microcytotoxicity tests did not indicate a second HLA-DR antigen being expressed on these cells, therefore the phenotype is HLA-DR (2,X). Confirmation of the HLA-A and B phenotype indicates that the sublines are progeny of the original parent U937 cell line and can not be attributed to the presence of a contaminating cell. Although the microcytotoxicity reactions of the sublines for HLA-DR were occasionally weak, they consistently indicated the presence of HLA-DR2 from their first testing in 1984. When sublines were tested for HLA antigens by microcytotoxicity in the fall of 1987, however, HLA-DR antigens could not be determined by microcytotoxicity. Decreased expression of HLA-DR was indicated as the probable cause and indeed this was confirmed by data obtained from immunofluorescence tests discussed below.

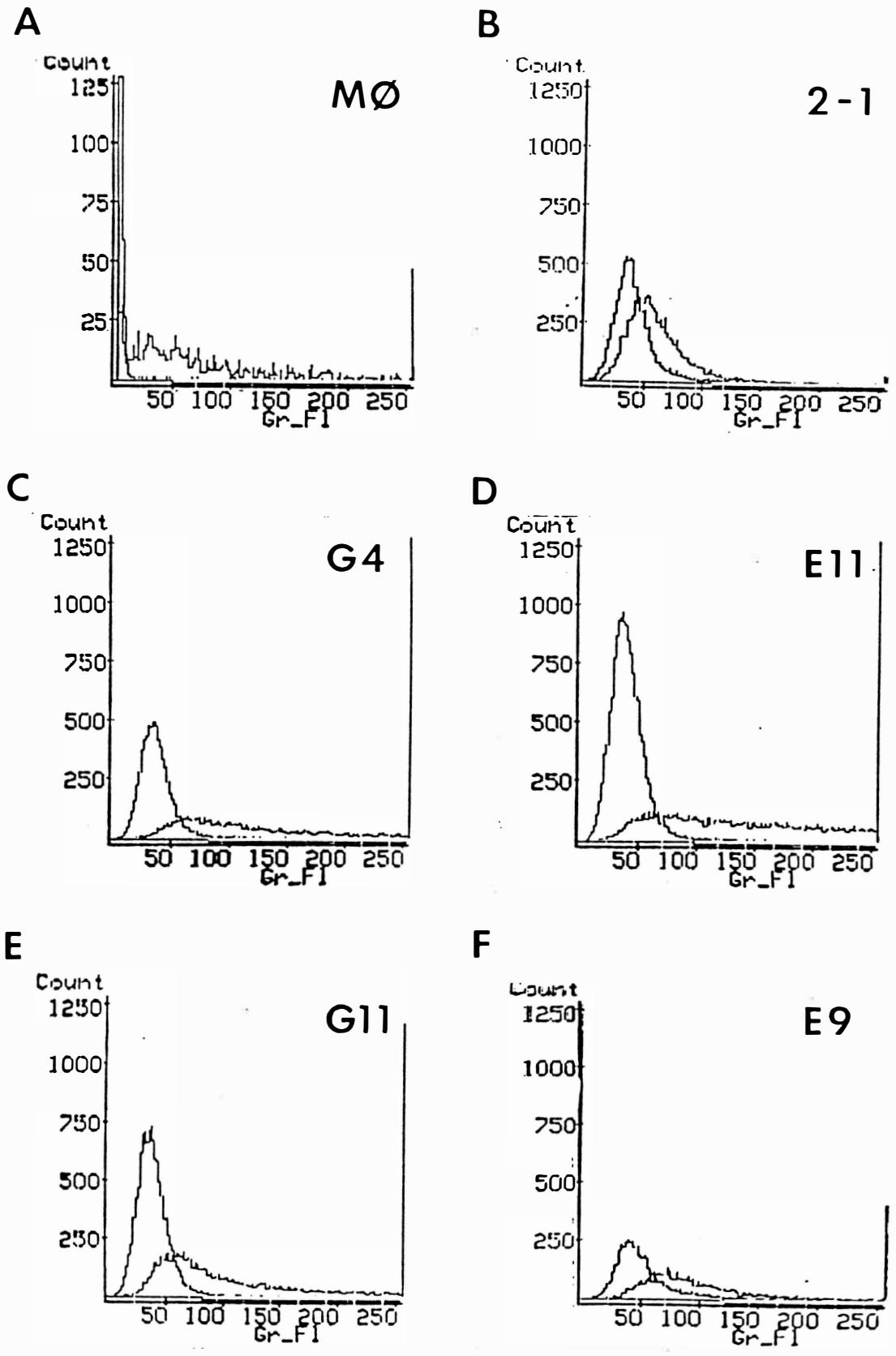
U937 parent and sublines were examined for expression of Ia using fluorescent antibody techniques.

Indirect immunofluorescence techniques were used to examine the expression of Ia on the surface of the U937 parent and sublines many times during the course of the project. The cells were stained with mAb Kula2, incubated, washed and incubated with a second antibody labeled with FITC, either $F(ab')_2$ goat anti-mouse IgG or $F(ab')_2$ sheep anti-mouse IgG, prior to examination by laser activated flow cytometry using the Ortho Spectrum III. Controls consisted of cells preincubated with MOPC 11 supernate which is an isotype matched irrelevant monoclonal antibody prior to incubation with the fluorescent second antibody. Normal peripheral blood mononuclear cells (PBMC), or selected gated populations of PBMC such as lymphocytes or monocytes, were also examined under the same conditions to ascertain the relevance of the U937 staining on normal populations.

A representative experiment is shown in Figure 9 in which peripheral blood

FIGURE 9. CYTOFLUOROGRAPHS OF U937 2-1 PARENT, FOUR SUBLINES AND NORMAL HUMAN MONOCYTES ± Kula2

Representative cytofluorographs of normal human monocytes (A), parent U937 cells (B) and four sublines, E11 (C), G4 (D), G11 (E) and E9 (F), isolated from U937 Ia+ and Ia- parent cell line by limiting dilution techniques. Cells were tested with Kula2 monoclonal antibody to a framework determinant of human Ia followed by FITC conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin. Cells were evaluated by flow cytometry using the Ortho Spectrum III. Abscissa = mean channel fluorescence (MCF); ordinate = cell number (Count).


A
Count
125
100
75
50
25
MØ
50 100 150 200 250
Gr_FI
B
Count
1250
1000
750
500
250
2-1
50 100 150 200 250
Gr_FI
C
Count
1250
1000
750
500
250
G4
50 100 150 200 250
Gr_FI
D
Count
1250
1000
750
500
250
E11
50 100 150 200 250
Gr_FI
E
Count
1250
1000
750
500
250
G11
50 100 150 200 250
Gr_FI
F
Count
1250
1000
750
500
250
E9
50 100 150 200 250
Gr_FI

monocytes, the U937 2-1 parent cell line and sublines E11, G4, G11 and E9 were examined for the expression of MHC class II using Kula2. The cytograms shown indicate the sharp peak of the negative control region to the left of the graph and the broad range of the positive region extending the full range of the green fluorescence. This pattern is typical of MHC class II expression on both normal monocytes and on the Ia^+ U937 cells and indicates that within both cell populations there is a wide range of expression of the MHC class II antigens. This positive reactivity is measured as fluorescence intensity which is expressed as percent positive cells and also as mean channel fluorescence ± standard deviation (MCF±SD) which provides a relative measurement of degree of positivity. The large standard deviations attached to mean channel fluorescence measurements shown for both normal monocytes and U937 cells are indicative of the heterogeneity within each population. The U937 2-1 parent line was found to be only 5.8% positive with a MCF±SD of149.5±42.0 for the small number of positive cells measured. The E9 subline tested in this experiment was found to be 30.2% positive with MCF±SD 193.0±50.6. Sublines G4, E11 and G11 were found to be strongly positive with 73.9%, 80.3% and 53.4% positive, respectively. Their corresponding MCF±SD values were 195.8±65.0, 220.7±50.6 and 169.8±64.0, respectively, indicating that the percent positive cells correlated with the degree of positivity of those cells, i.e. E11>G4>G11 for both percent positive and MCF values. In general this was found to be true throughout the study for cells having greater than fifty percent positive cells. In cells having less than fifty percent positive cells, such as the E9 cells in this case, the MCF was frequently found to be high relative to the percent positive cells. There are two possible explanations for this phenomenon. The small percent positive may reflect dead cells or cells which have taken up the FITC labeled antibody in a non-specific manner and although controlled for in setting the negative control parameters are still available in the positive region of the gated cell population where their bright, but non-specific fluorescence gives an artificially high MCF value. The

second explanation is the obvious possibility that there may be a small but very intensely positive population of cells present. Although there are undoubtedly positive cells present in the E9 subline population tested, the high MCF value seen is believed to reflect background non-specific staining which was found for the E9 subline in most experiments and not for either the 2-1 parent line or for the other sublines. The normal human monocytes tested are clearly positive for Kula2 (76.4%) and have a MCF±SD value (185.9±70.1) comparable to sublines E11, G4, and G11.

The 2-1 parent U937 and the sublines were tested with a battery of antibodies to establish a phenotypic characterization. U937 parent lines and primarily four sublines, E11, G4, G11 and E9 were examined by flow cytometry using a variety of monoclonal and polyclonal antibodies to human cell surface antigens. The antibodies and their respective specificities are listed in Tables 3A and 3B. The antibodies were chosen to represent a broad range of specificities typical of immunologically relevant cell types.

Cells were tested with antibodies specific for MHC class II. As previously noted the cells were tested with monoclonal antibody Kula2 which is specific for a framework determinant of human HLA-DR. Table 4 indicates the results of multiple testings using several antibodies specific for MHC class II including Kula2. Although there was wide variability over the four years involved in the project, the mean percent positive cells are listed. Both the 2-1 parent U937 and the E9 subline were weakly positive in all experiments, 16.7% and 18.3% positive, respectively. The D10 subline which was investigated early in the project was very strongly positive for Kula2 (96.9% positive). Sublines G4 and E11 were found to be positive for Kula2 averaging 62.6% and 61.6% positive, although somewhat less positive than the D10 subline. The G11 subline, originally isolated from the Ia negative parent line, was *marginally* positive when isolated (5.8% positive); however, the cell line became increasingly Ia positive

TABLE 3A. SPECIFICITIES OF ANTIBODIES USED FOR FLOW CYTOMETRIC ANALYSIS OF U937 PARENT AND SUBLINES

ANTI-MHC & ANTI-MØ

NAME	SPECIFICITY	REFERENCE
ANTI-MHC CLASS II		
Kula2	HLA-DR	unpublished
Kula3	HLA-DQ?	unpublished
L227	HLA-DR,DP (DQ)	313, 453
L203	HLA-DR	313, 453
ANTI-HLA(A, B, C)		
W6/32	HLA-A, B, C	454
ANTI-ß2M		
BBM.1	ß2 microglobulin	439
ANTI-MONOCYTE/MACROPHAGE		
M1	mature monocytes	455
MMA	monocyte/granulocyte	456
Mac 1	CR3, mature monocytes, PMN, LGL	457
63D3	mature monocytes	458
Mo 1	CD11b; monocytes, granulocytes, null cells	459
Mo 2	CD14; monocytes, macrophages, granulocytes	460
My 4	myeloid cells	461
My 7	myeloid cells	462
OKM5	human monocyte, platelet	463

TABLE 3B. SPECIFICITIES OF ANTIBODIES USED FOR FLOW CYTOMETRIC ANALYSIS OF U937 PARENT AND SUBLINES

ANTI-ENDOTHELIAL CELL, T CELL, B CELL, NK CELL & OTHERS

NAME	SPECIFICITY	REFERENCE
ENDOTHELIAL CELL		
2F11	endothelial cell	464
2H10	endothelial cell	464
3A1	endothelial cell	464
T, B, NK, AND OTHERS		
TA-1	activated T cells, monocytes	465
OKT3	CD3; T cell	466
OKT4	CD4; helper T cell	467
KT69-7	CD4; helper T cell	440
OKT8	CD8; cytotoxic T cell	468
3a1	T cell	469
Leu 4	CD4; T helper cell	470
Leu 5	CD2; S-RBC receptor	471
sIg	B cell surface Ig	
Leu 11a	NK (natural killer cells)	472
KuFc79	human Fc receptor	431
OKT9	human transferrin receptor	473
IL-2R	human interleukin 2 receptor	474
MOPC 11	fibronectin-control	

TABLE 4. U937 SUBLINES AND PARENT CELL LINE EXPRESS SURFACE MOLECULES ENCODED BY THE MAJOR HISTOMPATIBILITY COMPLEX

Antibody Specificity	Normal PBMØ	Parent 2-1	Cells G4	Cells E11	Sublines G11	Sublines E9	Sublines D10
anti-Ia							
Kula2	76.4	16.7	62.6	61.6	44.0	18.3	96.9
Kula3	n.d.	2.9	15.4	11.4	16.0	4.8	n.d.
L227	56.5	1.8	40.3	38.2	35.9	7.0	n.d.
L203	78.7	2.0	55.9	54.2	53.3	12.2	n.d.
anti-HLA (A,B,C)	95.4	69.5	82.1	60.6	67.4	57.9	95.4
anti-β_2M	96.3	65.4	76.4	42.1	56.0	72.5	n.d.

over a period of several months and the average percent positive G11 cells was 44.0% throughout the project, although as for other sublines, the percentages varied considerably. Normal human monocytes tested with Kula2 were found to be 76.4% positive.

The Ia positive sublines, G4 and E11, appeared to change during 1987. HLA-DR could no longer be determined by microcytotoxicity assay. When immunofluorescence data for Kula2 was compiled as shown in Figure 10, the variability in assays is apparent from the standard deviations. When the data was divided between assays performed prior to May, 1987, and assays performed after May, 1987, sublines G4, E11 and E9 were no longer reactive with Kula2 to the same degree in the later time period. Although there was a slight change in the reactivity of the G11 subline, it was not markedly different from those values shown for either the composite results or for the earlier time period; and the results shown for the 2-1 parent cell line indicate no real change in reactivity.

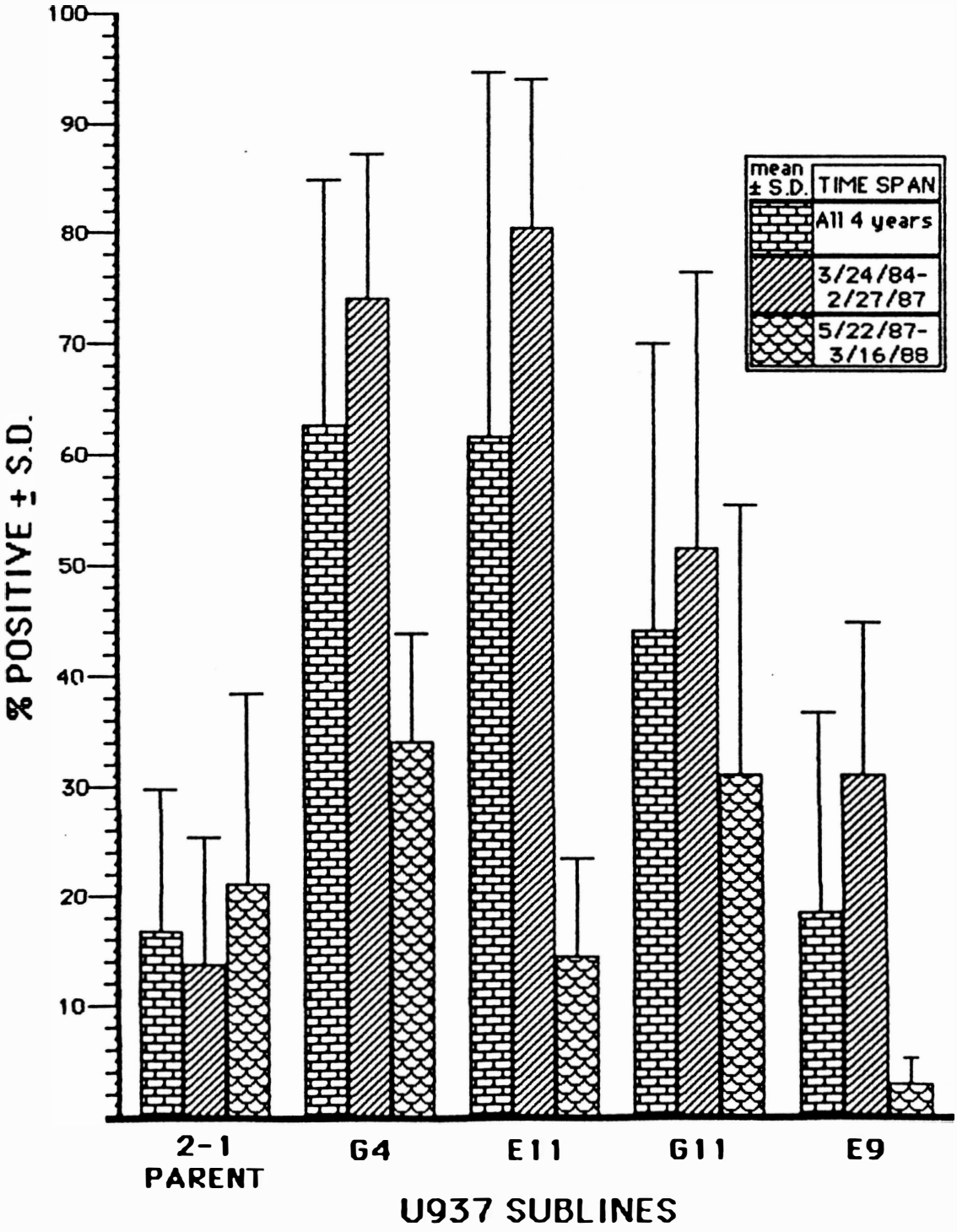
Reactivity to several other antibodies specific for MHC class II was also tested as shown in Table 4. The monoclonal antibodies L203, which is reported to be specific for HLA-DR, and L227, specific for determinants on HLA-DR, DP and also DQ, gave results similar to Kula2 in relative reactivity and intensity of staining with normal human monocytes, 2-1 parent U937 cells and sublines G4, E11, G11 and E9. In all cases, normal human monocytes were more reactive than sublines G4, E11 and G11 which were positive, and both parent 2-1 cells and subline E9 were negative. These values may indicate reactivity only or at least primarily with HLA-DR. The Kula3 antibody tested has an unconfirmed specificity for HLA-DQ and the cells tested gave weak reactions.

U937 parent cell line and sublines were tested with antibodies specific for MHC class I. Two antibodies were employed for the purpose of confirming the cell surface expression of MHC class I molecules by both parent U937 cells and by the sublines as

FIGURE 10. COMPARISON OF Kula2 REACTIVITY IN PARENT U937 AND SUBLINES OVER A FOUR YEAR PERIOD.

Immunofluorescence data was compiled from experiments analyzed on the Ortho Spectrum III using Kula2 + $F(ab')_2$ FITC labeled goat (or sheep) anti-mouse immunoglobulin. The first column is a composite of all the determinations performed. The second column includes only those determinations done between March, 1984, at the beginning of the project, and March, 1987. The third column includes all determinations done after March, 1987. Data is expressed as mean ± standard deviation for each of the cell types examined.

FIGURE 10. COMPARISON OF Ku1a2 REACTIVITY IN PARENT U937 AND SUBLINES OVER A FOUR YEAR PERIOD



shown in Table 4. Although the U937 cells gave somewhat lower percent positive reactions than normal human monocytes, they are all clearly positive for MHC class I as indicated by results with both anti HLA-A,B,C (W6/32) and with anti-ß2 microglobulin, which although not encoded by the MHC is held in close association with the MHC class I molecule on the cell surface. Normal human monocytes are virtually all positive for anti HLA-A,B,C (95.4%) and for anti-ß2 microglobulin (96.3%). The U937 parent and sublines were not markedly different from each other in reactivity, although the G4 and D10 sublines were 82.1% and 95.4% positive with anti HLA-A,B,C whereas the 2-1 parent and sublines E11, G11 and E9 were approximately 60% positive. This slight hierarchy of expression was not confirmed by the results seen with the anti-ß2 microglobulin antibody results also shown in Table 4. Although the G4 subline showed a slightly higher percent positive cells than the 2-1 parent, the E11 or G11 sublines, it was not higher than the findings with the E9 subline. The differences seen in reactivity to anti MHC class I, therefore, probably are not significant and no further investigation was made of this point. The D10 subline was not tested with anti-ß2 microglobulin.

The 2-1 parent U937 and the sublines were tested with nine monoclonal antibodies characterized as specific for monocytes and macrophages. Although the antibodies used were originally characterized as specific for monocytes and macrophages, they are generally specific for mature monocyte-macrophage (MØ) lineage cells as well as some other cells as indicated in Table 3A. The U937 cells, both parent line and sublines, were generally found to be negative or marginally positive with antibodies specific for mature monocytes and macrophages as shown in Table 5. The normal human monocytes tested were positive with all MØ specific antibodies with which they were tested. The U937 cells and sublines were very slightly reactive with antibody M1, giving a range of positive reactions from 6.4% for subline E11 to 10.5% for subline E9 with values for all others falling between those values. Normal

monocytes were 59.8% positive for M1. Antibody MMA which has a broader reactivity than M1, i.e. monocytes and granulocytes rather than mature monocytes, was 64.0% reactive with normal human monocytes and 31.2% and 30.8% reactive with the 2-1 parent U937 and the E9 subline, respectively. Sublines G4 and G11 were weakly reactive, indicated by 18.2% and 15.2% positive cells, respectively, and the E11 subline was not reactive. Both Mac 1 and 63D3 antibodies were negative for all parent lines and sublines except the D10 subline which was marginally positive for both antibodies, 16.9% and 13.3%, but which was tested only once. Antibodies Mo 1, Mo 2 and OKM5 were all negative for both parent and subline U937 cells. Both My4 and My7 monoclonal antibodies which are specific for cells of myeloid lineage were positive for 2-1 parent cells and for the sublines. Reactivity to My7 ranged from a minimum of 50.6% positive for the E11 subline to a maximum of 69.5% positive for the 2-1 parent line. Normal human monocytes were found to be 72.4% positive for My7. There were more varied results with My4 which was 85.4% positive with normal human monocytes. The G4, G11 and E9 sublines and the 2-1 parent line were also clearly positive, although the relative reactivity varied from 56.0% for the G4 subline to 29.3% for the 2-1 parent cell line. The E11 subline was weakly positive.

Results obtained by immunofluorescence techniques using antibodies specific for surface structures characteristic of mature monocyte-macrophage lineage cells which were positive for normal monocytes were generally negative for both parent U937 cells and subline cells. However, antibodies specific for structures characteristic of immature monocytic or myeloid cells were found to be reactive with the U937 although reactivity was variable.

Three monoclonal antibodies specific for endothelial cells were also tested. As seen in Table 5, there was variable reactivity with monoclonal antibodies specific for endothelial antigens. Lowest reactivity was seen with mAb 3A1 (8.9%-15.9% positive). Reactivity to 2F11 was 23.1- 29.4% for the parent line and sublines E11,

TABLE 5. U937 SUBLINES AND PARENT CELL LINE EXPRESS SURFACE MOLECULES CHARACTERISTIC OF IMMATURE MONOCYTES AND ENDOTHELIAL CELLS

			Cells				
Antibody	Normal	Parent			Sublines		
Specificity	PBMØ	2-1	G4	E11	G11	E9	D10
anti-monocyte							
M1	59.8	9.9	8.5	6.4	9.4	10.5	5.3
MMA	64.0	31.2	18.2	4.9	15.2	30.8	4.4
Mac 1	32.0	5.3	4.9	3.0	3.3	4.1	16.9
63D3	n.d.	4.7	0.8	3.2	2.6	1.3	13.3
Mo1	59.4	3.5	5.1	1.7	2.7	3.7	n.d.
Mo 2	75.1	5.0	4.6	1.6	2.8	3.9	n.d.
My 4	85.4	29.3	56.0	13.8	40.8	32.3	n.d.
My 7	72.4	69.5	55.8	50.6	61.0	57.4	n.d.
OKM5	n.d.	0.7	2.0	1.0	0.5	6.0	n.d.
anti-endothelial cell							
2F11	n.d.	23.1	46.4	27.2	23.2	29.4	n.d.
2H10	n.d.	51.7	79.3	65.6	43.5	72.0	n.d.
3A1	n.d.	8.9	15.9	13.5	15.1	10.7	n.d.

G11, and E9. Subline G4 was approximately twice as positive at 46.4% positive cells. Of the three antibodies tested, antibody 2H10 was found to be the most reactive with U937 cells (43.5%-79.3% positive). Despite the variable reactivity with the three endothelial antibodies, the U937 parent cells and sublines were clearly positive with antibodies 2F11 and 2H10, and therefore express surface structures in common with endothelial cells.

U937 sublines and parent cell line were tested with a diverse panel of antibodies specific for T cells, B cells and other cell surface antigens. Table 6 indicates reactivities of U937 parent cells and sublines when examined by immunofluorescence using flow cytometry with antibodies with diverse specificities. As shown in Table 6, both 2-1 parent cells and all sublines were found to be strongly positive for antibodies TA-1, which is specific for both T lymphocytes and monocytes; OKT4 and KT69-7, which are specific for the CD4 molecule found on helper T cells and at low density on human monocytes; and KuFc79, which is specific for the human Fc receptor. U937 showed no reactivity with NK specific Leu 11A, antibody to IL-2 receptor, surface immunoglobulin (sIg), S-RBC receptor specific Leu 5, thymocyte specific OKT10 and MOPC 11 anti fibronectin control. Several antibodies showed weakly positive reactions with some of the U937 cells but not with others. OKT3, 3a1 and Leu 4 which are T lymphocyte specific, OKT 8 which is specific for the CD8 molecule on cytotoxic T lymphocytes and OKT9 which is specific for the transferrin receptor were found to be weakly reactive (<20% positive) with some but not all U937 parent and sublines. The G4 subline was the only subline found to be weakly reactive with OKT3, 3a1, Leu 4, OKT8 and OKT9.

MHC class II antigens expressed by U937 sublines were demonstrated by SDS-PAGE techniques. Cell surface proteins on the U937 parent line (2-1), *a second Ia* negative U937 parent line (H-K) and several sublines were radioiodinated by the

TABLE 6. U937 SUBLINES AND PARENT CELL LINE WERE TESTED WITH A DIVERSE PANEL OF ANTIBODIES SPECIFIC FOR T CELLS, B CELLS, NK CELLS AND OTHER SURFACE ANTIGENS

		Cells					
Antibody	Normal	Parent			Sublines		
Specificity	PBMØ	2-1	G4	E11	G11	E9	D10
Ta1	n.d.	94.9	79.1	80.3	87.1	85.8	86.5
OKT3	11.1	13.8	16.0	2.9	3.3	11.7	n.d.
OKT4	0.2	50.7	36.2	33.6	39.1	32.1	n.d.
KT69-7	46.6	45.2	47.4	41.9	27.5	32.8	32.3
OKT8	0.9	14.6	13.1	6.2	2.0	8.7	n.d.
OKT10	n.d.	1.7	6.6	6.8	n.d.	n.d.	10.8
3a1	n.d.	5.1	24.0	13.0	4.3	10.2	n.d.
Leu 4	3.4	7.8	12.4	5.3	16.4	18.5	n.d.
Leu 5	23.0	3.2	3.8	1.7	2.4	3.0	n.d.
sIg	n.d.	6.1	7.9	4.6	6.6	8.5	n.d.
Leu 11A	n.d.	0.3	0.6	n.d.	n.d.	n.d.	n.d.
KuFc79	86.9	60.7	87.1	76.0	69.3	62.1	n.d.
OKT9	4.5	8.6	13.8	3.9	25.2	7.8	23.1
IL-2R	0.1	0.1	0.5	0.1	0.3	0.1	n.d.
MOPC 11	1.4	1.4	3.3	0.7	1.7	2.5	n.d.

lactoperoxidase method, extracted and immunoprecipitated with a monoclonal antibody to a framework determinant of human HLA-DR (Kula2). As seen in Figure 11, the autoradiograph shows bands at molecular weights corresponding to 29,000 and 34,000 for sublines G4, E11, and D10, with highest intensity for the D10 subline. The 2-1 parent and the H-K parent U937 cell lines are negative for these bands. The molecular weights of the proteins immunoprecipitated by Kula2 on the sublines are consistent with molecular weights for the beta and alpha chains of the heterodimeric MHC class II molecules. Thus human MHC class II antigens are demonstrable both by immunoprecipitation of iodinated cell surface molecules and by immunofluorescence techniques.

Cell surface expression of MHC class II molecules by U937 sublines can be modulated by gamma interferon. The level of expression of MHC class II molecules by the sublines is increased by treatment with gamma interferon. Sublines were grown in the presence of 100 Units/ml. of purified (natural) gamma interferon for various times to examine the kinetics of the effects of gamma interferon on the expression of both surface Ia and expression of Fc receptor. After incubation for the prescribed time periods cells were stained with Kula2 (anti-human Ia) or with KuFc79 (anti-human Fc receptor) followed by incubation with FITC coupled $F(ab')_2$ fragment of antibody to mouse immunoglobulin. These cells were then examined by flow cytometry using the Ortho Spectrum III. The results can be seen in Figure 12. Time points examined were 0, 1, 6, 12, 24, 36, 48, and 72 hours. By 24 hours, the expression of cell surface Ia was maximal and only a slight increase was noted after 24 hours with subline E9. In all cases the levels of Ia expressed was markedly increased. The most dramatic increase was with subline E9 which was 22% positive without gamma interferon treatment and reached 70% positive cells by 48 hours. Sublines E11 *and* G11 showed an increase from 45-55% positive to 75-80% positive cells. Subline G4 was

FIGURE 11. CELL SURFACE EXPRESSION OF MHC CLASS II MOLECULES DEMONSTRATED ON SUBLINES OF U937 CELL LINE.

Cells from two U937 parent cell lines (lanes 1 and 5) and sublines G4, E11 and D10 (lanes 2, 3, & 4) were radioiodinated with ^{125}I by the lactoperoxidase method. Cell surface proteins were extracted and immunoprecipitated with Kula2 (mAb anti-human Ia). Following electrophoresis on a 10-16% polyacrylamide gel under reducing conditions, proteins having molecular weights characteristic of human MHC class II alpha (34K) and beta (29K) were demonstrated by autoradiography.

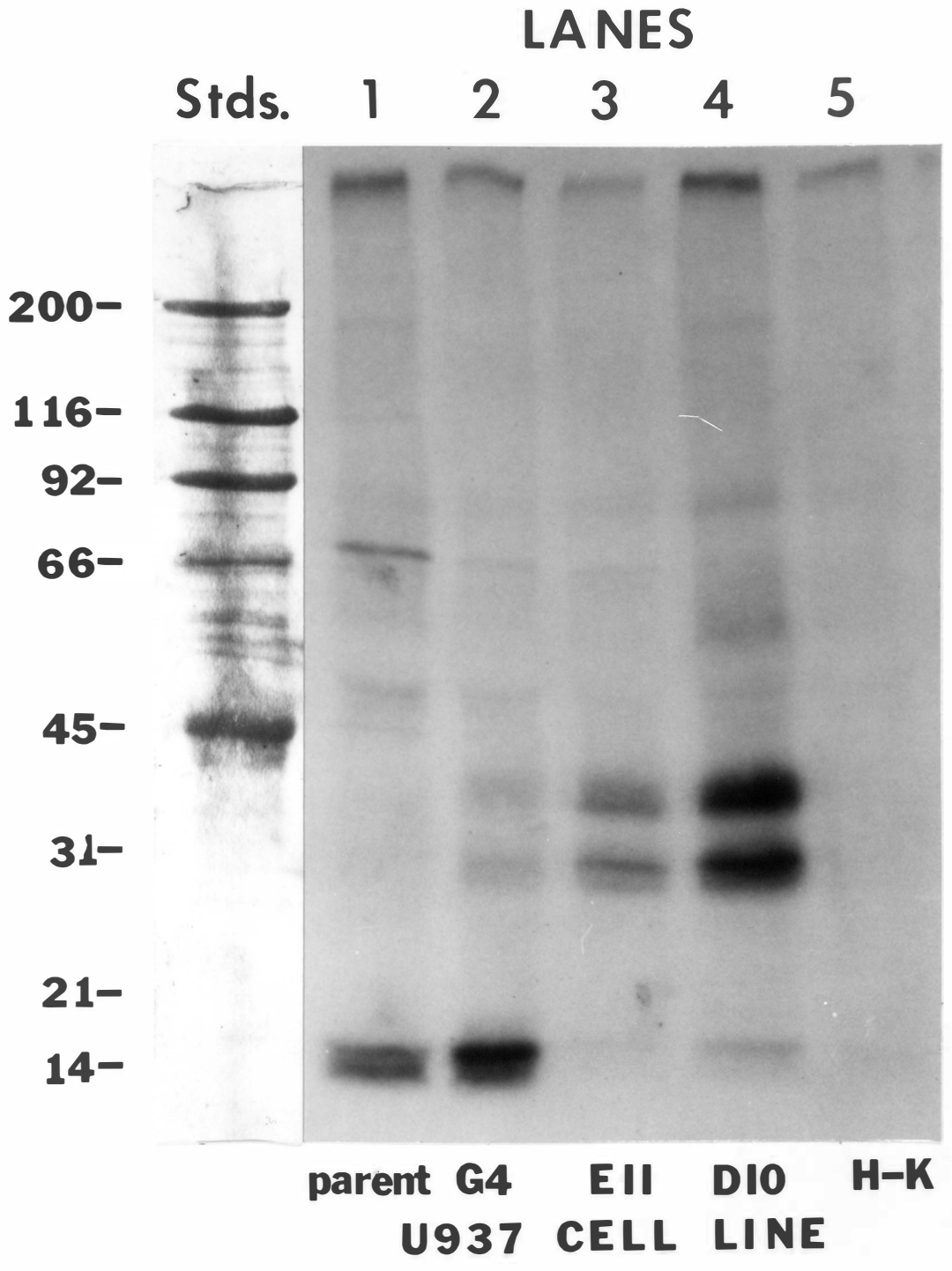
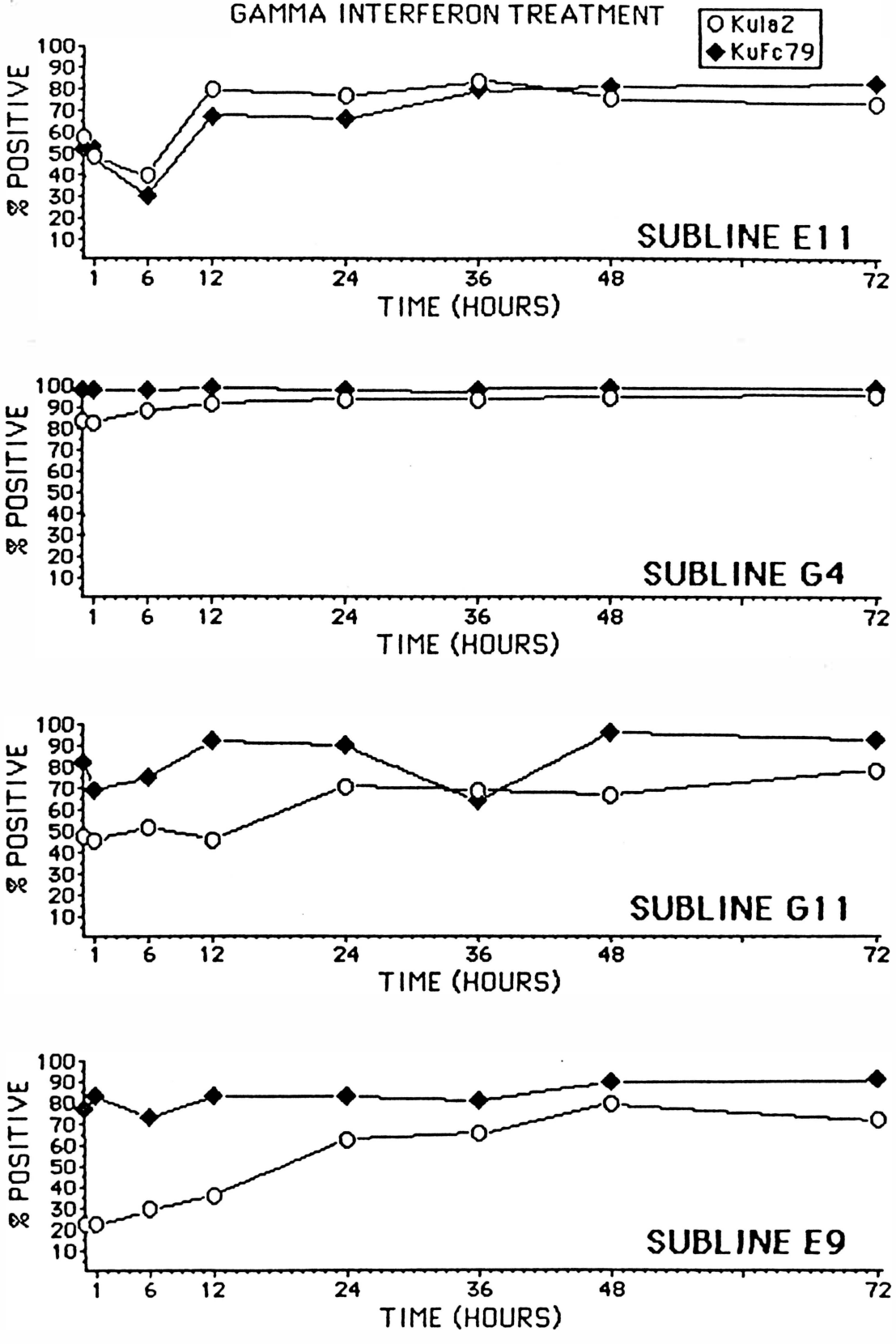

LANES
Stds.
1
2
3
4
5
200-
116-
92-
66-
45-
31-
21-
14-
parent G4
E11
D10
H-K
U937 CELL LINE

FIGURE 12. KINETICS OF INDUCTION OF EXPRESSION OF Ia AND FcR ON THE CELL SURFACE OF U937 SUBLINES AFTER GAMMA INTERFERON TREATMENT

Gamma interferon increases expression of Ia on U937 sublines and reaches maximal effect by 24-48 hours. Gamma interferon (100 U/ml.) was added to culture flasks containing U937 cells and cells were incubated for 0, 1, 6, 12, 24, 36, 48, 72 hours. Cells were harvested and stained with Kula2 (mAb anti-human Ia) or KuFc79 (mAb anti-Fc receptor) and examined by Ortho Spectrum III to determine percent Ia and FcR positive cells at each time point.

FIGURE 12. KINETICS OF EXPRESSION OF Ia AND FcR FOLLOWING GAMMA INTERFERON TREATMENT



strongly positive without gamma interferon treatment; however it became nearly 100% positive within 24 hours. In all cases the density of Ia expression measured as mean channel fluorescence also increased with gamma interferon treatment. Fc receptor expression was found to be slightly increased by gamma interferon treatment with similar kinetics. Several sublines already expressed Fc receptor at maximal levels (sublines G4 and E9) and were not induced to increase the percent of positive cells, although the mean channel fluorescence, i.e. FcR density, did increase slightly.

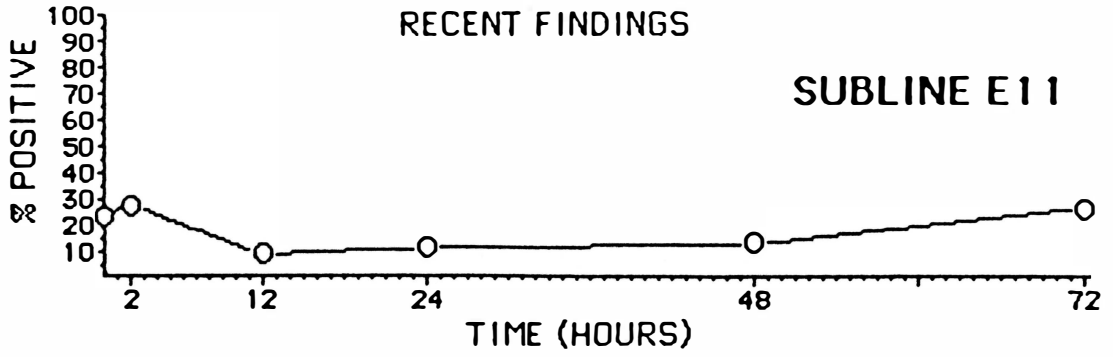
In more recent experiments using U937 sublines having decreased levels of constituitive MHC class II expression, treatment with gamma interferon increased the level of expression in all sublines except E11 as shown in Figure 13. Cells were treated as described above except that Fc receptor expression was not assayed. Time points at which Ia expression was measured were 0, 2, 12, 24, 48 and 72 hours. Initial levels of expression were much lower than in previously described experiments; however, the kinetics of induction of expression were similar as indicated by maximal levels of expression obtained at 24 hours for subline G4 and at 48 hours for sublines G11 and E9. The E11 subline, which in the previous experiments (Figure 12) appeared to decrease Ia expression as a primary result of gamma interferon treatment prior to demonstration of an enhanced Ia expression by 12 hours, again showed decreased Ia expression which was never succeeded by enhanced expression; and indeed, initial levels of Ia expression were not reached until 72 hours of gamma interferon treatment (Figure 13). Although not shown, treatment of the 2-1 parent line with gamma interferon increased Ia expression with kinetics similar to subline E9 beginning with an initial level of 11% and reaching 24% positive cells by 48 hours.

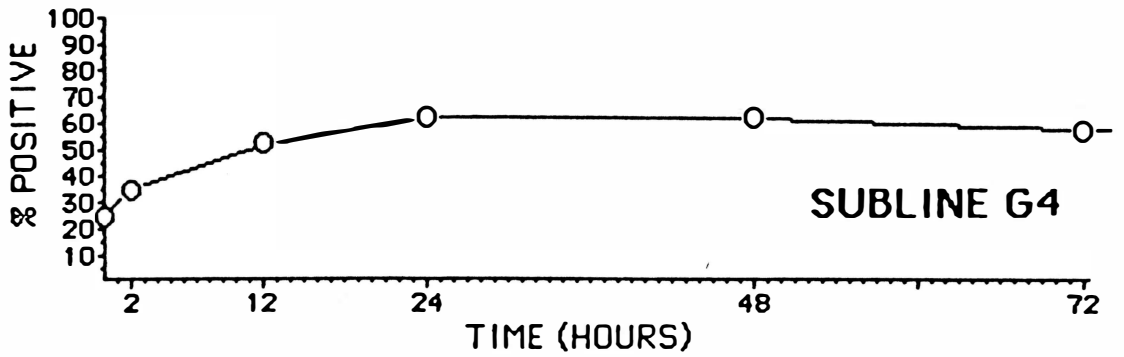
Functional capacity of the U937 cell line and sublines. The second aspect of the investigation of the U937 sublines involved the determination of their functional

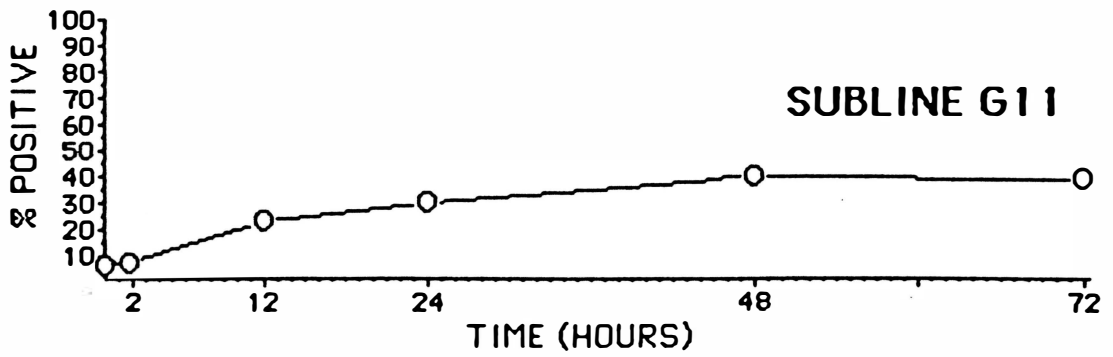
FIGURE 13. KINETICS OF EXPRESSION OF Ia FOLLOWING GAMMA INTERFERON TREATMENT ON U937 SUBLINES-RECENT FINDINGS

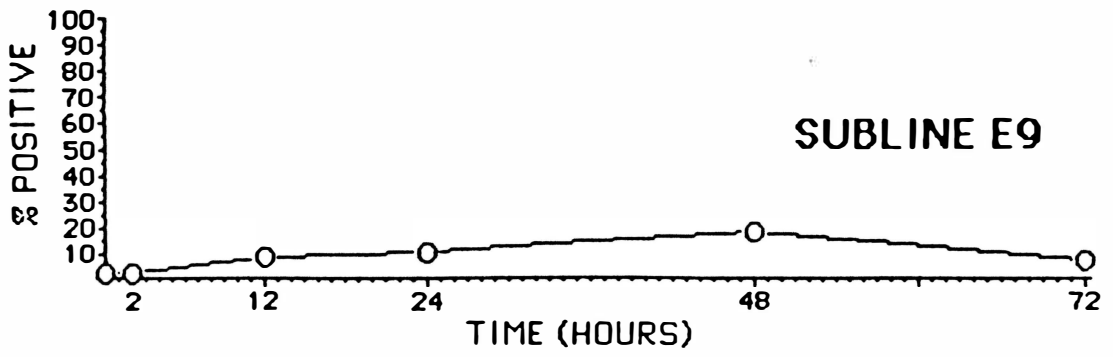
Gamma interferon increased expression of Ia on U937 sublines and reached maximal effect by 24-48 hours. Gamma interferon (100 U/ml.) was added to culture flasks containing U937 cells at 0, 2, 12, 24, 48 and 72 hours and cells were harvested and stained with Kula2 (mAb anti-human Ia) as described in Figure 12. Cells examined in this experiment were taken from culture in May, 1987.

