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THE RESPONSIVENESS OF MURINE TUMORS TO MALEIC VINYL ETHER

by
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B.A., Norwich University, 1972

Thesis

submitted in partial fulfillment for the requirements for the

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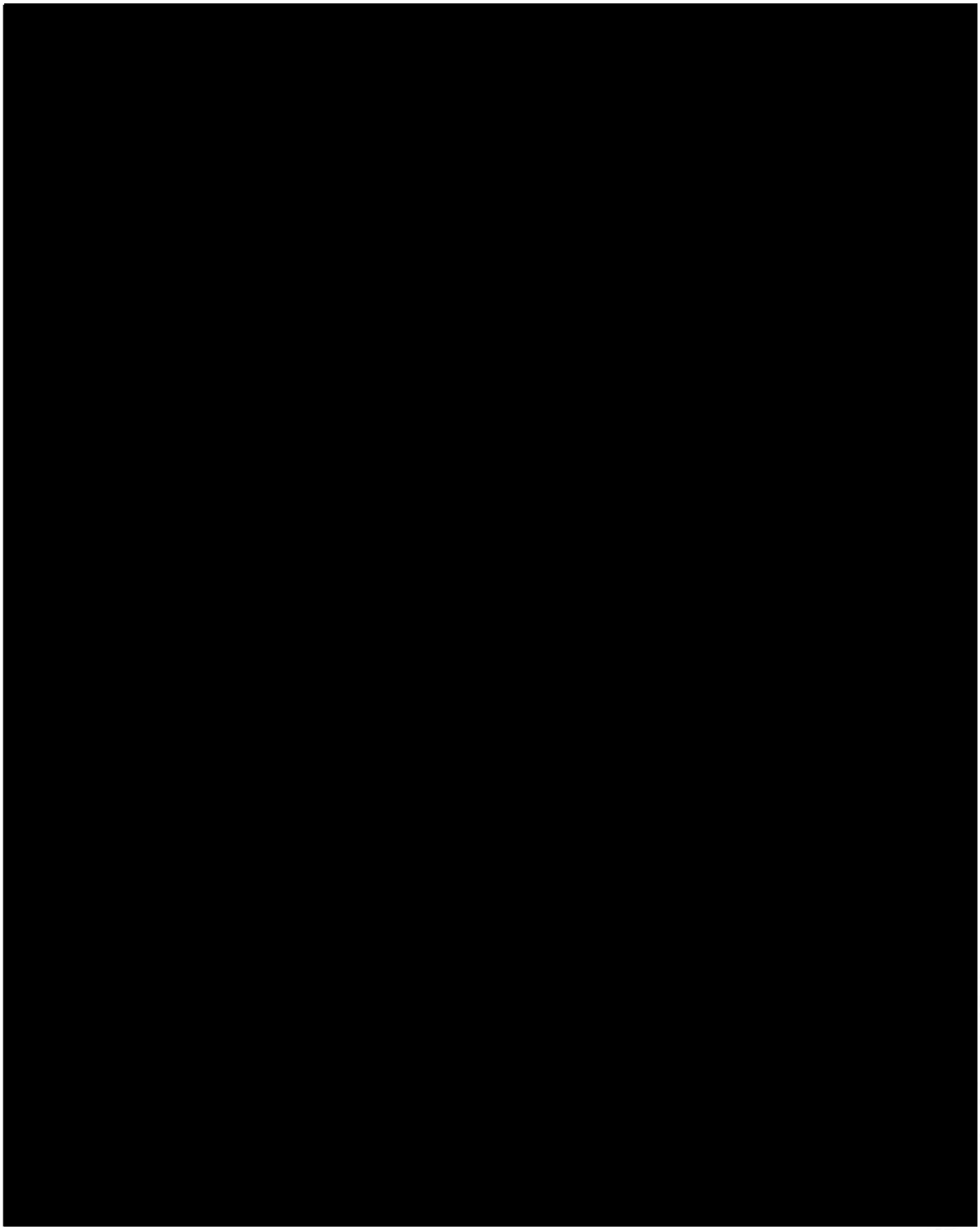