FIGURE 13. KINETICS OF EXPRESSION OF Ia FOLLOWING GAMMA INTERFERON TREATMENT ON U937 SUBLINES
RECENT FINDINGS









capabilities. The sublines were shown to be able to mediate a response normally attributed to monocytes or accessory cells in a variety of cellular assays.

The Ia+ U937 cell line was able to stimulate a mixed lymphocyte response and to stimulate the generation of cytotoxic T cells. Stimulation of a mixed lymphocyte reaction was measured as ^{3}H-thymidine incorporation by responder cells in culture with U937 stimulator cells in a six day assay in microtiter plates. Generation of cytotoxic T cells was measured as cell mediated lympholysis and expressed as % Cytotoxicity of radiolabeled target cells, which were identical to the stimulator cells, in a six hour chromium release assay following a six day culture of responder and stimulator cells. Responder cells in both mixed lymphocyte reaction and cytotoxicity assays were comprised of peripheral blood mononuclear cells prepared from normal blood donors by ficoll hypaque techniques as previously described. U937 stimulator cells were treated with mitomycin C prior to addition to the assays unless otherwise indicated.

The U937 cells were able to stimulate a mixed lymphocyte response by several responders. Ia+ and Ia- parent U937 cells and several sublines were used as stimulators in mixed lymphocyte reactions to determine whether the expression of Ia would correlate with a change in functional capacity. The responders included cells from three different individuals. Responder cells 1 and 2 were isolated as previously described. Responder 3 and responder 2 are the same individual, however, cells used as responder 3 were kept in culture without stimulators for three days prior to the assay. Cells from responder 4 were isolated as previously described and subsequently monocytes were removed by counterflow elutriation prior to addition to the assay. The results are given in Figure 14. In general, it became apparent that cells which had been either cultured or elutriated were less reactive to U937 cells than freshly isolated cells. Stimulator A, which was the Ia+ parent cell line U937 cell line from which sublines G4 and E11 were isolated as indicated in Table 1, was found to be able to

FIGURE 14. Ia POSITIVE U937 CELL LINES AND SUBLINES STIMULATE A MIXED LYMPHOCYTE REACTION

Five U937 cell lines and sublines were tested as stimulators in mixed lymphocyte reactions with four responder cell populations. Responder cells (R1 and R2) were isolated from normal donor blood by ficoll-hypaque techniques as previously described. Responder 3 cells were isolated from responder 2 and isolated; however, the cells were kept in culture for 3 days prior to addition to the assay. Responder 4 cells were isolated from a normal individual as previously described; however, cells were further purified by counterflow elutriation and only the lymphocyte fraction was used in the assay. Stimulator U937 cells were treated with mitomycin C prior to addition to microtiter plates. The cells were incubated for six days, pulsed for the last 16 hours with ^{3}H-thymidine, harvested and counted. Results are expressed as cpm. Stimulator populations were as follows:

(A) U937 Ia+ parent cell line

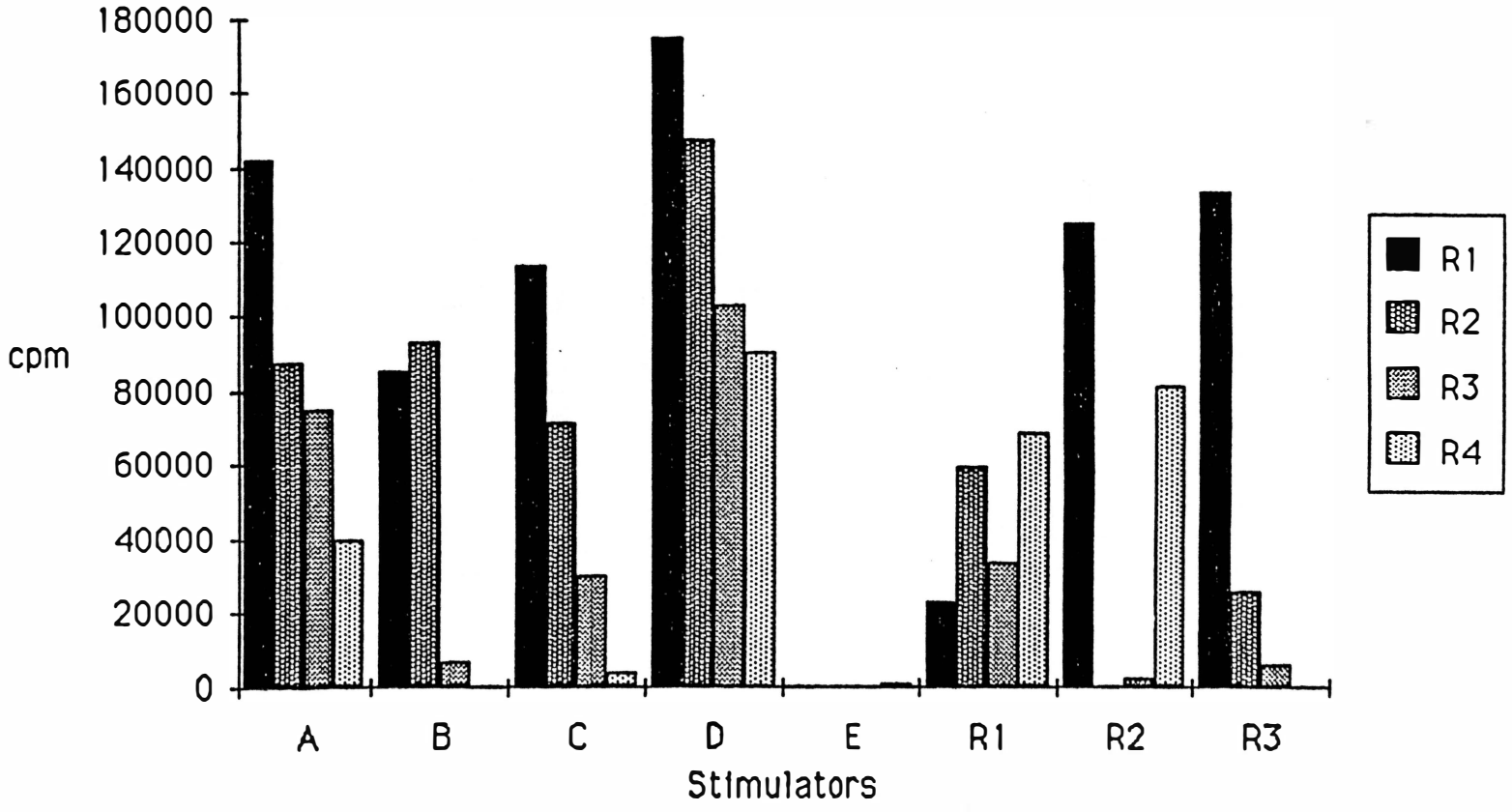
(B) U937 subline F7, Ia±

(C) U937 subline G4, Ia+

(D) U937 parent line, recently converted to Ia+

(E) U937 parent line, Ia-

FIGURE 14. Ia POSITIVE U937 CELL LINES AND SUBLINES STIMULATE A MIXED LYMPHOCYTE REACTION



stimulate a strong response ranging from 40,000 cpm with responder 4 to 140,000 cpm with responder 1. Stimulator B, which was a marginally Ia+ subline (F7) and which was not used beyond this experiment, gave strong stimulation to responders 1 and 2, but did not stimulate either the previously cultured cells or the elutriated cells. Stimulator C, the Ia+ G4 subline, also stimulated strongly with responders 1 and 2 but very little with responders 3 and 4. Stimulator D, which was an Ia+ parent U937 which had previously been an Ia- parent and rapidly converted to become an Ia+ parent cell line, was also a strong stimulator of all responders. Stimulator E, which was an Ia- parent U937 which remained Ia-, was found to lack the ability to stimulate any of the responders. The normal mixed lymphocyte reactions are shown with stimulators R1, R2 and R3. These preliminary results suggested a correlation between the expression of cell surface Ia on the U937 cell line and their ability to stimulate a mixed lymphocyte reaction.

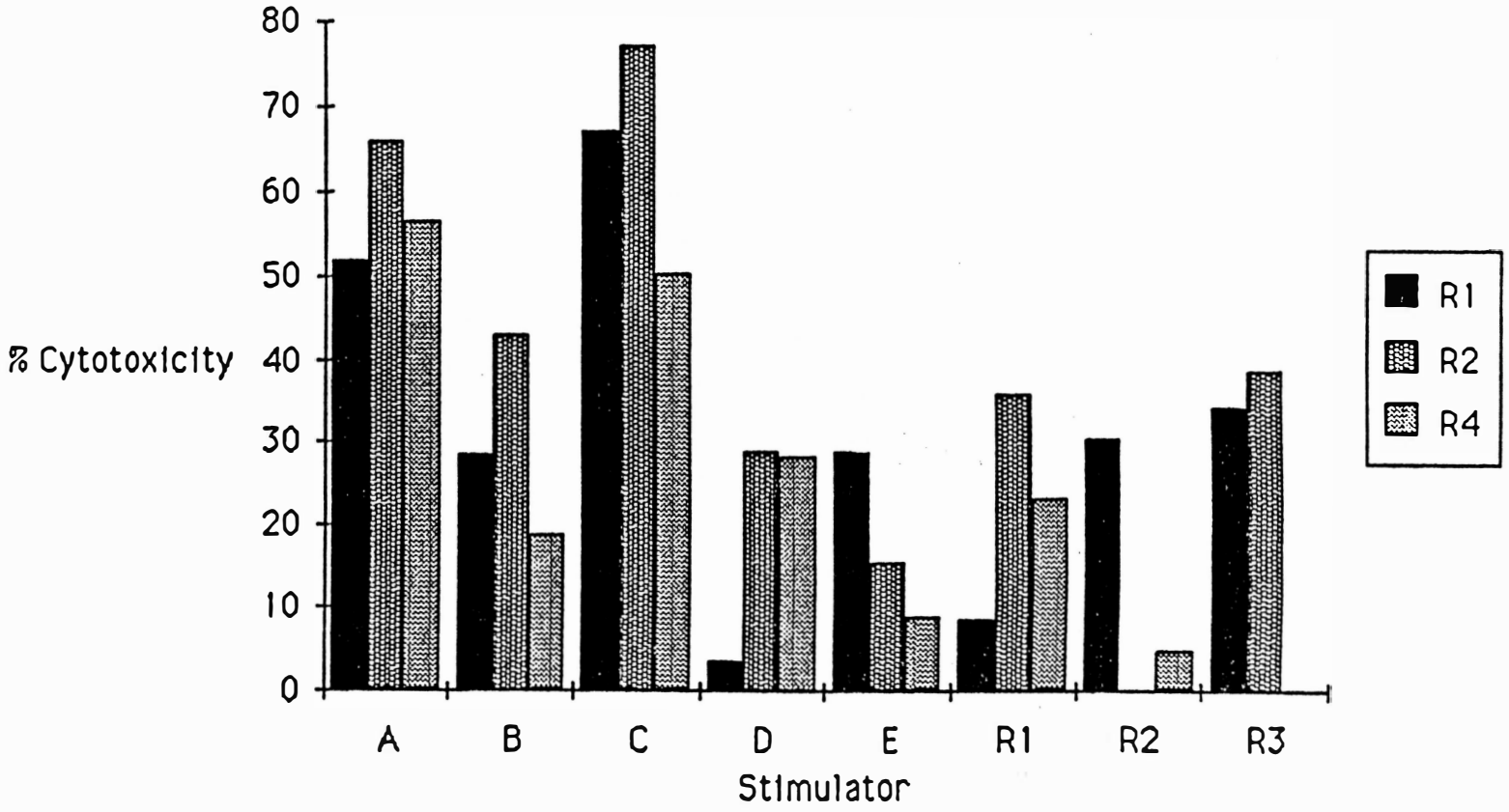
The U937 parent lines and the sublines tested were also found to be capable of generating specific cytolytic T cells. As shown in Figure 15, U937 Ia+ parent cell line (A) and the G4 subline were able to stimulate three different responders to greater than 50% cytotoxicity after a six day incubation. The other lines tested were capable of stimulating a smaller cytotoxic response as shown with stimulators B, D and E. Unlike the mixed lymphocyte reaction where no stimulation was noted, the Ia- parent line (E) was able to stimulate a small cytotoxic reaction.

From these results we concluded that the Ia+ U937 cell line was capable of stimulating a mixed lymphocyte reaction and also stimulating the generation of specific cytolytic T cells. At this point in time, the isolation and characterization of sublines or clones of Ia+ and Ia- U937 cells was undertaken for the dual purpose of (1) characterizing phenotypically (described above) and functionally the two types of cells and (2) discerning the role of the expressed Ia and the nature of the Ia expressed.

FIGURE 15. BOTH Ia POSITIVE AND Ia NEGATIVE U937 CELL LINES AND SUBLINES STIMULATE THE GENERATION OF CYTOLYTIC T CELLS

Responder cells from three normal donors (R1, R2 & R4) were isolated as described in Figure 14. Stimulator U937 cells were also the same as those described in Figure 14. Cells were incubated together for six days followed by a six hour incubation with stimulator-identical, ^{51}chromium-labeled target cells. Supernatants were counted for chromium released. Results are expressed as % Cytotoxicity and calculated as previously described.

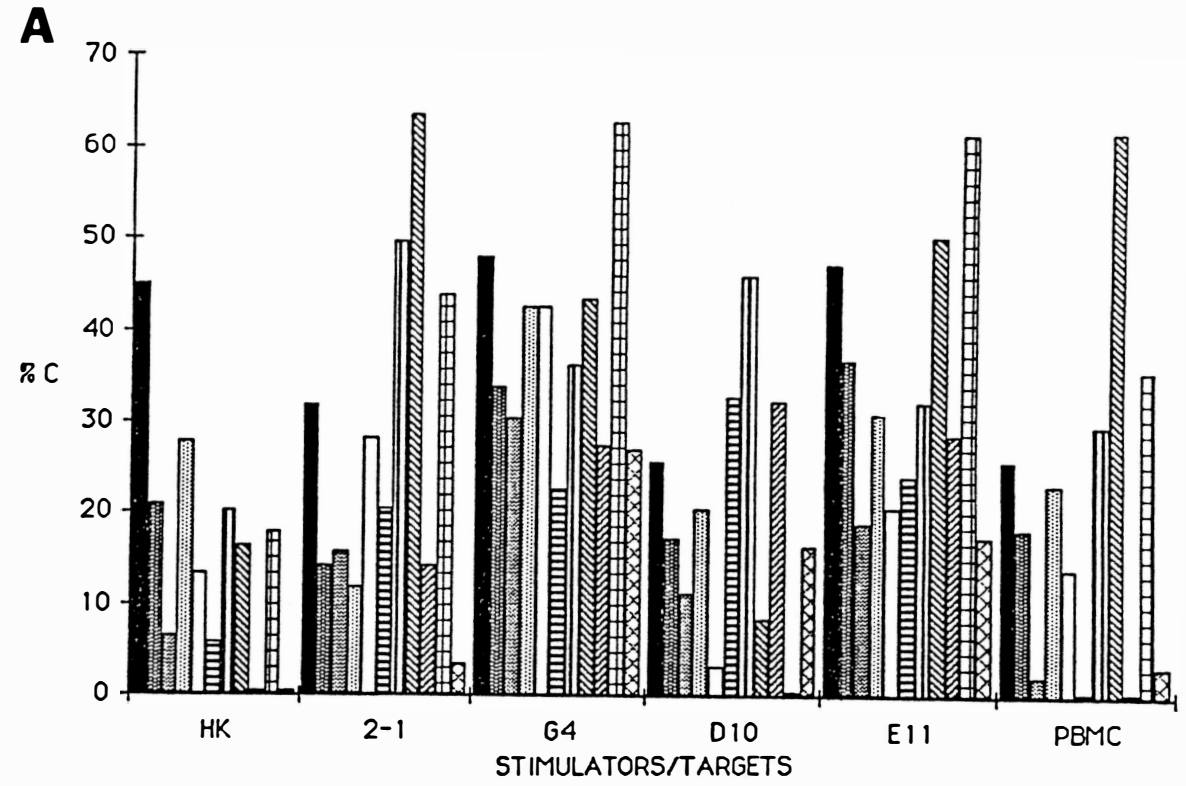
FIGURE 15. BOTH Ia POSITIVE AND NEGATIVE U937 CELL LINES AND SUBLINES STIMULATE THE GENERATION OF CYTOLYTIC T CELLS

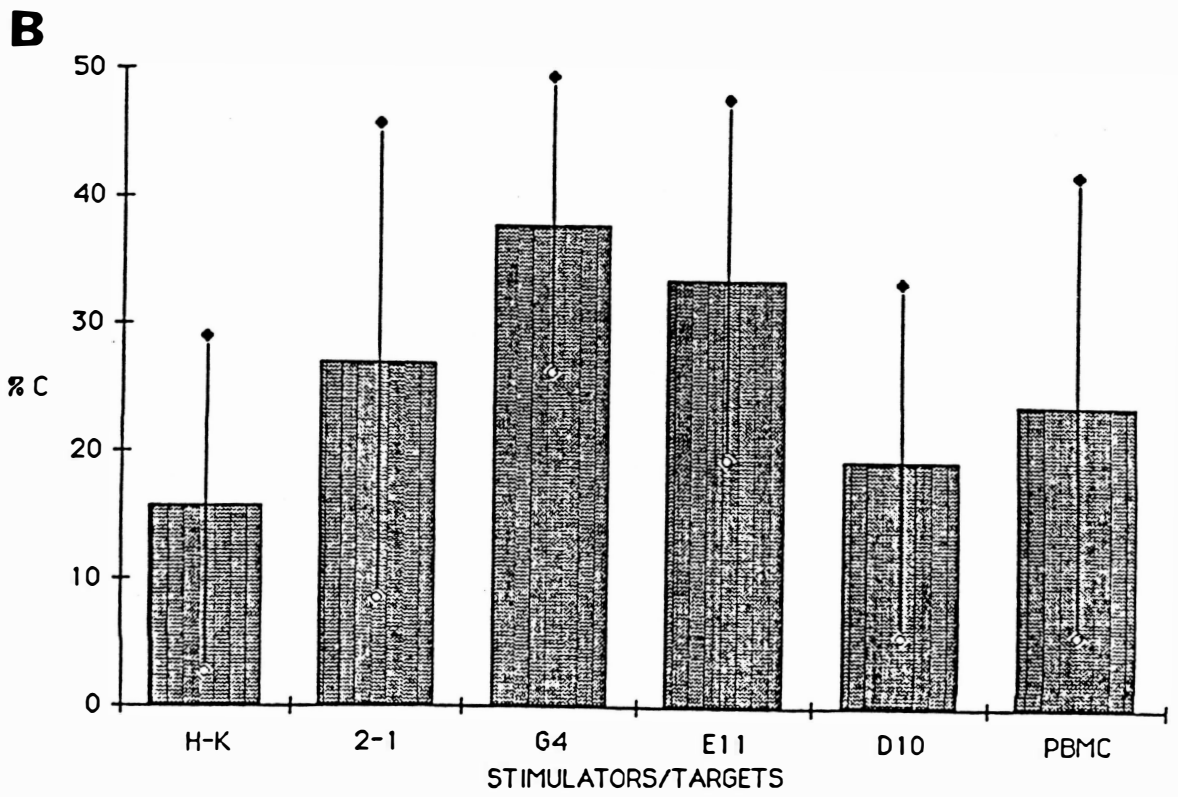


The U937 sublines were capable of stimulating the generation of cytolytic T cells. Figure 16 represents a compilation of several CML experiments in which the U937 parent line and sublines served as stimulator cells with eleven normal responders. U937 stimulators were incubated with normal peripheral blood mononuclear cell responders for six days followed by addition of ^{51}Cr-labeled target cells in a six hour chromium release assay as previously described. There was considerable variability in % Cytotoxicity from one responder to another as shown in Figure 16A; however, the CML response stimulated by U937 parent and subline cells is comparable to the response seen when normal PBMC were used as stimulators. To compare the different parent lines and sublines shown in Figure 16A, the mean and standard deviations were determined for the responses with each stimulator as shown in Figure 16B. The standard deviations indicate the high degree of variability in CTL generation among responders; however, all parent and subline U937 cells were capable of stimulating the generation of a CML response comparable to the normal response in both magnitude and variability. Figure 16A indicates a more consistent CML response by both G4 and E11 sublines than the other sublines or parents and considerably more consistant CML than normal PBMC stimulated CML responses. Differences in stimulatory capacity in the CML response may correlate with the level of expression of MHC class I on the cell surface of the stimuating cells, although as indicated in Table 4, the cell surface expression of MHC class I as measured by anti-HLA-A,B,C and by anti-ß2 microglobulin antibodies is higher for the G4 subline than for the other U937 cell lines, that is not found to be true for the E11 subline which was shown to be less positive for MHC class I than other sublines. The D10 subline which was very strongly positive for MHC class I as measured by anti-HLA- A,B,C appears to be a less efficient stimulator than either subline E11 or the 2-1 parent. This finding may actually reflect an enhanced resistance of the D10 subline targets to be *lysed by the CTL*'s generated. In the experiments summarized in Figures 16A and 16B, the % Cytotoxicity

FIGURE 16. U937 PARENT AND SUBLINES STIMULATED THE GENERATION OF SPECIFIC CYTOLYTIC T CELLS IN CML ASSAYS.

U937 parent cell lines (2-1 and HK), sublines (G4, D10 and E11) and normal peripheral blood mononuclear cells (PBMC) were added as stimulator cells to allogeneic peripheral blood mononuclear cells in CML assays. The results were variable as indicated. Stimulators (1 X 10^5 cells/well) were incubated with responder cells (1 X 10^5 cells/well) for six days followed by a six hour incubation with stimulator-identical ^{51}Cr-labeled target cells. Results are expressed as % cytotoxicity (% C). Individual responses with eleven different responders (A) showed marked variability for U937 stimulators as well as normal PBMC stimulators. Responses shown in (A) were compiled (B) and the data is presented as mean ± s.d.


A
70
60
50
40
30
20
10
0
% C
HK
2-1
G4
D10
E11
PBMC
STIMULATORS/TARGETS


B
50
40
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20
10
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% C
H-K
2-1
G4
E11
D10
PBMC
STIMULATORS/TARGETS

is based on cytotoxicity of stimulator-identical targets rather than a single type of labeled U937 target. In other experiments, the D10 subline was found to be a potent stimulator.

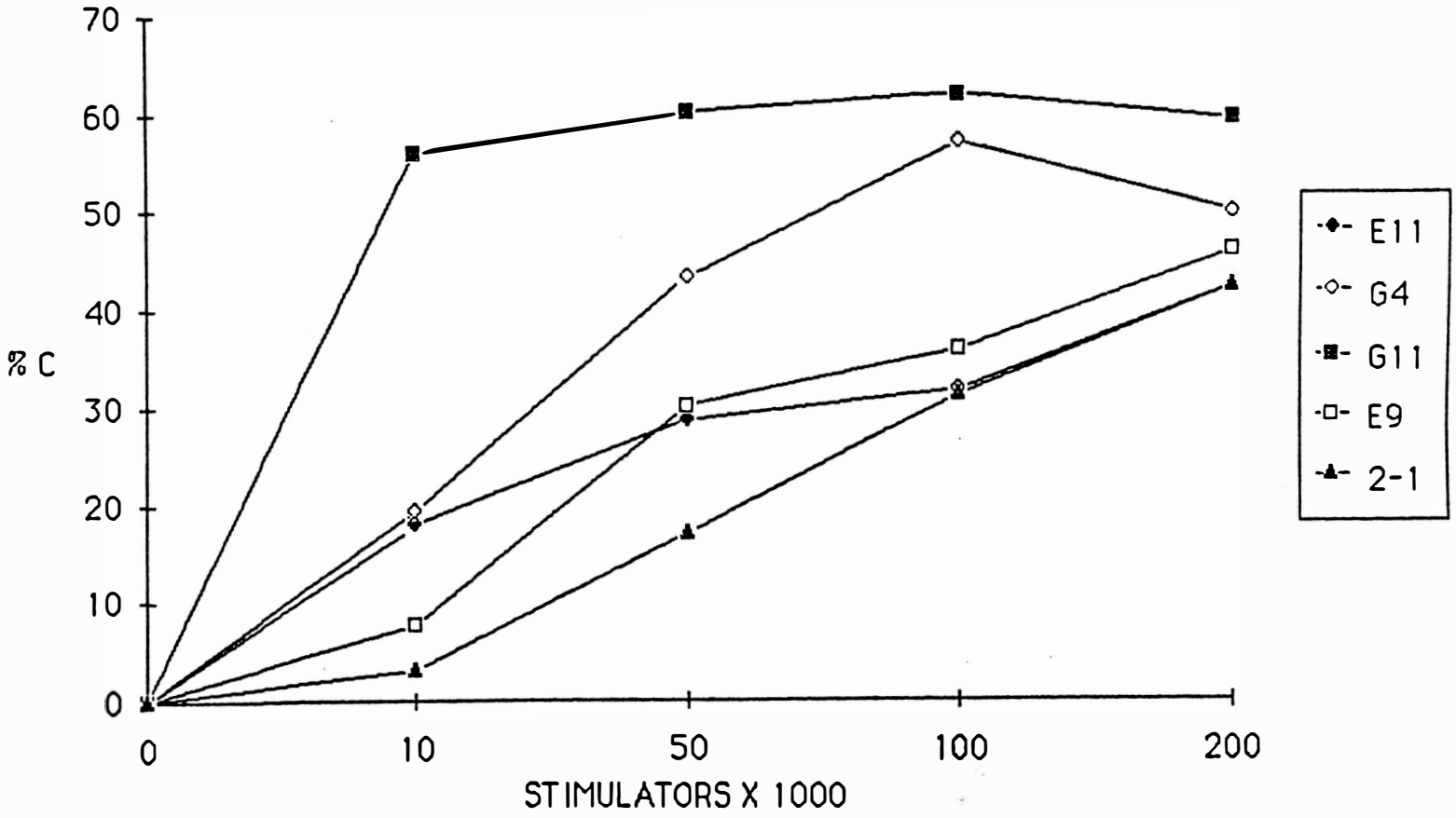
In order to further examine the capacity of the U937 cells to mediate a CML response, the effect of different doses of stimulators was examined. As shown in Figure 17, the magnitude of a CML response generated by the U937 sublines and 2-1 parent line was dose dependent. Also illustrated in Figure 17 is the CML response generated when sublines G11 and E9 were utilized as stimulators. As indicated the G11 subline was found to be a strong stimulator in CML assays even at cell doses as low as 5×10^3 cells/well. Routinely the stimulator cell dose was 1×10^5 cells/well and at that dose the G4 and G11 sublines were somewhat stronger stimulators than the other sublines and parent 2-1 cell line; however, at the highest cell dose all sublines stimulated to approximately the same degree.

U937 sublines were able to stimulate a mixed lymphocyte reaction. As previously indicated in Figure 14, the Ia+ U937 cell line and the Ia+ G4 subline were capable of stimulating a mixed lymphocyte reaction in early experiments. The generation of a mixed lymphocyte reaction was deemed to be an important finding for the Ia+ U937 cells because its generation is a function of the expression of MHC class II molecules. Because the Ia- U937 cells were not found to be capable of stimulating an MLR, the Ia+ U937 sublines were seen as a potentially useful tool for the elucidation of the role of MHC on monocyte-like cells. Mixed lymphocyte experiments were set up frequently over the whole course of the project and the results continued to be somewhat problematic in that in repeated MLR assays using a relatively small pool of normal responders, the results have been unpredicatable. Stimulation of an MLR by the Ia+ sublines E11 and G4 were more frequent than by Ia negative sublines G11 and E9 and the 2-1 and H-K parent cell lines; however, using the same responders rarely

FIGURE 17. U937 PARENT AND SUBLINES STIMULATED THE GENERATION OF SPECIFIC CYTOLYTIC T CELLS IN CML ASSAYS IN A DOSE DEPENDENT MANNER.

U937 parent 2-1 and sublines E11, G4, G11 and E9 were added as stimulator cells at four cell doses(1 X 10^4 cells/well, 5 X 10^4 cells/well, 1 X 10^5 cells/well, and 2 X 10^5 cells/well) to normal PBMC (1 X 10^5 cells/well) in a six day assay as previously described. Results are expressed as average % cytotoxicity (%C) based on release of ^{51}Cr from stimulator-identical target cells in triplicate wells in three experiments.

FIGURE 17. CML DOSE RESPONSE
AVERAGE % CYTOTOXICITY



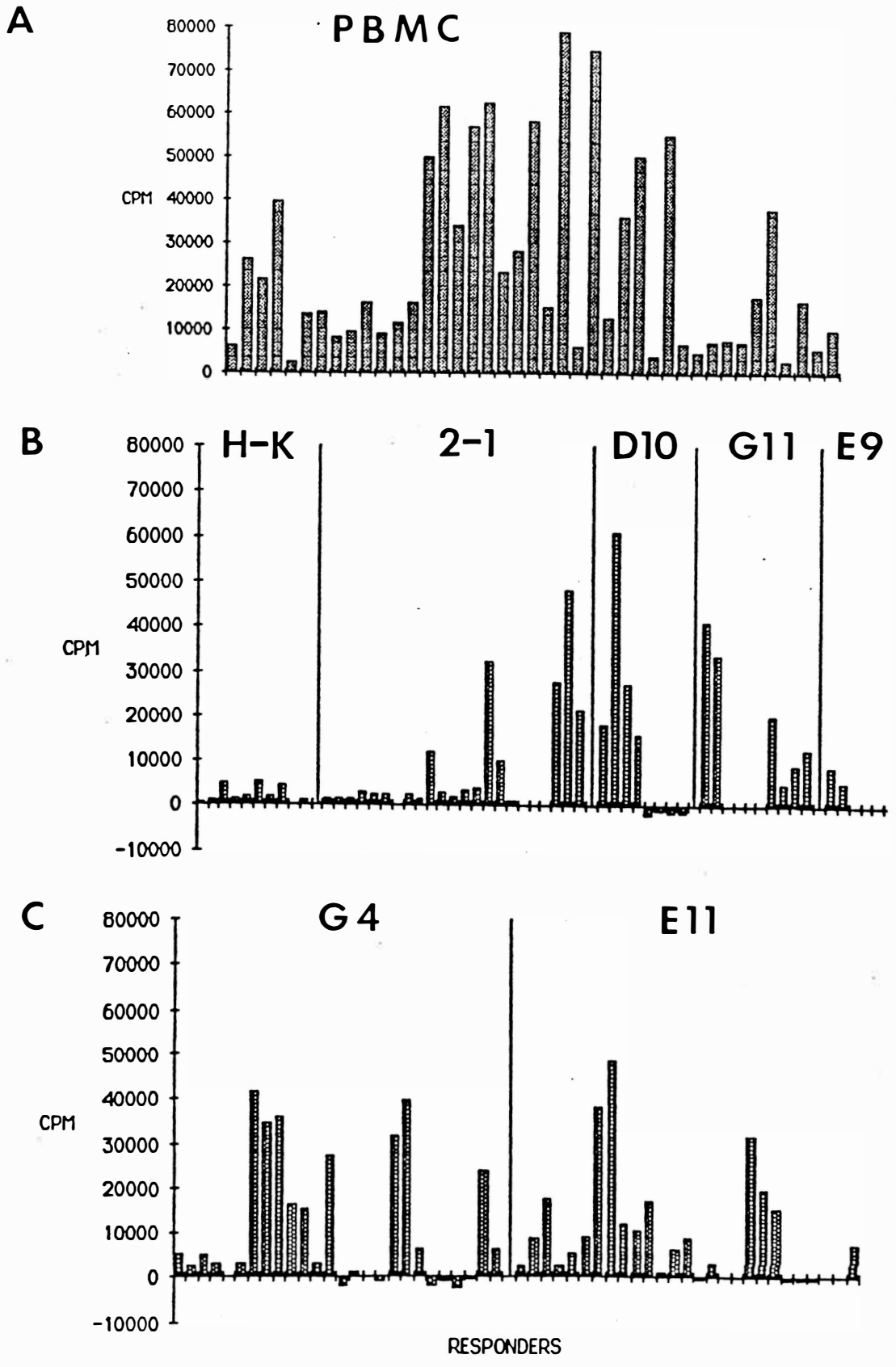
were the same results obtained in successive experiments and as the G11 subline became strongly Ia+ it too began to stimulate a strong MLR.

As indicated in Figure 18A in which normal responders cells were stimulated by normal PBMC stimulator cells, there is marked variability in the response. The results are expressed simply as cpm to indicate the degree of ^{3}H-thymidine incorporation by the responder cells in a six day assay. The magnitude of the response is related to two factors: disparity of MHC class II molecules expressed on responders and stimulators and inherent responsiveness of individuals used as responders. The most obvious responses are those greater than 20,000 cpm; however, there are many positive responses less than 20,000 cpm and indeed less than 10,000 cpm which were deemed to be positive responses because they were greater than three standard deviations above the negative control values.

A compilation of MLR responses for the U937 parent cell lines H-K and 2-1 and sublines D10, G11 and E9 is illustrated in Figure 18B. The H-K parent line never stimulated a strong MLR response, although with at least three individials a response in excess of 5000 cpm was obtained. The 2-1 parent line proved to be a variable stimulator in MLR assays. In the earliest assays the response was generally very low. Recent MLR findings with parent 2-1 indicate two patterns: strong stimulation or no stimulation with few values in between. The D10 subline was used only in the first several MLR assays, and this subline showed a pattern similar to that exhibited by the 2-1 parent line in that there was either strong stimulation or no stimulation, indeed, ^{3}H-thymidine incorporation measured as cpm fell below that of negative controls. The G11 subline also gave variable results in MLR assays. The E9 subline never gave strong stimulation; however, this subline was utilized as a stimulator in relatively few MLR assays, thus it may be coincidental that it appears to be a poor stimulator. The G11 subline, although originally isolated as an Ia- subline, became *rapidly Ia+ and* was probably Ia+ in all of the MLR assays in which it was added as a stimulator. Therefore,

FIGURE 18. U937 SUBLINES STIMULATED A MIXED LYMPHOCYTE REACTION.

Normal PBMC responders (1 X 10^5 cells/well) were incubated with allogeneic PBMC stimulators (A) or U937 parent or subline stimulators (B & C) (1 X 10^5 cells/well) as described. Individual responses are indicated as the mean of ^3H-thymidine incorporation of triplicate wells expressed as cpm.


A
PBMC
80000
70000
60000
50000
CPM 40000
30000
20000
10000
0
B
H-K
2-1
D10
G11
E9
80000
70000
60000
50000
40000
CPM
30000
20000
10000
0
-10000
C
G4
E11
80000
70000
60000
50000
40000
CPM
30000
20000
10000
0
-10000
RESPONDERS

lack of expression of MHC class II molecules does not explain the negative results seen in approximately half of the MLR assays in which G11 subline cells were added as stimulators.

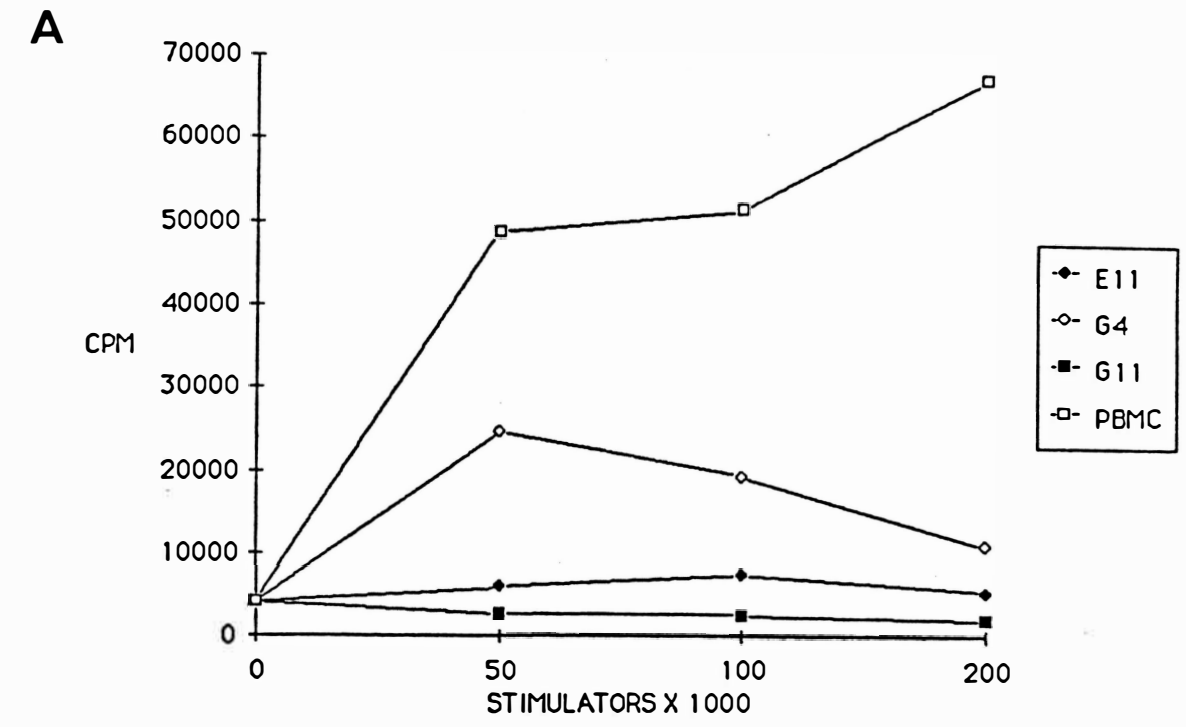
Shown in Figure 18C are the compiled MLR results for sublines G4 and E11 in which there are many strong responses. Alternating with the strong positive responses, however, are very negative responses in which the net cpm is negative. Both sublines express MHC class II molecules constituitively and would therefore be expected to stimulate an MLR. The negative results can not be explained by concurrence of HLA-DR antigens on both responders and stimulators and in many cases the same responders were found to give strong responses in some experiments and very negative responses in other experiments.

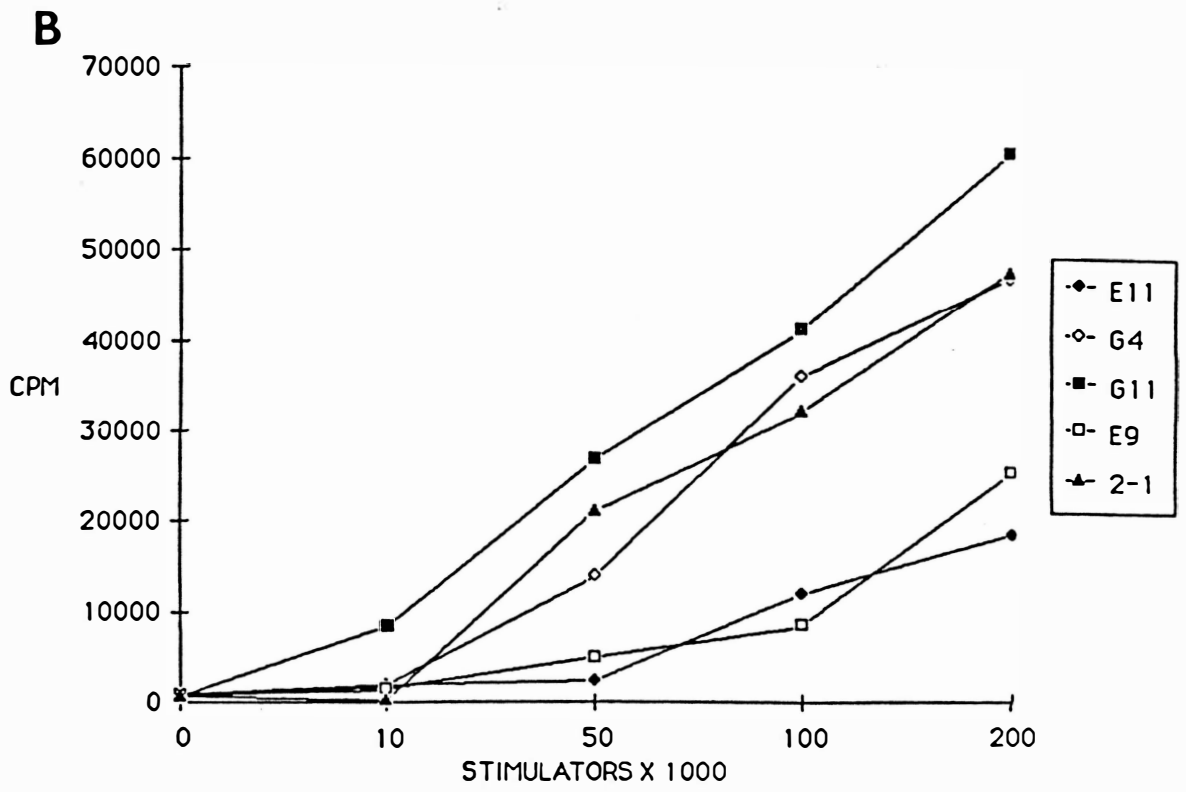
In general, if the MLR response was very low with one responder in a single assay using a particular U937 stimulator, all responses were low using that same preparation of stimulator cells. Although the earliest MLR responses were suggestive of MHC class II restriction phenomena, that notion was rapidly dismissed by the inconsistancy in reaction with the same pool of responders. Although the Ia+ sublines were generally more stimulatory in MLR assays than the Ia- sublines or parent cell line, the results were variable and did not correlate with Ia expression on the U937 sublines or Ia disparity between responders and U937 stimulators. The generation of a positive response might well require that MHC class II molecules be present; however, *it did* not insure that a response would be generated.

The effect of stimulator dose on the generation of an MLR was examined. The propensity of macrophages to act in a stimulatory capacity at low concentrations and in an inhibitory capacity at slightly higher concentrations was well known. It therefore became necessary to examine the effect of stimulator cell dose on the generation of a response. The standard stimulator cell dose used in the MLR assays was 1×10^5 cells/well. Figures 19A and 19B represent two patterns which emerged from these

FIGURE 19. U937 STIMULATOR DOSE RESPONSE WAS VARIABLE IN MIXED LYMPHOCYTE REACTIONS.

Representative experiments (A & B) exemplify the two types of response encountered in MLR in which U937 parent and subline cells were added as stimulators at several doses to normal PBMC (1 X 10^5 cells/well) in a six day assay. Results are expressed as the mean of triplicate wells. In panel A, a maximum response with normal allogeneic PBMC stimulators was obtained at the maximum stimulator cell dose (2 X 10^5 cells/well); however, U937 subline G4 stimulated a MLR response at the minimum cell dose examined (5 X 10^4 cells/well) and the stimulation decreased at increasing cell doses. In panel B, a positive dose response was noted for all U937 parent and sublines tested at increasing cell doses.


A
70000
60000
50000
40000
30000
20000
10000
0
CPM
0
50
100
200
STIMULATORS X 1000
E11
G4
G11
PBMC


B
70000
60000
50000
40000
30000
20000
10000
0
CPM
0
10
50
100
200
STIMULATORS X 1000
E11
G4
G11
E9
2-1

experiments. Both higher and lower doses of stimulators were added to the MLR assay to assess the effect of stimulator dose. The first pattern as shown in Figure 19A shows maximal stimulation at low cell dose, in this case only with the G4 subline. The E11 and G11 sublines showed no capacity to stimulate in this assay and with the G11 subline in particular, as higher doses of cells were added to the assay, the net cpm decreased below the negative control. The G4 subline stimulated well at a dose of 0.5 X 10^5 and the stimulation decreased with each successively higher dose. Although there continued to be a positive MLR at 2 X 10^5 cells/well, it is significantly lower than seen with 0.5 X 10^5 cells/well. The normal PBMC generated MLR is shown for comparison. As indicated by the normal MLR in this experiment, the responder cells used were capable of exhibiting a strong response.

The second pattern seen in response to increasing doses of stimulator U937 cells is shown in Figure 19B in which ^{3}H-thymidine incorporation increases in a dose dependent fashion from the lowest stimulator dose of 0.1 X 10^5 cells/well to the highest dose of 2 X 10^5 cells/well. Although all U937 stimulators ellicit a response at doses of 0.5 X 10^5 cells/well and above, the 2-1 parent cell line and the G4 and G11 sublines ellicit a slightly higher response than either the E11 or E9 sublines.

From the preceeding experimental data it seemed apparent that the 2-1 parent U937 cells and sublines E11, G4, G11 and E9 were capable of stimulating the generation of an MLR to some degree and that this stimulatory capacity might be dependent on the level of expression of MHC class II molecules if some other inhibitory capacity could be explained and eliminated.

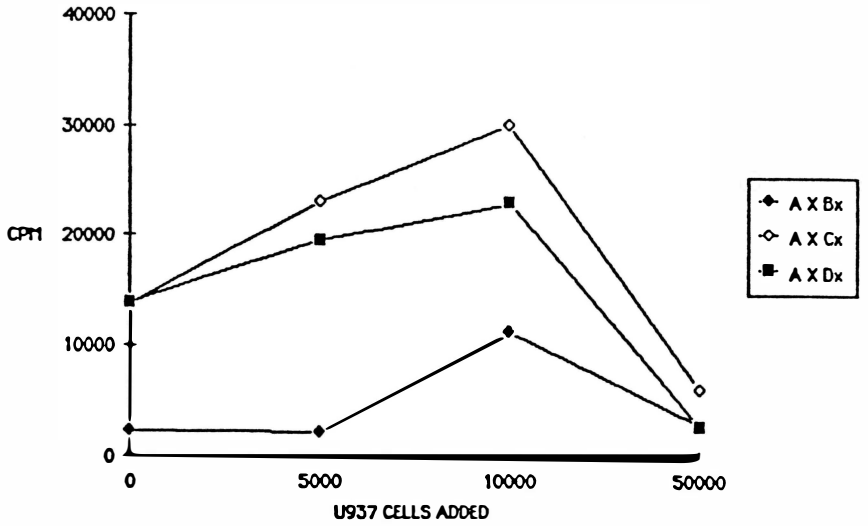
Inconsistency in the MLR response was attributed to an inhibitory capacity inherent in the U937 cell line. In order to investigate the nature of the inhibitory capacity exhibited by these cells experiments were conducted in which U937 sublines were treated with mitomycin C as they were for use as stimulator cells and subsequently added in different doses to a normal MLR reaction between HLA-DR

mismatched individuals in an assay in which T cell proliferation would be stimulated to ascertain whether the inhibitory effect could be mediated by the cells (or their products while in culture) or whether the addition of U937 subline cells would costimulate producing an additive effect on ^{3}H-thymidine incorporation. The results are shown in Figure 20. When no U937 cells were added, the MLR reaction produced by one of the three combinations in which the same responder PBMC were used with three different HLA mismatched stimulator PBMC populations was very weak (2200 cpm) whereas the MLR reaction for the second and third combinations were 13600 cpm and 13900 cpm, respectively. When E11 subline cells were added to this MLR there was augmented ^{3}H-thymidine incorporation at low E11 cell dose. When 5000 E11 cells were added to the stimulated combinations (A X Cx and A X Dx), there was significant increase in the cpm. When 10,000 E11 cells were added, ^{3}H-thymidine incorporation for all three combinations was increased; however, at high cell dose the E11 cells added were clearly inhibitory and reduced ^{3}H-thymidine incorporation to original MLR levels for [A X B_x] and below baseline MLR levels for the other combinations. In Figure 20B, G4 subline cells were added to these same MLR cell combinations. In this case, addition to [A X B_x] and [A X C_x] caused a transient increase in ^{3}H-thymidine incorporation when 5 X 10^3 U937 cells were added followed by a dramatic inhibition of the normal MLC at 1 X 10^4 and 5 X 10^4 added U937 cells. When G4 subline cells were added to [A X D_x], all doses of G4 cells were markedly inhibitory, although the 1 X 10^4 dose was not more inhibitory than 5 X 10^3 U937 cells. In Figure 20C, the addition of G11 subline cells was stimulatory at all doses added. The finding that the addition of G11 subline cells at all concentrations was stimulatory appears to rule out the possibility that addition of moderate to high doses of U937 cells to wells already containing reactive responder and stimulator cells inhibit MLR by mechanical means by sheer weight or senescence due to crowding or use of available nutrients. Thus the inhibitory effects are mediated either by the cells themselves possibly

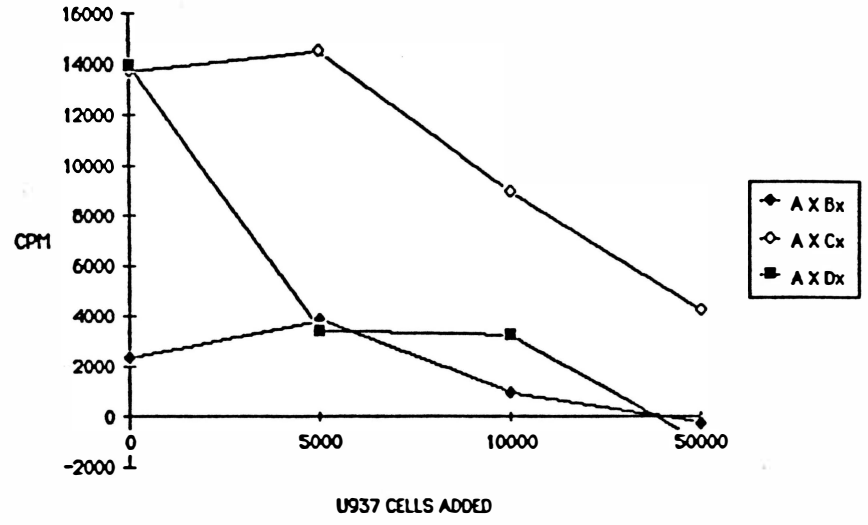
FIGURE 20. SUPPRESSIVE ACTIVITY OF U937 CELLS WAS INVESTIGATED BY ADDITION OF U937 SUBLINE CELLS TO A NORMAL MIXED LYMPHOCYTE REACTION.

MLR assays were set up between three normal PBMC responders and three normal PBMC stimulators (1 X 10^5 cells/well for each). Several doses of U937 subline E11 cells (A), subline G4 cells (B), and subline G11 cells (C) were added to each combination (5 X 10^3, 1 X 10^4, 5 X 10^4 U937 cells added/well). Results are expressed as the mean of ^{3}H-thymidine incorporation as cpm in triplicate wells.

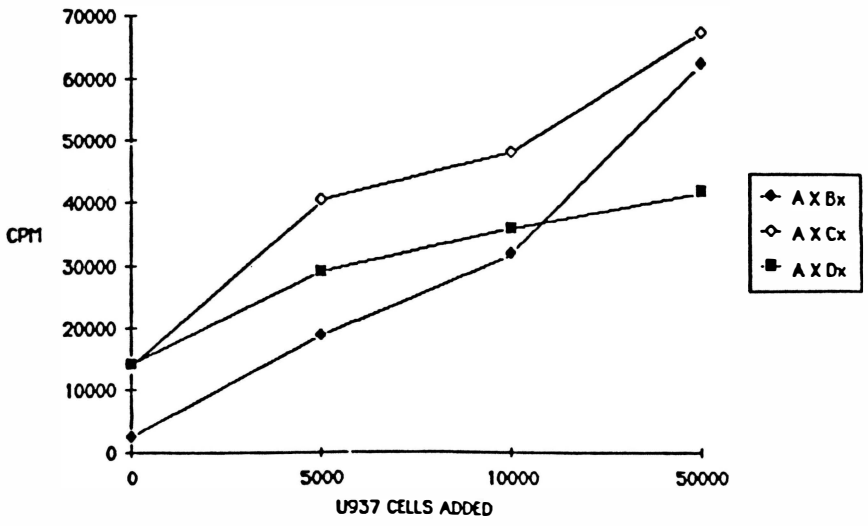
A


40000
30000
20000
10000
0
CPM
0
5000
10000
50000
U937 CELLS ADDED
A X Bx
A X Cx
A X Dx

B


16000
14000
12000
10000
8000
6000
4000
2000
0
-2000
CPM
0
5000
10000
50000
U937 CELLS ADDED
A X Bx
A X Cx
A X Dx

C


70000
60000
50000
40000
30000
20000
10000
0
CPM
0
5000
10000
50000
U937 CELLS ADDED
A X Bx
A X Cx
A X Dx

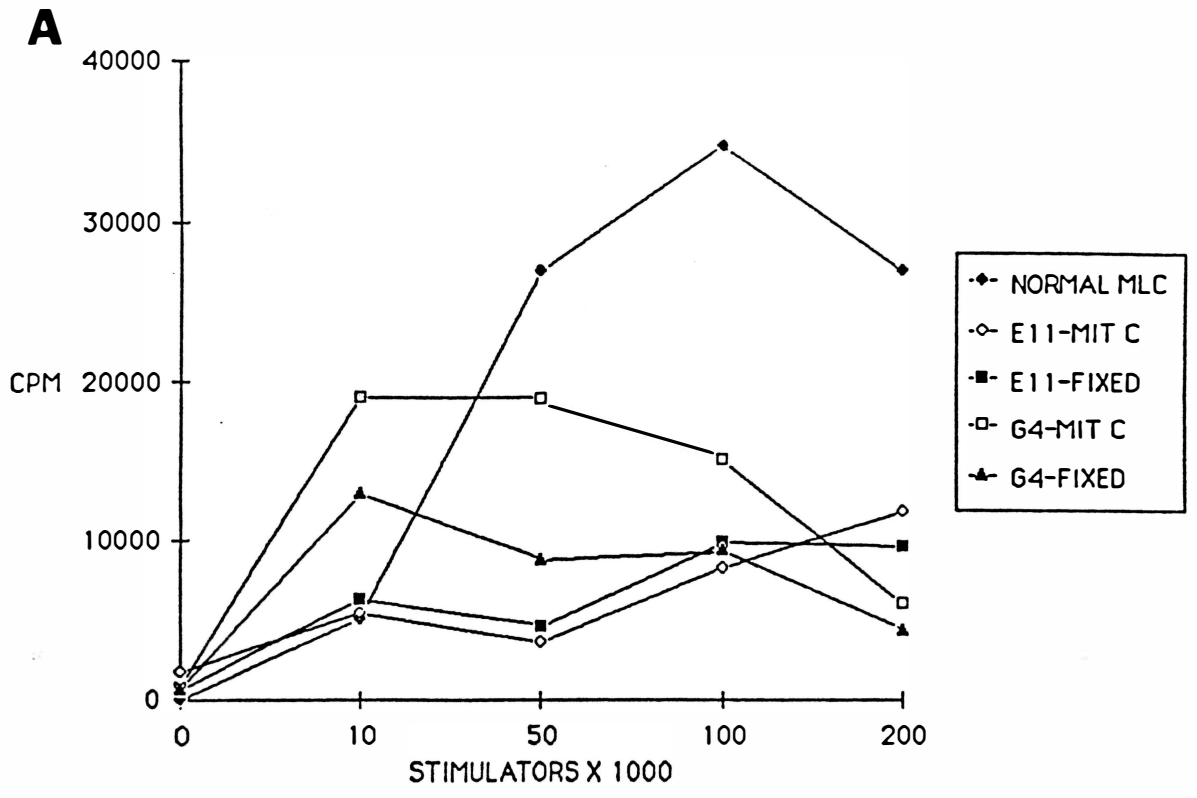
through interaction with a cell surface receptor or by some product secreted by the U937 cells in spite of their replicative inactivation by treatment with mitocycin C.

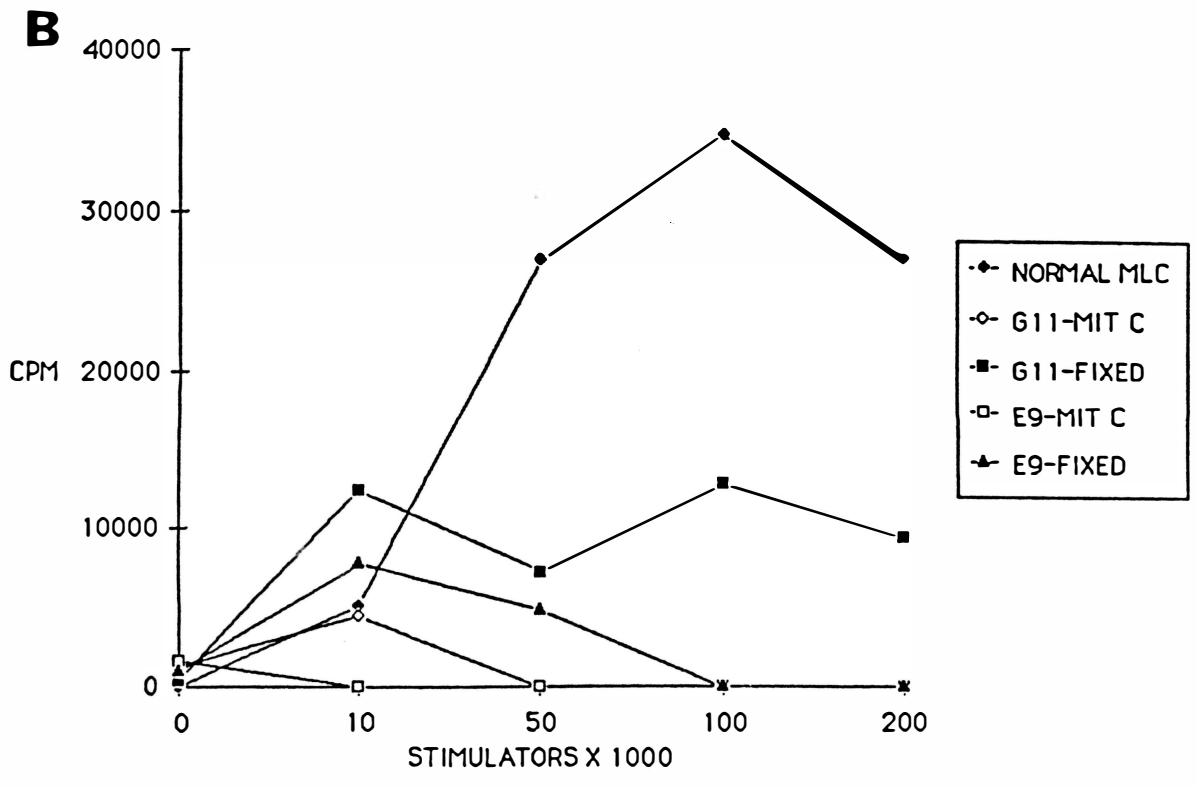
To further study the ability of the U937 parent and sublines to mediate an MLR and discover the nature of the inhibition commonly seen, cells were treated in two ways prior to addition to MLR assays. Gentle fixation with 1% paraformaldehyde has been shown to preserve the cell surface receptor architecture while inactivating any further metabolic function (98). Sublines G4, E11, G11 and E9 were treated in the normal manner with mitomycin C or by overnight fixation with 1% paraformaldehye followed in both cases by extensive washing. When these cells were added to responder cells in a series of experiments illustrated in Figure 21, it was found that the paraformaldehyde fixed cells were able to stimulate the generation of an MLR. Although the level of stimulation of the responders by fixed G4 subline cells was less than that seen with mitomycin C treated G4 subline cells except at the highest dose, stimulation by fixed E11 cells was virtually identical to mitomycin C treated E11 cells and stimulation by fixed G11 and E9 sublines exceeded that seen with mitomycin C treated cells at several concentrations. The normal MLR is shown for comparison to the U937 stimulated MLR and to indicate that the responder cells were not maximally stimulated by any of the U937 cells. Thus paraformaldehyde fixed U937 cells are capable of stimulating an MLR to approximately the same degree as mitomycin C treated cells and in the case of the G11 subline, an MLR is produced at the usual dosage of 1 X 10^5 cells/well with fixed cells but not with mitomycin C treated cells. Thus it postulated that the production of secreted inhibitory factors might be responsible for the inconsistencies seen in generation of MLR.

The effect of dose of normal PBMC stimulators in MLR to which U937 cells were added was examined. To further investigate the effect of addition of U937 cells to a normal MLR between two individuals known to have different MHC class *II and to expand* on the information gained from the experiments shown in Figure 20, responder cells

FIGURE 21. STIMULATORY CAPACITY OF PARAFORMALDEHYDE FIXED AND MITOMYCIN C-TREATED U937 SUBLINE CELLS WAS COMPARED IN MIXED LYMPHOCYTE REACTION.

U937 subline cells were treated with mitomycin C, as previously described, or gently fixed with 1% paraformaldehye prior to addition to normal PBMC responder cells (1 X 10^5 cells/well). U937 sublines E11 and G4 (A) and sublines G11 and E9 (B) were treated and added at four doses: 1 X 10^4, 5 X 10^4, 1 X 10^5, and 2 X 10^5 cells/well. Normal allogeneic MLR reaction between responder and PBMC stimulator cells is illustrated for comparison. Results are presented as the mean of triplicate wells in three assays.


A
40000
30000
20000
10000
0
CPM
0
10
50
100
200
STIMULATORS X 1000
NORMAL MLC
E11-MIT C
E11-FIXED
G4-MIT C
G4-FIXED


B
40000
30000
20000
10000
0
CPM
0
10
50
100
200
STIMULATORS X 1000
NORMAL MLC
G11-MIT C
G11-FIXED
E9-MIT C
E9-FIXED

(1 X 10^5 cells/well) were incubated with a series of doses of normal PBMC stimulator cells (5 X 10^3, 2.5 X 10^4, 5 X 10^4 and 1 X 10^5 cells/well) as shown in Figure 22. Addition of 1 X 10^5 normal PBMC stimulator cells ellicited the highest ^{3}H-thymidine incorporation by the responders and stimulators alone, although, at all doses there was a strong MLR. When various doses of mitomycin C treated E11 subline cells were added to this MLR, a dose dependent increase in ^{3}H-thymidine incorporation was noted when low doses of normal stimulators were added. At normal MLR stimulator concentration (1 X 10^5 cells/well) the addition of mitomycin C-treated E11 cells at all concentrations failed to significantly increase the MLR generated. When 5 X 10^4 paraformaldehyde fixed E11 subline cells were added to the normal MLR, there was marked enhancement of stimulation at all stimulator cell doses and even at the highest normal stimulator cell dose, the addition of paraformaldehyde fixed cells significantly enhanced ^{3}H-thymidine incorporation. Conversely, when U937 supernatant pooled from tissue culture flasks of 3-day cultures was added to the normal MLR at a final concentration of 1:4 or 1:40, the MLR was ablatted at all cell concentrations. Findings in this series of experiments that addition of mitomycin C treated or paraformaldehyde fixed U937 E11 subline cells to a normal MLR was capable of stimulating enhanced ^{3}H-thymidine incorporation indicated further that the U937 cells were capable of stimulating an MLR. The finding that addition of paraformaldehyde treated, metabolically inactive E11 subline cells enhanced to a marked degree even at the highest doses further indicated that there might be something secreted by partially inactivated, mitomycin C treated cells. Also the finding that direct addition of pooled fresh U937 supernatant in the form of spent culture medium caused loss of MLR at all stimulator doses confirmed that U937 cells constituitively produce inhibitory substances.

Addition of U937 cell supernatant inhibited MLR assays. Figure 23 illustrates a representative experiment in which supernatant from parent 2-1 and sublines E11,

FIGURE 22. PARAFORMALDEHYDE-FIXED OR MITOMYCIN C-TREATED U937 SUBLINE E11 CELLS AUGMENT A NORMAL MIXED LYMPHOCYTE REACTION WHEN NORMAL STIMULATORS ARE ADDED AT SUBOPTIMAL DOSES.

MLR assays were set up with normal PBMC responders (1 X 10^5 cells/well) and four doses of normal PBMC stimulator cells: 5 X 10^3, 2.5 X 10^4, 5 X 10^4 and 1 X 10^5 cells/well. U937 subline E11 cells were treated with mitomycin C and added to MLR assay wells at three doses (5 X 10^3, 1 X 10^4, 5 X 10^4, and 1 X 10^5 cells/well); fixed with 1% paraformaldehyde and added to MLR assays at a single dose of 5 X 10^4 cells/well; or supernatant from U937 subline E11 was added to MLR assay wells at a final concentration of 1:4 or 1:40. Results are expressed as the mean of ^{3}H-thymidine incorporation as cpm of triplicate wells in two assays.

FIGURE 22. NORMAL MLC + U937 SUBLINE E11 CELLS OR E11 SUPERNATE

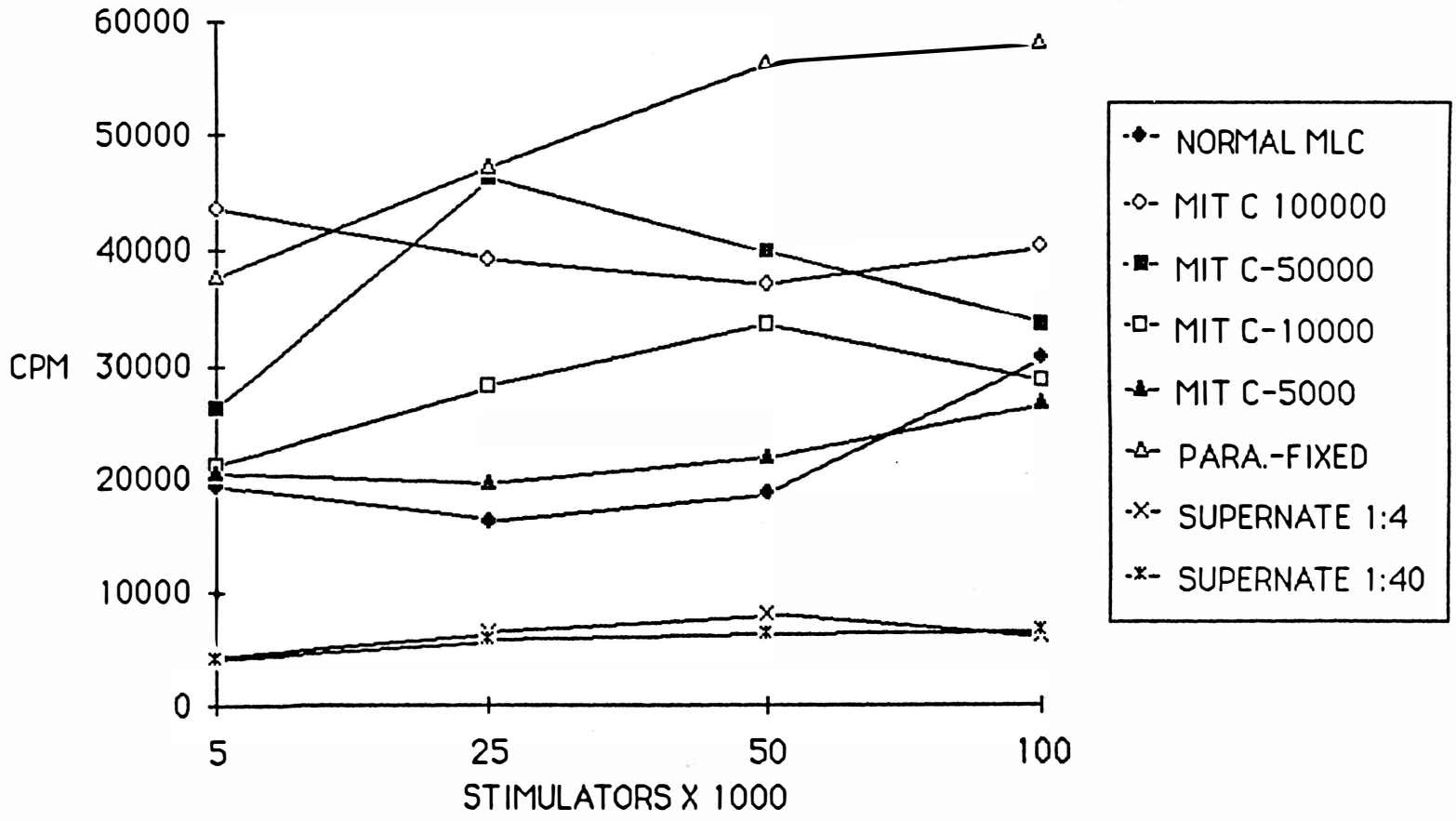
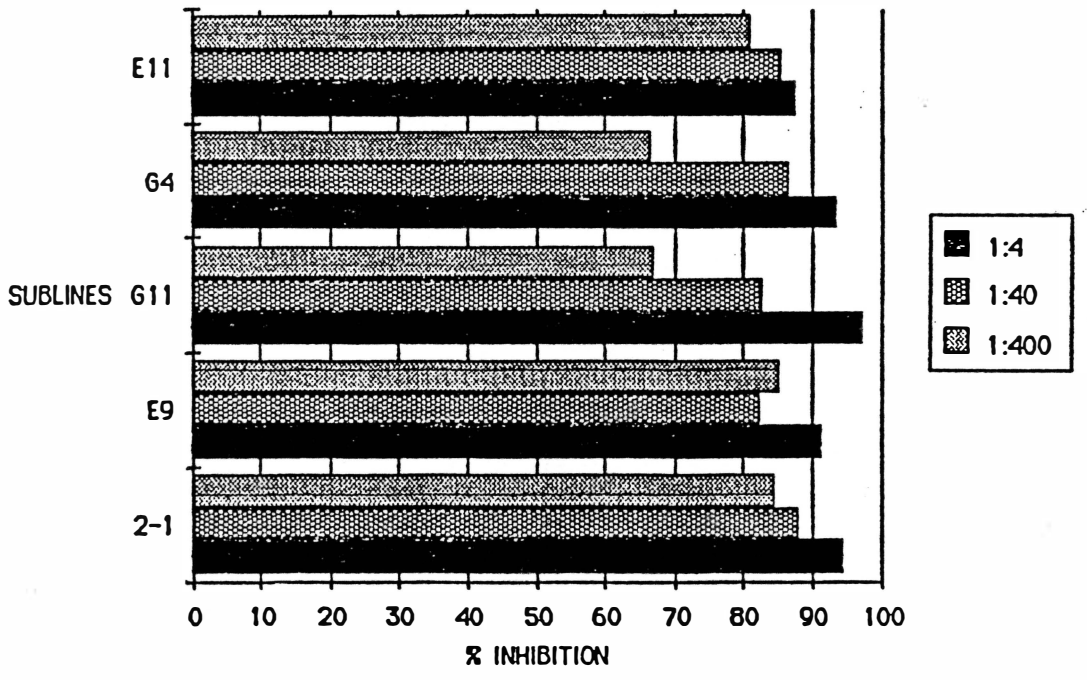


FIGURE 23. SUPERNATANT FROM U937 PARENT AND SUBLINES IS A POTENT INHIBITOR OF NORMAL MIXED LYMPHOCYTE REACTION.

Supernatant from U937 parent 2-1 and sublines E11, G4, G11, and E9 was added to MLR assay wells containing normal PBMC responder cells (1 X 10^5 cells/well) and normal allogeneic PBMC stimulator cells (1 X 10^5 cells/well) at final concentrations of 1:4, 1:40 and 1:400. Results are expressed as % Inhibition of the normal allogeneic MLR as measured by ^{3}H-thymidine incorporation in triplicate wells in three assays.


E11
G4
SUBLINES G11
E9
2-1
0
10
20
30
40
50
60
70
80
90
100
% INHIBITION
1:4
1:40
1:400

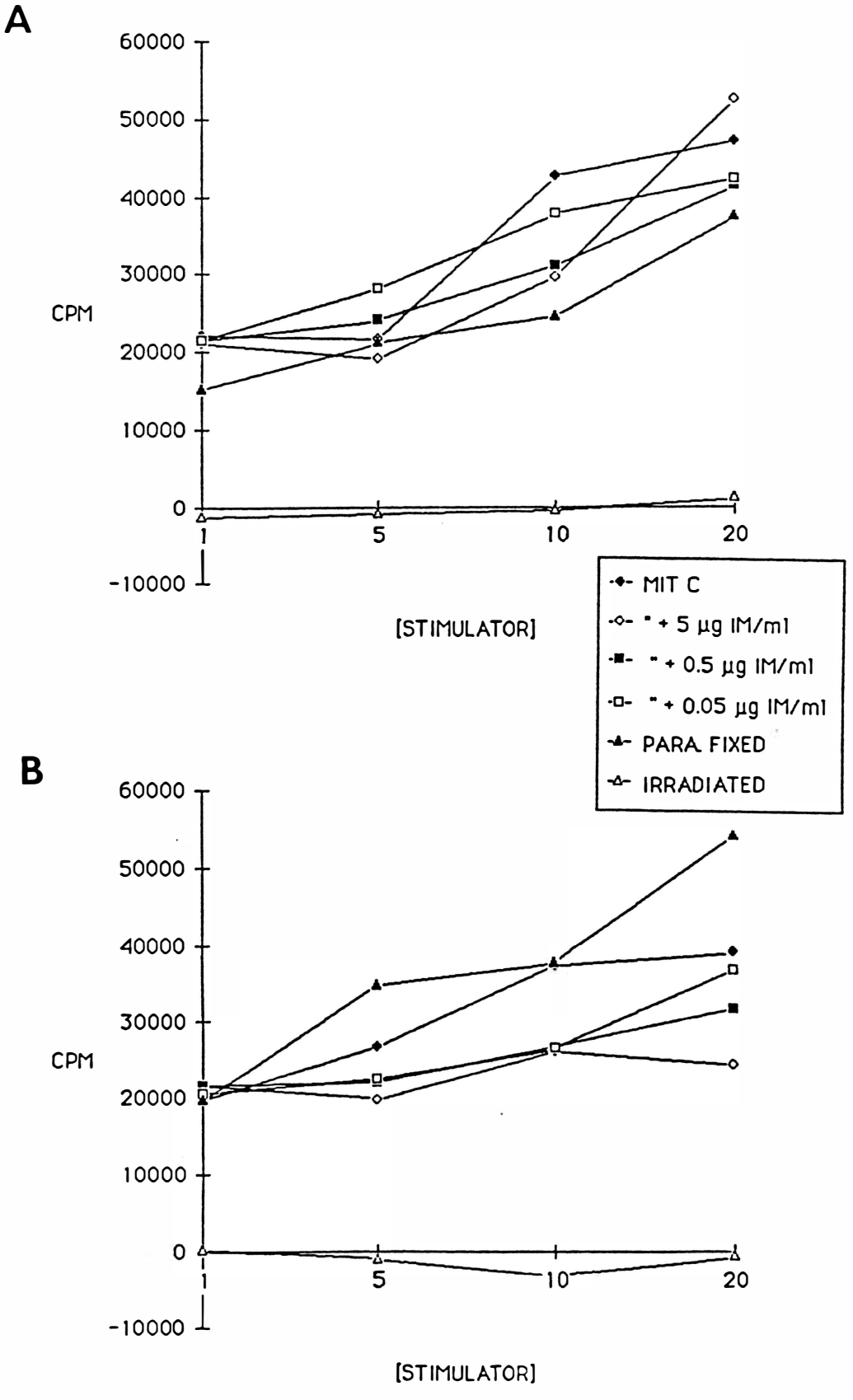
G4, G11 and E9 was added to normal MLR assays at final concentrations of 1:4, 1:40 and 1:400. For all supernatants at a 1:4 dilution, greater than 80% inhibition was seen. Inhibitory activity persisted at 1:40 and 1:400 dilutions and only with G11 and G4 supernatants was there any diminution in inhibitory capacity at 1:400 dilution. Thus the inhibitory substances secreted by U937 cells are secreted by all of the sublines and the parent line and are very potent.

Addition of indomethacin to the MLR did not affect the response. Prostaglandins, particularly PGE_2, have been shown to be small molecular weight inhibitory molecules produced by macrophages. Because the U937 cell line has macrophage-like characteristics, it seemed probable that inhibition of the MLR might be related to the production of PGE_2 by the U937 cells. To investigate the role of PGE_2 in U937 supernatant inhibition, indomethacin, a PGE_2 inhibitor, was added to the MLR at three concentrations as shown in Figure 24A and 24B. Addition of indomethacin to the assay had no significant effect on the generation of the MLR by the mitomycin C treated G4 subline stimulator cells, Figure 24A, or E11 subline stimulator cells, Figure 24B, and the level of stimulation was similar to the level obtained with the addition of paraformaldehyde fixed stimulator cells. Thus, production of prostaglandin which may occur is not responsible for the MLR inhibition seen when supernatant is added. Addition of stimulator U937 subline G4 or subline E11 cells inactivated by irradiation with 3000 rads in a ^{137}Cs source cell irradiator, abrogated any MLR response indicating that irradiation of U937 with the standard dose used for inactivation of PBMC stimulators does not inactivate U937 production and secretion of inhibitory substances.

Pretreatment of stimulator U937 cells with immunomodulators did not affect the MLR. It was reasoned that if the Ia+ U937 sublines G4 and E11 are capable of mediating an MLR, then treatment with gamma interferon, which *increases MHC class* II expression, or PDBu, which increases membrane IL-1 expression (to be discussed

FIGURE 24. ADDITION OF INDOMETHACIN TO MIXED LYMPHOCYTE REACTION IN WHICH U937 SUBLINE CELLS WERE ADDED AS STIMULATOR CELLS HAD NO EFFECT.

U937 subline G4 (A) and subline E11 (B) cells were mitomycin C-treated and added to normal PBMC responder cells (1 X 10^5 cells/well) at three doses: 5 X 10^4, 1 X 10^5 and 2 X 10^5. Indomethacin at three concentrations indicated was added at the initiation of MLR assay to wells containing mitomycin C-treated U937 cells + normal PBMC. In addition U937 cells treated by fixation with 1% paraformaldehyde or by irradiation with 3000 rads using a ^{137}Cs irradiator were also added as stimulator cells to normal PBMC responders. Results are expressed as the mean of ^3H-thymidine incorporation as cpm of triplicate wells in two assays.


A
60000
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1
5
10
20
[STIMULATOR]
MIT C
" + 5 µg IM/ml
" + 0.5 µg IM/ml
" + 0.05 µg IM/ml
PARA FIXED
IRRADIATED
B
60000
50000
40000
30000
CPM
20000
10000
0
-10000
1
5
10
20
[STIMULATOR]

below), might enhance the ability of these sublines to stimulate an MLR. As shown in Figure 25, this was not found to be true with sublines G4 and E11. U937 cells were treated with gamma interferon for 24 hours or treated with PDBu for 72 hours prior to addition to an MLR assay. As indicated there was no change in ^{3}H-thymidine incorporation after treatment with either gamma interferon or PDBu. The normal MLR in shown to emphasize the fact that the responder cells used in these experiments were not maximally stimulated by the U937 cells, either treated or non-treated.

U937 sublines have been shown to mediate macrophage dependent functions in several cellular assays.

U937 sublines were able to replace monocytes in CD3(T3) induced mitogenesis. Monoclonal antibody to the CD3 molecules of the CD3-T cell (T3-TcR) receptor complex on the surface of T cells has been shown to bind to the CD3 molecule and cause a mitogenic proliferation of the T cells (99). This proliferation, however, is dependent on the presence of monocytes in the culture (103). It has been claimed that the requirement for monocytes may also be overcome by addition of exogenous IL-1 (104). Figure 26 represents a titration experiment in which different concentrations of normal human monocytes isolated from PBMC by adherence followed by scraping from plastic petri dishes were added to nylon-wool purified lymphocytes to reconstitute the mitogenic response caused by the addition of CD3 specific antibody (OKT 3) to peripheral blood mononuclear cells. Nylon-wool purified lymphocytes alone incorporated less than 10000 cpm ^{3}H-thymidine during a 16-18 hour pulse, whereas addition of monocytes increased the ^{3}H-thymidine incorporation in a dose dependent fashion for doses of 5×10^{3} and 1×10^{4} monocytes. The addition of 2×10^{4} monocytes did not significantly increase the mitogenic response seen with 1×10^{4} monocytes and 5×10^{4} monocytes inhibited the response. Thus a dose of 1×10^{4} monocytes/well appeared to mediate optimal mitogenic response.

FIGURE 25. PRETREATMENT OF U937 SUBLINE STIMULATOR CELLS WITH GAMMA INTERFERON OR PDBU DID NOT AUGMENT THEIR ABILITY TO STIMULATE AN MLR.

U937 subline G4 cells and subline E11 cells were treated with gamma interferon (100 units/ml.) for 24 hours or PDBu (10^{-8}M) for 72 hours prior to mitomycin C treatment and addition to MLR assay wells containing normal PBMC responder cells (1 X 10^5 cells/well). Results are expressed as the mean of ^{3}H-thymidine incorporation as cpm of triplicate wells in two assays.

FIGURE 25. EFFECT OF PRETREATMENT OF U937 STIMULATOR CELLS ON GENERATION OF MLC

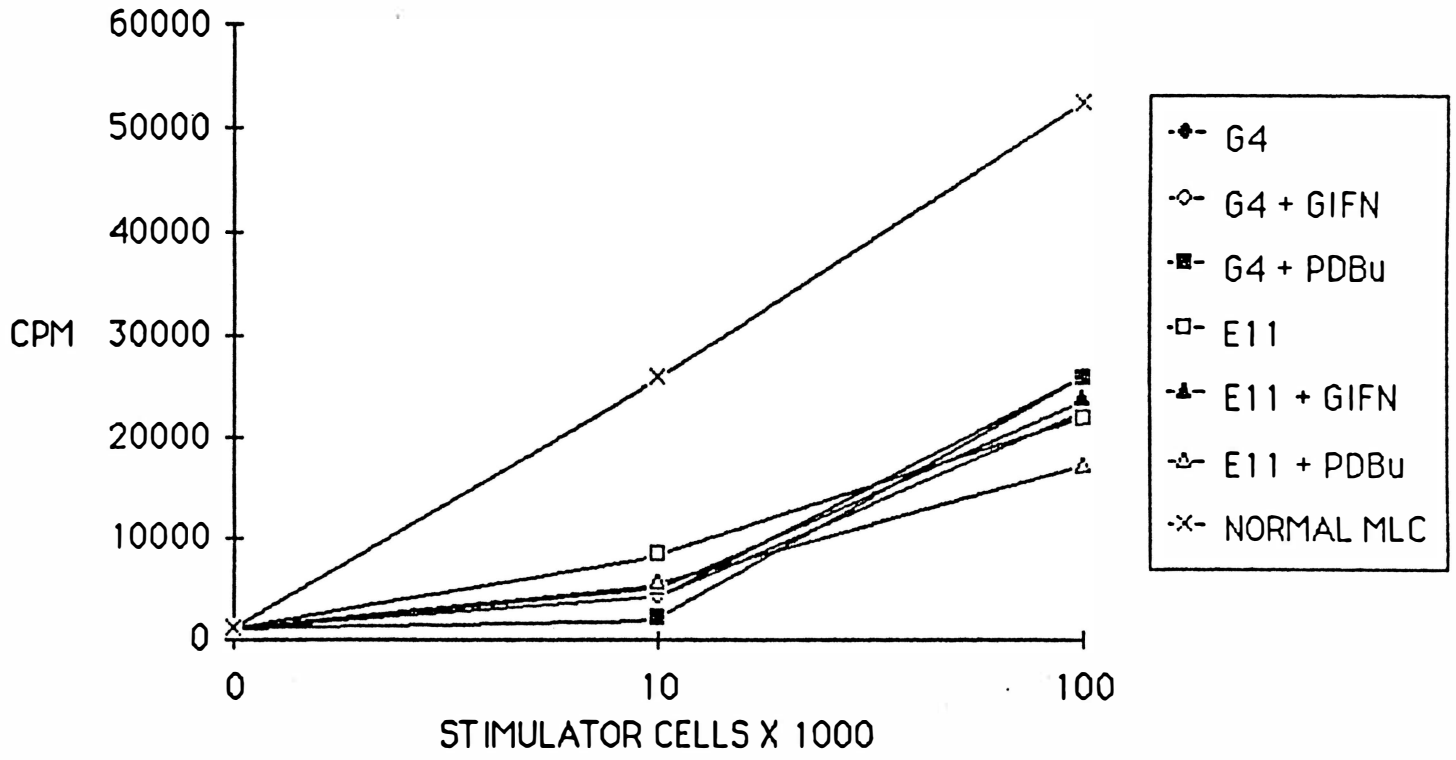
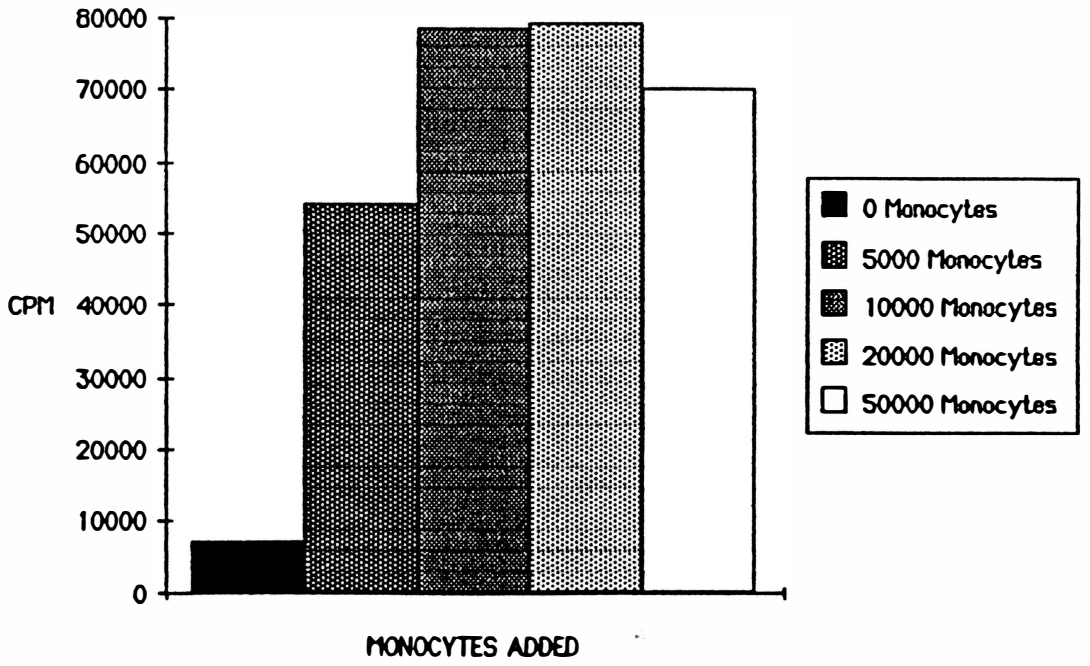


FIGURE 26. MONOCYTES ADDED TO NYLON-WOOL PURIFIED LYMPHOCYTES RECONSTITUTES THE CD3 MITOGENIC RESPONSE.

Peripheral blood mononuclear cells isolated by ficoll-hypaque techniques were incubated in plastic petri dishes for 1-2 hours. Non-adherent cells were removed by pipet, concentrated by centrifuging and added to nylon wool columns to remove residual B cells and any remaining monocytes. The petri dishes were washed with media and adherent monocytes were removed with a rubber policeman. Cells were counted and an appropriate number of monocytes was added to wells containing nylon-wool purified lymphocytes. OKT3 antibody (0.1 μg/well) was added to wells containing unseparated PBMC and lymphocytes+monocytes. Microtiter plates were incubated for 72 hours with a 16-18 hour ^{3}H-thymidine pulse.


80000
70000
60000
50000
40000
30000
20000
10000
0
CPM
MONOCYTES ADDED
0 Monocytes
5000 Monocytes
10000 Monocytes
20000 Monocytes
50000 Monocytes

Experiments were conducted to determine whether U937 sublines were capable of substituting for monocytes in the mediation of CD3 induced mitogenesis. Partially purified T cells were prepared by passage over nylon-wool columns, added to microtiter plates to which monoclonal anti CD3 (OKT3) was added at a concentration of 0.1 μg/ml and U937 sublines were added at doses of either 1 X 10^4 or 5 X 10^4 cells/well. As seen in Figure 27, the U937 sublines were capable of substituting for monocytes in the mitogenic response. Subline G4 was found to mediate levels of ^{3}H-thymidine uptake comparable to the level seen with monocytes at cell doses of both 1 X 10^4 and 5 X 10^4. Sublines G11 and E9 were effective accessory cells at the higher cell concentration but much less able to mediate the mitogenic response at the lower cell concentration. Subline E11 and 2-1 parent U937 cell lines mediated strong responses at both cell doses but in both cases the response was slightly less than that seen for either added monocytes or added G4 subline cells. T cells incubated with U937's alone did not take up ^{3}H-thymidine (data not shown) and T cells incubated with antibody alone incorporated less than 10,000 cpm.