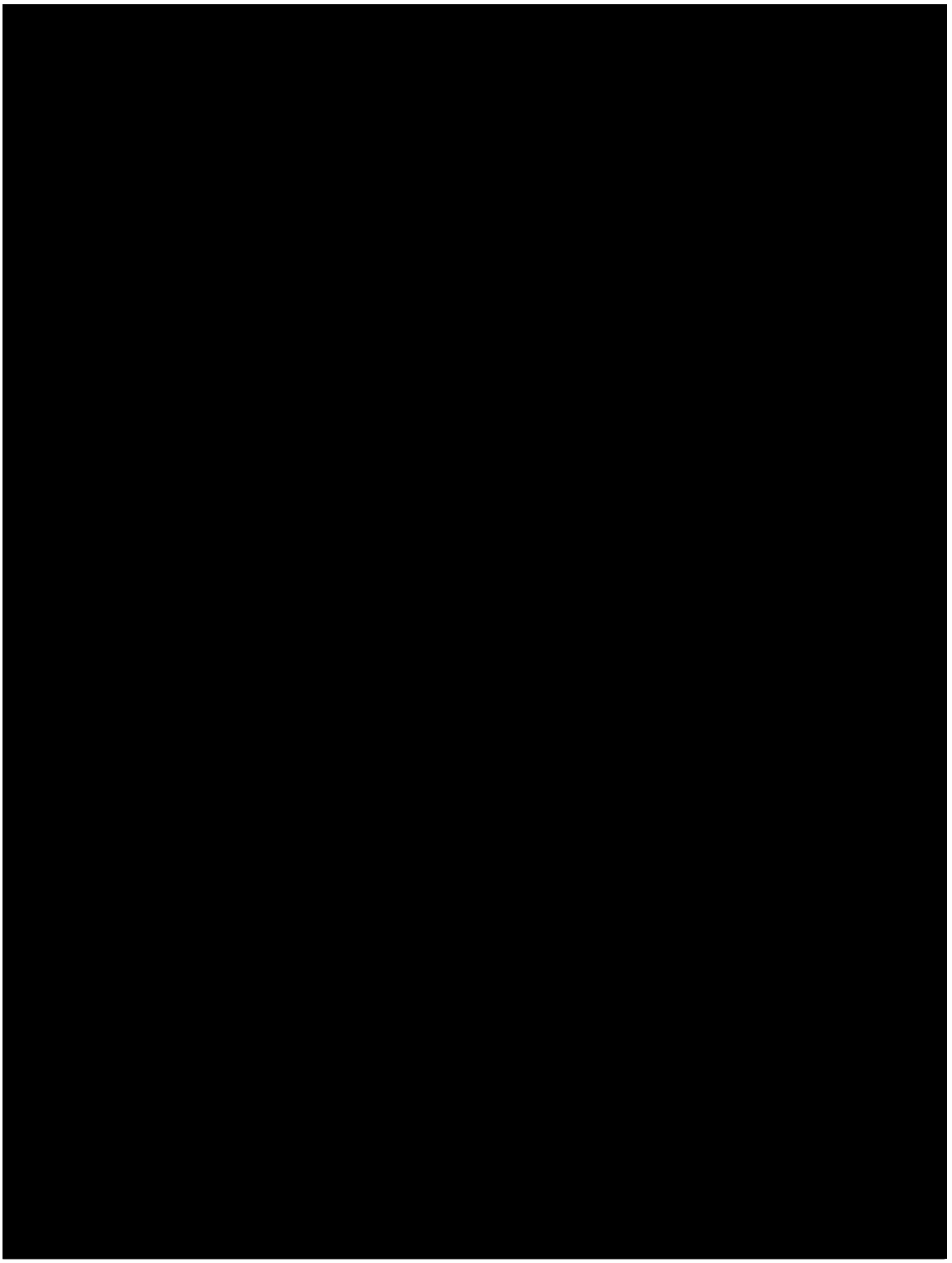

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DEDICATION

To Chris goes my deepest appreciation for the unselfish encouragement and support she has readily given me throughout the course of my graduate training.