Pretreatment of U937 parent and subline cells with gamma interferon did not significantly alter their relative abilities to mediate the mitogenic response to CD3 specific antibody. U937 parent and sublines were pretreated with gamma interferon at a concentration of 100 Units/ml. for 24 hours as shown in Figure 28 at an accessory cell concentration of 5 X 10^4 cells/well, except for the G11 subline for which gamma interferon pretreatment decreased the accessory cell function. The ^{3}H-thymidine incorporation exhibited by nylon-wool purified lymphocytes in wells where U937 2-1 parent cells, E11 subline and G4 subline cells were added as accessory cells was slightly higher for pretreated cells than for non-treated cells; and the ^{3}H-thymidine incorporation in the presence of pretreated E9 subline cells was slightly lower than non-treated E9 subline cells. U937 cells were washed after incubation with gamma interferon for 24 hours and gamma interferon was not present during the CD3

FIGURE 27. U937 SUBLINES AND PARENT LINE ACT AS ACCESSORY CELLS IN CD3 MITOGENESIS.

U937 sublines were added to nylon wool purified peripheral blood mononuclear cells as accessory cells in a CD3 mitogenesis assay. Mononuclear leukocytes prepared by standard ficoll hypaque density gradient techniques were incubated in plastic petri dishes for 1-2 hours to allow adherence of monocytes. Non-adherent cells were removed and further purified by passage over nylon-wool columns. These nylon wool non-adherent cells were then pipetted into microtiter plates (1 X 10^5/well) to which monoclonal anti-CD3 (OKT3) was added at a concentration of 0.1 μg/ml. U937 sublines which were pretreated with mitomycin C to prevent ^{3}H-thymidine incorporation were added at doses of either 1 X 10^4 or 5 X 10^4 cells/well. Controls included T cells alone, T cells + OKT3, T cells + U937's, U937's + OKT3, and T cells + monocytes (removed from the petri dishes by scraping with a rubber policeman) at concentrations of 5 X 10^3, 1 X 10^4 or 5 X 10^4 cells/well ± OKT3. The cells were incubated for 72 hours with a ^{3}H-thymidine pulse for the final 16-18 hours. Cells were harvested and counted in a liquid scintillation counter to measure ^{3}H-thymidine uptake.

FIGURE 27. U937 SUBLINES AND PARENT LINE
ACT AS ACCESSORY CELLS IN CD3 MITOGENESIS

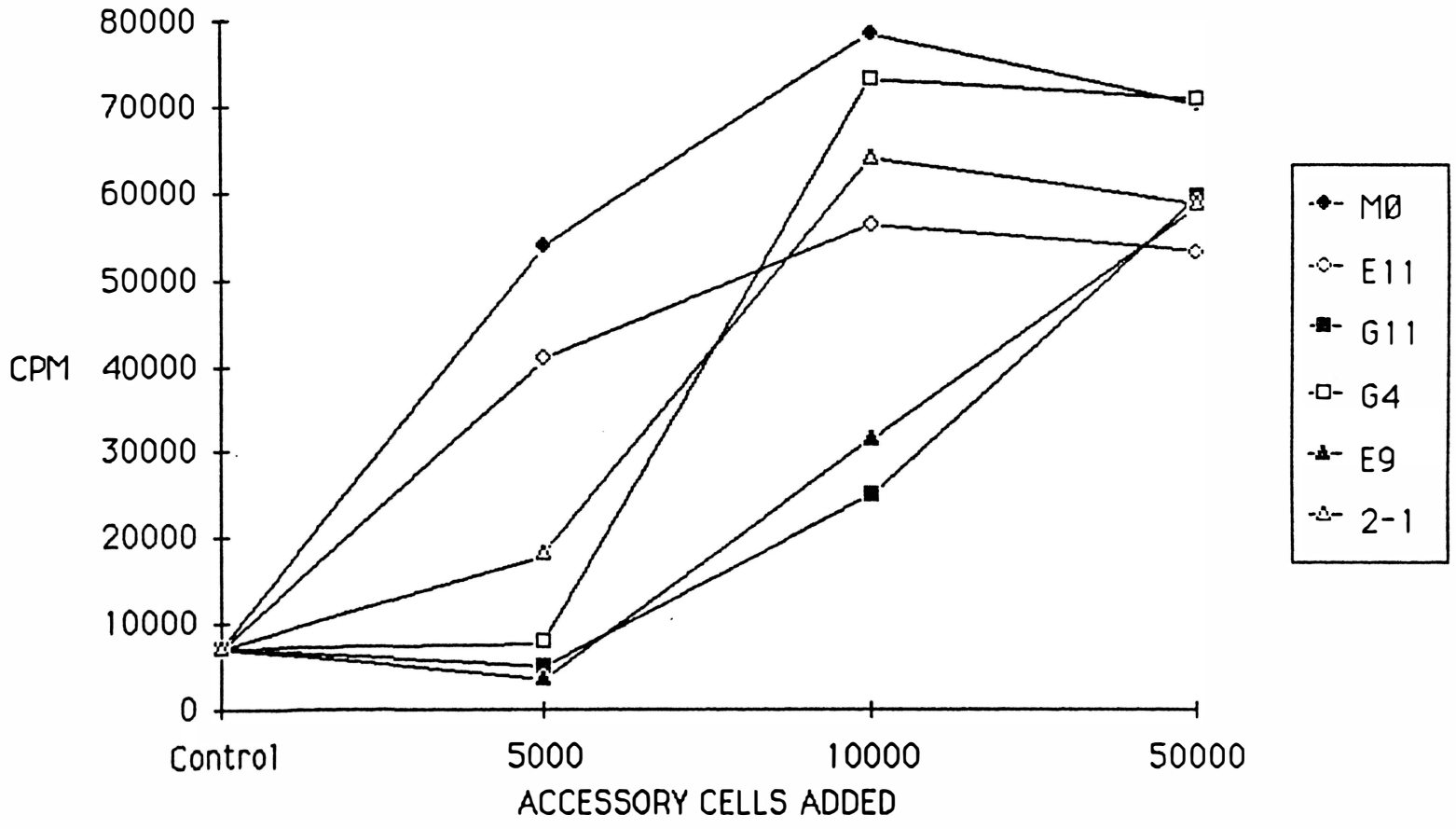
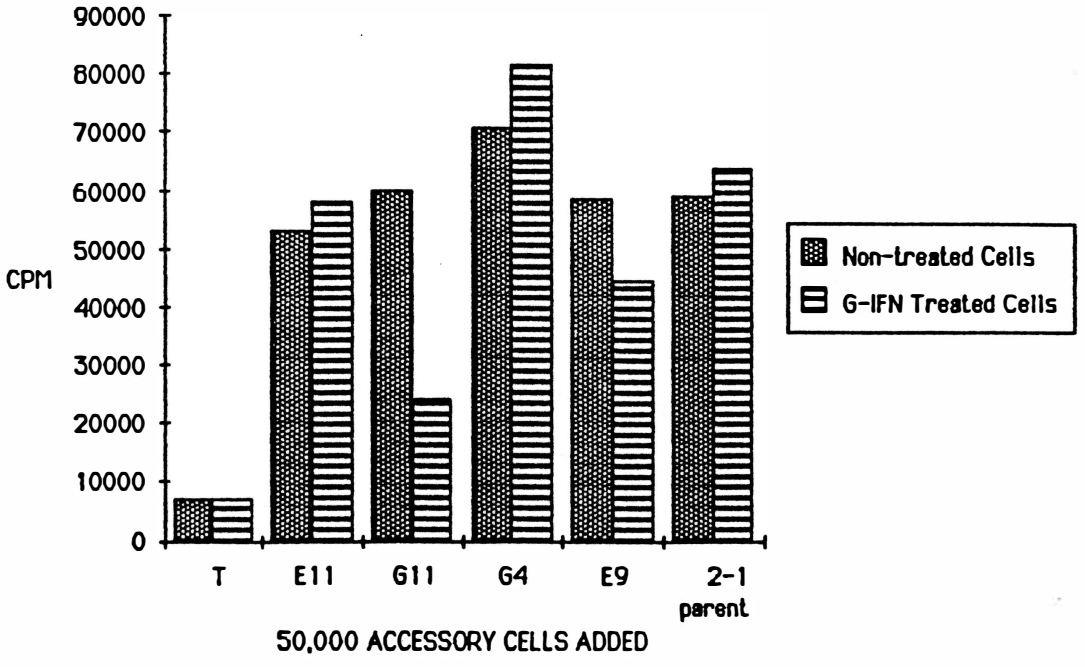


FIGURE 28. EFFECTS OF GAMMA INTERFERON PRETREATMENT ON U937 RECONSTITUTION OF CD3 MITOGENESIS.

U937 parent 2-1 cell line and sublines were incubated with gamma interferon at a concentration of 100 U/ml. for 24 hours followed by washing to remove any residual gamma interferon. Cells were resuspended in media and treated with mitomycin C. CD3 mitogenesis assay was set up in the same manner as for Figure 23, except that only one cell dose was utilized. Gamma interferon was not present during the CD3 mitogenesis assay.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80000
70000
60000
50000
40000
30000
20000
10000
0
CPM
T
E11
G11
G4
E9
2-1
parent
50,000 ACCESSORY CELLS ADDED
Non-treated Cells
G-IFN Treated Cells

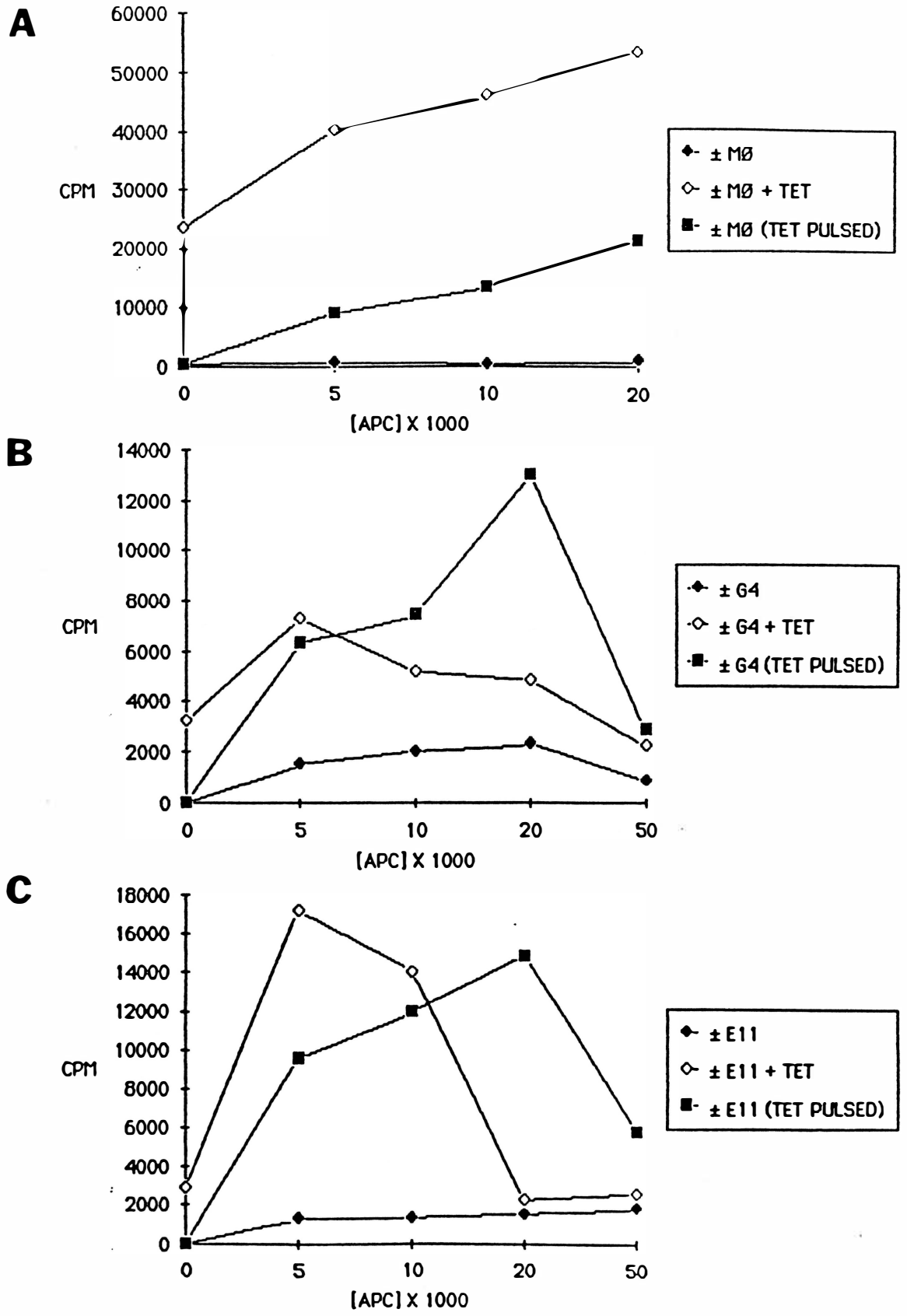
mitogenic assay.

U937 parent and sublines were able to present antigen to nylon wool purified lymphocytes. The antigen chosen for presentation was tetanus toxoid because all of the available donors had been immunized with tetanus toxoid within 24 months of this study. Donors were tested for a cellular response to tetanus toxoid by addition of dilutions of tetanus toxoid to ficoll-hypaque isolated PBMC (438). Response was measured as ^{3}H-thymidine incorporation after a 16 hour pulse at the end of a 96 hour incubation. In all cases where a response was noted, a 1/200 dilution of antigen was found to be optimal (data not shown).

Although the standard method for assay of tetanus reactivity simply required addition of the dilution of tetanus toxoid directly to the microtiter well containing the T cells and accessory cells, this method proved to be unsatisfactory for testing the antigen presentation capacity of U937 cells. A continuing problem with the antigen presentation experiments was the residual tetanus reactivity of the nylon-wool purified lymphocytes, which were probably contaminated with autologous accessory cells as indicated in Figure 29. When normal monocytes were added to the T cells + tetanus toxoid, there was a dose dependent increase in ^{3}H-thymidine incorporation (Figure 29A); however, when U937 subline G4 cells were added, there was an increase in ^{3}H-thymidine incorporation at low G4 dose and suppression of ^{3}H-thymidine incorporation at higher G4 doses as shown in Figure 29B. This phenomenon was even more striking when subline E11 cells were added as indicated in Figure 29C. In order to circumvent autologous antigen processing and presentation by residual accessory cells as much as possible, cells to be used as accessory cells were incubated overnight with tetanus toxoid at a concentration of 1/200 prior to mitomycin C treatment followed by extensive washing . Monocytes and U937 sublines G4 and E11 treated in this manner were able to present tetanus toxoid to T cells as shown in Figure 29.

FIGURE 29. U937 SUBLINE G4 AND E11 CELLS CAN PRESENT ANTIGEN TO NYLON WOOL-PURIFIED T CELLS.

Nylon wool purified responder lymphocytes were prepared from normal PBMC by adherence followed by passage of non-adherent cells over a nylon wool column. Donors selected had been reimmunized to tetanus toxoid within the previous 24 months. Monocytes were isolated by retrieval of adherent cells from petri dishes by scraping. Monocytes (A) and U937 subline G4 (B) and E11 (C) cells were pulsed with tetanus toxoid at a concentration of 1:200, incubated overnight, washed 4-5 times, treated with mitomycin C and added to responder lymphocytes in microtiter plates (2×10^5 cells/well) at stated doses. Alternatively, U937 subline G4 and E11 cells and monocytes were added to wells containing responder lymphocytes and tetanus toxoid at a final concentration of 1:200 was added directly to the wells. Assays were incubated for six days with ^{3}H-thymidine pulse for the final 16-18 hours. Results as cpm are expressed as the mean of triplicate wells in two experiments.


A
60000
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40000
CPM 30000
20000
10000
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10
20
[APC] X 1000
± MØ
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± MØ (TET PULSED)
B
14000
12000
10000
8000
CPM
6000
4000
2000
0
0
5
10
20
50
[APC] X 1000
± G4
± G4 + TET
± G4 (TET PULSED)
C
18000
16000
14000
12000
CPM 10000
8000
6000
4000
2000
0
0
5
10
20
50
[APC] X 1000
± E11
± E11 + TET
± E11 (TET PULSED)

Tritiated-thymidine incorporation increased in a dose dependent fashion for monocytes and increased to a maximum level at 1 X 10^4-2 X 10^4 cells/well for U937 sublines G4 and E11 prior to becoming suppressive at 5 X 10^4 cells/well. Figure 30 shows a compilation of several experiments in which antigen pulsed monocytes and antigen pulsed U937 sublines G4 and E11 were added to nylon-wool purified T cells. As indicated, the tetanus response mediated by both the sublines and monocytes is comparable in magnitude of ^{3}H-thymidine incorporation and cell dose response. Non-antigen pulsed U937 sublines and monocytes failed to stimulate ^{3}H-thymidine incorporation.

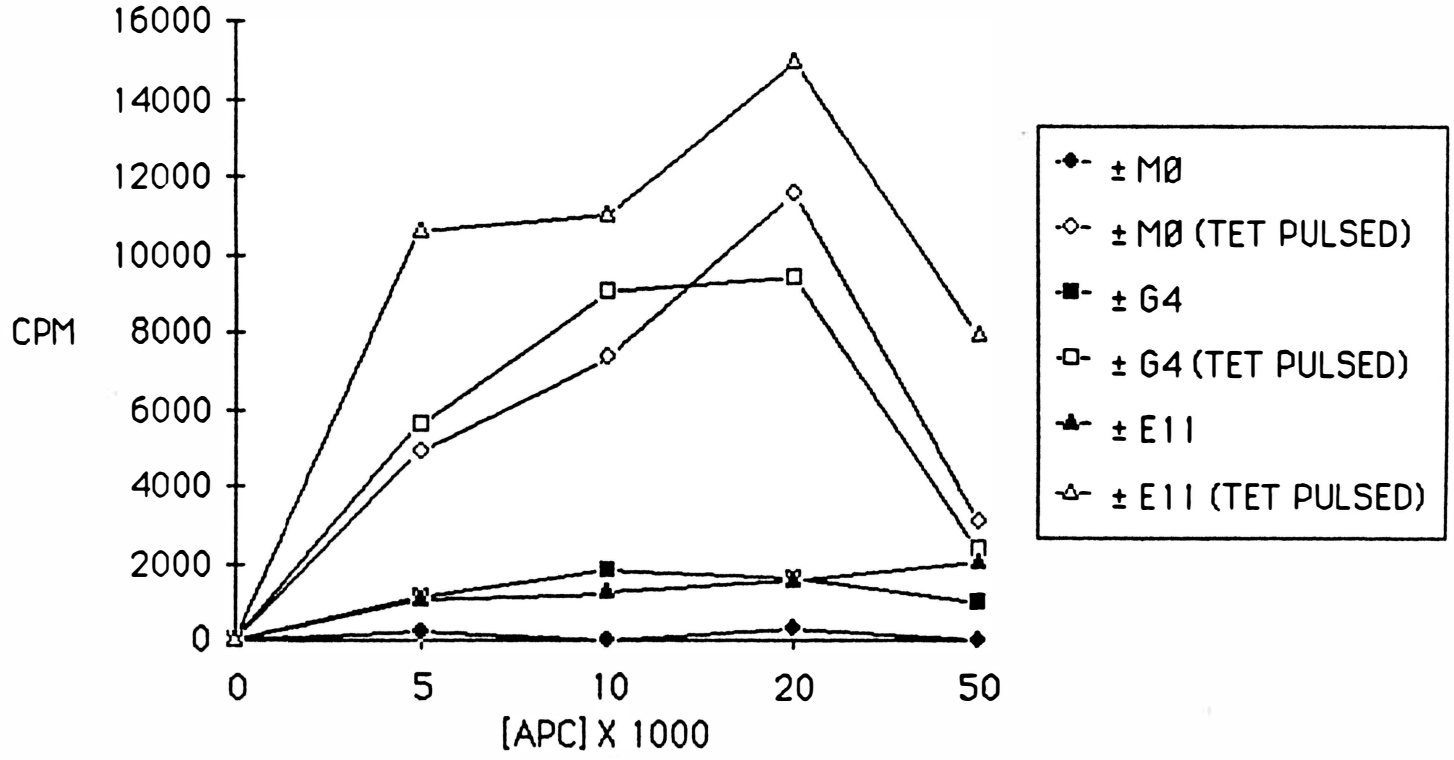
Preliminary data indicated that treatment of U937 sublines with tuftsin did not alter antigen presentation. Tuftsin, which is a small molecular weight polypeptide, has been shown to stimulate phagocytic uptake by monocytes or macrophages (475). It was reasoned that monocytes and U937 cells might be able to process and present antigen to a greater degree if more antigen could be endocytosed. This was tested with subline G4 cells and normal monocytes. Cells were treated with tuftsin just prior to addition of antigen. Although a slight increase in tetanus response by to tuftsin-treated antigen pulsed monocytes, no comparable response was seen with subline G4 cells (data not shown).

Pretreatment of U937 parent cells and sublines G4 and E11 with either gamma interferon or PDBu did not significantly alter their capacity to present tetanus toxoid. Further attempts to augment the antigen presenting capacity of the U937 cells by pretreatment of the U937 sublines G4 and E11 and the 2-1 parent cell line with either gamma interferon for 24 hours or PDBu for 72 hours prior to addition of these cells to nylon wool purified lymphocytes had no significant effect on the magnitude of the response (data not shown). Thus pretreatment with either gamma interferon or PDBu neither enhances the antigen presenting capacity of the U937 cells *nor does it inhibit* that capacity.

FIGURE 30. ANTIGEN PULSED U937 SUBLINE G4 AND E11 CELLS EFFECTIVELY PRESENT ANTIGEN TO NYLON-WOOL PURIFIED T LYMPHOCYTES IN A MANNER COMPARABLE TO AUTOLOGOUS MONOCYTES.

Cells were isolated and antigen pulsed with tetanus toxoid and assays were conducted as described for Figure 29. Results as cpm are expressed as the mean of triplicate wells in three experiments.

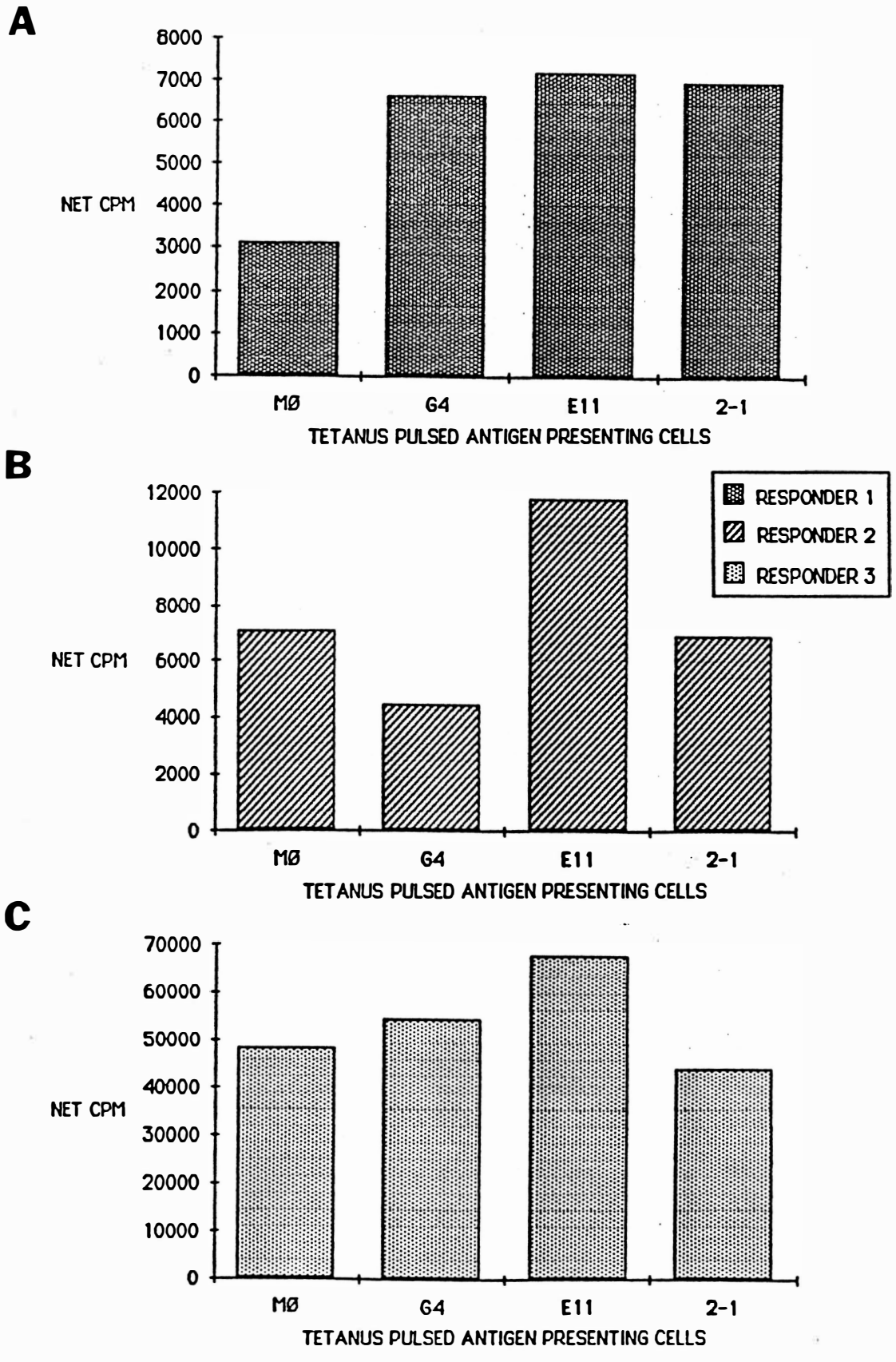
FIGURE 30. ANTIGEN PRESENTATION
RESPONDER T CELLS ± MØ, U937 G4 OR E11
PRETREATED WITH TETANUS TOXOID



The antigen presenting capacity of U937 is inhibited by antibody specific for MHC class II. Figure 31 illustrates the response of three donors to tetanus toxoid pulsed autologous monocytes and to tetanus toxoid pulsed U937 subline G4 and E11 cells and parent 2-1 cells. The levels of response were varied as indicated by the vast difference in ordinate scale. Responders CS and CJ, Figures 31A and 31B, were relatively low responders and responder CD, Figure 31C, was a high responder. In all cases, however, the response to antigen presentation by U937 cells was comparable to antigen presentation by autologous monocytes. Antibodies specific for framework determinants of human Ia (Kula2), ß2 microglobulin (BBM.1) which associates with MHC class I molecules, CD4 (KT69-7) which is in association with the T cell receptor and also present on U937 cells and at a low concentration on normal human monocytes, and heterologous antibodies specific for particular HLA-DR specificities and presumably specific for the polymorphic regions of those MHC class II molecules were added to the antigen presentation assay. Tetanus toxoid antigen was added to the antigen presenting cells and incubated overnight as previously described, followed by extensive washing prior to addition to the nylon-wool purified lymphocytes. Thus only antigen in association with the antigen presenting cells was added to the assay. As shown in Figure 32, antibody to human Ia (Kula2) effectively inhibited the response in all cases both at a 1:10 dilution and at 1:25 in a dose dependent manner. A second antibody which was also broadly inhibitory was the antibody specific for CD4 (KT69-7) which has previously been shown to inhibit the autologous MLR but not the allogeneic MLR (440). The 2-1 parent U937 antigen presentation reaction was not inhibited to the same degree by this antibody for any of the three responders tested. As indicated in Table 5, the 2-1 parent cell line expresses CD4 as measured by KT69-7 using immunofluorescence techniques to the same degree as the sublines G4 and E11. No significant inhibition of the tetanus response by the antibody to ß2 microglobulin was observed in antigen presentation by U937 cells or autologous monocytes. Although

FIGURE 31. ANTIGEN PULSED U937 PARENT 2-1 AND SUBLINES G4 AND E11 PRESENTED TETANUS TOXOID TO THREE RESPONDER NYLON-WOOL PURIFIED T LYMPHOCYTE PREPARATIONS.

Nylon wool purified T lymphocytes were prepared as previously described and added to assay wells at 2 X 10^5 cells/well. Accessory cells (APC) were pulsed, washed and treated with mitomycin C prior to addition to the assay wells at a dose of 5 X 10^3 cells/well. Responder 1 (A) and responder 2 (B) were moderately responsive to antigen presentation by all APC; however, responder 3 (C) gave a significantly higher response. Control values included addition of non-pulsed APC to responder cells and addition of tetanus toxoid directly to responder cells. All values shown have been corrected for response to non-pulsed APC at the same dose and for response to tetanus without addition of APC. Values illustrated are the mean of cpm for individual responders assayed in triplicate wells.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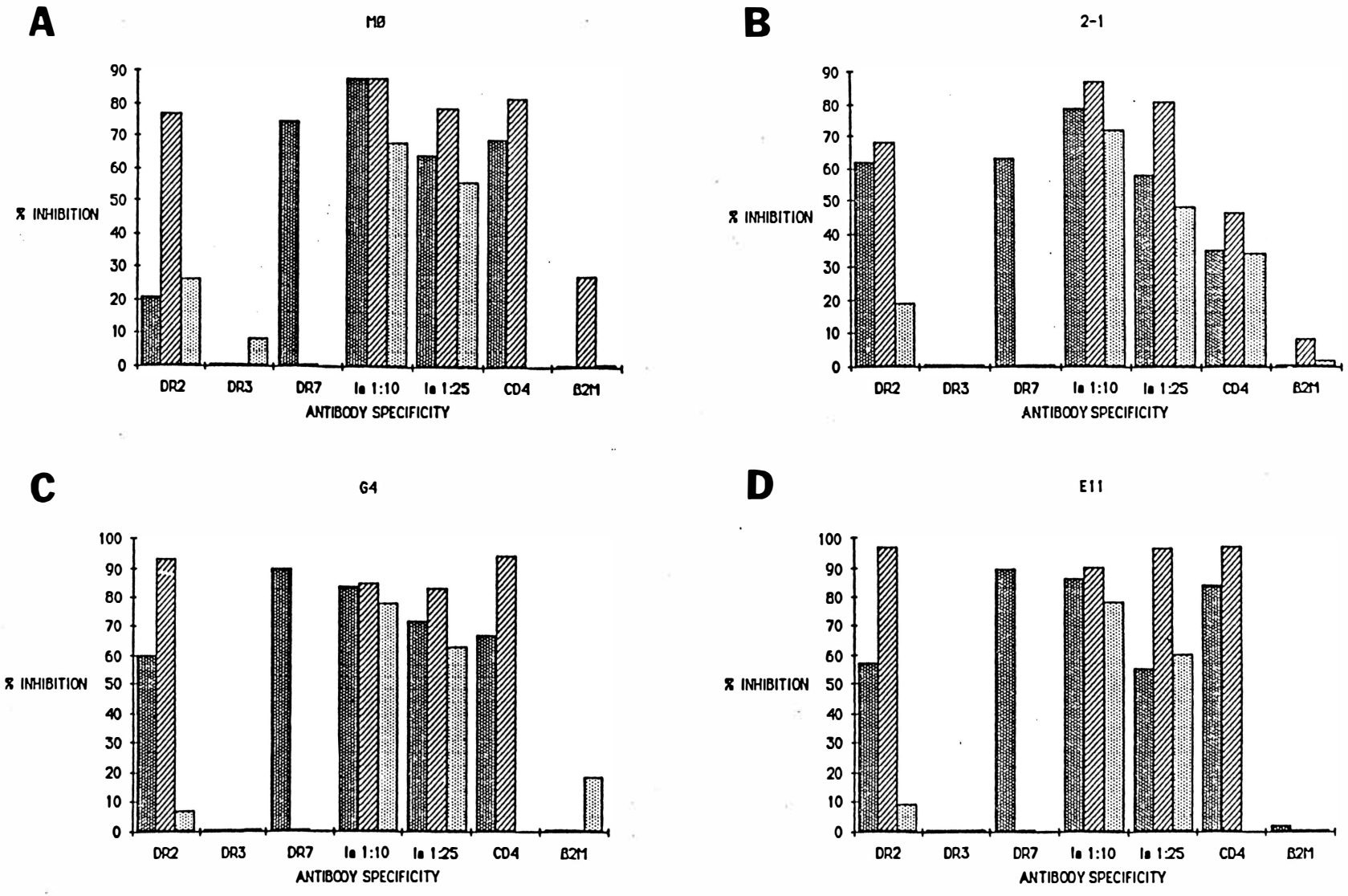
G4
E11
2-1
TETANUS PULSED ANTIGEN PRESENTING CELLS
B
12000
10000
8000
NET CPM 6000
4000
2000
0
RESPONDER 1
RESPONDER 2
RESPONDER 3
MØ
G4
E11
2-1
TETANUS PULSED ANTIGEN PRESENTING CELLS
C
70000
60000
50000
NET CPM 40000
30000
20000
10000
0
MØ
G4
E11
2-1
TETANUS PULSED ANTIGEN PRESENTING CELLS

FIGURE 32. ANTIGEN PRESENTATION BY MONOCYTES AND U937 2-1 PARENT AND SUBLINE G4 AND E11 CELLS IS ABROGATED BY ADDITION OF ANTIBODY TO MHC CLASS II OR CD4.

Responder lymphocytes and antigen presenting cells were those previously described in Figure 31. Heteroantibodies specific for HLA-DR2, HLA-DR3, and HLA-DR7 were added to wells at a concentration of 1:10. Monoclonal antibody specific for framework determinant of human Ia, Kula2 was added at 1:10 and 1:25; monoclonal antibody specific for CD4, KT69-7, was added at a concentration of 1:10; and monoclonal antibody to ß2-microglobulin, BBM, was also added at a concentration of 1:10. Values illustrated indicate the % Inhibition of the maximal response seen in Figure 31 for monocytes (MØ) (A), U937 parent 2-1 (B), U937 subline G4 (C) and U937 subline E11 (D).


A
MØ
% INHIBITION
90
80
70
60
50
40
30
20
10
0
DR2
DR3
DR7
Ia 1:10
Ia 1:25
CD4
B2M
ANTIBODY SPECIFICITY
B
2-1
% INHIBITION
ANTIBODY SPECIFICITY
C
G4
% INHIBITION
100
ANTIBODY SPECIFICITY
D
E11
% INHIBITION
ANTIBODY SPECIFICITY

slight inhibition was seen, the responses were small and inconsistent.

When heterologous HLA-DR specificity specific antibodies were added to the assays, the results were very interesting. As shown in the chart of HLA specificities of the responders and the U937 cell line shown in Figure 32, only responder CS shares the HLA-DR2 specificity with the U937 cell line; however, all of the responders respond to the addition of antigen pulsed U937 cells to the assay. HLA-DR2 specific antibody was strongly inhibitory for responders CS and CJ and was small with responder CD, who it must be remembered from Figure 31C, was hyperresponsive to tetanus toxoid. HLA-DR3 specific antibody did not inhibit antigen presentation by either G4 or E11 subline or parent 2-1 cells. The effect of HLA-DR7 specific antibody on U937 mediated antigen presentation was variable, strongly inhibiting the response by CS but not the response by CJ. Unfortunately, the HLA-DR7 antibody was utilized only on two of the three responders. When these same antibodies were added to antigen presentation assays in which antigen pulsed autologous monocytes were added, anti-HLA-DR2 antibody blocked all responses somewhat with strongest inhibition of HLA-DR(5,6) responder CJ. Although this can not be explained at this time, heterologous antibodies used for typing reagents are polyclonal reagents which frequently have specificity for Dw52 and Dw53 supertypic specificities which react with HLA-DQ. In spite of their high correlation coefficients (R values) for a particular specificity, they are also reactive with these broader specificities, although in a microcytotoxicity assay they would not be strongly cytotoxic for the supertypic specificities. Anti-HLA-DR3 was slightly inhibitory of antigen presentation by autologous monocytes only from responder CD, [HLA-DR(3,5)] and anti-DR7 strongly inhibited responder CS, [HLA-DR(2,7)]. Inhibition of the antigen presentation by U937 cells by the addition of anti-HLA-DR7 indicates the possibility that in these assays there are sufficient residual antigen presenting cells to mediate a response by presenting antigen provided by the addition of the antigen pulsed U937 cells. Although the response appears to

correlate with the HLA-DR7 specificity known to be expressed by the responder, the presence of other specificity interactions can not be ruled out. This data, particularly with regard to HLA-DR specificity specific blocking, must be viewed as preliminary due to the small number of responders examined and their HLA disparate nature; however, there was strong inhibition of antigen-pulsed U937 stimulation of 3H-thymidine incorporation, i.e. antigen presentation, by monoclonal antibodies specific for human Ia and for CD4.

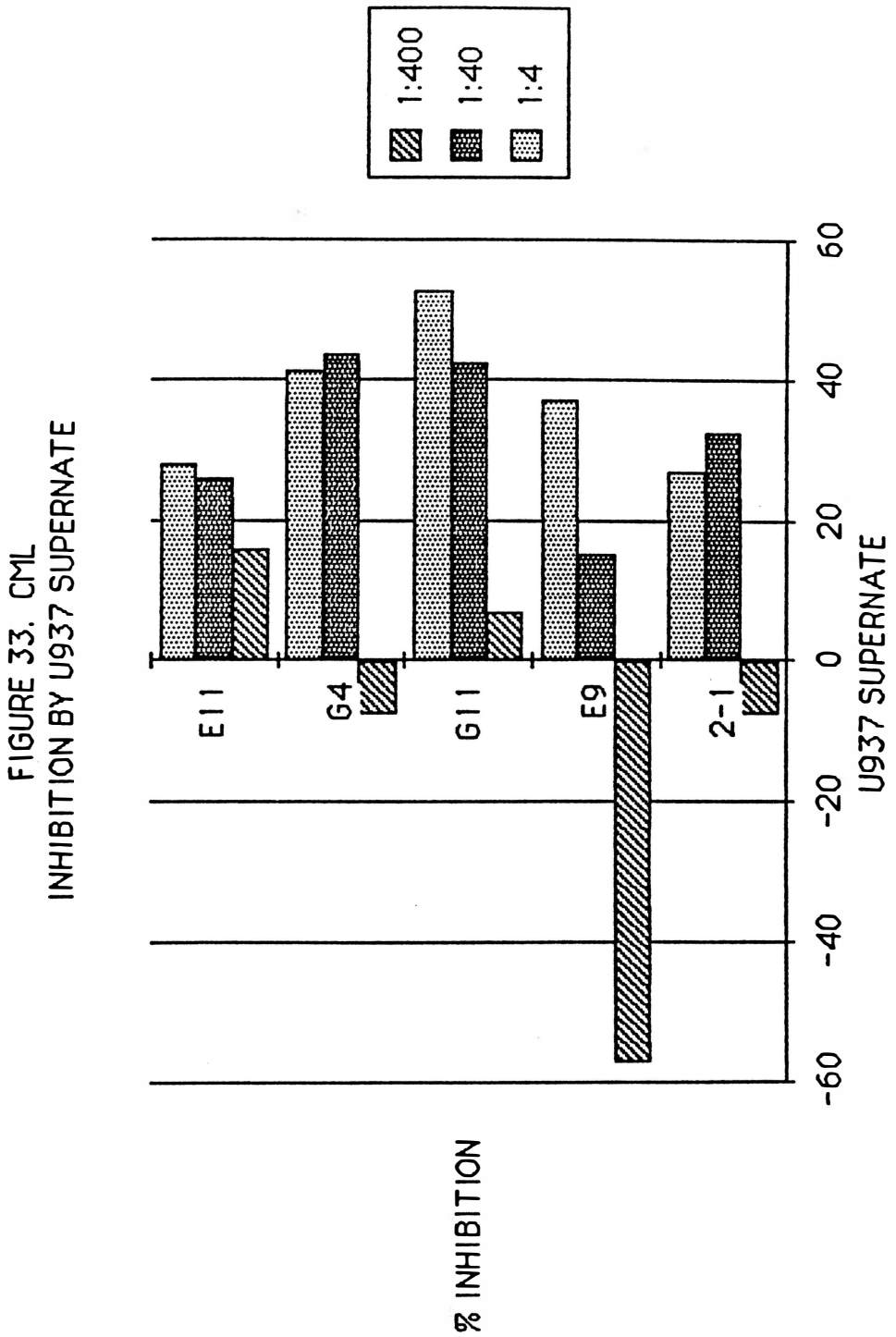
The inhibitory capacity of U937 supernatant was investigated.

Supernatant from U937 cells was found to inhibit the CML response. Previously it had been shown in Figure 23 that supernatants from the parent and all sublines tested were able to inhibit a normal MLR when added at concentrations as high as 1:400. Further investigation of the inhibitory properties of the supernatant were undertaken. Figure 33 summarizes the inhibitory effects of added supernatant in the generation of a CML response. As shown, the generation of a CML was relatively less sensitive to the supernatant effects than the MLR in that the % inhibition was less than 60% in all cases in the CML rather than the 85% for the MLR. Because the CML is quantitated by measurement of chromium release and the MLR is quantitated as ^{3}H-thymidine incorporation, the two values are not exactly comparable but give a general idea about relative sensitivity. Because a strong proliferative response is not necessary for the generation of the CML, the relative insensitivity of the CML may reside in reduction of cell numbers of cytolytic T cell precursors (CTLp) which can respond. Inhibition seen in the MLR in which the incorporation of radionuclide is correlated with cellular proliferation may indicate either a cytostatic effect or a cytotoxic effect and probably both. Also seen in Figure 33 is the markedly decreased inhibition for sublines E11 and G11 and enhancement with sublines G4 and E9 and the parent 2-1 cells of the CML response with the addition of small amounts of

FIGURE 33. ADDITION OF SUPERNATANT FROM U937 PARENT 2-1 AND SUBLINES E11, G4, G11 AND E9 INHIBITS THE CML RESPONSE.

Cell mediated lympholysis assays (CML) were set up between normal PBMC responders and stimulators as previously described. Supernatant from U937 parent 2-1 and sublines E11, G4, G11 and E9 were added to assay wells at concentrations of 1:4, 1:40 and 1:400 at the initiation of the assay. Results are indicated as the % Inhibition of the % Cytotoxicity in the normal CML in the six hour ^{51}Cr release assay.

FIGURE 33. CML
INHIBITION BY U937 SUPERNATE

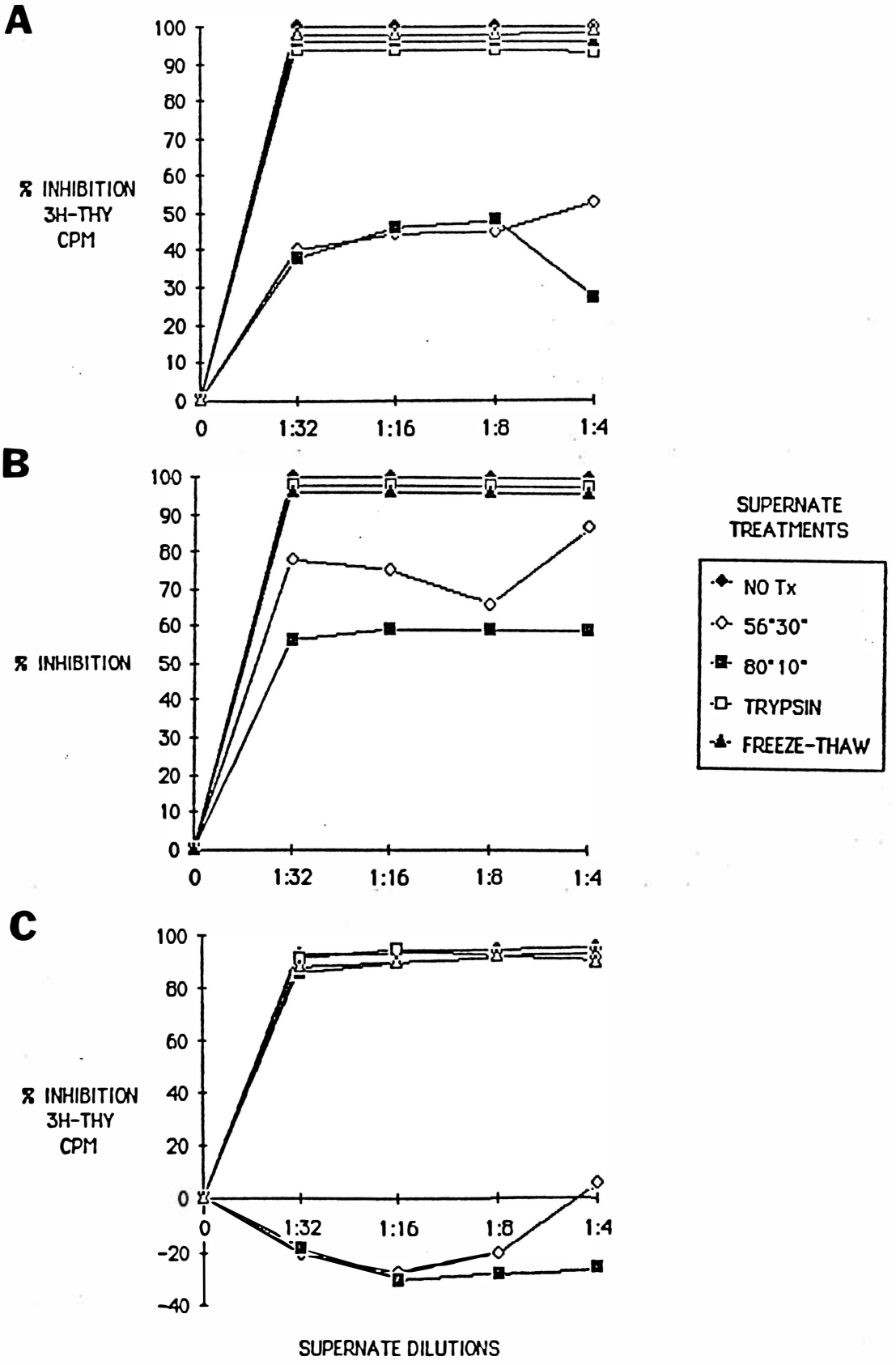


supernatant, 1:400 dilution. This indicates that the supernatant probably contains a complex mixture of different secreted products some of which are inhibitory and some stimulatory having specific activities which are diluted out at different rates.

Supernatant from U937 also inhibits tritiated-thymidine incorporation in other assays and is partially heat labile. Figure 34 illustrates typical inhibitory patterns in three assays. The MLR as previously shown in Figure 23 is inhibited by addition of supernatant from parent 2-1 U937 cells and from all sublines tested. Supernatants added to assays shown in Figure 34 were pooled from equal quantities of supernatant from parent 2-1 and sublines E11, G4, G11, and E9. Pooled supernatant added to both MLR and PHA responses, Figure 34 (A and B) inhibit those assays, both of which utilize PBMC isolated from normal donors. When supernatants were subjected to digestion by trypsin or chymotrypsin coated beads for 30-60 minutes at 37°C no diminution in inhibitory capacity was noted. Also no loss of inhibitory capacity was noted after three cycles of freezing and thawing which involved 30 minutes at -40°C followed by 30 minutes at 37°C. Significant decrease in inhibition of both assays was seen, however, following either 30 minute incubation at 56°C or 10 minutes at 80°C. In the PHA response incubation at 80°C for 10 minutes was clearly more effective than the 56°C incubation, but the differences are seen only at the highest concentration in the MLR assay. In contrast, the assay shown in Figure 34C illustrates ^{3}H-thymidine incorporation by a lymphoid cell line, REH, which was diluted in normal culture medium and plated in 96 well plates at approximately 2 X 10^{5} cells/ml. Plates were incubated for 72 hours and pulsed with ^{3}H-thymidine for the final 16 hours. The REH cell line was found to be very sensitive to the effects of supernatant and ^{3}H-thymidine incorporation was inhibited by non-treated supernatant, enzyme digested supernatant and supernatant subjected to freezing and thawing. Unlike the PHA and MLR assays, supernatant which had been incubated at 56°C or 80°C acted as a growth factor and

FIGURE 34. INHIBITORY EFFECTS OF POOLED SUPERNATANT FROM U937 CELLS ARE PARTIALLY ABROGATED BY HEAT.

Supernatants were pooled from U937 parent 2-1 and sublines E11, G4, G11 and E9 and aliquots were subjected to digestion with trypsin or chymotrypsin coated beads, three cycles of freezing and thawing, heating at 56°C for 30 min., or heating at 80°C for 10 minutes. After treatment supernatants were added at stated dilutions to a normal MLR (A), PHA stimulation (B), or to REH cells (human null cell leukemia cell line) in culture (C). All assays were dependent on ^{3}H-thymidine incorporation during the final 16 hours of the assay. % Inhibition was calculated based on the response in the various assays in the absence of supernatant. All assays were repeated three times and values expressed are the mean of triplicate wells.

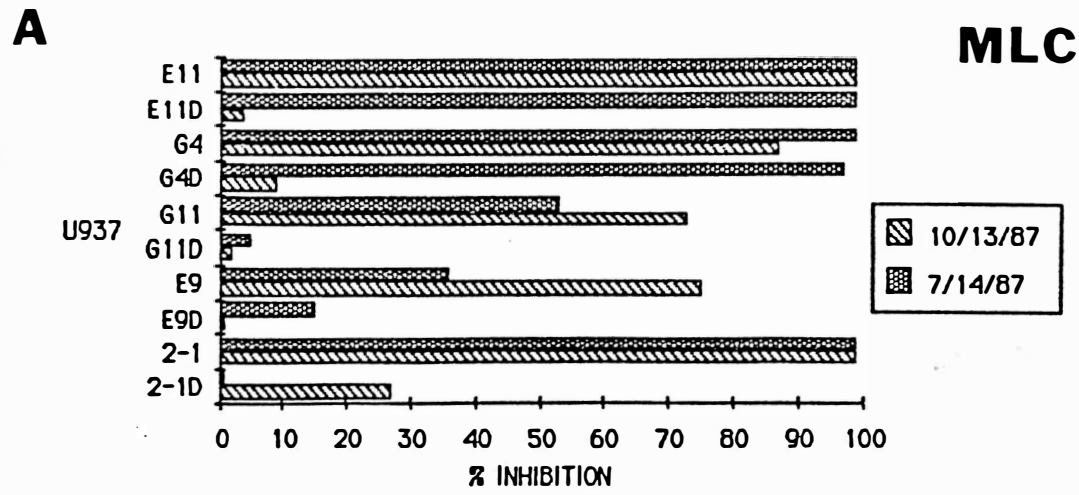

A
% INHIBITION
3H-THY
CPM
100
90
80
70
60
50
40
30
20
10
0
0
1:32
1:16
1:8
1:4
B
% INHIBITION
SUPERNATE
TREATMENTS
NO Tx
56°30"
80°10"
TRYPSIN
FREEZE-THAW
C
% INHIBITION
3H-THY
CPM
-20
-40
SUPERNATE DILUTIONS

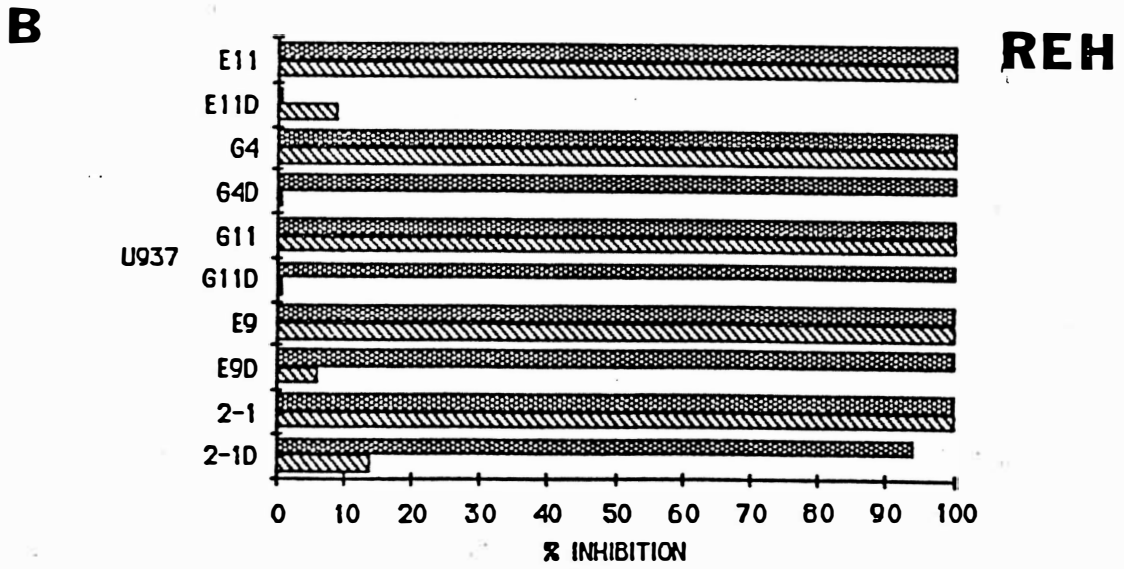
increased the ^{3}H-thymidine incorporation showing maximal effect at a 1:16 dilution. At a 1:4 dilution the supernatant treated at 56°C was very slightly inhibitory. These assays illustrate that the inhibitory factor is either not digested by trypsin or chymotrypsin or that digestion does not diminish potency. Also illustrated is the sensitivity to inactivation by heating but insensitivity to short cycles of freezing and thawing.

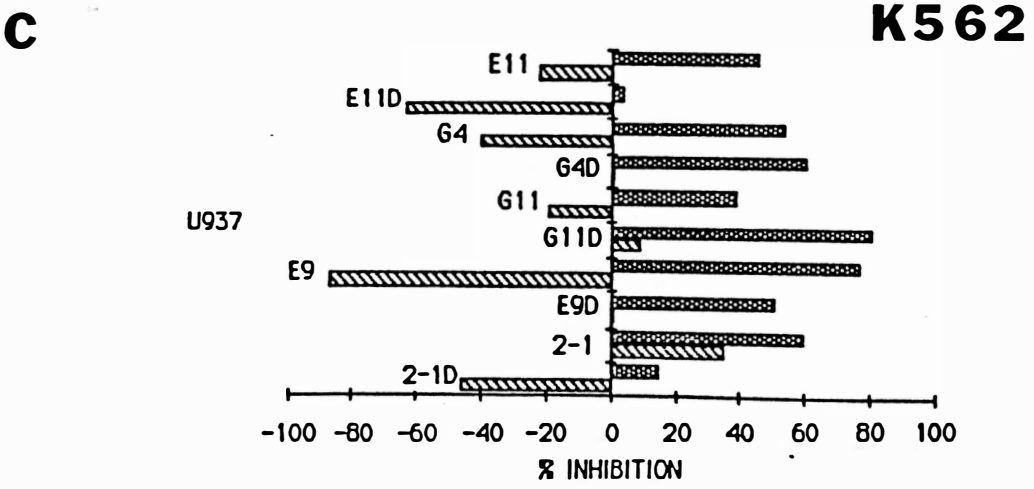
Dialyzed samples of supernatant were more labile than non-dialyzed samples over a three month period of storage. Two potentially inhibitory characteristics of the supernatants, i.e. the low pH of the supernatants removed from U937 cell cultures and the possibility that the U937 cells were producing prostaglandins or other small molecular weight inhibitory factors such as platelet activating factor (PAF-acether), were investigated by subjecting samples of the supernatants to dialysis against RPMI 1640 culture medium containing HEPES buffer to maintain a neutral pH during the period of dialysis at 4°C. Although sterile conditions were maintained during the dialysis, the dialyzed samples and other supernatant samples were sterilized by filtration following 24 hours at 4°C and aliquoted in small samples prior to freezing to obviate the necessicity for freezing and thawing samples repeatedly. The supernatants treated in this manner were added to various cellular assays over a three month period. As shown in Figure 35A, dialyzed supernatants from subline E11 and G4 cells were still strongly inhibitory when added to normal MLR assays; however, dialyzed supernatants from sublines G11 and E9 and parent 2-1 U937 were less inhibitory than non-dialyzed samples. The supernatant concentrations in all cases were 25% of the total well volume. The results with non-dialyzed supernatants from sublines G11 and E9 were surprising in that in previous assays using fresh supernatants at this high concentration, inhibition had always been greater than 80%. Supernatants used in these assays and tested on 7/14 had been in storage at -20°C for several months. Non

FIGURE 35. SUPPRESSIVE CAPACITY IN DIALYZED SAMPLES OF SUPERNATANT WAS MORE LABILE OVER THREE MONTHS IN STORAGE THAN NON-DIALYZED SAMPLES.

Supernatant from U937 parent 2-1 and sublines E11, G4, G11 and E9 were collected and divided. Half of each supernatant sample was dialyzed against fresh RPMI 1640 culture medium overnight. After dialysis, samples of dialyzed and non-dialyzed supernatants were aliquoted and frozen. These samples were then removed from storage at two time points and added to normal assays for MLR (A), REH cells in culture (B) and K562 (erythrleukemia cell line) cells in culture at a 1:4 concentration. ^{3}H-thymidine incorporation as cpm was measured and % Inhibition was calculated using values in assays to which no supernatant had been added. The MLR results are a composite of two assays for each date, each assay set up in triplicate wells. REH and K562 cells were set up as a single assay for each date, also set up in triplicate wells.


A
MLC
E11
E11D
G4
G4D
G11
U937
G11D
E9
E9D
2-1
2-1D
10/13/87
7/14/87
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION


B
REH
E11
E11D
G4
G4D
G11
U937
G11D
E9
E9D
2-1
2-1D
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION


C
K562
E11
E11D
G4
G4D
G11
U937
G11D
E9
E9D
2-1
2-1D
-100 -80 -60 -40 -20 0 20 40 60 80 100
% INHIBITION

dialyzed supernatants from parent 2-1 and subline E11 removed from the freezer three months later (10/13) and added to the same type of MLR assays were found to be as inhibitory as previously found at the same dilution (1:4); however, supernatant from the G4 subline was slightly less inhibitory and supernatants from sublines G11 and E9 appeared to be slightly more inhibitory, although the difference in the response with G11 and E9 supernatants was possibly attributable to differences in responsiveness of the responding cells and therefore resistance to inhibition. Results shown for dialyzed supernatants indicate that in all cases little inhibitory capacity remained. The somewhat higher inhibitions seen with dialyzed 2-1 parent supernatant may also reflect responder resistance rather than inherent supernatant change. As shown in Figure 35B in which supernatant was added to REH cells in culture as previously described, all supernatants, both non-dialyzed and dialyzed, except subline E11 were strongly inhibitory when tested on 7/14; however, when retested on 10/13, all dialyzed supernatants had lost most of their ability to inhibit the growth of REH cells whereas the non-dialyzed supernatants maintained inhibitory capacity. Unlike results seen in Figure 34C with heated supernatants, stored dialyzed supernatants showed no capacity for enhanced ^{3}H-thymidine incorporation. Results obtained when supernatants were added to the erythroleukemia cell line, K562, shown in Figure 35C indicate that supernatants whether dialyzed or not were never as inhibitory for K562 cells as for REH cells, although supernatant from G11 subline cells inhibited ^{3}H-thymidine incorporation by K562 cells by approximately 80%. Except for subline G4 supernatant where there were no differences between dialyzed and non-dialyzed supernatant inhibition, all dialyzed supernatants were less inhibitory than non-dialyzed supernatants when tested on 7/14. Most supernatants, both dialyzed and non-dialyzed, tested on 10/13 showed stimulatory capacity rather than inhibition, except for the small inhibition mediated by supernatant from the 2-1 parent and subline G11. Figure 35C illustrates that the inhibitory capacity of the supernatants changes over

time in culture and, as illustrated in Figure 35B, the inhibitory capacity in dialyzed supernatants is more labile than in non-dialyzed supernatants.

Effects of pH and possible prostaglandin production by U937 cells were examined. Parent 2-1 cells and sublines G4 and E11 cells were grown in media containing either 10mM HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) (Sigma) which has an efficient buffering range of 6.8-8.2 or 10^{-5}M indomethacin (1-[p-chlorobenzoyl]-5-methoxy-2-methylindole-3-acetic acid) (Sigma) which is an inhibitor of prostaglandin synthesis. Cells were washed and added to fresh media containing either addition and incubated for 72 hours. Supernatants were removed from these flasks and added to assays at dilutions of 1:4, 1:8, 1:16 and 1:32. As shown in Figure 36 (A, B, C) the inhibition of MLR, PHA stimulation and REH proliferation was nearly 100% for all supernatants at all dilutions and addition of HEPES and indomethacin to culture media had no effect. Although the concentration of indomethacin may not have been sufficient to prevent prostaglandin production for the entire three days during which the cells were in culture if the U937 cells were capable of metabolizing the indomethacin, it appears that the profound inhibition seen with supernatants from U937 cells is not attributable to either production of an excessively acidic environment or to production of prostaglandins. As indicated in Figure 35, however, some fraction of the inhibitory capacity is probably attributable to the effects of pH and dialyzable, i.e. small molecular weight, moieties which may or may not include prostaglandins. It is suggested that the specific evaluation of prostaglandin production would require the use of a radioimmunoassay.

Inhibition of tritiated-thymidine incorporation was shown to parallel a decrease in viable cells present. As shown in Figure 37, three of the assays in which the mode of assessment is evaluation of incorporation of ^{3}H-thymidine measured as cpm by liquid scintillation were set up in duplicate and evaluated *in two ways:* by standard methods of isotope counting and by cell counts of cells/well using a

FIGURE 36. PRODUCTION OF SUPPRESSIVE ACTIVITY WAS NOT DECREASED BY ADDITION OF HEPES BUFFER OR INDOMETHACIN TO U937 TISSUE CULTURE MEDIA.

U937 parent 2-1 cells and subline G4 and E11 cells were grown in the presence of 10 mM HEPES buffer or 10^{-5}M indomethacin for 72 hours. Cells incubated in normal tissue culture media were removed from culture, washed with fresh media, and added to fresh normal media or media containing either HEPES (+H) or indomethacin (+IM). Cells were incubated for an additional 72 hours after which supernatants were removed and tested at two dilutions (1:4 and 1:32) in three types of assays: (A) MLR, (B) PHA mitogen stimulation, and (C) REH cells in culture. Suppressive activity was determined as % Inhibition of ^{3}H-thymidine incorporation as previously described.

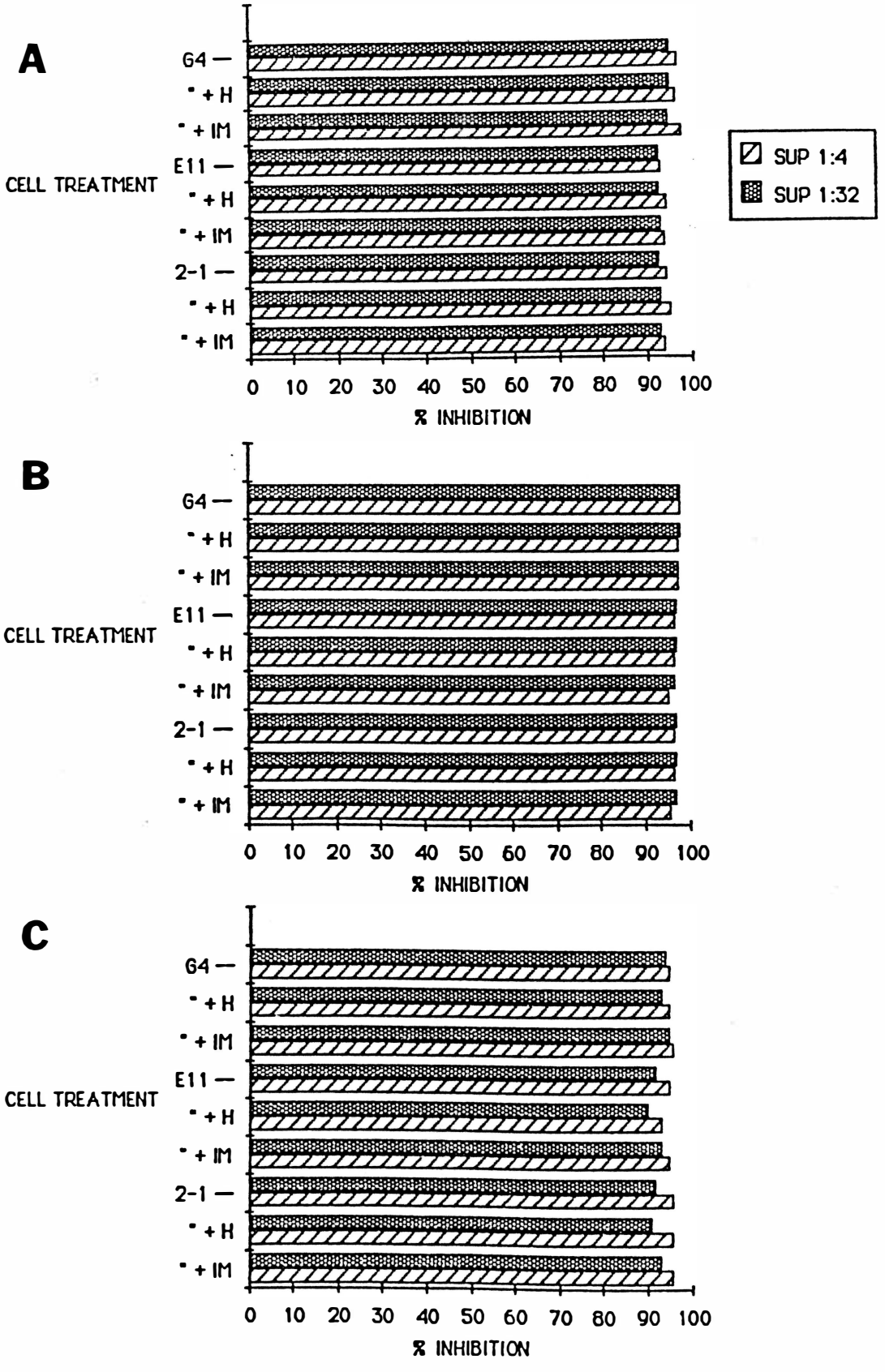
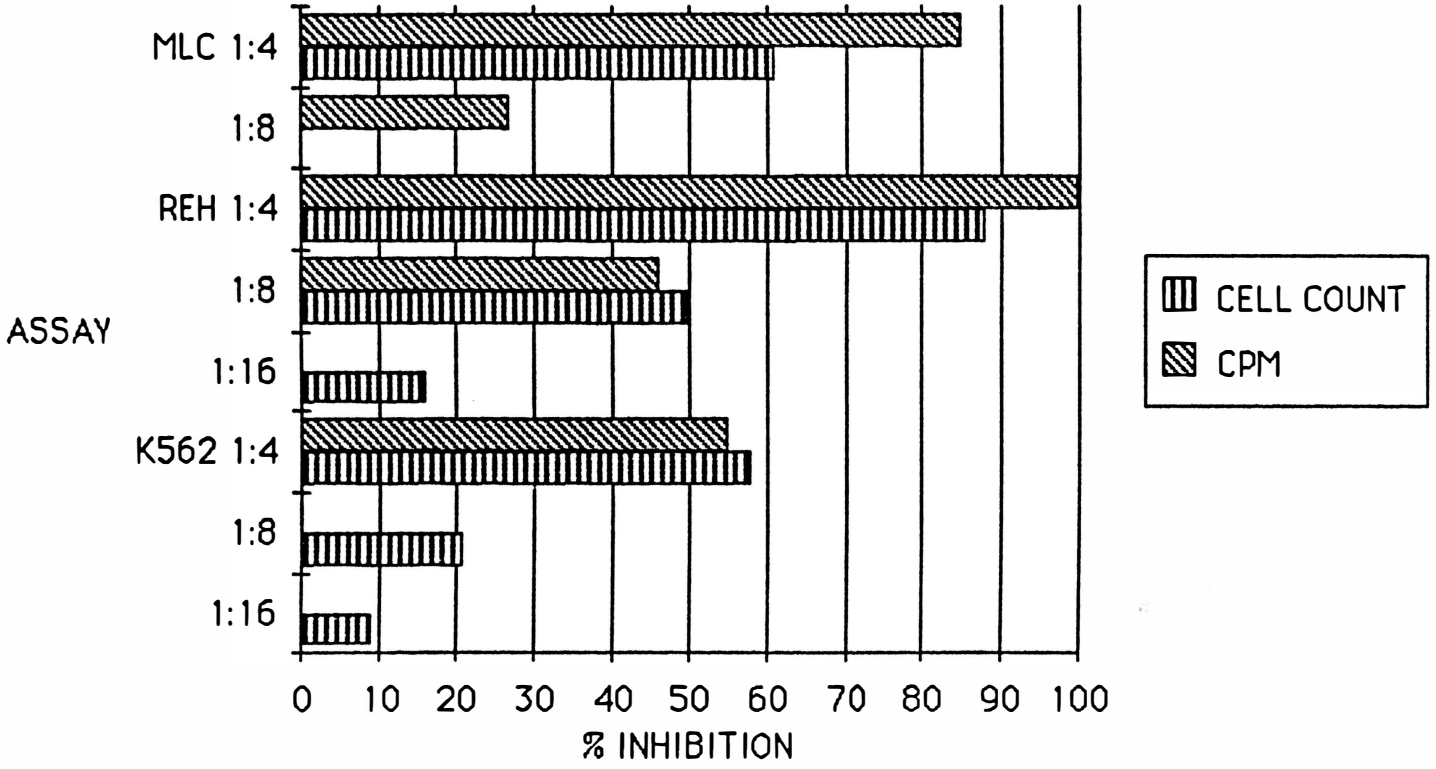

A
G4 —
" + H
" + IM
E11 —
" + H
" + IM
2-1 —
" + H
" + IM
CELL TREATMENT
SUP 1:4
SUP 1:32
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION
B
G4 —
" + H
" + IM
E11 —
" + H
" + IM
2-1 —
" + H
" + IM
CELL TREATMENT
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION
C
G4 —
" + H
" + IM
E11 —
" + H
" + IM
2-1 —
" + H
" + IM
CELL TREATMENT
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION

FIGURE 37. SUPPRESSIVE ACTIVITY AS ASSESSED BY 3H-THYMIDINE INCORPORATION CORRELATED WITH CYTOSTASIS.

Parallel assays were set up for MLR, REH cell culture and K562 cell culture. In one set of assays % Inhibition was assessed by ^{3}H-thymidine incorporation as described. In the second set of assays the % Inhibition was assessed by counting cells in a hemocytometer. Cell viability was determined by trypan blue exclusion.

FIGURE 37. SUPERNATE SUPPRESSION OF PROLIFERATION AND 3H-THYMIDINE INCORPORATION



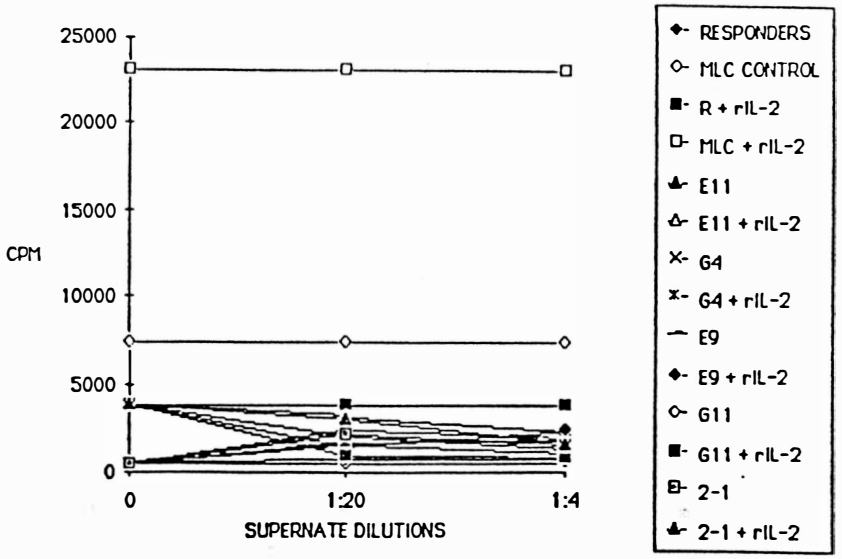
hemocytometer and determination of viable cells by trypan blue exclusion. The results in all three assays, (MLR, REH proliferation and K562 proliferation) indicate that where strong inhibition was seen using isotope counting, a marked decrease was also seen in viable cells present. When the MLR was examined, supernatant at a 1:8 dilution was found to inhibit ^{3}H-thymidine incorporation with no concomitant decrease in viable cells; however, because of the nature of the MLR, i.e. that there is a population of supposedly viable responder cells and a senescent population of irradiated stimulator cells, evaluation of viable cell counts involves large errors in counting which on average were the same as wells to which no supernatant had been added. In assays for proliferation of cell lines, counting of cells was simplified by the presence of only one cell type. In these assays, changes in cell number appeared to be a more sensitive measure of supernatant added than ^{3}H-thymidine incorporation as the decrease in cell count was dose dependent. It must be noted that in all assays except proliferation of K562, a significant increase in the percentage of dead cells present was noted when supernatants were added (data not shown). The supernatants which were cytotoxic for both normal PBMC and REH cells were not cytotoxic for K562 cells, although there was clearly a cytostatic effect on K562 cells. The supernatants added to all assays were not completely cytotoxic to any cell population and although many cells appeared to be more granular and vacuolated in the presence of supernatant, they continued to exclude trypan blue. The supernatants used in this series of experiments exhibited decreased inhibitory capacity as shown by the marked decrease in inhibition at 1:8 dilution. This decrease is attributed to their long term storage as pooled supernatant kept for several months at -20°C.

Inhibition of cellular assays was not overcome by addition of recombinant IL-2. Using fresh supernatants from sublines E11, G4, G11 and E9 and from the 2-1 parent U937 cells, ^{3}H-thymidine incorporation by normal PBMC in normal MLR (Figure 38A), PHA stimulations (Figure 38B) and Con A stimulations (Figure 38C) was

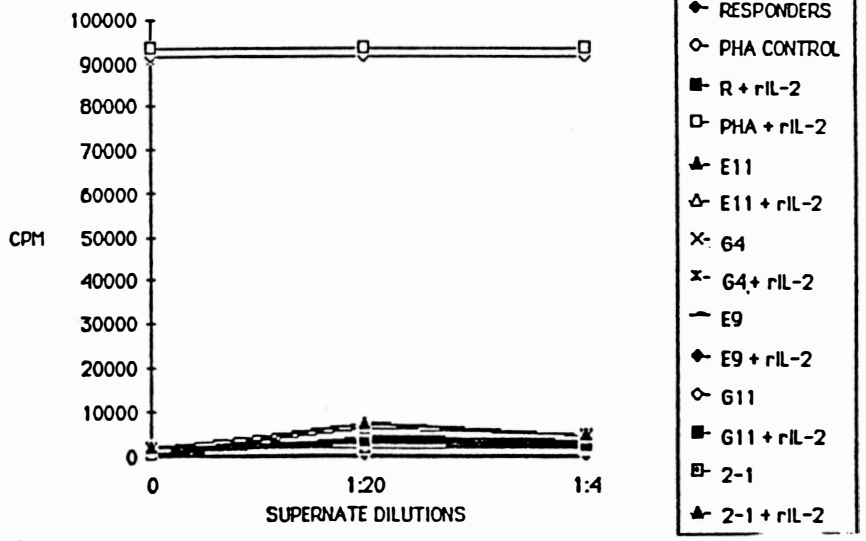
FIGURE 38. INHIBITION OF CELLULAR ASSAYS WAS NOT OVERCOME BY ADDITION OF RECOMBINANT IL-2.

Cellular assays using normal PBMC were set up as described. Supernatants from U937 2-1 parent and sublines E11, G4, G11 and E9 were added to microtiter wells at two dilutions: 1:4 and 1:20. Recombinant IL-2 (100 u/well) was added at initiation of the assays and 15 minutes prior to addition of supernatant. Results are presented as the mean of ^{3}H-thymidine cpm from triplicate wells in two assays.

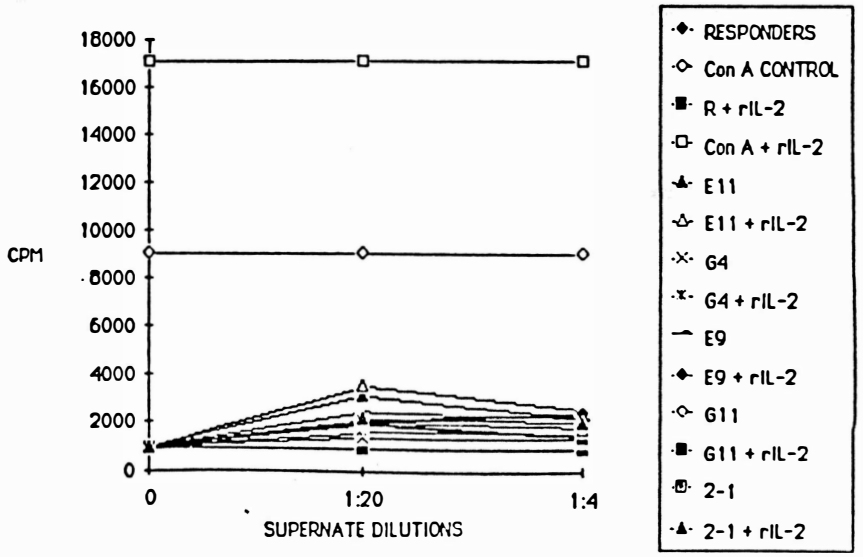
A


25000
20000
15000
CPM
10000
5000
0
0
1:20
1:4
SUPERNATE DILUTIONS
RESPONDERS
MLC CONTROL
R + rIL-2
MLC + rIL-2
E11
E11 + rIL-2
G4
G4 + rIL-2
E9
E9 + rIL-2
G11
G11 + rIL-2
2-1
2-1 + rIL-2

B


100000
90000
80000
70000
60000
CPM
50000
40000
30000
20000
10000
0
0
1:20
1:4
SUPERNATE DILUTIONS
RESPONDERS
PHA CONTROL
R + rIL-2
PHA + rIL-2
E11
E11 + rIL-2
G4
G4 + rIL-2
E9
E9 + rIL-2
G11
G11 + rIL-2
2-1
2-1 + rIL-2

C


18000
16000
14000
12000
10000
CPM
8000
6000
4000
2000
0
0
1:20
1:4
SUPERNATE DILUTIONS
RESPONDERS
Con A CONTROL
R + rIL-2
Con A + rIL-2
E11
E11 + rIL-2
G4
G4 + rIL-2
E9
E9 + rIL-2
G11
G11 + rIL-2
2-1
2-1 + rIL-2

abrogated by supernatants from all U937 cell sources at dilutions of 1:4 and 1:20. Addition of recombinant IL-2 did not overcome the effects of the added supernatants in any case, although addition of IL-2 significantly increased the weak MLR and the Con A response. The PHA response was not augmented by the addition of IL-2; however, the PBMC may have been maximally stimulated without IL-2 and unable to mount any increase in response.

A partial fractionation of supernatant indicated that the primary inhibitory factor eluted was a relatively high molecular weight. U937 cells were transfered to media containing 1% fetal bovine serum after growing routinely in 10% fetal bovine serum. Cells were incubated for 48 hours in reduced fetal bovine serum after which supernatant was collected, pooled and concentrated 50 fold by vacuum dialysis as shown in Figure 39. Concentrated supernatant and a sample of pooled, neat supernatant were dialyzed against PBS. Concentrated supernatant was applied to an 85 cm^3 column of Sephacryl S-200 equilibrated in PBS and 1 ml. fractions were collected. Absorbance was measured spectrophotometrically at 280 nm. as shown in Figure 40A and fractions were pooled as shown in Figure 40B. It is apparent from the elution pattern shown in Figure 40 that fractions pooled would contain a mixture of components; however, it was hoped that the inhibitory capacity would be found to reside in one or perhaps two fractions.

As indicated in Figure 41, inhibitory capacity was found to some degree in all fractions eluted from the column; however, the most potent fractions eluted early. Inhibition of normal MLR was chosen as the assay system for assessing the distribution of inhibitory capacity in column fractions. Column fractions initially screened were added to assay wells at a final dilution of 1:4. Maximal inhibition was observed in fractions II and III as indicated in Figure 41A. To confirm this initial finding and to examine the potency of the inhibitory fractions, a second series of *MLR assays were* performed and fractions I-IV were added at a series of dilutions, 1:4, 1:8, 1:16 and

FIGURE 39. SCHEMATIC PROCEDURE FOR COLLECTION, CONCENTRATION & FRACTIONATION OF CELL SUPERNATANT BY SEPHACRYL S-200 COLUMN CHROMATOGRAPHY.

U937 cells (parent 2-1 and sublines E11, G4, G11 and E9) were grown in media containing 1% fetal bovine serum for 48 hours. Supernatant was collected by centrifuging cells, and equal amounts were pooled from each cell source. Pooled supernatant was concentrated by vacuum dialysis using dialysis tubing with a molecular weight exclusion of 12,000-14,000. Following 50X concentration and dialysis against PBS, concentrated supernatant was added to an 85 cm^3 column of Sephacryl S-200 and 1 ml. fractions were collected.

SUPERNATE COLLECTION, CONCENTRATION & FRACTIONATION

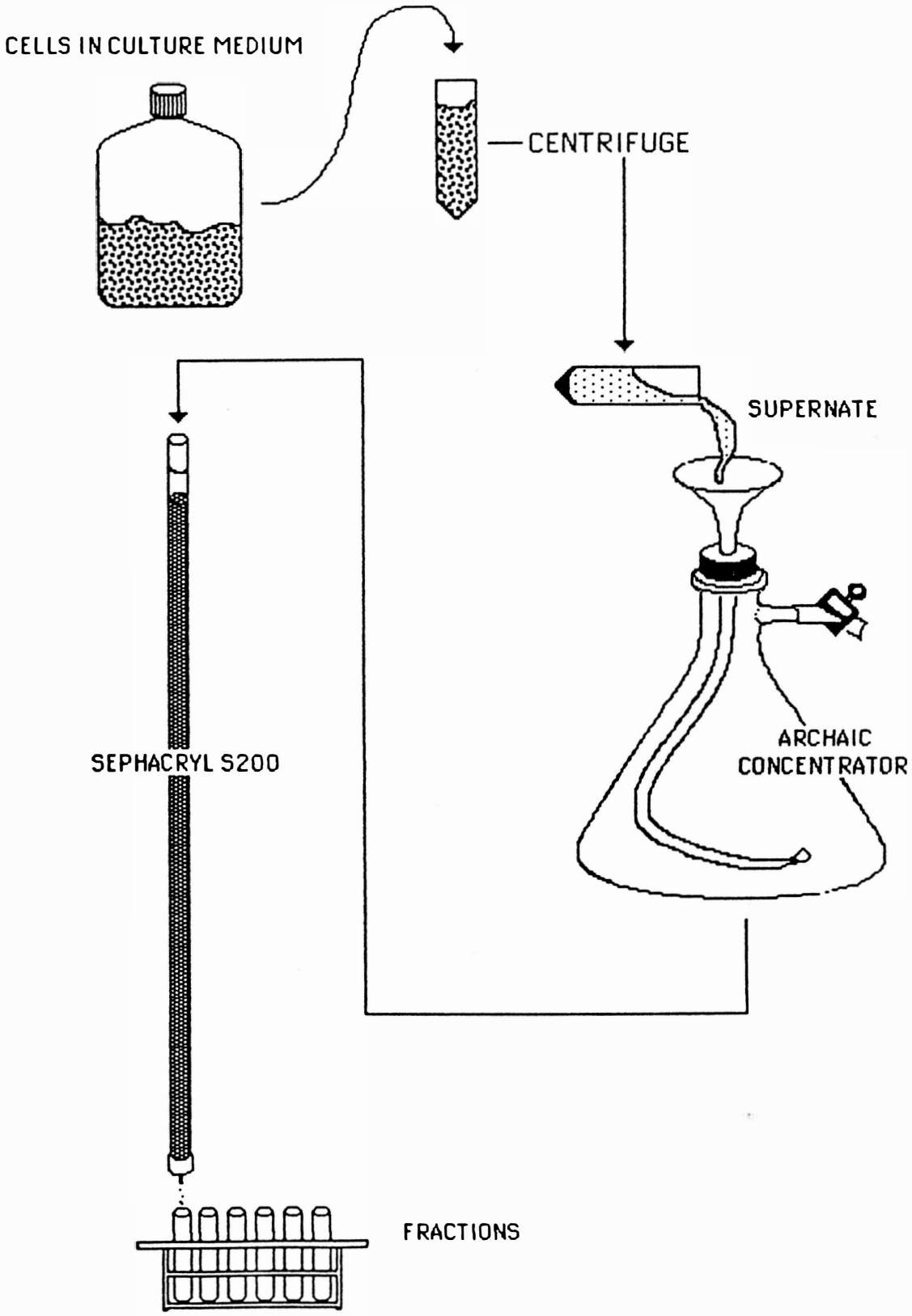
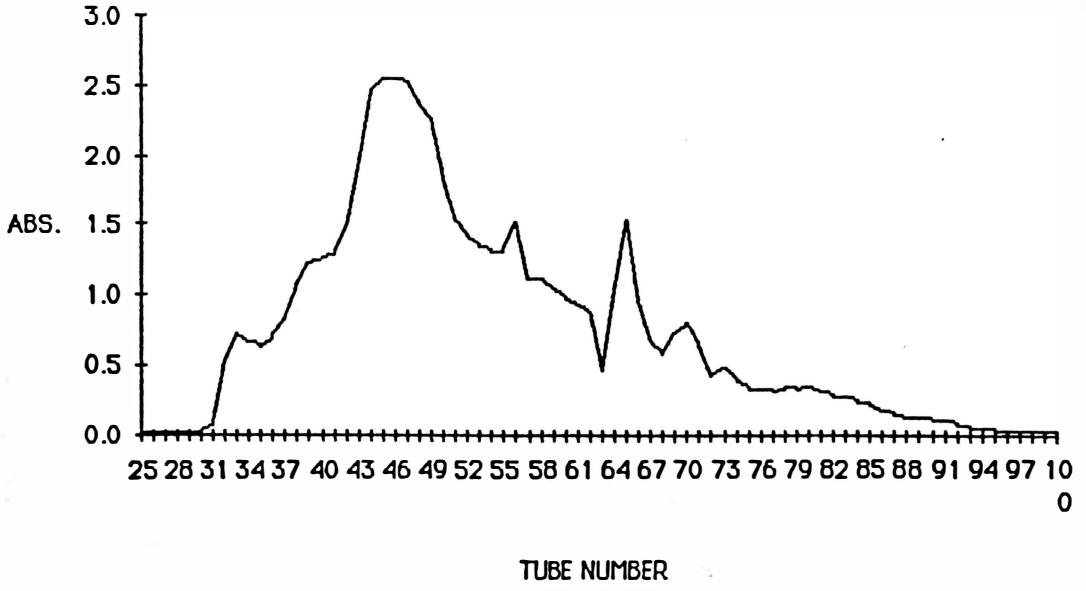


FIGURE 40. SUPERNATANT FRACTIONS WERE ELUTED FROM SEPHACRYL S-200 AND POOLED.

Fractions (1 ml. each) collected from the column were eluted as illustrated in (A). Absorbance was measured by spectrophotometry at 280 nm. Fractions were pooled as indicated in (B).