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LIST OF ABBREVIATIONS

B-	bone marrow derived
BCG	bacillus Calmette-Guerin
BHI	brain heart infusion broth
CFU	colony forming units
Ci	curie
cm	centimeter
^{60}Co	cobalt-60
Con A	concanavalin A
cpm	counts per minute
^{51}Cr	chromium-51
CTX	cyclophosphamide
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
EMEM	Eagle's minimal essential medium
g	gravity
gm	gram
HSA	human serum albumin
I	iodine
IgM	immunoglobulin M
ILS	increased life span
i.m.	intramuscular
i.p.	intraperitoneal
i.t.	intratumor
IUDR	iododeoxyuridine

i.v.	intravenous
K	phagocytic index
kg	kilogram
LD_{50}	mean lethal dose
LLC	Lewis lung carcinoma
LPS	lipopolysaccharide
M	molar
M109	Madison 109 lung carcinoma
mg	milligram
ml	milliliter
mm	millimeter
MST	mean survival time
MVE	maleic vinyl ether
MW	molecular weight
nm	nanometer
PBS	phosphate buffered saline
PFC	plaque forming cell
PHA	phytohemagglutinin
PI	propidium iodide
PMN	polymorphonuclear cell
r	rad
RE	reticuloendothelial
RES	reticuloendothelial system
RNA	ribonucleic acid
rpm	revolutions per minute
s.c.	subcutaneous

SE . . . . . standard error

SI . . . . . stimulation index

SRBC . . . . . sheep erythrocytes

Sx . . . . . surgery

T- . . . . . thymus derived

TI . . . . . thymidine index

THE RESPONSIVENESS OF MURINE TUMORS TO MALEIC VINYL ETHER

Paal Christian Klykken, Ph.D.
Medical College of Virginia - Virginia Commonwealth University, 1979
Major Professor: Albert E. Munson, Ph.D.

Studies were undertaken to characterize the immune status of mice bearing the Lewis lung carcinoma (LLC) and to maximize the cure rate of LLC and Madison 109 carcinoma (M109) bearing mice by employing the immunomodulator maleic vinyl ether (MVE) in combination with surgery or radiotherapy.

The pattern of immunodeficiency in mice bearing the LLC appeared to be in contrast to most studies. LLC mice with a minimal tumor burden were found to have a diminished ability to phagocytize sheep erythorcytes (SRBC) or elicit an antibody response to the same antigen. Vascular clearance and phagocytic uptake of ^{51}Cr-labeled SRBC into reticuloendothelial (RE) organs exhibited a triphasic response. Liver phagocytosis and vascular clearance were markedly suppressed 24 and 48 hours after tumor inoculation. The initial decrease in RE function was followed by a phase of increased activity which progressively decreased to control levels with increasing tumor burden. IgM antibody forming cells to SRBC were reduced to 18% of control values 7 days after inoculation and by day 27 no antibody forming cells could be detected in the LLC bearing mice. In contrast, delayed type hypersensitivity to SRBC remained intact until day 17 and no significant changes in inflammatory activity were noted. Surgical excision of the primary tumor burden on day 14 had no effect on survival time but partially restored the suppressed immune functions.

Accordingly, immunotherapeutic regimens were designed to treat early in the disease state or after cytoreductive therapy in an effort to have immunocompetent animals with a minimal tumor burden. Yet, systemic MVE treatment by itself, or in combination with surgical excision or local radiotherapy of the primary tumor was ineffective in prolonging life span. Intravenous MVE treatment also failed to exhibit antitumor activity against the LLC when the metastatic tumor burden was markedly reduced by multiple cyclophosphamide injections. In contrast to systemic MVE treatment, an acute intralesional injection of MVE proved to be efficacious by increasing life span by 36%. Moreover, the combination of intralesional MVE with local radiation of the primary LLC tumor significantly prolonged survival time over the radiation controls.

The M109 was shown to be relatively responsive to MVE treatment. Multiple injections of MVE in doses ranging from 10 to 50 mg/kg significantly prolonged the life span of the host and inhibited the growth of the primary tumor. The efficacy of MVE therapy appeared to be dependent on the distribution of the polymer. Weekly intralesional injections of MVE were most effective in prolonging life span early in the disease state, presumably by inhibiting the metastatic process. As the tumor metastasized to the lung only MVE introduced directly into the metastatic tumor bed by intrapleural or inhalation administration proved to be efficacious in prolonging life span. These data emphasize the importance of drug distribution in the treatment of neoplasia with nonspecific immunotherapy.

INTRODUCTION

The basic assumption in cancer treatment is that the achievement of cures or normal life expectancy requires the destruction or removal of all malignant cells. Four therapeutic modalities have the potential of accomplishing this end: surgery, radiotherapy, chemotherapy and immunotherapy. Surgery and radiotherapy are the most frequently used and most effective approaches currently available, but neither of these modalities can be considered curative when the neoplasm has metastasized beyond the primary tumor site. Chemotherapy has proven to be effective in selected forms of cancer, yet its ability to eradicate residual tumor cells is presently limited by the toxicities associated with the treatment schedules.