A


SUPERNATE FRACTIONATION-SEPHACRYL S-200
3.0
2.5
2.0
ABS. 1.5
1.0
0.5
0.0
25 28 31 34 37 40 43 46 49 52 55 58 61 64 67 70 73 76 79 82 85 88 91 94 97 10
0
TUBE NUMBER

B

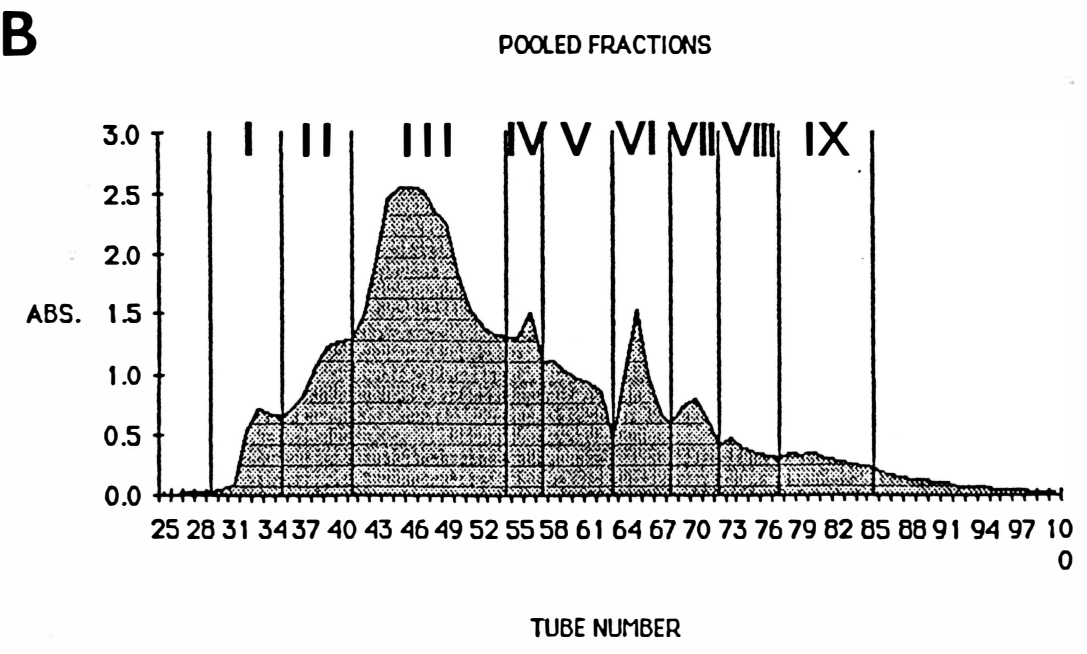
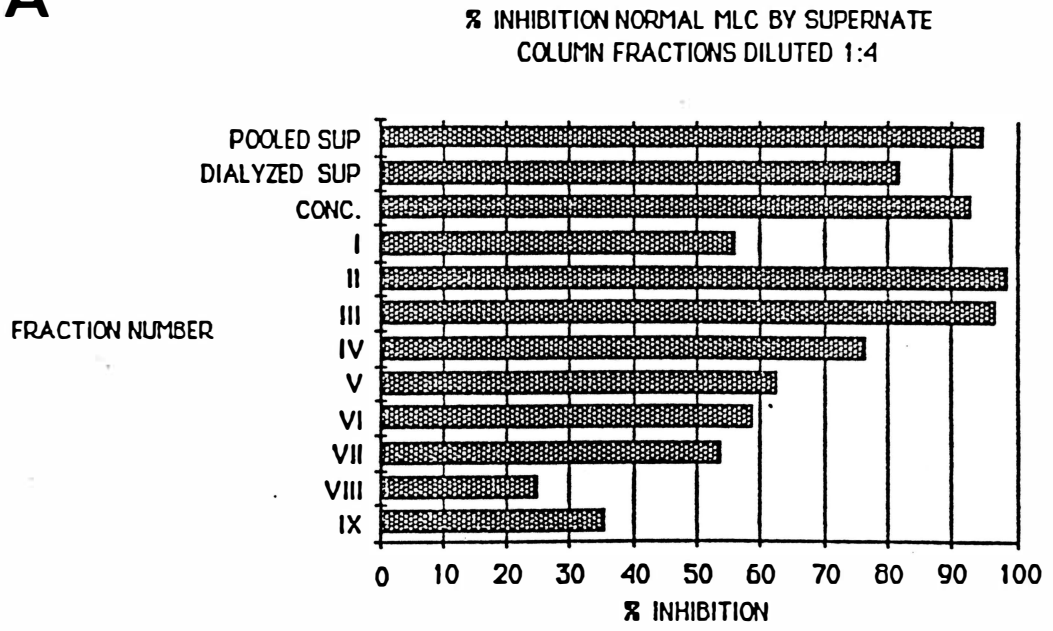

POOLED FRACTIONS
I II III IV V VI VII VIII IX
3.0
2.5
2.0
ABS. 1.5
1.0
0.5
0.0
25 28 31 34 37 40 43 46 49 52 55 58 61 64 67 70 73 76 79 82 85 88 91 94 97 10
0
TUBE NUMBER

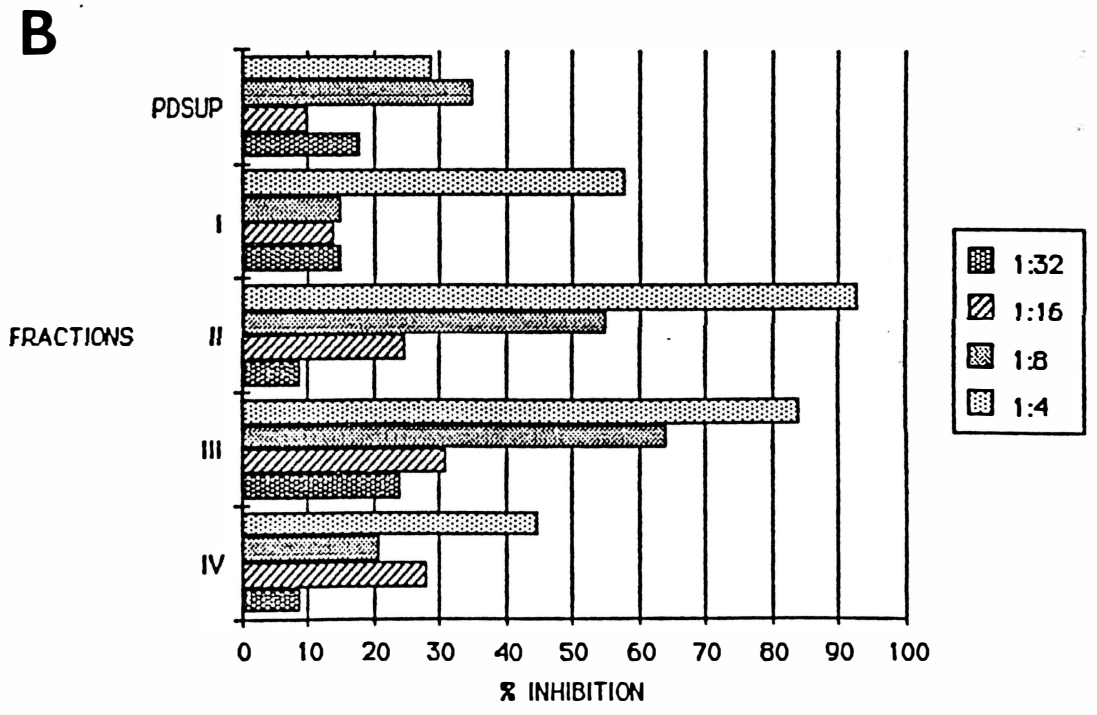
FIGURE 41. POOLED FRACTIONS WERE TESTED FOR SUPPRESSIVE ACTIVITY IN NORMAL MLR.

Fractions of supernatant were added to normal MLR set up as described. Suppressive activity indicated as % Inhibition was determined by ^{3}H-thymidine incorporation relative to MLR to which no supernatant was added. Results represent the mean of triplicate wells for each fraction in two assays. As indicated in (A) all fractions were tested at a dilution of 1:4. In (B) fractions I, II, III, and IV which had appeared to contain the most potent activity in (A) were further tested at dilutions of 1:8, 1:16, and 1:32.

A


% INHIBITION NORMAL MLC BY SUPERNATE
COLUMN FRACTIONS DILUTED 1:4
POOLED SUP
DIALYZED SUP
CONC.
I
II
III
IV
V
VI
VII
VIII
IX
FRACTION NUMBER
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION

B


PDSUP
I
II
III
IV
FRACTIONS
1:32
1:16
1:8
1:4
0 10 20 30 40 50 60 70 80 90 100
% INHIBITION

1:32. As indicated in Figure 41B, maximal inhibition was again observed in fractions II and III, although as previously seen at the highest concentration strong inhibition was also seen in fractions I and IV. Inhibition in fractions I and IV was not as strong as that seen in fractions II and III and was rapidly lost at higher dilutions. Thus the inhibitory component appears to have eluted in early fractions (tubes 36-41 and tubes 42-54) indicating from calculated Kav values based on void volume determination and the separation capacity of Sephacryl S-200 that it probably has a molecular weight between 50,000-100,000. Fraction II was a pool of six tubes containing 1 ml. each. Although not cleanly separated it was projected by calculated values to contain a mixture of components with molecular weights ranging from approximately 120,000 to 60,000. Fraction III contained the whole large major peak and probably contained the major portions of everything having molecular weights from above 60,000 to approximately 20,000 including as expected the highest protein content, probably including albumin from the fetal bovine serum. Unfortunately, due to the archaic nature of the chromatographic procedure,the column was not calibrated prior to use and when retrieved for calibration after use, was found in two pieces--perhaps for discontinuous separations. Samples of the column fractions were run under reducing conditions on Pharmacia-LKB Phastgel. Fraction I had too little protein content to reveal any band. As assessed by molecular weight standards the major band seen in fraction II had a molecular weight of approximatly 90,000. This band appeared also in fraction III, although at a lower concentration, and the major band in fraction III was a large band with molcular weight of approximately 66,000. This band is very probably albumin attributable to the fetal bovine serum present in the medium. The "albumin" band, but not the 90,000 kd band, persisted throughout the next five fractions at progressively decreasing concentration. It is postulated that the inhibitory component is the 90,000 kd band seen in both fractions II and III.

Interleukin 1 production by macrophages and other cell types constitutes an immunologically important product, both soluble and membrane bound. As monocyte-like cells, it was expected that the U937 parent and subline cells would produce IL-1 as had been previously been reported (397). IL-1 activity was measured in a costimulator assay using the IL-1 dependent T cell line, D10.G4.1 developed by Janeway et. al. (444) with a submitogenic dose of con A. This proved to be an unsuitable method for determination of soluble IL-1 because of its dependence on proliferation as indicated by ^{3}H-thymidine incorporation as seen in Figure 42. The assay was performed by addition of dilutions of supernatant from the U937 cells to D10.G4.1 cells and incubation with a submitogenic dose of con A for 72 hours with a ^{3}H-thymidine pulse for the last 16-18 hours. When samples were counted for ^{3}H-thymidine incorporation, it was found that the supernatants from 2-1 parent and sublines E11, G4, G11 and E9 had inhibited the background incorporation to such an extent that the stimulation indices were approximately 0.1. The control stimulation of the D10.G4.1 cell line mediated by the addition of dilutions of purified human IL-1 (0.5 µl.-0.125 µl.) is also shown to indicate that indeed the cells were capable of mounting a significant response to human IL-1. Quantitation of the specific activity of the human IL-1 (Genzyme) used was prevented by the finding that maximal stimulation reached a plateau at low concentration which was maintained throughout several different dilutions and abruptly became inhibitory of D10.G4.1 stimulation at higher doses. A dose response curve was not run over sufficient number of different dilutions to warrant assignment of IL-1 units, although the original preparation was assayed to contain 1 unit of IL-1 activity/µl.

Heat treatment of supernatant abrogated its inhibitory capacity in the IL-1 assay. As indicated in Figure 43, non-treated supernatant completely inhibited even the background ^{3}H-thymidine incorporation by the D10.G4.1 cells and this inhibition

FIGURE 42. U937 SUPERNATANT SUPPRESSED ^{3}H-THYMIDINE INCORPORATION BY IL-1 INDICATOR CELL LINE, D10.G4.1.

Fresh supernatant from U937 parent 2-1 and sublines E11, G4 and G11 was added at dilutions of 1:2, 1:4, and 1:8 to D10.G4.1 cells in the presence of a submitogenic dose of con A (2.5 μg/ml.) in microtiter wells. Plates were incubated for 72 hours and pulsed with 1 μCi ^{3}H-thymidine for the final 16 hours. Results represent stimulation indices determined by comparison of cpm (the mean of triplicate wells) of control wells to which no additions were made to wells containing either (a) 0.5 μl. (1:2), 0.25 μl. (1:4), and 0.125 μl.(1:8) of purified IL-1 or (b) dilutions of supernatant.

U937 SUPERNATE SUPPRESSION OF 3H-
THYMDINE INCORPORATION BY D10.G4.1 CELLS

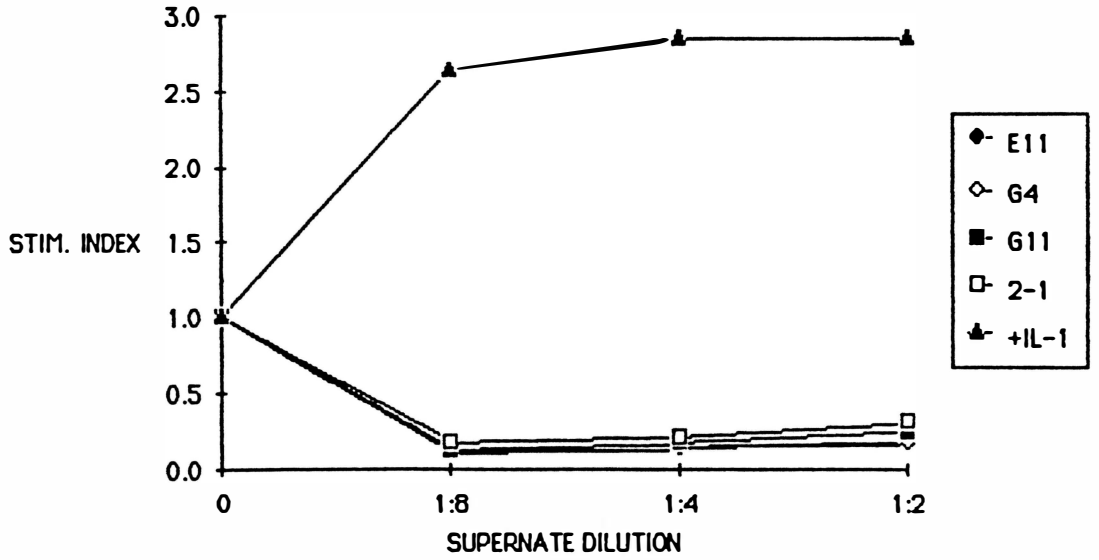
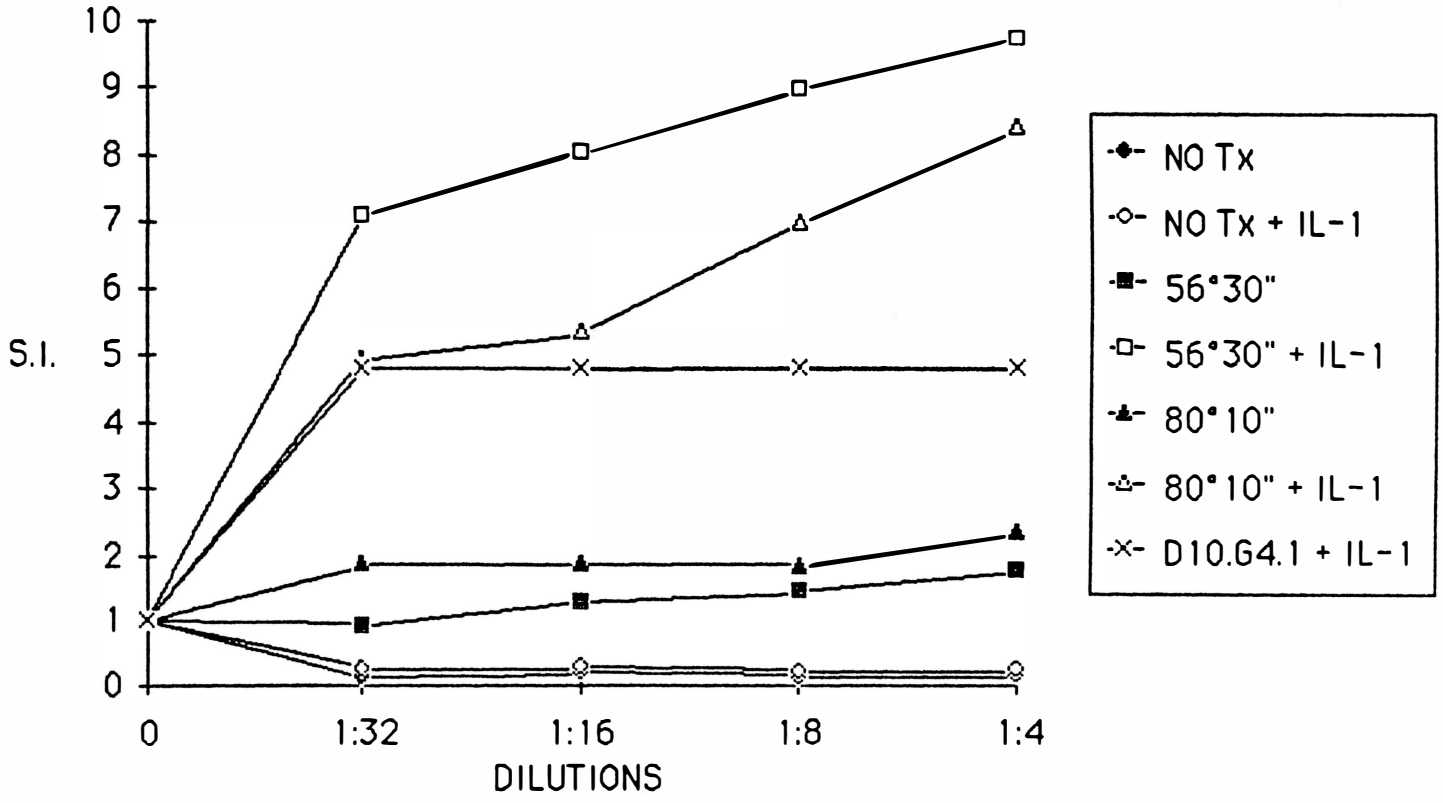


FIGURE 43. HEAT TREATMENT OF SUPERNATANT ABROGATED ITS INHIBITORY CAPACITY IN THE IL-1 ASSAY.

Supernatant, at dilutions of 1:4, 1:8, 1:16, and 1:32, was added to IL-1 assay using D10.G4.1 indicator cell line. Supernatant had been pooled, aliquoted and subjected to two heat treatments: 56°C for 30 minutes (56°30") and 80°C for 10 minutes (80°10"). Purified IL-1 added to wells was added as 1 µl./well of dilutions stated. Results are presented as stimulation index (S.I.) determined by comparison of the mean of control triplicate wells to which no additions were made with the mean of triplicate wells containing stated additions in three assays.

FIGURE 43. IL-1 DETERMINATION U937
SUPERNATE



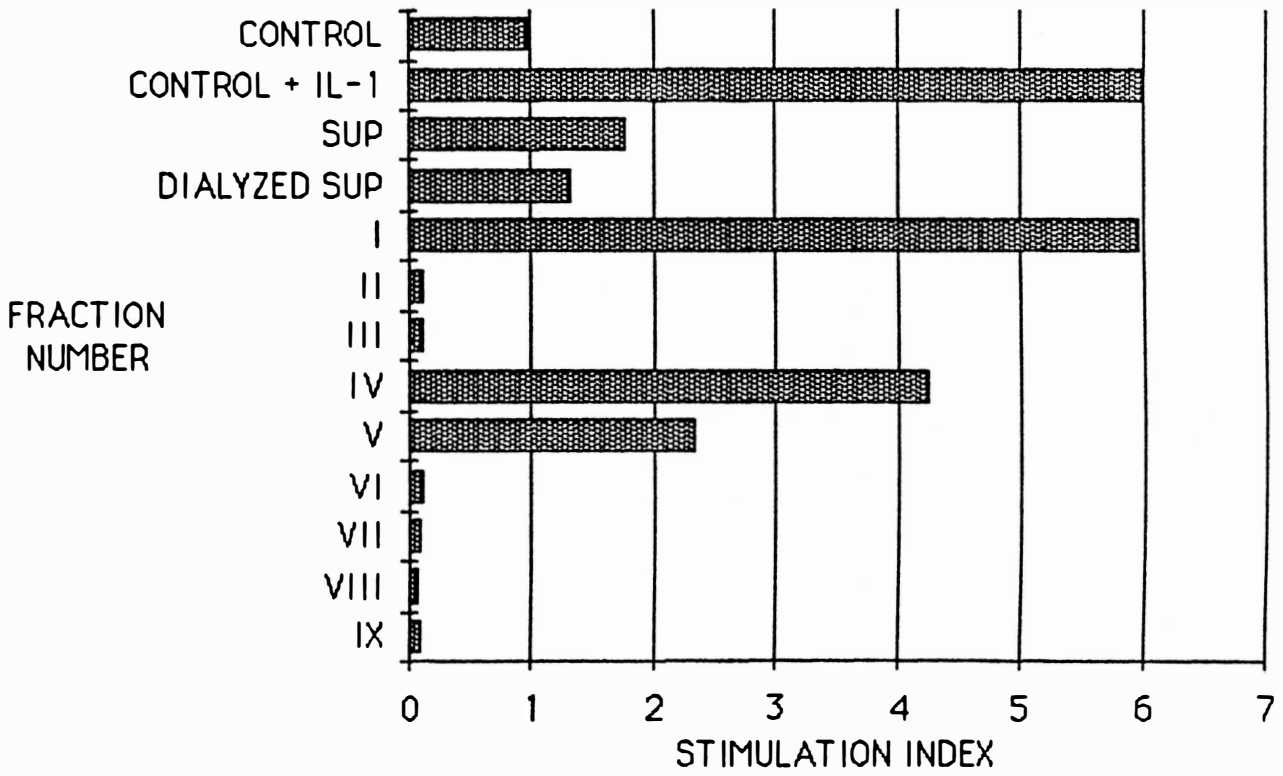
was not overcome by addition of exogenous purified IL-1. However, supernatant treated by heating at 56°C for 30 minutes or 80°C for 10 minutes was no longer inhibitory and instead was found at highest concentration (1:4) to be slightly stimulatory in the IL-1 assay. Addition of purified IL-1 to the treated supernatants vastly augments the stimulation of the D10.G4.1 cells. As previously described, the typical pattern of stimulation seen after the addition of human IL-1 is readily apparent in the plateau achieved after the addition of a 1:8 dilution of 1 µl. of purified IL-1. This plateau is clearly overcome or exceeded by the addition of heat treated pooled U937 supernatant. The implication from this data is that there is some factor or component present in the U937 supernatant which is masked by the inhibitory factor and which is not the same as purified IL-1 but which acts as a costimulator or growth factor in the IL-1 assay.

Column fractions obtained by molecular sieving of concentrated pooled supernatant were also tested for IL-1 activity. As indicated from Figure 41, the major inhibitory capacity eluted in the second and third pooled fractions although other fractions exhibited significant inhibition of MLR assays. As indicated in Figure 43, heat treated supernatant showed abrogated inhibition of the IL-1 assay and a small degree of inherent IL-1 activity. The column fractions tested showed an interesting pattern of reactivity shown in Figure 44. Fractions II and III which had exhibited maximal MLR inhibition also inhibited the IL-1 assay and background ^{3}H-thymidine incorporation to give a stimulation index of approximately 0.2. Fractions VI-IX which were proposed to comprise the small molecular weight components found in the pooled supernatant also exhibited strong inhibitory capacity in the IL-1 assay to a slightly higher degree even than fractions II and III to give a stimulation index close to 0.1 for fractions VII-IX. Strong stimulation of ^{3}H-thymidine incorporation by D10.G4.1 cells mediated by addition of fraction I, and to a somewhat lesser degree, by addition of fractions IV and V, indicates that perhaps soluble IL-1 is constituitively produced by

FIGURE 44. IL-1 ACTIVITY WAS DETECTED IN SUPERNATANT SEPHACRYL S-200 COLUMN FRACTIONS.

Column fractions of pooled supernatant obtained by fractionation on Sephacryl S-200 were added at a 1:4 dilution to IL-1 assays using D10.G4.1 indicator cells. Results are expressed as Stimulation Index determined by comparison of cpm of 3H-thymidine incorporation (the mean of triplicate wells) of control wells to which no additions were made with cpm of wells containing supernatant in three assays. Purified IL-1 (1 µl./well) was added to assess the capacity of the D10.G4.1 cells to respond in the assay.

FIGURE 44. IL-1 DETERMINATION ON SUPERNATE SEPHACRYL S-200 COLUMN FRACTIONS



the U937 cells. If the factor produced is not strictly synonymous with IL-1, then it may have IL-1-like activity and synergize with IL-1 as indicated in Figure 43. Clearly indicated from the preceeding experiments is the finding that assessment of IL-1 presence and its quantitation can not be satisfactorily achieved in an assay dependent on proliferation as measured by ^{3}H-thymidine incorporation especially in supernatant samples in which the inhibitor factor(s) is present in high concentration.

Assessment of membrane IL-1 on U937 cells was facilitated by the use of paraformaldehyde-fixed cells. Figure 45 illustrates experiments in which constituitively expressed membrane IL-1 was assessed on the 2-1 parent and sublines E11, G4 and G11. Paraformaldehyde fixed PBMC were used as controls for normal constituitive membrane IL-1 expression. Apparent from Figure 45 is the finding that at low doses of fixed U937 cells, 5 X 10^3 and 1 X 10^4 cells/well, there is small but consistent stimulation of D10.G4.1 cells. Maximal stimulation reached with 1 X 10^4cells/well decreases at higher cell concentrations and becomes slightly inhibitory at 1 X 10^5 cells/well for sublines E11 and G4. The 2-1 parent cell line reaches a maximal stimulation at 5 X 10^4 cells/well but stimulation with 2-1 parent cells also decreases at 1 X 10^5 cells/well. Unlike the U937 cells, the fixed PBMC continue to increase their stimulation of D10.G4.1 in a dose dependent manner throughout all doses of cells added. Thus a low level of membrane IL-1 is suggested for all the U937 cells tested.

Treatments with immunomodulators generally were not effective inducers of increased membrane IL-1 activity. As illustrated in Figure 46A, U937 cells were treated with 100 units/ml. purified gamma interferon for 24 hours, 10 µg./ml. *E. coli* LPS for 24 hours, gamma interferon + LPS for 24 hours or PMA for 72 hours. Only PMA augmented the detectable membrane IL-1. Incubation with PMA (10^{-9}M) increased the ability of E11 and G4 subline cells fixed with paraformaldehyde to

FIGURE 45. UNTREATED U937 CELLS DID NOT DISPLAY SIGNIFICANT MEMBRANE IL-1.

U937 2-1 parent, sublines E11, G4, G11, and normal PBMC were fixed with 1% paraformaldehyde and incubated in fresh media overnight before addition to D10.G4.1 indicator cells. Fixed cells were added at 5 X 10^3, 1 X 10^4, 5 X 10^4, and 1 X 10^5 cells/well. Results are presented as Stimulation Index (Stim. Index) determined by comparison of wells to which no fixed cells were added with wells containing appropriate numbers of fixed cells, either U937 cells or PBMC, as previously described.

FIGURE 45. MEMBRANE IL-1 DETERMINATION

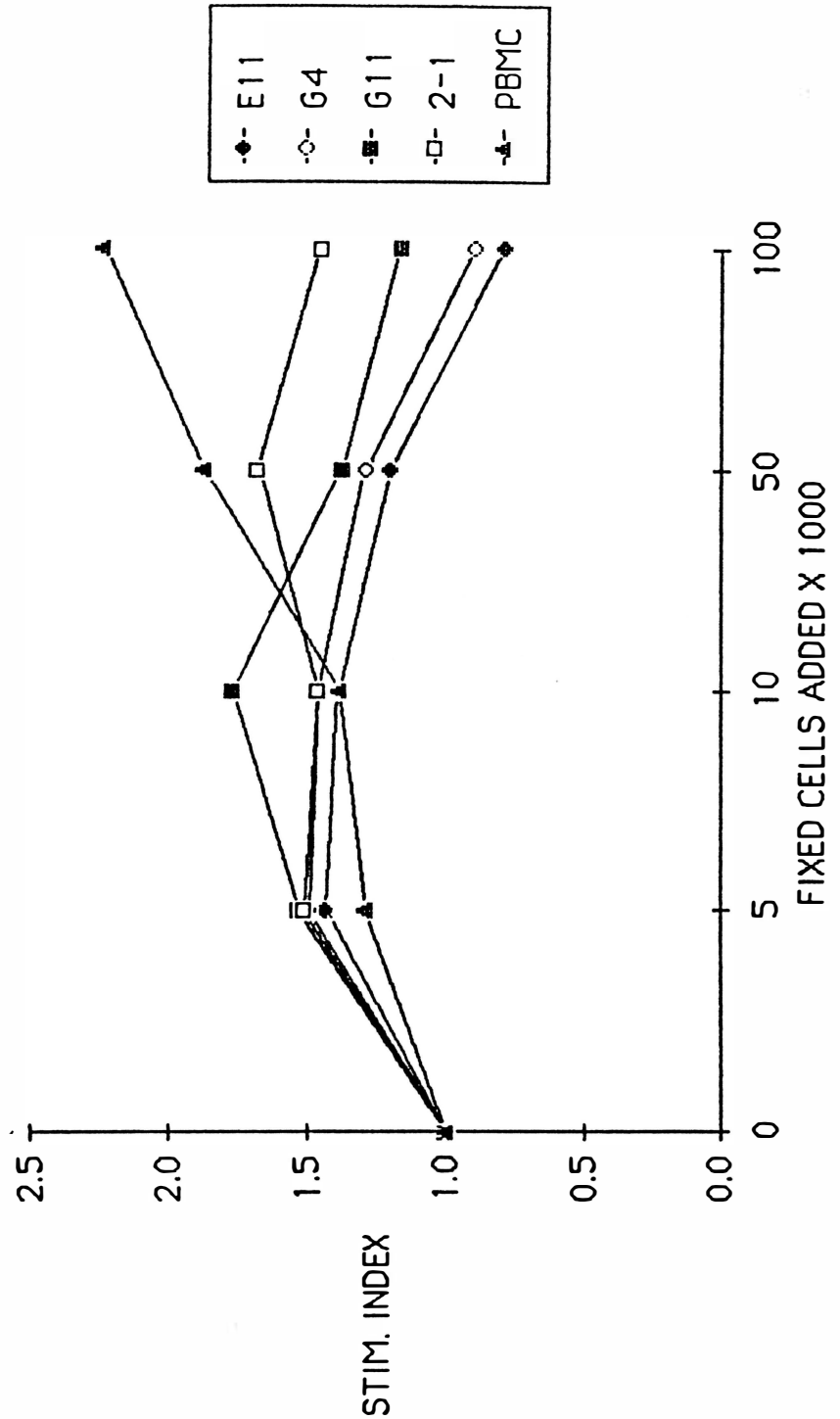
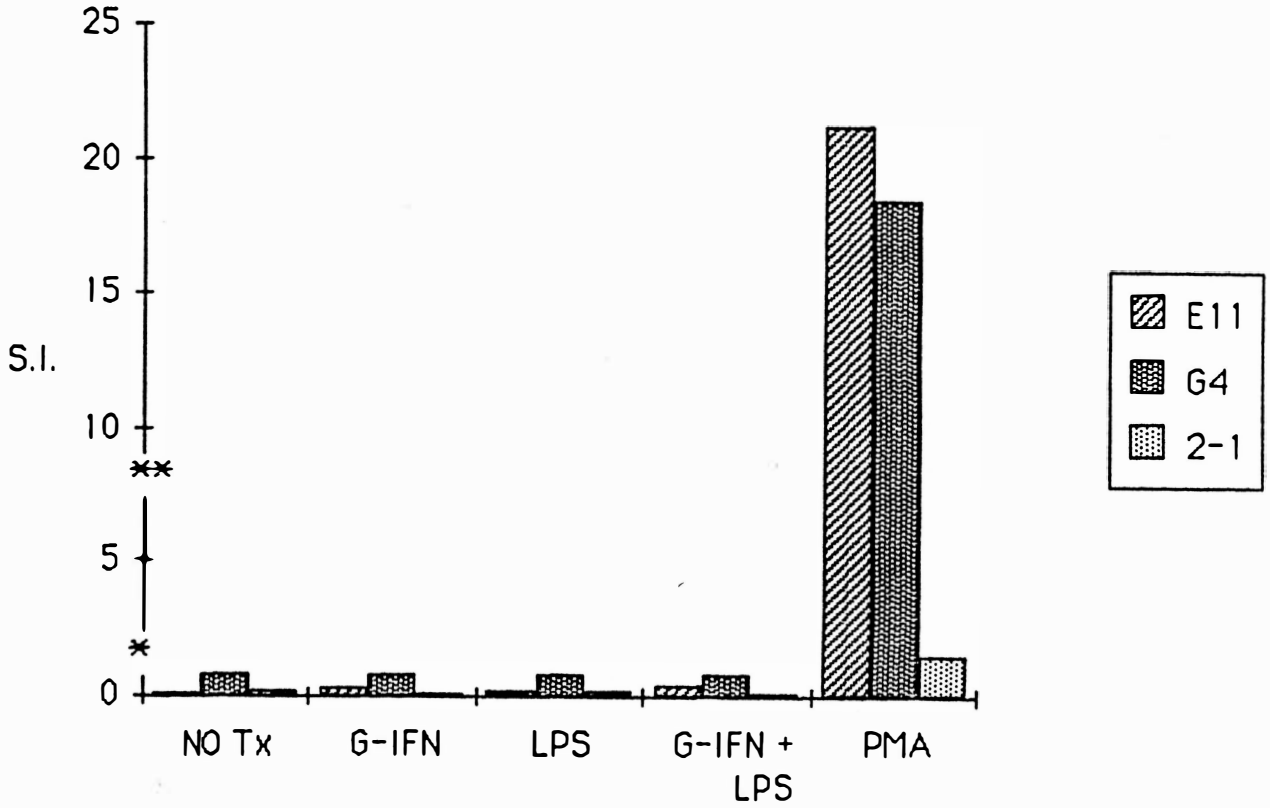


FIGURE 46. PRETREATMENT WITH PMA STIMULATED MARKED INCREASE IN MEMBRANE IL-1 IN SUBLINES E11 AND G4.

U937 parent 2-1 and sublines E11 and G4 were treated with gamma interferon (G-IFN) at a concentration of 100 u/ml. for 24 hours; E. coli lipopolysaccharide (LPS) at a concentration of 10 μg./ml. for 24 hours; combined G-IFN and LPS for 24 hours; or phorbol myristate acetate (PMA) at a concentration of 10^{-9}M for 72 hours prior to fixation with 1% paraformaldehyde. Fixed cells (1 X 10^5 cells/well) were added to D10.G4.1 indicator cells. Results are presented as Stimulation Index (S.I.) as previously described. * control D10.G4.1 cells without additional cells; S.I.=1.0. **D10.G4.1 cells + 1 μl. purified IL-1.

FIGURE 46. MEMBRANE IL-1 DETERMINATION ON PRETREATED, FIXED U937



stimulate proliferation by D10.G4.1 cells to a stimulation index of approximately 20. The 2-1 parent was induced by PMA to stimulate to a very small degee. Cells in these experiments, except subline G4 cells, were found to be very inhibitory to background ^{3}H-thymidine incorporation, thus, the stimulations indices for E11 subline and 2-1 parent line fell below 1.0. Inhibition by fixed cells is not explained. Because PMA is known to be highly lipophilic and bypasses signal for activation in varied cell systems, it was possible that the dose of PMA used to treat the U937 cells had merely inserted into the U937 membrane where it could directly stimulate the D10.G4.1 cells. Therefore, a dose response curve was set up for addition of PMA directly to the IL-1 assay. As shown in Figure 47, at 10^{-9}M PMA is beginning to directly stimulate the D10.G4.1 cells to a very small degree. At the next higher dose, 10^{-8}M PMA, there is more clearly a stimulatory response. Because the cells pretreated with PMA were extensively washed prior to addition to the IL-1 assay, it appears unlikely that a sufficiently high concentration of PMA was carried over to directly stimulate the D10.G4.1 cells. If on the other hand, PMA selectively seeks to enter the membrane from the aqueous medium, it seems possible but improbable that a high concentration of PMA might be available in the membrane microenvironment and that this concentration might locally exceed the minimum requirements for direct stimulation of D10.G4.1 cells. To circumvent this problem, the more water soluble and more easily washed out phorbol ester, PDBu (10^{-8}M), was utilized to pretreat U937 cells prior to their addition to IL-1 assays. As indicated in Figure 48, PDBu proved to be a potent stimulator of membrane IL-1 expression. It must also be noted that in this series of IL-1 assays, it appears that the fixed but non-pretreated subline E11 and G4 cells expressed significant membrane IL-1 as shown by stimulation indices greater than 5. Gamma interferon pretreatment for 24 hours apparently abrogated any membrane IL-1 expression by sublines E11 and G4, however, it slightly increased the membrane IL-1 on parent 2-1 cells which did not exhibit any membrane IL-1 activity if not treated.

FIGURE 47. PMA HAS NO DIRECT EFFECT ON D10.G4.1 CELLS WHEN ADDED AT LOW CONCENTRATION.

Dose response curve was determined for response of D10.G4.1 (IL-1) indicator cells to direct addition of PMA at concentrations stated. Assays were conducted as described and results are presented as Stimulation Index (S.I).

FIGURE 47. IL-1 DETERMINATION
PMA DOSE RESPONSE CURVE FOR D10.G4.1
CELLS

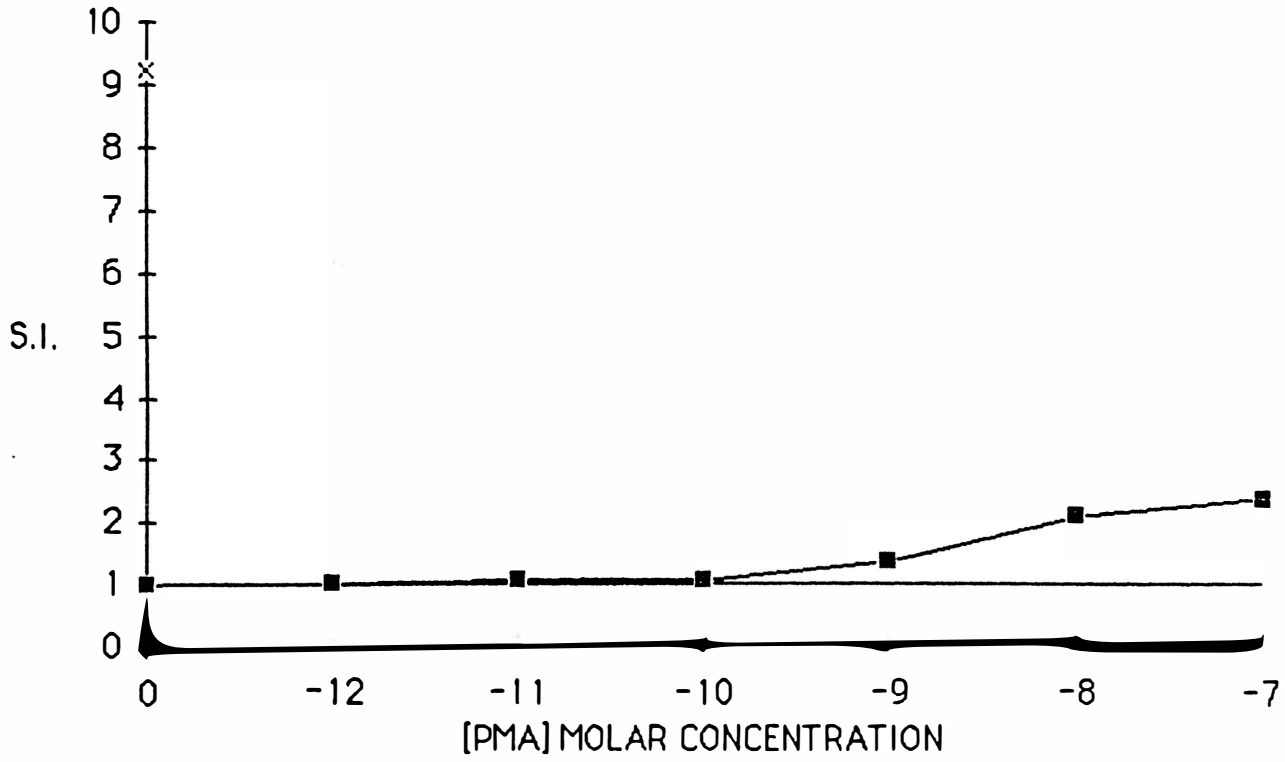
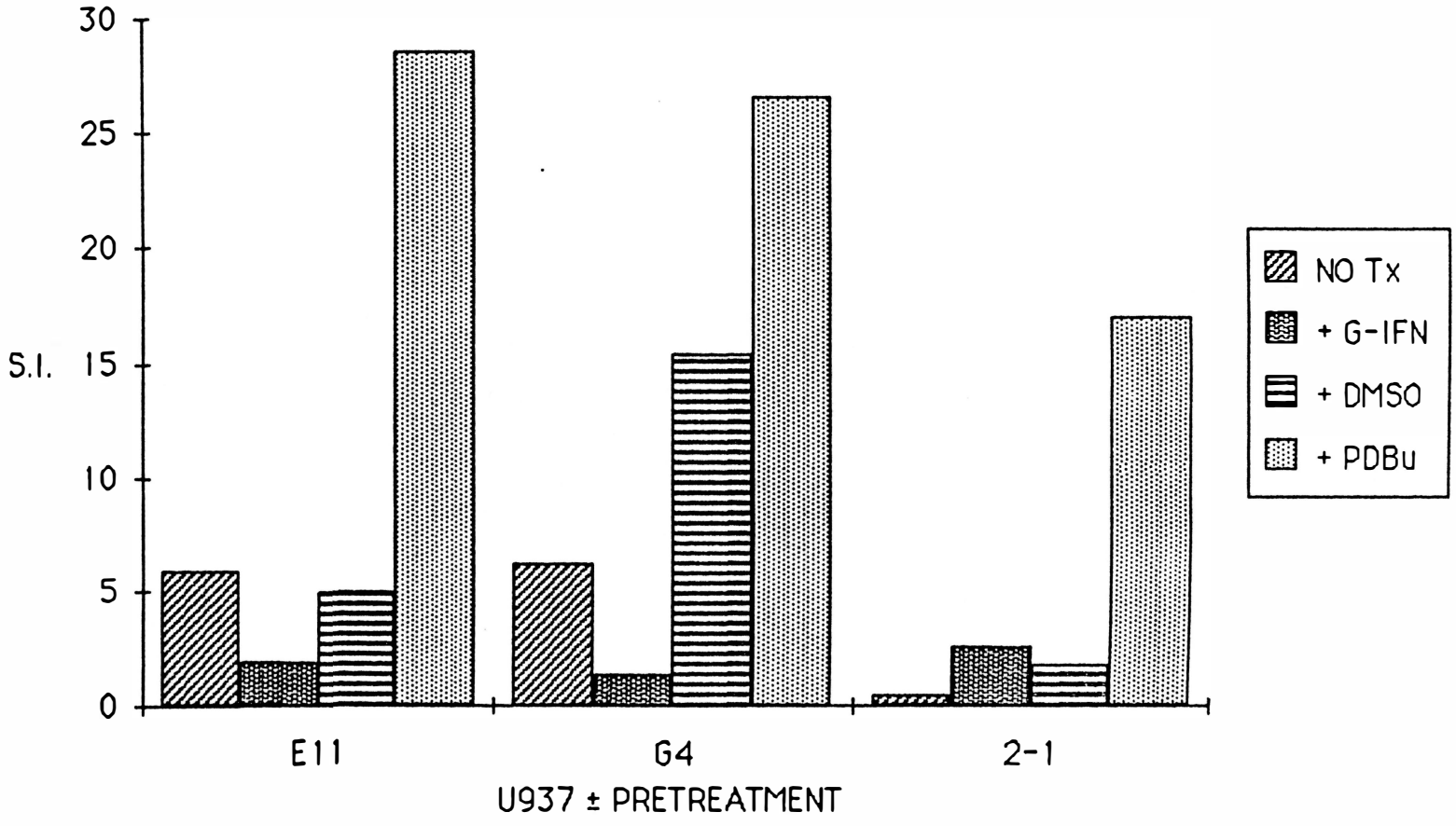


FIGURE 48. PRETREATMENT WITH PDBu AUGMENTS MEMBRANE IL-1 ON U937 CELLS.

U937 2-1 parent and sublines E11 and G4 were treated with gamma interferon (G-IFN) for 24 hours with 100 u/ml.; dimethyl sulfoxide (DMSO) at 1:1000 for 72 hours; or phorbol dibutyrate (PDBu) at 10^{-8}M for 72 hours prior to fixation with 1% paraformaldehyde and addition to IL-1 assay. Results are presented as Stimulation Index (S.I.) as previously described.

FIGURE 48. MEMBRANE IL-1 DETERMINATIONS
ON U937 CELLS ± PRETREATMENT



DMSO, which was used as a diluent for dissolving PDBu initially, was found at low concentration to have no effect on membrane IL-1 expression by subline E11, to markedly increase membrane IL-1 by subline G4 and to slightly increase membrane IL-1 on 2-1 parent cells.

Thus it appears that U937 cells may constituitively express very low levels of membrane IL-1 and that this level is not increased significantly by gamma interferon or *E. coli* LPS. Membrane IL-1 expression is markedly increased by pretreatment with phorbol esters which change the membrane characteristics and morphology of U937 cells causing them to adhere to surfaces and to become more macrophage-like. DMSO at low concentrations also causes U937 cells to become adherent and a concomitant effect was also seen on membrane IL-1 expression in the G4 subline. The concentration of DMSO utilized (0.001 µl./ml.) may represent a threshold amount in U937 activation thus causing a positive effect in the G4 subline and a negative effect in the E11 cell line.

MHC class II specific mRNA was examined by Northern blot analysis using two cDNA probes specific for human HLA-DR alpha and HLA-DQ alpha. As a final aspect of the characterization of the U937 parent and sublines, the Northern blot analysis was carried out using probe pDCH1, specific for HLA-DQ alpha (446), and probe T-33, specific for HLA-DR alpha (unpublished). Both probes were obtained and were prepared as previously described.

Autoradiograms were examined by scanning densitometer and the results of the scan were normalized for 28S rRNA present. Normalization using ribosomal RNA was chosen as the preferred method for two reasons: first, cell lines but not normal cells have been shown to produce a relatively constant level of rRNA in spite of treatment with activating agents (476) and second, it is unclear what effect activating agents may have on the levels of actin production in a monocytic cell line being treated with agents which clearly have profound effects on morphology and adherence properties which in

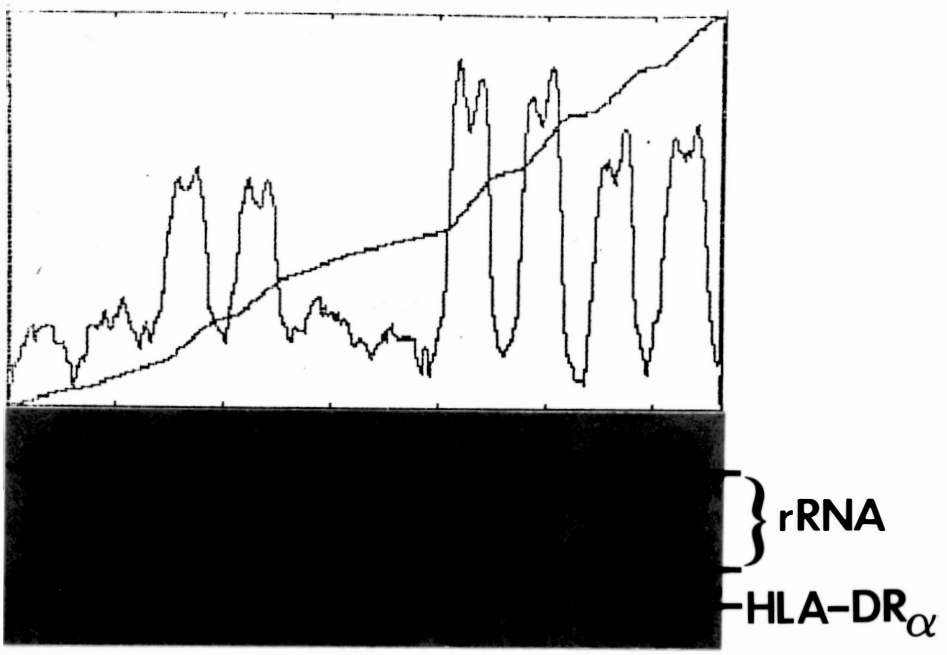
all probability involve increased production of cytoskeletal elements. A probe for 28S rRNA was obtained (pAD-19.IIa) (447) and prepared; however, when labeled and hybridized with previously hybridized-stripped blots, the results were very inconsistent. It is believed that the pattern of hybridization seen with the rRNA probe is consistent with compression of the blots during incubation with the labeled probe in a manner which precluded probe from areas of the blots due to the considerable pressure exerted by large numbers of sealed pouches stacked on each other and weighted down to keep them submerged in the 42°C water bath. A few of the large number of blots hybridized at the same time with the rRNA probe gave hybridization patterns consistent with the 28S rRNA demonstrated by ethidium bromide staining on the original gels as photographed prior to transfer. These autoradiograms were examined by scanning densitometer. Thus because the results of the rRNA probing were unsuccessful in most cases, the photographic negatives taken of the original gels served as an alternative means for controlling for the amount of rRNA in the particular lanes of the blots. The 28S rRNA band was measured by scanning densitometer in the same manner as the autoradiograms. Here again a dilemma presented itself in that the film background was found to vary in its density in a regular manner with the lightest area appearing at the edges of the negative. Background readings were obtained across the base of each negative and these values were subtracted from the densitometer readings for the rRNA bands measured. Because the densitometer had to be reset to measure these background areas, the values did not correlate with values obtained for the actual rRNA bands measured, but instead were relative amounts and varied according to the darkness of the negative. Therefore, a third means of correcting for the amount of 28S rRNA present was developed as a modification of the measurement of the 28S rRNA on the photographic negative and was judged to be closer to the actual background observed. Because the film background varied in a regular manner, the background readings and the rRNA readings could be fitted to a normal curve and thus adjusted. The values

obtained in this way correlated well with visual assessment of the relative size and intensity of the rRNA bands on the photographic negatives and thus this method of background correction and rRNA normalization was utilized for most calculations in the absence of usable rRNA probe data. The values obtained were further normalized to either a fraction of the total, the total being 1, or fraction of the total, the total being [0.1 X no. of lanes], to facilitate comparison of duplicate or near duplicate blots. Thus the numbers obtained for the hybridization in any lane are relative only to the hybridization seen in all other lanes on that particular blot and are not comparable to other blots containing that RNA sample unless all other samples are also present. Therefore, the values obtained are qualitative rather than quantitative.

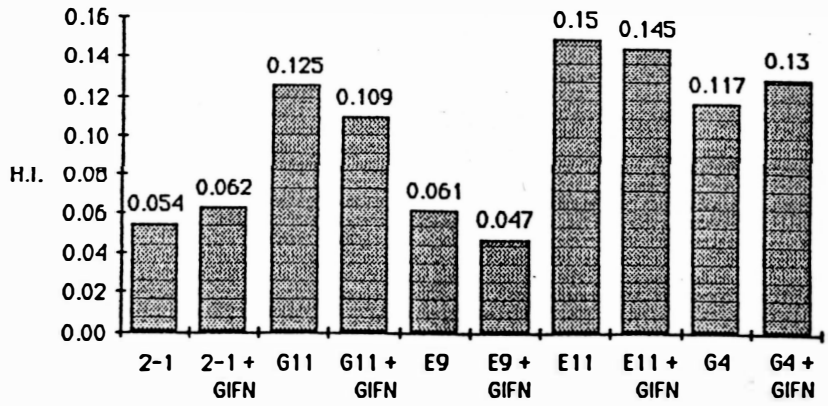
Ia+ sublines of U937 produce detectable mRNA specific for HLA-DR and DQ. The initial hybridization is shown in Figure 49 in which RNA samples from parent 2-1 U937 cells and sublines G11, E9, E11 and G4 which received no treatment or which were treated for 48 hours with purified gamma interferon (100 units/ml.) were hybridized with probe T-33, specific for HLA-DR alpha. This is an "early" experiment as the RNA was extracted from these cells in 1986 at a time when the 2-1 parent and the E9 subline were still Ia-, but when sublines G11, G4 and E11 were strongly Ia+ as measured by immunofluorescence. The probe was nick translated and the membrane was hybridized for 24 hours, stringently washed and autoradiographed for three days. The values shown for both the 2-1 parent and the E9 subline in Figure 49B represent values for these samples which are visible only to the scanning densitometer as shown in Figure 49A. Hybridization bands were apparent for the G11, E11 and G4 sublines, for both untreated samples and cells pretreated for 48 hours with gamma interferon. There were small changes in the amounts of mRNA produced after gamma interferon treatment; however, they were not consistent as there was a slight decrease with sublines G11 and E11 [(0.125->0.109) and (0.150->0.145) for G11 and E11, respectively] and a slight increase for subline G4 (0.117->0.130). Thus,

FIGURE 49. U937 SUBLINES G11, E11 AND G4 PRODUCE mRNA SPECIFIC FOR HLA-DRa AND THE LEVEL OF EXPRESSION IS NOT SIGNIFICANTLY CHANGED BY PRETREATMENT WITH G-IFN FOR 48 HOURS.

Scanning densitometer tracing of blot hybridization autoradiogram and autoradiogram show hybridization bands with nick translated T-33 probe. 28S and 18S rRNA are indicated. Hybridization Index (H.I.) was calculated as described (see Materials and Methods) by comparison between scanning densitometer values for gel photograph and hybridized probe autoradiogram.



U937 PARENT 2-1 AND SUBLINES
T33 (DR alpha) PROBE



U937 SUBLINES ± GAMMA INTERFERON (48 HOURS)

although gamma interferon treatment maximally increases the expression of MHC class II molcules on the cell surface after 48 hours, no concomitant increase in specific mRNA is seen at that time. It was postulated that there were several explanations for this finding. The most compelling explanation was that perhaps 48 hours is too late to observe an increase in mRNA as an increase in transcription would temporally precede translation and subsequent transport and expression. Although there was no significant change after gamma interferon treatment for 48 hours, HLA-DR alpha and DQ alpha mRNA could be demonstrated for sublines which by other means had been found to be Ia+.

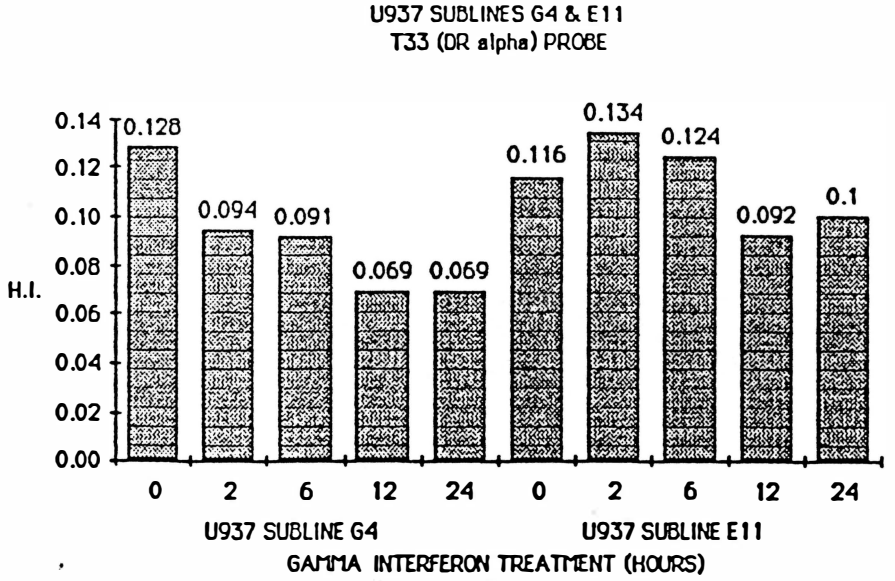
A more extensive kinetic analysis was attempted to examine whether gamma interferon treatment of U937 parent and subline cells could be shown to regulate the transcription of HLA-DR alpha or DQ alpha mRNA. The 2-1 parent U937 cell line and sublines were incubated with gamma interferon for 0, 2, 6, 12 and 24 hours prior to RNA extraction. RNA samples obtained from the G4 and E11 sublines were blotted and probed also with pDCH1 and T-33 probes and the results are shown in Figure 50. In Figure 50C, HLA-DR alpha specific hybridization gave bands of approximately 1.2 kb just below but distinctly separate from the position of the 18 S rRNA bands visualized by ethidium bromide staining of blotted RNA. As indicated the E11 subline hybridized with the probe to a somewhat higher degree than the G4 subline and showed opposite effects of gamma interferon treatment. As shown in Figure 50A, the G4 subline hybridization decreased during the whole course of gamma interferon treatment, reaching a steady level of 0.069 at 12 hours. The E11 subline showed an initial level of 0.116 but rapidly increased to 0.134 at 2 hours followed by an apparent decline which reached a minimum level at 12 hours before rising again at 24 hours. For both G4 and E11 sublines, the HLA-DR specific mRNA demonstrated at 24 hours is clearly lower than the initial levels. Although it is possible that at that time the cells have either metabolized all of the available gamma interferon via endocytosis through their

FIGURE 50. HYBRIDIZATION OF HLA-DRa AND HLA-DQa SPECIFIC PROBES WITH TOTAL CELLULAR RNA FROM SUBLINES G4 AND E11 INDICATE KINETICS OF mRNA EXPRESSION AFTER G-IFN TREATMENT.

U937 sublines G4 and E11 were treated with gamma interferon (100 u/ml.)for 0, 2, 6, 12, and 24 hours and total cellular RNA was extracted, electrophoresed in agarose gel, stained with ethidium bromide, photographed, electrically transblotted to nylon membranes and hybridized with probes (T-33/HLA-DRa and pDCH1/HLA-DQa) labeled by random priming techniques. Measurements were made by scanning densitometer from autoradiograms of probe hybridizations and photographs of ethidium bromide stained gels. Hybridization Index (H.I.) was calculated as described (see Materials and Methods) for hybridization with probe T-33 (A) and pDCH1 (B). Scanning densitometer tracings and autoradiograms of probe hybridizations as well as scanning densitometer tracings of gel photographs and photographs are shown for T-33 probe (C) and for pDCH1 probe (D).

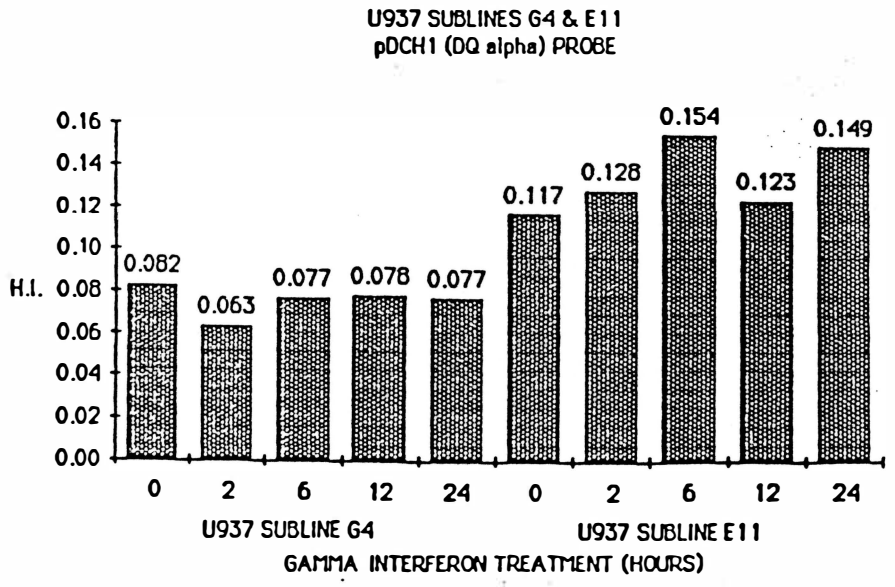
A

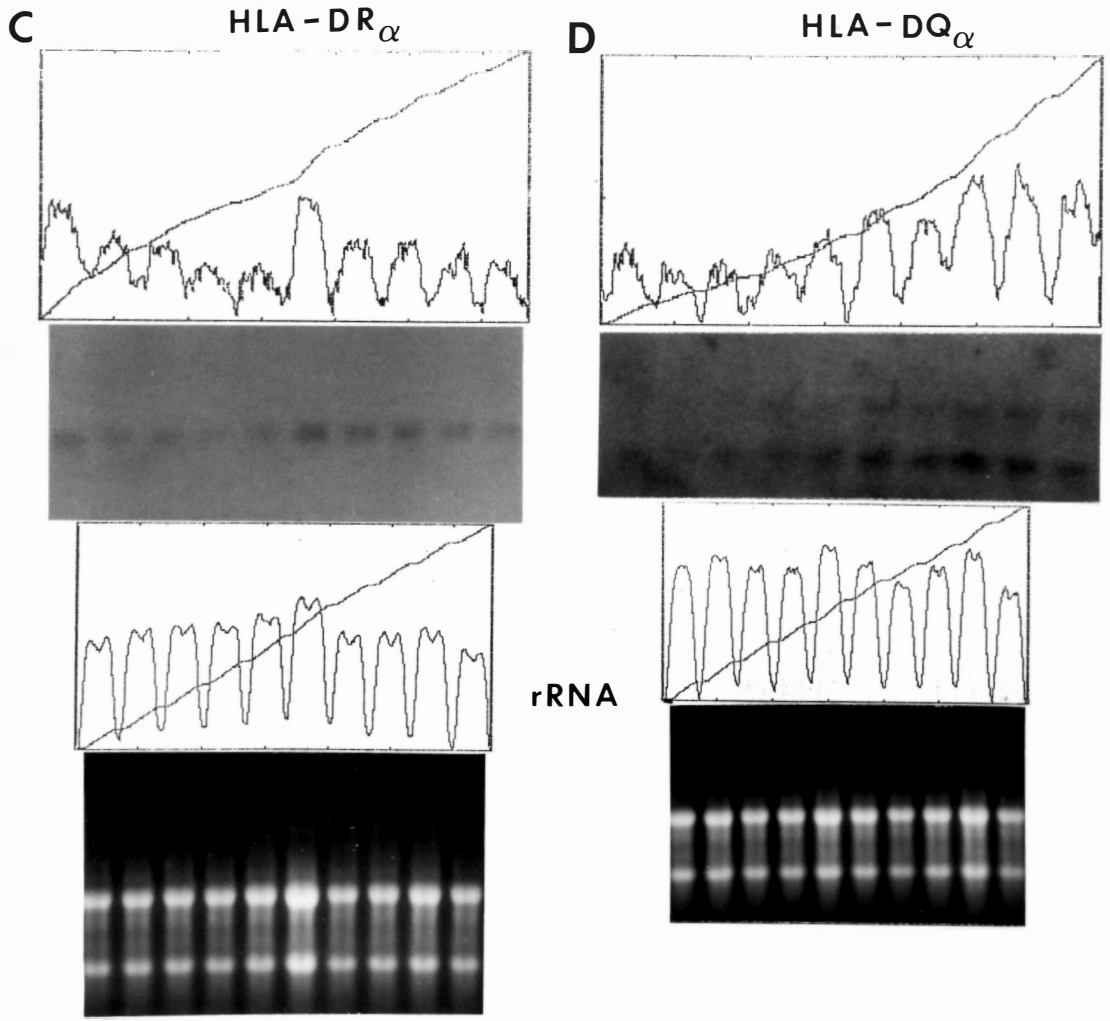
HLA-DR$_\alpha$



B

HLA-DQ$_\alpha$



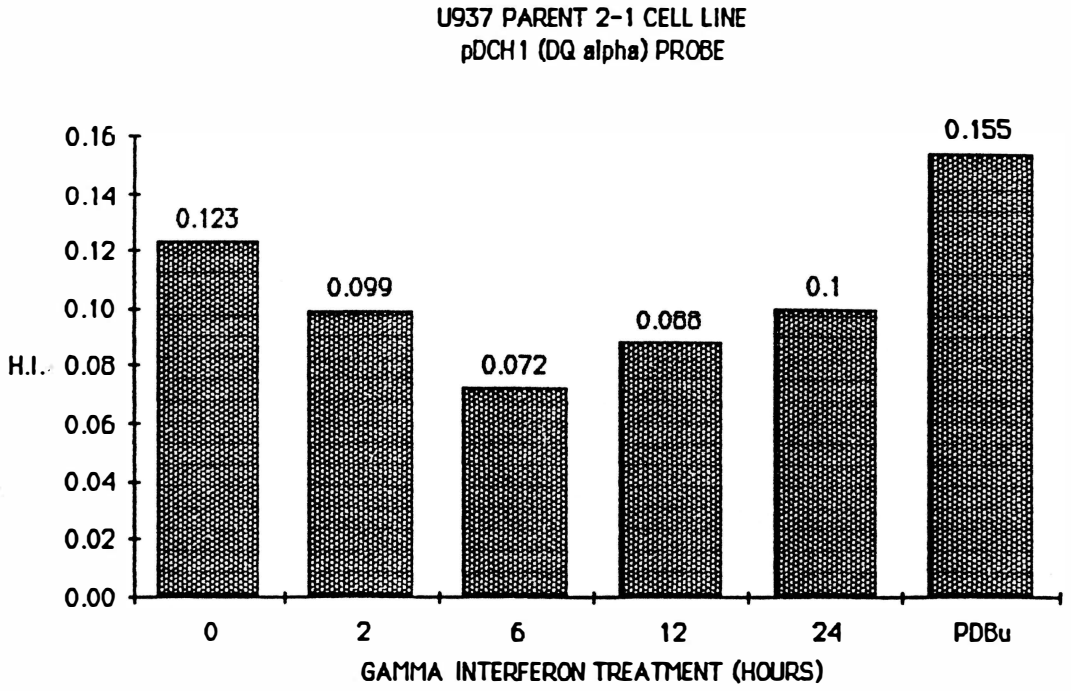

C
HLA-DR$_\alpha$
D
HLA-DQ$_\alpha$
rRNA

gamma interferon receptors or that the cells are refractory to further stimulation, these possibilities are not supported by findings with the HLA-DQ alpha probe, pDCH1. Figure 50B illustrates the mRNA hybridizable with the HLA-DQ alpha probe in RNA samples also from sublines G4 and E11. The G4 subline shows an initial hybridization with HLA-DQ alpha (0.082) which rapidly decreases at 2 hours during gamma interferon treatment (0.063) but returns rapidly to a level near that of the untreated cells (0.077). The E11 subline, in contrast, shows a higher level of initial hybridization (0.117) and increases to a much higher level (0.154) at 6 hours followed by a decrease at 12 hours and return to high level after 24 hours of treatment. There is no explanation from the available data for the apparently biphasic level of HLA-DQ alpha mRNA at 12 hours. Figure 50C illustrates a representative hybridization of RNA samples from sublines G4 and E11 with the T-33 (HLA-DR alpha) cDNA probe. This probe was isolated from the plasmid (pUC) by restriction endonuclease digestion followed by electroelution from a preparative gel. The purified probe was labeled by random priming techniques and is shown to give clean, single bands when hybridized. Figure 50D illustrates a duplicate blot hybridized with pDCH1 (HLA-DQ alpha) probe which was random primed directly in the plasmid prior to hybridization. The results with this probe were very messy and difficult to interpret by scanning densitometer because of the high background; however, after many scans and constant attention to whether the band intensity correlated with the band or with a random spot, results were tabulated. A second finding with this probe is that there are always two hybridization bands. This has been previously reported (477) and has been attributed to different transcriptional lengths seen with HLA-DQ alpha and also with the corresponding murine MHC equivalent, I-A (478).

No HLA-DR alpha specific mRNA could be demonstrated in RNA extracted from the 2-1 parent U937 cell line. In contrast to this finding, the 2-1 parent line produced clearly discernable HLA-DQ alpha specific mRNA, as shown in Figure 51,

FIGURE 51. mRNA SPECIFIC FOR HLA-DQa BUT NOT HLA-DRa WAS DETECTABLE IN TOTAL CELLULAR RNA ISOLATED FROM U937 PARENT 2-1 CELL LINE.

U937 parent 2-1 cell line was treated with gamma interferon (100 u/ml.) for 0, 2, 6, 12, and 24 hours and with 10^{-8}M PDBu for 72 hours prior to extraction of total cellular RNA. RNA was electrophoresed in agarose, stained with ethidium bromide, photographed, transblotted to nylon membranes and hybridized with cDNA probes specific for HLA-DRa (T-33) and HLA-DQa (pDCH1) labeled by random priming techniques. Hybridization was indicated by autoradiography. No hybridization was seen with probe T-33 (data not shown); however, hybridization indices (H.I.) are indicated for pDCH1 probe. H.I. were determined as previously described.


U937 PARENT 2-1 CELL LINE
pDCH1 (DQ alpha) PROBE
H.I.
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.123
0.099
0.072
0.088
0.1
0.155
0
2
6
12
24
PDBu
GAMMA INTERFERON TREATMENT (HOURS)

which was decreased by gamma interferon treatment during the first six hours followed by a slight increase at twelve hours and again at 24 hours to reach a value slightly below that seen in non-treated cells. As indicated in Figure 51, treatment of the 2-1 parent cell line with 10^{-8}M PDBu for 72 hours caused a marked increase in the amount of HLA-DQ alpha specific mRNA produced.

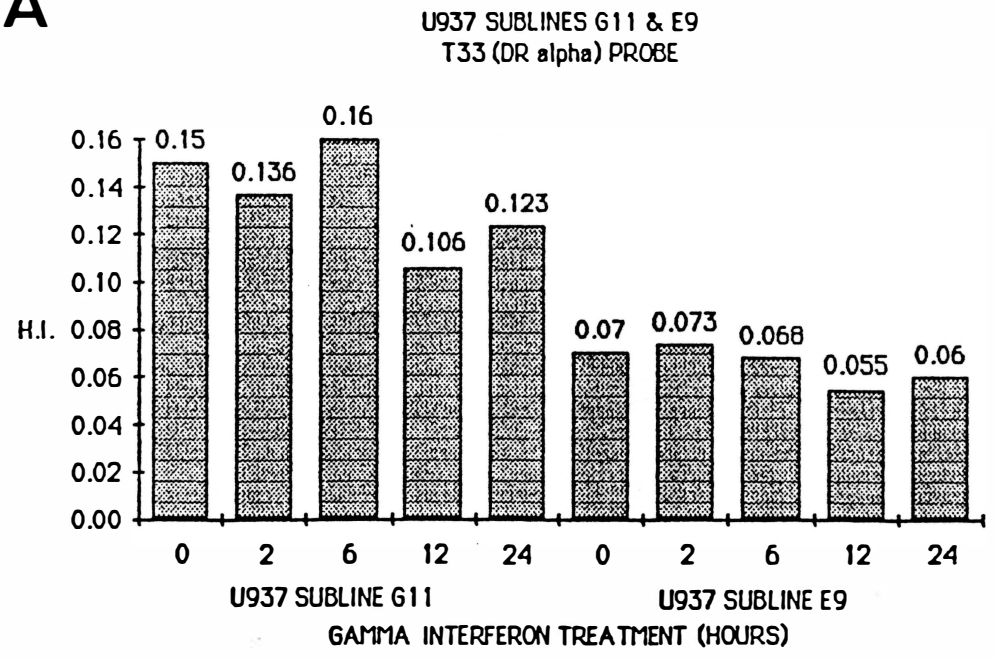
Sublines G11 and E9 were also treated with gamma interferon using the same protocol and total cellular RNA was extracted. When hybridized with the T-33 probe as shown in Figure 52A, the G11 subline demonstrated the presence of HLA-DR alpha specific mRNA, although at low levels. Values indicated for the E9 subline indicate the presence of HLA-DR alpha message also; however, there was very little hybridization apparent and illustrated values give an impression of greater degree of hybridization for both G11 and E9 than was actually apparent. Hybridization with pDCH1 (HLA-DQ alpha) gave more striking results for subline G11 as illustrated in Figure 52B. There was little or no apparent hybridization with E9 subline RNA; however, the G11 subline showed a pattern of hybridization similar to the E11 subline in that there was an initial level of HLA-DQ alpha specific mRNA which was clearly visible and which rapidly rose to very high level after 2 hours of gamma interferon treatment. This rapid rise was followed by a sharp decrease in HLA-DQ alpha specific mRNA present by 12 hours of treatment followed by an increase at 24 hours, again illustrating a biphasic level of specific mRNA.

Gamma interferon treatment of U937 cells has been shown to markedly increase Ia expression as measured by immunofluorescence using Kula2 monoclonal antibody with specificity for a framework determinant of human Ia, probably HLA-DR, as illustrated in Figures 12 and 13. All sublines except E11 were found to express increased Ia after 6 hours of incubation with gamma interferon. The E11 subline in early experiments showed a decrease in expression at 6 hours and a rise thereafter to reach a maximum expression after 24 hours of treatment (Figure 12). In more recent

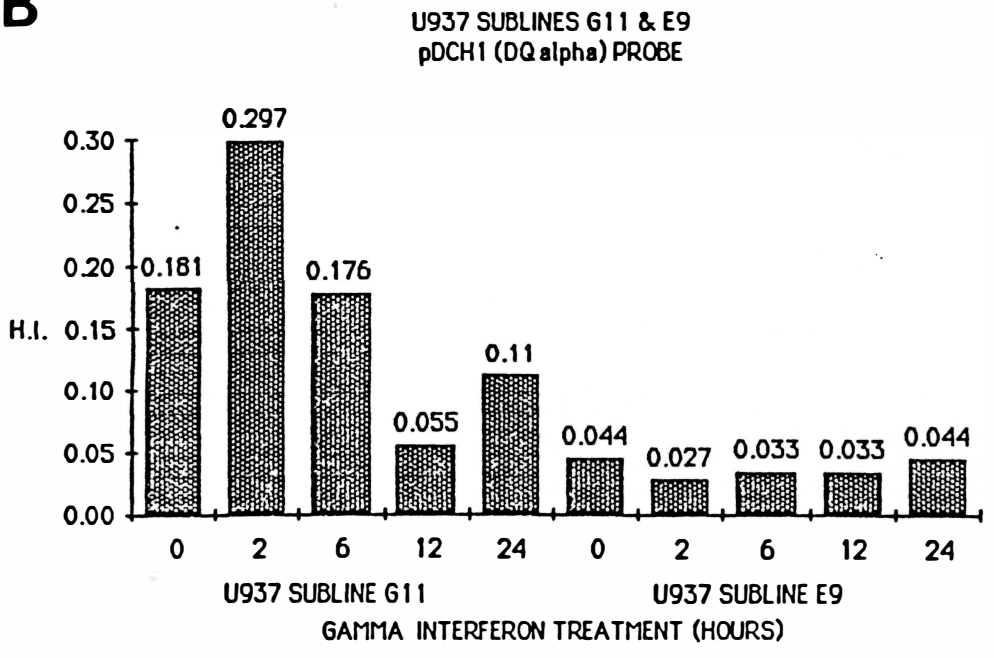
FIGURE 52. RNA FROM U937 SUBLINE G11 BUT NOT SUBLINE E9 HYBRIDIZED SIGNIFICANTLY WITH PROBES FOR HLA-DRa AND HLA-DQa.

U937 sublines G11 and E9 were treated with gamma interferon (100 u/ml.) for 0, 2, 6, 12, and 24 hours prior to extraction of total cellular RNA. RNA was electrophoresed, stained with ethidium bromide, photographed, transblotted to nylon membranes and hybridized with probes for HLA-DRa (T-33) (A) and HLA-DQa (pDCH1) (B). Hybridization Index (H.I.) was calculated as previously described.

A


U937 SUBLINES G11 & E9
T33 (DR alpha) PROBE
H.I.
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.15
0.136
0.16
0.106
0.123
0.07
0.073
0.068
0.055
0.06
0
2
6
12
24
0
2
6
12
24
U937 SUBLINE G11
U937 SUBLINE E9
GAMMA INTERFERON TREATMENT (HOURS)

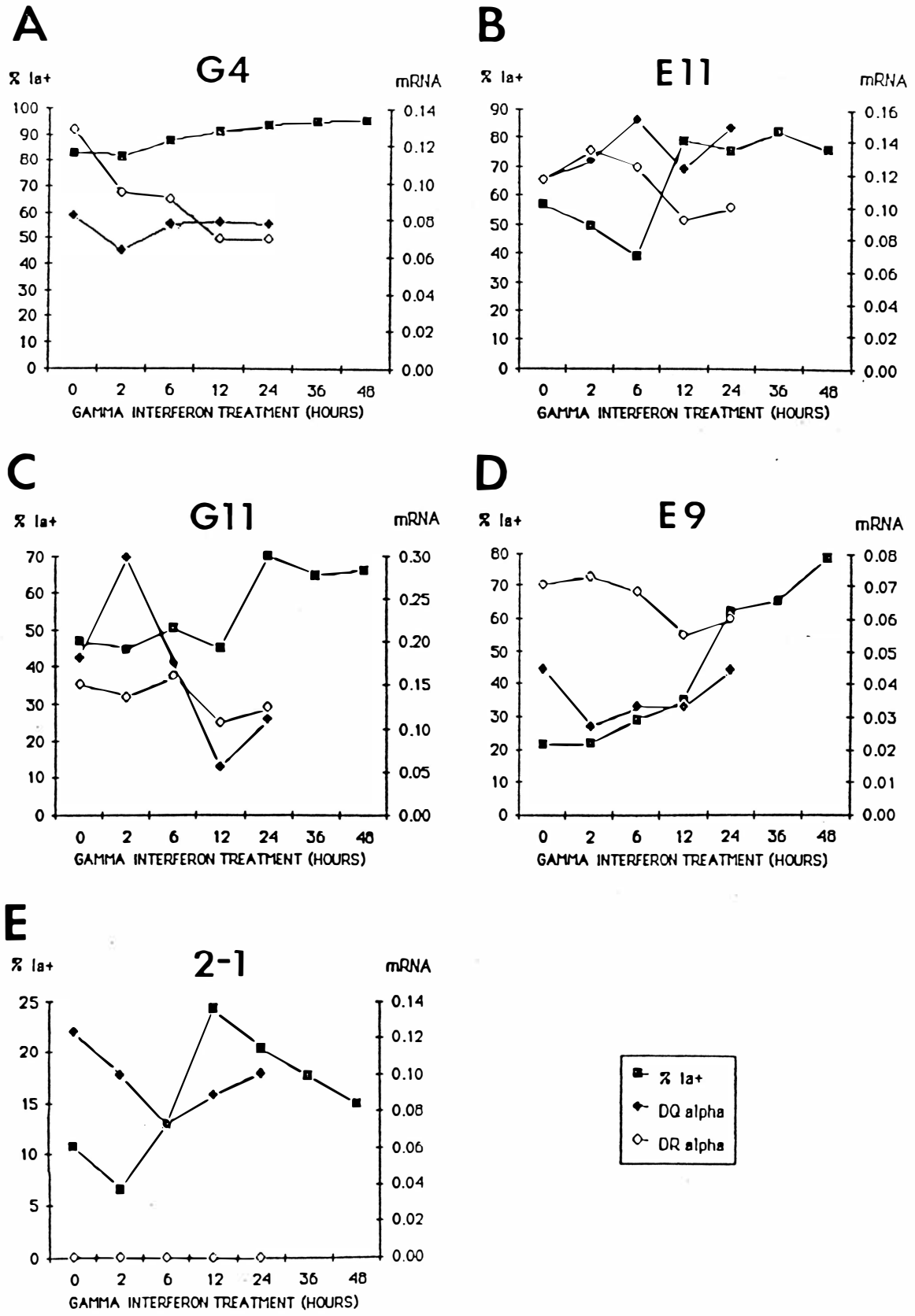
B


U937 SUBLINES G11 & E9
pDCH1 (DQ alpha) PROBE
H.I.
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.181
0.297
0.176
0.055
0.11
0.044
0.027
0.033
0.033
0.044
0
2
6
12
24
0
2
6
12
24
U937 SUBLINE G11
U937 SUBLINE E9
GAMMA INTERFERON TREATMENT (HOURS)

kinetic studies the E11 cell line was found to decrease Ia expression as a result of gamma interferon treatment and the expression did not reach non-treated levels until cells were treated for 72 hours. The findings with immunofluorescence and Northern blotting, therefore, are clearly contradictory for the expression of MHC class II as shown in Figure 53. Whereas the levels of cell surface expression of Ia initially decreased slightly for subline G4 as shown in Figure 53A, then rose to maximum levels by 24-48 hours, the detectable mRNA for HLA-DR alpha clearly decreased for the G4 subline and continued to decrease to its lowest level by 12 hour of gamma interferon treatment. HLA-DQ alpha initially decreased but returned to a sustained level slightly lower than the initial level found. These results seem to be contradictory if one expects a temporal relationship between regulation of transcription and translation. The E11 subline shown in Figure 53B, which exhibited the most profound decrease in Ia expression at 6 hours of gamma interferon treatment, also exhibited the most profound increase in HLA-DR alpha and HLA-DQ alpha mRNA at 2-6 hours. This pattern of expression lagging behind transcription of increased mRNA is the expected pattern. The G11 subline shown in Figure 53C exhibited a long lag period in MHC class II expression on the cell surface with a very slight decrease in expression following 2-6 hour treatment with gamma interferon. Also shown was a very slight decrease in HLA-DR alpha mRNA at 2 hours followed by a very slight increase at 6 hours and subsequent larger decrease, but subline G11 exhibited a marked increase in HLA-DQ alpha at 2 hours followed by an equally marked decrease at 6 hours. It appears from these results that there is an apparent increase in HLA-DQ alpha specific mRNA in the G11 subline and concomitant decrease in HLA-DR alpha mRNA. The level of MHC class II expression by the E9 subline, shown in Figure 53D, as with subline G11, lagged for the first 12 hours of gamma interferon treatment but increased steadily thereafter. The E9 subline expressed levels of mRNA determined by probe hybridization for HLA-DR alpha and HLA-DQ alpha which were barely detectable. HLA-DR alpha mRNA decreased

FIGURE 53. COMPARISON OF Ia EXPRESSION ON THE CELL SURFACE AFTER GAMMA INTERFERON TREATMENT DID NOT NECESSARILY CORRELATE WITH MHC CLASS II mRNA HYBRIDIZATION.

Data obtained from kinetic study of Ia expression on the cell surface as measured by flow cytometry using monoclonal antibody Kula2 (% Ia+) was compared with hybridization index (mRNA) data obtained from HLA-DRa (T-33) and HLA-DQa (pDCH1) probe hybridizations for U937 subline G4 (A), subline E11 (B), subline G11 (C), subline E9 (D), and parent 2-1 (E).

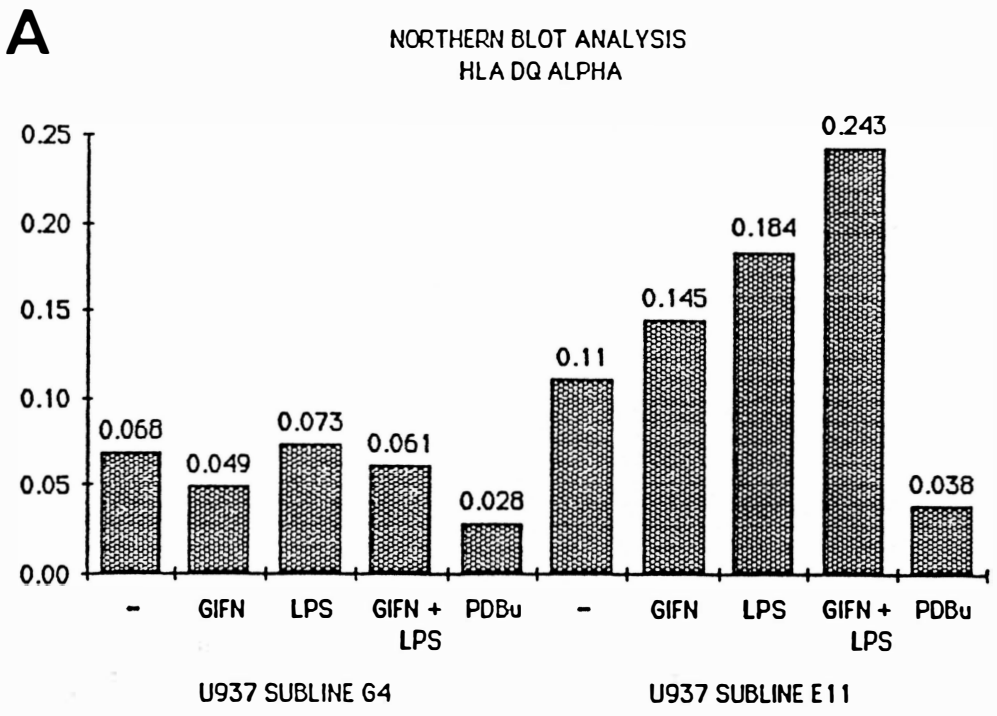

A
G4
% Ia+
mRNA
GAMMA INTERFERON TREATMENT (HOURS)
B
E11
% Ia+
mRNA
GAMMA INTERFERON TREATMENT (HOURS)
C
G11
% Ia+
mRNA
GAMMA INTERFERON TREATMENT (HOURS)
D
E9
% Ia+
mRNA
GAMMA INTERFERON TREATMENT (HOURS)
E
2-1
% Ia+
mRNA
GAMMA INTERFERON TREATMENT (HOURS)
% Ia+
DQ alpha
DR alpha

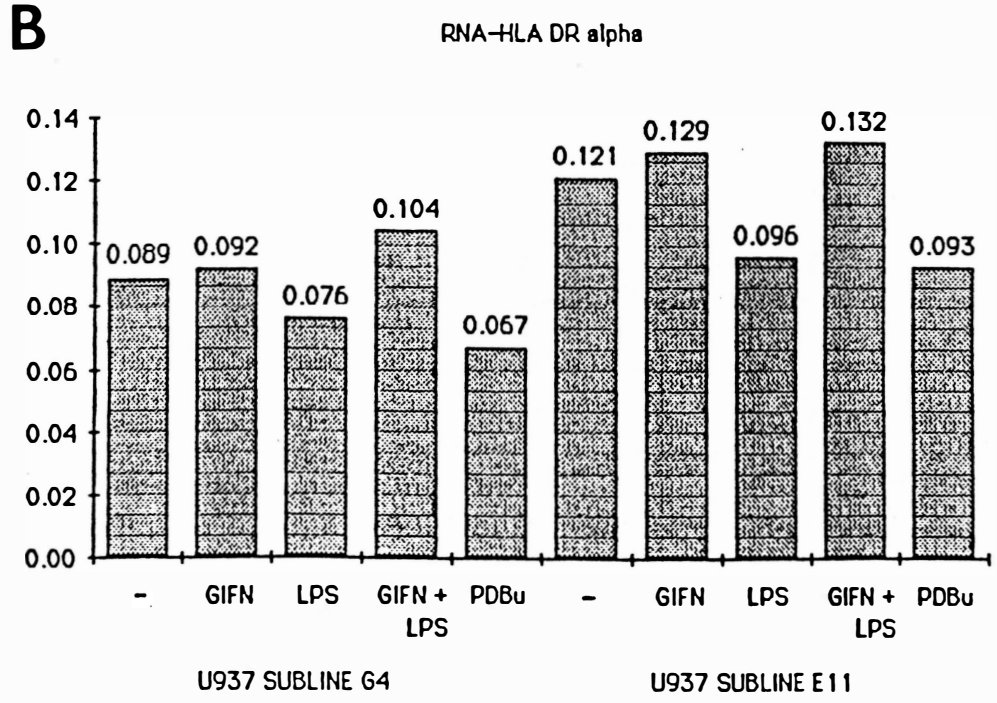
steadily until after 12 hours of gamma interferon treatment; however, HLA-DQ alpha decreased initially but by 6 hours appeared to be rising again, reaching nearly non-treated levels by 24 hours. Also shown in Figure 53E, the level of expression of MHC class II on the parent 2-1 cell line initially decreased, but by 6 hours had begun to rise quite substantially to reach a maximum at 12 hours followed by a steady decline to slightly above non-treated levels at 48 hours. As indicated, no HLA-DR alpha mRNA was detected at any time point. In all hybridizations using RNA isolated from the 2-1 parent cell line, HLA-DR alpha was never seen as more than a very weak smudge. HLA-DQ alpha was seen however, quite clearly. As shown, HLA-DQ alpha mRNA also decreased, however, with gamma interferon treatment and did not appear to begin to increase again until 6 hours after the observed rise in cell surface Ia expression. Here again, the levels of expression and transcription do not appear to be temporally related in the expected manner.

The effects of other immunomodulators on regulation of HLA-DR alpha and HLA-DQ alpha RNA transcription were also examined. Figure 54 illustrates the results obtained from experiments in which cells from sublines G4 and E11 were treated with gamma interferon, LPS (*E. coli*), gamma interferon + LPS for 24 hours or PDBu for 72 hours prior to extraction of total cellular RNA. When examined for HLA-DR alpha using the T-33 probe, hybridization bands were apparent for the non-treated G4 and E11 sublines. Treatment with gamma interferon alone increased the levels of mRNA very slightly for subline G4 and more substantially for subline E11. Treatment of both sublines with LPS alone decreased HLA-DR alpha specific mRNA transcription; however, treatment with a mixture of gamma interferon and LPS caused a synergistic response manifested as an increase in HLA-DR alpha specific mRNA which exceeded that seen with gamma interferon alone. Treatment with PDBu for 72 hours also decreased the HLA-DR alpha mRNA level markedly in the G4 subline and to about the same degree

FIGURE 54. IMMUNOMODULATOR PRETREATMENT HAD VARIABLE EFFECTS ON EXPRESSION OF HLA-DRa AND HLA-DQa mRNA BY U937 SUBLINE G4 AND SUBLINE E11.