Immunotherapy is a new and exciting fourth treatment modality being developed to aid the host in controlling cancer. Its chief value lies in the specificity of the immune response which may enable immunotherapy to destroy tumor cells not amenable to killing by conventional treatment modes. The underlying concept of tumor immunology is that there are characteristic antigens in or on tumor cells that distinguish them from normal host cells, and that these antigens are capable of eliciting immune responses. It is the hope that immunotherapeutic manipulation of the host's immune response will allow for destruction of tumor cells through specific immunologic mechanisms mediated by thymus derived lymphocytes, cytotoxic antibodies, null cells, or armed macrophages, or through non-specific mechanisms mediated by activated macrophages.

Immunity against cancer, however, is relative rather than absolute (Mathe et al., 1969; Zbar et al., 1972). Host defenses are capable of destroying small numbers of tumor cells (10^6-10^7), but 10^8 tumor cells almost always result in tumor progression. Because a neoplasm only 1 cm in diameter contains approximately 10^9 tumor cells, most tumors have already overcome or avoided immune defenses by the time they are clinically detectable (Carter, 1976). Therefore, the practical future of immunotherapy appears to be part of a combined modality approach, as it is unlikely that immunotherapy alone will bolster host defenses sufficiently to reverse tumor growth in individuals with advanced disease.

The immune status, or immune competence of the tumor bearing host can also constitute a critical limitation to the success of immunotherapy. Immunosuppression of mice with antithymocyte serum (Hanna et al., 1973) cortisone acetate (Chung et al., 1973), or by thymectomy and sublethal irradiation (Bartlett et al., 1972) delayed the development of tuberculin hypersensitivity and abrogated the ability of Bacillus Calmette-Guerin (BCG) to suppress fibrosarcoma growth. Scott (1975a) has produced similar findings with Corynebacterium parvum in that C. parvum was somewhat less effective in thymectomized, irradiated, bone marrow reconstituted mice than in intact recipients. Thymectomy has also been reported to reduce the ability of pyran-copolymer to protect mice against the Lewis lung carcinoma or B16 melanoma (Morahan et al., 1974).

Following this line of reasoning, the overall objectives of this research were to (1) characterize the immune status of mice bearing

the Lewis lung carcinoma and (2) develop a rational approach, consisting of surgery or radiotherapy combined with immunotherapy to maximize the cure rate of mice bearing the Lewis lung carcinoma or Madison 109 carcinoma.

I. REVIEW OF THE LITERATURE

A. Tumor Systems

1. Lewis lung carcinoma

The tumor models employed in these investigations were the Lewis lung carcinoma (LLC) and the Madison 109 carcinoma (M109). The LLC was discovered by Dr. Margaret R. Lewis in 1951 as a spontaneously occurring carcinoma of the lung of a C57BL/6 mouse. Tumor take rate is 100% and the median day of death is 25 days after subcutaneous (s.c.) implantation in the F_1 hybrid (Mayo, 1972a; Simpson-Herren et al., 1974). The growth of the s.c. implanted LLC fits a Gompertzian function with an increase in doubling time from 1.7 days for a 0.1 g tumor to 7.7 days when the tumor reaches 5.0 g (Mayo, 1972a). The length of the cell cycle (Tc = 16 hrs) and the S phase (Ts = 9 hrs) are not significantly altered when mean tumor weight increases from 0.3 to 2.7 g (Mayo, 1972a), yet the majority of primary s.c. tumors ulcerate by day 14 and by day 21 essentially all of the tumors have necrotic cores surrounded by a proliferating shell (Simpson-Herren et al., 1974). In addition, the thymidine index (TI) decreases from 35% on day 6 to 18% on day 16. These data indicate that a decrease in the proliferative fraction as the tumor grows larger accounts for the observed increase in doubling time from 0.4 days with a 350 mg tumor on day 6 to 23 days with a 6400 mg tumor on day 24 (Mayo, 1972a). After implant the LLC quickly metastasizes, primarily to the lungs, with over 50% of the mice succumbing to pulmonary insufficiency when the primary tumors are removed on day 2

posttumor implant. Surgical removal of the primary tumor more than 6 days postimplant has no effect on the lifespan of the tumor-bearing host (Mayo, 1972a).

The growth kinetics of the metastatic LLC is also characterized by a progressive increase in tumor doubling time with increasing tumor mass and age. In comparison with the subcutaneously implanted primary tumor the metastatic tumors have a shorter doubling time (0.5 days vs. 4.7 days on day 15 postimplant) and the TI is consistently higher. In addition, the cell cycle and S phase are of shorter duration in the metastatic tumor measured at day 25 than in the primary tumor on day 5 or later (Simpson-Herren et al., 1974). Each of these factors is consistent with greater drug sensitivity in the metastatic foci.

The LLC is refractory to most chemotherapeutic agents with established activity against human cancer, yet is quite sensitive to cyclophosphamide and the nitrosoureas (Mayo et al., 1972b; Goldin et al., 1971). No optimal single dose schedule for these compounds has been developed since effective treatment varies with the extent of neoplasia (Humphreys et al., 1970).

Studies with cyclophosphamide (CTX) by Karrer et al. (1967) indicate that the LLC is not curable by treatment with CTX alone. However, surgical removal of the primary tumor in combination with CTX treatment results in long term tumor free survivors. Similar results have been obtained using irradiation treatment of the primary implant (Johnson, 1969). These results correlate with the tumor cell kinetic studies of Simpson-Herren et al., (1974) and are consistent with the conclusion reached by DeWys (1972) that the proliferative state of a tumor cell

population is a major determinant of sensitivity of CTX. Additional support for this concept is derived from the observation that metastatic foci are considerably more sensitive to gamma irradiation than the primary tumor (Shipley et al., 1975). An alternative explanation for the divergent effect of CTX on the primary and metastatic LLC has been published by Houghton et al. (1976). The results obtained from this distribution study imply that the greater sensitivity of pulmonary as compared to s.c. Lewis lung tumors is due to the attainment of greater CTX concentrations in the lung tumors. In comparison with CTX, methyl-CCNU is superior in the treatment of LLC. Although equally effective in "curing" widely desseminated Lewis lung tumor cells, methyl-CCNU shows greater effectiveness against solid tumor masses (Mayo et al., 1972b). Marked activity has been documented by a number of reliable end points such as increases in life-span, tumor regressions, some cures and bioassay of tumor cells for viability after methyl-CCNU treatment.

The responsiveness of the LLC to immunotherapy has been shown to be dependent on the dose, dosing regimen and route of administration of the immunomodulator, and more importantly on the tumor burden of the mice at the time of therapy (Bast et al., 1976). Mathe et al. (1973, 1975) have reported that one mg of BCG administered intravenously displayed antitumor activity against the LLC whereas the same dose injected subcutaneously resulted in enhancement of tumor growth. In the first case the manifestations of septicemia were much more intense than in the second, as the antitumor activity of

BCG seems to be correlated with the degree of septicemia produced (Khalil et al., 1975; Mathe, 1976). Systemic i.p. or i.v. administration of C. parvum has been ineffective in retarding primary tumor growth and prolonging life span (Castro and Sadler, 1975; Morahan and Kaplan, 1976a) although daily intralesional administration of C. parvum initiated 24 hrs after tumor inoculation has proven to be effective against the LLC (Morahan and Kaplan, 1976b). Numerous reports have also documented the antineoplastic activity of pyran in the LLC tumor model (Morahan et al., 1974; Snodgrass et al., 1975; Morahan and Kaplan, 1976a). Yet, as with BCG and C. parvum, a moderate increase in life span was afforded only when daily pyran treatment was initiated early in the disease state when the tumor burden was minimal.

The limited success of immunotherapy against the LLC can partially be attributed to the inability of the host to develop an immune response to tumor-associated antigens, since Morahan and Kaplan (1977) and Otu et al. (1977) have been unable to detect any concomitant immunity or transplantation resistance to the LLC. When tumors of different immunogenicities have been compared for susceptibility the more strongly immunogenic tumors have, in general, been more responsive to BCG immunotherapy (Parr, 1972; Baldwin and Pimm, 1973). A similar correlation has been obtained with C. parvum (Smith and Scott, 1972), C. granulosum (Milas et al., 1974a) and L. monocytogenes (Bast et al., 1976).