U937 subline G4 and subline E11 were treated with gamma interferon (GIFN) (100 u/ml.) for 24 hours; E. coli lipopolysaccharide (LPS) (10 µg./ml.) for 24 hours; a combination of GIFN and LPS (GIFN+LPS) for 24 hours; or phorbol dibutyrate (PDBu) (10-8M) for 72 hours prior to total cellular RNA extraction, electrophoresis, ethidium bromide staining, photography, and hybridization with HLA-DRa (T-33) (A) or HLA-DQa (pDCH1) (B) probe hybridization and autoradiography. Results are indicated as Hybridization Index (H.I.) calculated as previously described.


A
NORTHERN BLOT ANALYSIS
HLA DQ ALPHA
0.25
0.20
0.15
0.10
0.05
0.00
0.068
0.049
0.073
0.061
0.028
0.11
0.145
0.184
0.243
0.038
-
GIFN
LPS
GIFN + LPS
PDBu
-
GIFN
LPS
GIFN + LPS
PDBu
U937 SUBLINE G4
U937 SUBLINE E11


B
RNA-HLA DR alpha
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0.089
0.092
0.076
0.104
0.067
0.121
0.129
0.096
0.132
0.093
-
GIFN
LPS
GIFN + LPS
PDBu
-
GIFN
LPS
GIFN + LPS
PDBu
U937 SUBLINE G4
U937 SUBLINE E11

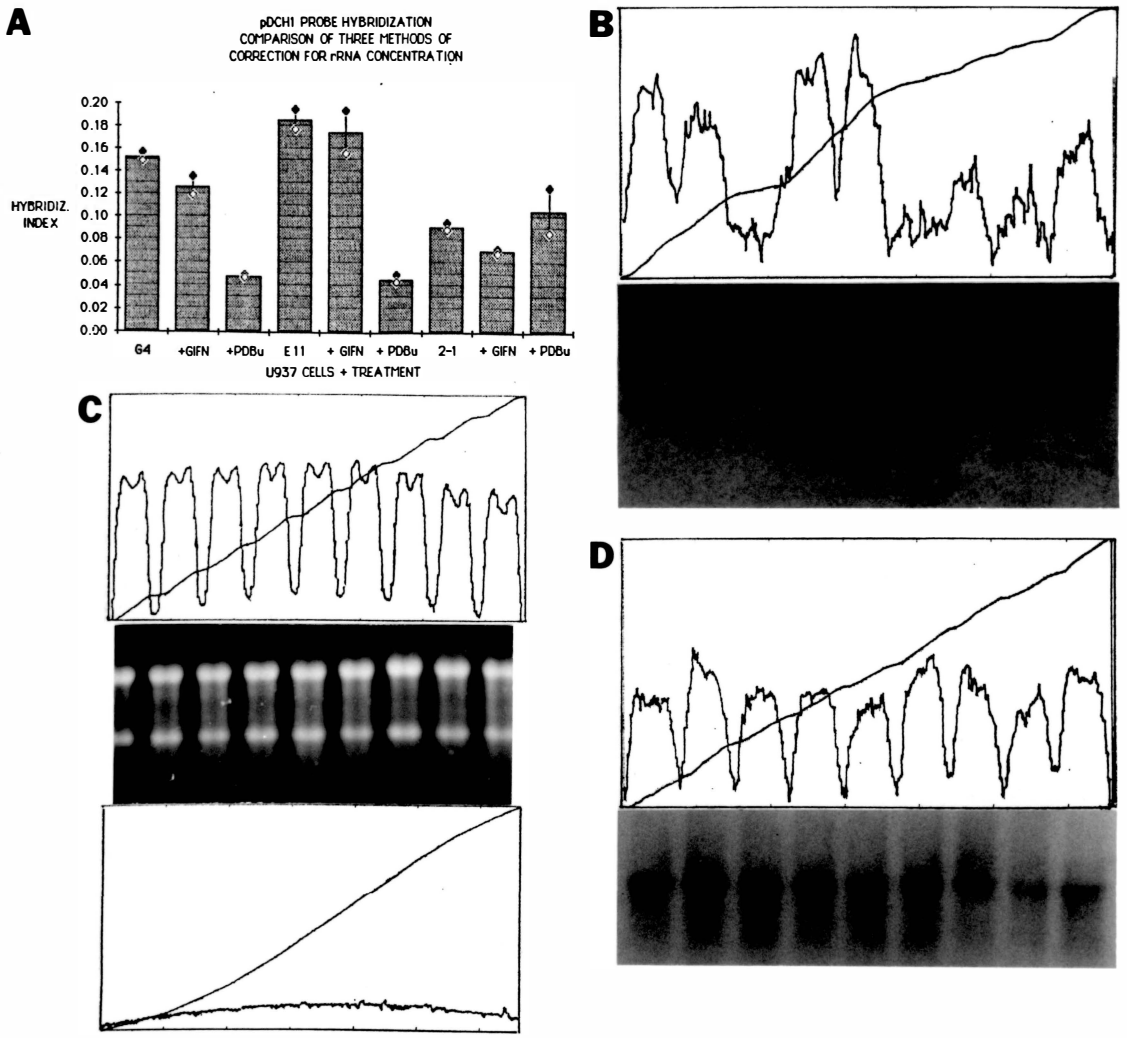
as the decrease seen after LPS treatment in the E11 subline. When hybridization with pDCH1 (HLA-DQ alpha) was examined there was greater disparity in response as indicated in Figure 54B. Both sublines G4 and E11 initially hybridized with the HLA-DQ alpha probe. Treatment with gamma interferon decreased the HLA-DQ alpha specific mRNA in the G4 subline cells but strongly increased the HLA-DQ alpha specific mRNA in the E11 subline cells, similar to the increase seen in Figure 50B. Treatment with of G4 subline cells with LPS increased the HLA-DQ alpha specific mRNA level slightly and treatment with a combination of LPS and gamma interferon had a small net negative effect. Treatment of the E11 subline with LPS alone strongly increased the level of HLA-DQ alpha specific mRNA present and treatment with a combination appeared to facilitate a hyperinduction of HLA-DQ alpha mRNA transcription. For both subline G4 and subline E11 cells, treatment with PDBu for 72 hours caused a significant loss of HLA-DQ alpha specific mRNA to levels which were only barely detectable.

Normalization by the three methods described gave similar results. An experiment illustrated in Figure 55 in which the blot was probed with pDCH1 (HLA-DQ alpha) and successfully with pAD-19.IIa (28S rRNA), the values obtained with three types of analysis using (1) scanning densitometer reading of the ^{32}P-rRNA hybridization autoradiogram, (2) scanning densitometer reading of rRNA on the photographic negative of the original gel corrected by subtraction of background obtained by directly scanning the film background and (3) scanning densitometer reading of rRNA on the photographic negative of the original gel corrected instead by subtraction of background determined by normal curve determined by curve fitting to rRNA readings, were comparable to each other.

Although the Northern blotting data is largely preliminary and requires further confirmation, the finding that there was comparative similarity of the values (Figure

FIGURE 55. HYBRIDIZATION INDEX (H.I.) CALCULATED FROM GEL PHOTOGRAPHS CORRELATED CLOSELY WITH H.I. CALCULATED FROM 29S rRNA SPECIFIC PROBE HYBRIDIZATION.

U937 sublines G4 and E11 and parent 2-1 cells were treated with gamma interferon (GIFN) (100 u/ml.) for 24 hours or with phorbol dibutyrate (PDBu) (10-8M) for 72 hours prior to extraction of total cellular RNA. RNA was subsequently electrophoresed in agarose, stained with ethidium bromide, photographed, electrophoretically transblotted onto nylon membranes and hybridized with probes for HLA-DQa (pDCH1) and 28S rRNA (pAD-19IIa) labeled by random priming techniques followed by autoradiography. Hybridization Index calculated as previously described (see Materials and Methods) was used as a means of normalizing data. H.I. as illustrated in (A) was calculated using comparison of scanning densitometer values for HLA-DQa specific hybridization with (1) scanning densitometer values from gel photographs with film background subtracted, (2) scanning densitometer values from gel photographs with film background determined by curve fitting, and (3) scanning densitometer values of rRNA probe specific hybridization. Results are indicated as the mean ± s.d. of these three values. Scanning densitometer tracing and probe hybridization autoradiogram for pDCH1 hyrbidization is shown in (B). Scanning densitometer tracings for gel photograph and for film background are shown in (C), and scanning densitometer tracing and probe hybridization autoradiogram for pAD-19IIa are shown in (D).


A
pDCH1 PROBE HYBRIDIZATION
COMPARISON OF THREE METHODS OF
CORRECTION FOR rRNA CONCENTRATION
HYBRIDIZ. INDEX
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
G4
+GIFN
+PDBu
E11
+ GIFN
+ PDBu
2-1
+ GIFN
+ PDBu
U937 CELLS + TREATMENT
B
C
D

55B) obtained in spite of the difficulties encountered in hybridization with the rRNA probe allowed the analysis of mRNA specific for the HLA-DR alpha and HLA-DQ alpha probes.

Summary. Sublines were derived from the U937 human monocytic cell line by limiting dilution techniques. Several of the sublines derived in this manner were found to constituitively express Ia, unlike most U937 cell lines previously examined. Thus the Ia+ and Ia- sublines were examined and characterized phenotypically and functionally. Morphologically the sublines, both Ia+ and Ia-, were found to be similar to the originally described U937 parent cell line with small differences in size among the sublines. They were positive for production of non-specific esterase and expressed the HLA phenotype A(3,X), B(51,18), DR(2,X). The sublines were examined by immunofluorescence techniques with a large number of antibodies specific for cell surface structures and found to express MHC class I, MHC class II the major type being HLA-DR and perhaps HLA-DQ in small amounts, Fc receptor, TA-1 structure typical of monocytes and T cells, endothelial antigens and CD4. The sublines and parent U937 cell line were found to express few cell surface antigens typical of mature monocytes/macrophages; however, they expressed myeloid antigens typical of early monocytic or promonocytic lineage. Although low levels of expression of several other cell surface antigens were variably detected, no surface immunoglobulin was detected. Expression of MHC class II by the sublines was confirmed by immunoprecipitation with monoclonal antibody to a framework determinant of human Ia and SDS-PAGE autoradiography which gave bands at p29:34 for Ia+ sublines but not for Ia- parent U937 cell lines. Cell surface expression of Ia was increased to a significant degree by treatment with gamma interferon which peaked at 24-48 hours of treatment. Functionally the parent U937 cell line and several Ia+ sublines were examined in several assays. The Ia- and Ia+ U937 cells were found to stimulate the generation of

specific cytolytic T cells in CML assays to approximately the same degree. The Ia+ sublines were found to stimulate in MLR assay, although variable results were obtained and indicated that the U937 parent and subline cells produced factors which were found to be inherently immunosuppressive. The sublines were found to be able to substitute for monocytes by reconstituting the CD3 mediated T cell mitogenic response. The Ia+ sublines and the parent U937 cell line (also weakly Ia+) were found to present tetanus toxoid antigen to nylon-wool purified T cells following an overnight pulse with antigen, and this response was found to be significantly abrogated by addition of antibody specific for CD4 and MHC class II, but not MHC class I. Preliminary characterization of the immunosuppressive factor produced by the U937 cell line and the sublines revealed that it was strongly antiproliferative and affected lymphoid cells somewhat more than non-lymphoid cells; that it had a molecular weight of approximately 90,000, was not inactivated by treatment with trypsin or chymotrypsin, was not immediately inactivated by freezing although dialysis and long term storage diminished its activity, and was partially inactivated by heat treatment at both 56°C and 80°C. Clear indication of soluble IL-1 production by the U937 parent cell line and the sublines was problematic due to the strong inhibition of proliferative assays by supernatants; however, partial inactivation of inhibitory activity by heat treatment as well as partial removal of the inhibitor fraction by gel filtration indicated that IL-1 or a cytokine with IL-1 activity was constituitively produced by the cells. Membrane IL-1 was detected in very low amounts in unstimulated cells and was significantly increased by treatment with phorbol esters but not other immunomodulators. Preliminary examination of Northern blots of HLA-DR alpha and HLA-DQ alpha mRNA production by the parent U937 cells and the sublines revealed that both HLA-DR alpha and HLA-DQ alpha mRNA were detectable for Ia+ sublines E11, G4 and G11, that only trace amounts were detectable for relatively Ia- subline E9 and that surprisingly, HLA-DQ alpha was detectable for the 2-1 parent cell line but no HLA-DR alpha mRNA

was detectable. The results of mRNA analysis following gamma interferon treatment, as well as treatment with LPS and phorbol esters, were variable for the different sublines and indicate that in some sublines gamma interferon treatment appears to augment Ia specific mRNA and in others the levels are decreased indicating that the expression of MHC class II specific mRNA may be differentially regulated in the different sublines.

DISCUSSION

Competent immune function is dependent on coordinated activity of a battery of cell types, some of which act in relatively non-specific ways and some of which show exquisite discrimination in their specificity. T cells and B cells which confer specificity to the immune response through interactions mediated by their respective receptors are extremely important regulators of immune function and also are the repository cells for maintenance of an anamnestic capability. Macrophages, on the other hand, mediate immune function in an apparently non-specific manner making use also of an array of cell surface receptors which, though specific for classes of molecules, are not specific for individual molecules. In other words, T cells and B cells are imbued with the capability of recognizing foreign molecules based on the sometimes infinitesimally small differences which they display which make these molecules non-self, and their ability to recognize these intruding molecules resides in a receptor which is clonally distributed and devised by a complex series of rearrangements of genes which themselves are similar in organization using common mechanisms. Macrophages make use of receptors which recognize molecules based on shared structural characteristics such as Fc portions of antibody molecules, mannose and fucose residues and, of primary importance, MHC molecules which make specific immune response mediated by T cells possible. Perhaps it would be more accurate,therefore, to describe the role of macrophages in immune function as semi-specific rather than non-specific. Certainly other cells, from the ubiquitous epithelial and endothelial cells to the highly specialized follicular dendritic cells, are also vitally important in protection of the organism from invasion and in the induction of an immune response. Their discussion is, however, beyond the scope of this project.

In conjunction with the cellular components possessing their respective arrays of membrane bound recognition structures, immune function is regulated by the complex interaction of soluble mediators, many of which are autocrine products. Most of the exocrine products of immune cells are tightly regulated and induce feedback mechanisms which modulate their production to delimit the immune response to the specific microenvironment where a response is required. These "interleukins," antibodies and other molecules are highly potent and unregulated production would cause generalized harm to the organism.

Macrophages play a central role in mediating immunologically important functions. As a cell type they are found widely distributed in tissues and organs as blood monocytes, histiocytes, alveolar macrophages and Kuppfer cells to catalogue just a few. In all tissues their function as mononuclear phagocytes is profoundly important for the removal and elimination of foreign matter, organisms and autologous effete cells and cellular debris. Their phagocytic activity is important in growth processes for such jobs as restructuring bone architecture and wound repair (241). Mononuclear phagocytes are important cytotoxic cells playing an essential role in elimination of bacterial invaders, parasites and tumor cells through processes of endocytosis, macrophage activation and subsequent release of cytolytic proteases and reactive oxygen compounds. In some tissue macrophages, particularly alveolar macrophages, long term localization in situ results in loss of capacity to release large amounts of cytotoxic substances as a result of phagocytosis perhaps as a mechanism of protecting the relatively sensitive surrounding normal tissues (241). Macrophages are extremely competent secretory cells and produce more than a hundred excreted products including enzymes, prostaglandins and interleukins (350). Macrophages are relatively impervious to physical and chemical harm and can withstand the effects of their own secretions as well as the effects of gamma radiation partially at least through their complement of enzymes capable of inactivating harmful peroxides and reactive oxygen

and perhaps also due in part to the nature of their membrane phospholipids which have been found to contain a high proportion of ether linkages rather than ester linkages (479). Thus because of their phagocytic function mediated by membrane-bound receptors for immunoglobulin Fc and for glycosylation products and their varied secretory function, macrophages are important in a broad sense in maintenance of organismic integrity. Phagocytosis and endocytosis also allow macrophages to participate in the generation of a specific immune response through partial digestion of antigenic substances and their reexpression in conjunction with MHC class II molecules on the cell surface where they become available for interaction with T cell receptors which specifically recognize the combination of a foreign antigen fragment with self-MHC. Although it may be argued that dendritic cells and B cells present large amounts of antigen to potentially reactive T cells through the expression of large amounts of MHC class II molecules, the capacity of the MHC class II expressed on macrophages to be modulated by immunologically relevant soluble mediators such as gamma interferon make it likely that macrophages serve an important antigen presenting function in vivo in areas to which they have been attracted by chemotactic stimuli, i.e. areas of infection.

Study of monocytes and macrophages has been carried out for many years and there are vast tomes of information related to this cell type. As a cell type, however, they have been relatively difficult to study in depth for several reasons. (1) Macrophages are characterized by their ability to adhere to surfaces; thus when removed from the peripheral circulation as cells in suspension, they rapidly marginate and adhere to any solid surfaces provided. Macrophage adherence is tenacious and virtually irreversible requiring that macrophages once adhered be treated harshly to remove them which invariably causes a loss of viability if scraping or high pressure liquid is used or change in cellular function if enzymes or xylocaine are used. Laboratories which have sought to remove macrophages for study have devised

"favorite" methods for doing so and have found that they must accept losses incurred by isolation procedures. (2) Macrophages normally occur as a minor population within a large population of other cells; thus, isolation of macrophages requires either prior stimulation to attract macrophages into a particilar area by use of sterile inflammatory agents for eliciting macrophages in the peritoneum or complex methods for enriching the population by such means as percoll gradient centrifugation. Each of these techniques has inherent difficulties. Injection of sterile inflammatory agents and, to an even greater degree, injection of activating agents such as LPS or C. parvum elicit macrophages which are not in a resting state. Although these agents has been successfully used in the study of murine macrophages, they are not satisfactory for the study of human macrophages and obtaining macrophages by peritoneal dialysis of normal individuals is not generally feasible. Macrophages obtained by percoll density gradient centrifugation have also been found to ingest the silica particles of percoll which changes their morphology and functional capacity to some degree (358). Monocytes isolated from human donors generally come from peripheral blood samples using enrichment of mononuclear cells by ficoll-hypaque density gradient centrifugation followed by adherence or further isolation by percoll gradient or centrifugal elutriation. Just as it has been difficult to remove the last monocyte from T cell preparations, it has been virtually impossible to remove the last T cell, B cell and dendritic cell from monocyte populations. None of these methods is wholly satisfactory making isolation of monocytes a troublesome aspect of their study. (3) Monocytes leaving the bone marrow following their maturation from stem cells do not generally undergo cell division in the periphery. Unlike T cells and B cells for which proliferative capacity is a function of activation, macrophages do not increase in number when activated except in the sense that increased numbers may be drawn into an area by a chemotactic stimulus. Thus, once isolated, macrophages do not make more of themselves, although while in culture, they may develop into large, multinucleated

giant cells and remain viable for long periods of time. Attempts to establish long term cell lines from human peripheral blood monocytes have been generally unsuccessful. Although there have been claims to the effect that it has been accomplished (364, 365, 366), these cell lines have not been widely used and do not often appear in the human macrophage literature. Attempts to immortalize peripheral blood monocytes by transfection with an SV40 construct with a defective origin of replication were tried and for a period of time held promise; however, these cells grew so slowly that they were not useful (personal communication). Attempts to use the same construct in murine cells appeared to be more successful (363), although in general attempts to establish macrophage cell lines from murine cells has been successful and numerous cell lines have been available for several years, many of which have been derived from tumor cells (359, 360, 362). Further attempts to produce human cell lines involved somatic cell fusion of human macrophages with other cell lines (367), but these attempts have not yielded cell lines which have found wide acceptability. The most successful methods for the establishment of human macrophage-like cell lines have been from macrophage tumors which are relatively rare. At the time when this project was begun there were very few macrophage-like cell lines of human origin. The HL-60 cell line was described as being of value because it could be differentiated in vitro along either granulocytic or monocytic pathways (369). The U937 cell line which was isolated from the pleural effusion of a human histiocytic lymphoma patient in 1976 was described as monocyte-like and appeared to have promonocytic characteristics (95). Since the beginning of this project to characterize the Ia+ U937 cell line in our laboratory, several other monocyte-like cell lines have become more widely used including the KG-1 (370) line and THP-1 line (371), both of which were derived from malignant cells.

The U937 cell line did not express MHC class II molecules when isolated and although several groups attempted to induce expression, these attempts were

unsuccessful. In 1981-1982, several laboratories reported finding that the U937 cell lines in their laboratories had begun to express MHC class II or could be induced to do so with gamma interferon treatment (422, 424). The U937 cell line in our laboratory was found to constituitively express MHC class II as measured by both immunofluorescence and by SDS-PAGE analysis using a monoclonal antibody to a framework determinant of human Ia. The U937 cell line had previously been shown to mediate several monocyte-like functions and to share monocyte specific cell surface molecules (452), to produce lysozyme (95), to bear Fc_E receptors (381), to be positive for non-specific esterase (95) and to mediate ADCC (404). Finding that the U937 cell line also constituitively produced Ia for unknown reasons made the cell line an attractive tool for study of monocyte functions which required interaction with T cells by means of the T cell receptor-MHC class II interaction. Thus this study was undertaken.

The U937 cell line in our laboratory was found to be heterogeneous in both MHC class II expression and cell size. It was decided that if clones of the cell line could be isolated, a more homogeneous expression of MHC class II and homogeneity of cell size would allow for evaluation and characterization of these cells and of the role of MHC class II molecules. It would allow for the selection of clones expressing a variety of levels of MHC class II molecules, thus affording the opportunity to look at MHC class II concentration effects on functional capacity. Clones isolated by limiting dilution techniques were found instead to continue to express MHC class II in a highly variable manner and cell size also remained highly variable, although the G11 subline averages slightly larger than the parent line and the E11 subline averages slightly smaller as indicated in Figure 2. Several explanations for this observed behavior are possible. T cell lines which express MHC class II molecules have been found to display a variable distribution (480) and indeed normal monocytes, although not a clonal population, express MHC at widely disparate levels as indicated in Figure 3. There is the

possibility that expression of MHC class II corresponds to position in the cell cycle; however, this was not examined for the U937 cells and sublines. The pattern of Ia expression exhibited by sublines G4 and E11 were found to remain constant over a period of time exceeding two years and cells kept frozen and recultured exhibited the same pattern of expression. Unfortunately, the parent cell lines were not completely Ia- when clonings were done; however, generally more Ia+ clones were derived from parent cell lines than Ia- clones. Also clones which were selected for their low level of Ia expression in the search for an Ia- clone such as G11 and E9, became progressively more Ia+, particularly the G11 subline which rapidly became a strongly Ia+ subline after its isolation at which time only 5.8% cells were Ia+. As previously stated the clones isolated by limiting dilution techniques were not subsequently subcloned and have therefore been referred to as sublines. There is no readily apparent explanation for the wide variability in cell size within populations of U937 cells except the difference in cell cycle which is supported only by the unquantified observation that U937 cells at near maximal cell concentration approximately three days after splitting of cultures, and presumably at a time when cell density and depletion of available nutrients might be retarding entry into mitosis thus causing a cultural synchrony, appeared to be more nearly homogeneous in size and shape. Recently reported karyotypic analysis of U937 cells obtained from three separate laboratories including the laboratory in which the cell line was originally derived indicates the genetic variability within the populations (373). The investigators stated that their first impression from the disparity in chromosome number and chromosomal aberrations was that the cells were derived from entirely different cell lines; and that it was only after analysis revealed that concordance was present for four chromosomal aberrations including a change known to associate with monocytic malignancy that it was concluded that all three cell lines had indeed been monoclonally derived. Therefore, although the original chromosomal aberration leading to malignant transformation was preserved,

there was extensive genetic variability among and within U937 parent lines which had arisen over the period of twelve years since the initial cell line isolation.

Morphologically the U937 cells examined were very similar to the U937 cells described by Sundstrom and Nilsson (95) in their original description of the cell line. The Ia+ and Ia- U937 cells examined in this project expressed the published HLA phenotype, except for the HLA-DR2 found on the Ia+ cells, thus dispelling any possibility that the Ia+ cell might have arisen from a contaminating cell. A further bit of evidence that these cells are related to original U937 cells was the finding that they were positive for non-specific esterase.

Phenotypic characterization was accomplished by immuno-fluorescence techniques utilizing a battery of antibodies having specificities for monocyte, T and B cell markers. As indicated in Table 4, the 2-1 parent U937 cell line and all sublines expressed MHC class I molecules at levels which were slightly lower than normal peripheral blood monocytes, except subline D10. The parent 2-1 line was found to express low but detectable levels of MHC class II molecules, as was the E9 subline. Sublines characterized as Ia+, G4, E11 and G11, were found to give positive reactions when incubated with antibodies specific for MHC class II as shown in Table 4 and there were no striking differences between sublines in reactivity. The presence of MHC class II molecules on the cell surface of sublines G4, E11 and D10 was confirmed by immunoprecipitation and SDS-PAGE techniques as shown in Figure 11; however, in this case there were clearly differences in amounts of Ia precipitated with no detectable Ia for parent cell lines and p29:34 detected in a hierarchy as follows: subline D10 > E11 > G4. In all cases, the percent positive U937 cells was lower than seen with normal human monocytes, except subline D10; therefore, the U937 sublines examined expressed less MHC class II than a comparable, normal population and as indicated in Figure 10, the level of expression was markedly diminished in the final year of the project for unknown reasons. The pattern of reactivity with antibodies specific for

monocytes/macrophages indicated that these cells lacked significant levels of antigens common to mature monocytic lineage cells in agreement with findings previously described by others (374). Further evidence that these cells represented an early monocytic stage was the finding that they were reactive with antibodies My4 and My7 but not Mo1 and Mo2 (Table 5). These cells also clearly shared surface antigens with endothelial cells and expressed these antigens at somewhat different levels. As indicated further in Table 6, the U937 cells strongly expressed CD4, Fc receptor and the TA-1 antigen reported on both T cells and monocytes. The U937 cells appeared to express few of the other antigens for which they were tested, although low levels of transferrin receptor and CD3 were detected. The G4 subline appeared to be somewhat more positive than other sublines for many of the antibodies tested. It is difficult to assess the significance of this finding without further examination; however, it is possible that at least in some cases the increase in reactivity may be related to the slightly higher level of Fc receptor expressed by this subline than the other U937 cells examined, although steps were taken to control for Fc binding of both directly conjugated reagents and reagents used in indirect assays.

The level of expression of MHC class II molecules on the cell surface was found to be modulated by treatment with gamma interferon as indicated in Figures 12 and 13. Although in more recent experiments using cells which have diminished Ia expression the increases seen in Ia expression with originally Ia+ sublines has been less, gamma interferon treatment was found to cause a maximal enhancement after 24 hours of incubation. There was an initial transient decrease in expression, seen most clearly in the E11 subline. In the recent studies shown in Figure 10, the E11 subline did not recover from this initial decrease until 72 hours of gamma interferon treatment. When compared with data obtained from Northern blotting experiments, no consistent correlation of levels of expression with levels of detectable mRNA is seen. Results seem to be contradictory if one expects a temporal relationship between regulation of

transcription and translation. Although gamma interferon has been shown to affect a region of MHC class II genes upstream from the coding region in an area known to contain regulatory elements (329, 330), endocytosis of gamma interferon via its receptor might also be expected to cause an increased rate of endocytosis of existing MHC class II molecules on the cell surface which would account for the initial decrease in MHC class II expression seen after short periods of gamma interferon treatment. Given the admittedly preliminary nature of the RNA data and the need for refinement of the questions being asked, it is postulated that gamma interferon affects MHC class II expression in several ways. The first step appears to be engagement of the gamma interferon receptor which has been shown to be expressed on the U937 cell line (384, 385). Engagement of this receptor appears to induce receptor mediated endocytosis in ways described for many receptors. Whether Ia is endocytosed at the same time by coincidence because it occupies the same area of the membrane and is therefore passively engulfed with the gamma interferon, or whether engagement of the gamma interferon receptor causes a coordinate clustering of gamma interferon and Ia on the surface in a manner similar to the phenomenon of capping or whether engagement of the gamma interferon receptor and the process of its endocytosis causes a coordinated stimulation of Ia endocytosis can not be discriminated; however, they explain the initial decrease in Ia expression seen after incubation with gamma interferon of short duration in some sublines. Increased endocytosis of Ia would allow for its clearing of bound ligand, which is likely to be self-derived, in the acidic compartment of the endocytic pathway and allow for attachment of available antigen fragments which would perhaps be non-self and also the cause of gamma interferon production in the first place. Interaction of gamma interferon with DNA as a DNA binding protein may then cause the release from repression of MHC class II transcription or perhaps promotion of transcription. This in turn leads to de novo transcription of mRNA for MHC class II. There also may be an increase in the rate of translation of MHC class II which would

increase the expression of Ia with little apparent increase in mRNA present. It is also possible that interaction with gamma interferon causes increased lability of existing mRNA and perhaps of newly transcribed mRNA such that in concert with the postulated increase in translational activity, a marked increase in expression of Ia is achieved which is of short self limiting duration. Although it is not shown from the data, it is postulated that cells previously stimulated with gamma interferon, enter a refractory period of unknown duration. Another explanation for the plateau in Ia expression may also be the loss of gamma interferon activity by being metabolized by the cells thus progressively removing it from the environment. Of interest is the finding that the different sublines and the parent 2-1 line behave in different ways when treated with gamma interferon, although they appear similar from Ia cell surface expression data. The sublines all show increased Ia expression; however, the G4 subline accomplishes this without indication of significant net change in mRNA for either HLA-DR alpha or DQ alpha and in fact there is a net decrease in both. In contrast, the E11 subline shows increased Ia expression and appears to exhibit profound increase in both HLA-DR alpha and DQ alpha mRNA levels. The G11 subline shows marked increase only in the HLA-DQ alpha mRNA available with little net change in the HLA-DR alpha mRNA. Little can be said of the E9 subline behavior given the low levels of expression, but the impression is that there is a small net increase in HLA-DR alpha and a decrease in DQ alpha. The parent 2-1 cell line is also of interest because it is clearly stimulated to increase Ia expression by gamma interferon treatment; however, HLA-DR alpha mRNA was not seen at all and DQ alpha mRNA was decreased. A possible complicating problem in the RNA hybridizations may be the fact that total cellular RNA was isolated and the quantitation was based on total RNA of which the overwhelming majority is ribosomal. Although there is evidence in cell lines that ribosomal RNA transcription is not significantly increased after gamma interferon treatment unlike normal cells (476), this particular cell line has not been examined. The mRNA for particular MHC class II

gene products would be expected to constitute an extremely small percentage of the total mRNA and therefore a vanishingly small percentage of total cellular RNA. If rRNA increased slightly with stimulation, the distortion seen in interpretation of mRNA results would be magnified giving the impression of decreased levels rather than what might in fact be sharply increased levels. Effects of other immunomodulators were also examined. As seen in Figure 54, sublines G4 and E11 exhibited decreased HLA-DR alpha mRNA when treated with LPS for 24 hours. HLA-DQ alpha mRNA was also decreased in the G4 subline, but was sharply increased in the E11 subline and combining LPS and gamma interferon was synergistic for HLA-DR alpha for both G4 and E11 sublines and HLA-DQ alpha for subline E11. Treatment of these sublines with PDBu for 72 hours markedly decreased HLA-DQ alpha mRNA and DR alpha mRNA to a smaller degree. Conversely, treatment of the parent 2-1 cell line with PDBu did not stimulated HLA-DR alpha mRNA transcription but DQ alpha mRNA was sharply increased. Thus Ia was expressed and there were clearly differences seen in the expression of Ia and regulation of transcription of Ia specific mRNA by the various populations of U937 cells in response to several substances previously shown to affect the immune response.

Functional studies were undertaken to characterize the ability of the U937 cells to mediate processes ascribed to macrophages. While all of these findings pointed to the monocytic lineage for the origins of the U937 cell line, the collateral finding that the cell line did not express Ia and apparently could not be induced to express Ia limited its usefulness as a monocyte analog. When it became apparent that the cell line in our laboratory expressed Ia and that sublines isolated from the relatively Ia negative parent U937 cell line also expressed Ia, it was postulated that the U937 cell line could be shown to mediate monocyte-like activation of T cells in an Ia dependent manner. As indicated in Figures 15 and 16, the U937 cell line and derived sublines were fully capable of stimulating the generation of specific cytolytic T cells in a CML response

which was comparable to the response seen with normal PBMC. Generation of the CML response was not, however, dependent on the presence of Ia on the surface of the cells but was instead dependent on MHC class I molecules which were detectable on all U937 cells. Generation of a mixed lymphocyte response, however, had been shown to be dependent on the expression of Ia (481), but may also be dependent on the presence of costimulating moieties (482) or differential glycosylation (483). Stimulation of an MLR by the U937 cell line and sublines has been problematic at least in part because of the production of secreted products having strong antiproliferative activity; however, in general the Ia+ U937 sublines were found to stimulate an MLR and the Ia- cell lines were less capable as shown in Figures 14 and 18. Inconsistencies in responses to U937 sublines at varying cell doses in the MLR response may also be at least partially attributable to the effects of soluble products. When stimulator U937 cells remained able to secrete suppressive factors it would be expected that at all cell doses, the stimulatory function, Ia, would be intact; however, at low cell doses the suppressor factor production would be insufficient to suppress the response but at high cell doses sufficient suppressor factor would be produced to completely depress the response. In experiments where a positive dose response curve was noted, it is postulated that the cells were sufficiently inactivated to prevent factor production at inhibitory concentrations. Support for this view was provided by several findings. Evidence that in some cases addition of cells to a known active normal MLR was suppressive and sometimes stimulatory as indicated in Figure 20 and that fixation, and therefore complete metabolic inactivation, of U937 cells allowed the production of an MLR response of the same or higher magnitude than mitomycin C treated cells as shown in Figures 21 and 22 indicated that the capacity for stimulation of the MLR was present. Although not quantitated, it was found during the course of the project that use of recently purchased, freshly prepared mitomycin C at high dose relative to cell number tended to correlate with production of an MLR response. Inhibition of cellular assays

by large numbers of normal monocytes or macrophages in both the human and murine systems is a well known phenomenon which may be caused by the production of suppressive levels of prostaglandins or combinations of other suppressive products, although the exact nature of the inhibition is not well characterized. The inhibition in the assays with U937 sublines was not found to be attributable to prostaglandin production as indicated in experiments in which indomethacin, a prostaglandin inhibitor, was contained in the cultures.

U937 cells and sublines were found to be fully competent in a dose dependent manner to reconstitute the T cell mitogenic response to OKT3, monoclonal antibody to the CD3 cell surface molecule, which has also been found to be monocyte dependent as shown in Figures 27 and 28. Although this function was attributable to the presence of the Fc receptor on the surface of the U937 cells and was not dependent on the presence of Ia, it is clearly a monocyte-like function which the U937 cells had not previously been shown to mediate.

The U937 sublines G4 and E11 were shown to present tetanus toxoid antigen to normal T cells as indicated in Figures 29-31. Because of the problem of residual reactivity in PBMC treated by adherence and nylon wool columns for the removal of monocytes and B cells, U937 cells were pulsed with tetanus toxoid, incubated and carefully washed prior to addition to the partially purified T cells. Therefore, the only antigen present in the assay was added in association with the U937 accessory cells. As indicated, the stimulation of ^{3}H-thymidine incorporation by the T cells was comparable to that seen with isolated human monocytes pulsed and added back in the same way. This stimulation was also found to be significantly abrogated by antibody both to human Ia (Kula 2 monoclonal antibody) and by antibody to the CD4 molecule (KT69-7 monoclonal antibody (440) which has been shown to interact in the binding of T cell receptor-antigen-Ia complex in the activation of T cells. Irrelevant antibodies and antibody to ß2 microglobulin were not significantly inhibitory. HLA-DR specific

antibody reactivity in inhibition of antigen presentation by U937 cells was found to correlate to some degree with the reaction except for the anti DR2 reagent which was found to block all normal reactions to some degree and strongly blocked the response by responders CS and CJ. Although this antiserum and the others used were found to have high reactivity for the stated HLA-DR specificities, they are heteroantisera with polyclonal reactivities which also include the public specificities Dw52 and Dw53 w;hich include HLA-DQ reactivity, and reactivity which would be predicted to block several other HLA-DR specificites. Thus it is not surprising that apparently inappropriate responses were noted. In order to delineate the HLA-DR and DQ specificity of the antigen presenting cells, monoclonal reagents to the private specificities found in the polymorphic portions of the MHC class II molecules would have to be employed. Unfortunately, these reagents are not currently available. It would also be advantageous to utilize antigen specific T cell clones to examine the capacity of U937 cells to present antigen. The use of T cell clones would obviate the necessity to purify the T cells and thus remove all autologous accessory cells. The use of T cell clones with known HLA phenotype also would allow for investigation of the MHC restriction of antigen presentation by the U937 cells.

Investigation of the suppressor activity found in the supernatants of the U937 cells included assessment of its effects on a number of proliferative assays including MLR and PHA and Con A mitogenesis, growth and ^{3}H-thymidine incorporation by other cell lines and the generation of CML reactivity. Preliminary characterization indicated that the suppressive activity was very potent, that it was not prostaglandin or a small molecular weight compound, although the production of such products may have contributed to the suppression seen, that it was not inactivated by treatment with either trypsin or chymotrypsin, but that partial inactivation could be accomplished by heating or long term storage and that when partially fractionated the suppressive activity correlated with elution of a 90 kd protein. Suppressive factor production by

the U937 cell line has been previously reported by several groups (393, 394) and molecular weights for these factors have variously been found to be approximately 56 kd (393) and 85 kd (394). The properties reported have been similar to those which were found in this study in that proliferative assays were strongly inhibited by addition of the supernatants. Our findings closely parallel those of Fujiwara and Ellner (394, 395) in similarity of molecular weight and character of the suppression seen. Admittedly the results obtained in investigation of the suppressive factors produced by these cells is preliminary and warrants more extensive treatment. It is not apparent at present whether the potent inhibitory activity, which seems to affect lymphoid cells to a greater degree than non-lymphoid cells as shown by differential effects on PBMC and REH cells of lymphoid origin as opposed to K562 cells of erythroleukemia origin, is characteristic of monocytic cells or whether it is a function of the malignant transformation which caused the development of these cells making it a tumor cell characteristic.

Production of soluble IL-1 by U937 cells after stimulation with bacterial extract from Staphylococcus aureus causing toxic shock syndrome has been previously reported (397). The IL-1 derived from U937 cells was found to be similar to but not exactly synonymous with human IL-1ß. In this study, measurement of soluble IL-1 activity using the standard costimulator assay with measures ^{3}H-thymidine incorporation by the IL-1 dependent T cell line, D10.G4.1, was found to be inappropriate because of the antiproliferative effect of other components of the supernatants tested. A surprising finding was the measurable IL-1 activity in column fractions from the Sephacryl S200 fractionation of concentrated supernatant which eluted either before or after the suppressive factor and also the synergistic activity seen in heat treated supernatants. It is unclear whether the stimulatory component in these supernatants or fractions of supernatants was normal human IL-1ß. In routine IL-1 assays, purified IL-1 added to the assays at high concentration was found to be

inhibitory and stimulation above a particular level could not be achieved. Addition of heated supernatant to these assays including optimal concentrations of purified human IL-1 appeared to synergize with the IL-1 rather than inhibit the response as one would expect with addition of higher concentration of purified human IL-1. In assays in which column fraction IL-1 activity was assessed, no purified IL-1 was added to the assay wells, thus the stimulation seen was not a synergistic response and the implication is that IL-1 activity was present. Membrane IL-1 which has been shown to be important in T cell stimulation was assessed by the method of Kurt-Jones et. al. (98). Unstimulated U937 cells were found to display small amounts of membrane IL-1 and treatment with immunomodulators including gamma interferon, LPS, gamma interferon + LPS and DMSO were not significantly stimulatory; however, phorbol esters including both PMA and PDBu were very effective stimulants of membrane IL-1 expression.

In summarizing the data obtained during the course of this project, it was apparent that although there were indeed small differences in the sublines in some parameters, notably in general cell size and cell surface phenotype, and there were differences noted in their expression of MHC class II specific mRNA in response to treatment with immunomodulators, these differences disappeared when functional capacity was investigated and no clear distinction could be made between sublines which could consistently be found. It became apparent early in the project that maintenance of an absolutely Ia negative U937 cell line was not possible over a long period of time. Although the impetus for expression of Ia by this cell line is not known, several groups have investigated mechanisms causing expression including methylation of the DNA and have found that hypomethylation may be necessary but not sufficient for MHC class II expression (424, 484). It appears that once negative regulation of Ia expression is removed, its expression is positively regulated to a variable degree by culture conditions. In MLR assays there appeared to be a correlation between the degree of Ia

expression and the frequency of MLR production. This is particularly apparent with the H-K parent cell line which remained Ia negative for the time that it was in culture and for sublines G4 and E11 which were isolated as Ia positive sublines and remained Ia positive to approximately the same level until very recently. The 2-1 parent and the E9 subline which were Ia- when first utilized, became measurably Ia+ over time, although the level of expression remained generally lower than other sublines used. The G11 subline became Ia+ rapidly after isolation but also continued to show more variability in expression than either the G4 or E11 sublines.

U937 cells have been investigated by numerous laboratories since their original isolation and description. Other laboratories have described finding Ia bearing variants of the cell line, notably Gitter et. al. (425) who isolated their clone, I937, by selection for a high level of Ia expression by cell sorting techniques. Their findings with the I937 cell line, that they would stimulate an MLR and that phenotypically they resembled promonocytes in their lack of Mo1 and Mo2 expression, were similar to our findings with the Ia+ sublines. A clear difference which has remained unresolved is their finding of HLA-DR3 positive cells rather than HLA-DR2 as we have found. Unfortunately the HLA-DR phenotype of the patient from whom the cell line originated is not known; however, it is possible that the HLA-DR genotype for the U937 cells is HLA-DR(2,3) and that the cell line in different laboratories selectively expresses one but not both HLA-DR specificities.

The U937 cell line has been found to be a useful tool for numerous avenues of investigation. Recent findings that the cell line can be chronically infected with HIV-I (417, 418, 419) has made it a possible tool for the investigation of the potentially lethal role of monocytes as repositories and disseminators of virus in AIDS. The U937 cell line has also been shown to ingest cholesterol esters to become analogous to foam cells making them potentially useful for the investigation of monocyte function in the establishment of atherosclerotic plaque formation (379, 380). In addition to these

studies, the U937 cell line has been useful in studies of the effects of metabolites of vitamin D and of phorbol ester induced differentiation in a large number of studies (399-414).

The sublines of U937 which have been characterized in this project represent an important adjunct to the study of Ia bearing cells in the human system. The relative ease with which they can be kept in culture makes them a readily available reagent. This area of investigation has previously been dependent on the isolation of human monocytes which is limited by the availability of suitable uninfected donors of large volumes of blood followed by tedious procedures which yield relatively small numbers of cells generally contaminated by significant numbers of T cells, large granular lymphocytes, B cells and dendritic cells. There are certainly differences between U937 cells and normal human monocytes and the use of tumor derived cells for discovering "normal" functions poses many problems of extrapolation; however, these problems once recognized may pose fewer problems than continuous isolations of cells from a battery of donors. The findings in this study that the G4 and E11 sublines appear to be able to process and present tetanus toxoid antigen to partially purified T cells and that this function can be abrogated by addition of Ia specific antibody makes them a potentially valuable tool for the investigation of the mechanisms involved in endocytosis and processing of antigens and of the reexpression of antigenic fragments in association with MHC molecules which have been difficult areas to study. Other areas of interest and potentially important investigation are the role of the Fc receptor and the role of CD4 molecules on monocytes in facilitation of Ia mediated function The finding that the U937 sublines were capable of reconstituting the mitogenic effect of anti-CD3 antibody in purified T cells indicates that these cells can mimic monocyte function in non-Ia dependent ways which nevertheless lead to fully activated T cells. The expression of CD4 by U937 cells and at low levels by normal *monocytes has been* previously reported (387, 388) but its function on these cells and U937 cells is not

known. The production of potent immunosuppressive factors partially described in this project, and the regulation of this factor production is also an area to which the U937 cells can make a contribution. Finally, preliminary findings indicate that there may be differences in the regulation of Ia expression among the sublines of U937 and the parent 2-1 cell line; therefore, investigation of regulation of Ia expression by addition of immunomodulating drugs, interleukins and other components of the microenvironment surrounding activated cells may lead to further understanding of the differential regulation of the HLA-DR and DQ gene products as well as HLA-DP, DO and DZ.

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Vita