2. Madison 109 lung carcinoma

The M109 was discovered in 1964 by Dr. Russel M. Madison as a spontaneously occurring lung tumor in a Balb/c mouse. As only a

few laboratories have utilized this tumor model, the M109 has been poorly characterized with regard to its tumorigenicity, growth characteristics, tumor cell kinetics and immunogenicity. Like the LLC the M109 tumor is a highly stable transplantable tumor which kills all recipient mice within a fairly narrow time range after inoculation with no evidence of spontaneous regression. The tumor metastasizes primarily to the lungs which results in the death of the host in about 35 days after implant (Marks et al., 1977). However, the M109 displays a different spectrum of activity than the LLC to chemotherapy and immunotherapy. Agents with confirmed activity against the M109 such as actinomycin D, adriamycin, daunorubicin, and procarbazine have not displayed activity against the LLC. Similarly, cytosine arabinoside, BCNU, methyl-CCNU and melphalan are active against the LLC but inactive in the M109 tumor model (Wood, 1977). However, pyran copolymer has been reported to be active against both of these experimental lung carcinomas (Snodgrass et al., 1975; Schultz et al., 1977).

B. Alterations in Immune Status of Tumor Bearing Animals

Modulation of immune responses has been repeatedly observed in cancer patients and tumor-bearing animals. Several studies have indicated enhanced immunologic responsiveness to foreign antigens or mitogens (Zolla, 1972; Konda et al., 1973). Absence of a detectable effect on immunity by tumors has also been reported (Fauve et al., 1974). However, the majority of investigations have noted a progressive immuno-

logic impairment in animals bearing primary tumors (Kamo and Ishida, 1971; Kamo et al., 1976), those infected with oncogenic viruses (Seigel and Morton, 1966; Ceglowski and Friedman, 1968), or those treated with carcinogens (Gericke et al., 1971).

1. Alterations in reticuloendothelial activity

The role of macrophages and the entire reticuloendothelial system (RES) in neoplastic disease has been extensively studied. Investigations using different laboratory animals and tumor lines have yielded conflicting data, however. The neoplastic process has been shown in some instances to have a stimulatory effect on the RES (Baum and Fisher, 1972; Old et al., 1960) whereas in other studies a significant RES depression has been observed in tumor-bearing animals (Stern et al., 1967; Franchi et al., 1972; DiLuzio et al., 1972).

Aside from the inherent differences in tumors and experimental conditions, one possible source of discrepancy may be that hepatic uptake of foreign particles, which accounts for the majority of RES activity, has been shown to be variably affected in tumor-bearing animals (Stern et al., 1967). Moreover, increased hepatic phagocytosis may be of limited benefit in the maintenance of immune responsiveness since the liver functions mainly in clearance and degradation of antigens (Frei et al., 1965; Franzl, 1972).

In contrast to Kupffer cell activity, splenic and pulmonary phagocytosis appear to be regularly and markedly reduced in tumor-bearing animals (Kampschmidt and Pulliam, 1972; Stern et al., 1967).

The work of Stern et al. (1967) with four spontaneous murine tumors suggested that a correlation exists between splenic uptake and tumor size. Other studies by Franchi et al. (1972) with a murine sarcomma transplanted in different sites indicated that RES activity is not influenced by tumor size since the s.c. tumor may reach 25% of the body weight without an impairment of colloidal carbon clearance. These investigators concluded that the suppression of the RES function is related to the degree of cancer cell dissemination in the blood.

2. Alterations in humoral immunity

Altered B-cell responsiveness to sheep erythrocytes (SRBC) in lymphomas, ascites and solid murine tumors has been measured at the level of the individual antibody cell in lymphoid tissues (Gericke et al., 1971; Kamo and Ishida, 1971; Ceglowski and Friedman, 1968; Kamo et al., 1976) and at the level of serum antibodies (Seigel and Morton, 1966; Ceglowski and Friedman, 1968). Generally, the results have indicated that the degree of depression of the primary antibody response is directly related to the extent of neoplasia at the time of immunization. In contrast, studies on the secondary or the amnestic response have yielded divergent results. Seigel and Morton (1966) reported that the secondary response to SRBC is not depressed in mice inflicted with Rauscher virus, whereas the work of Ceglowski and Friedman (1968) indicated that both 7s and 19s antibody forming cells are suppressed in secondary-stimulated mice bearing the same leukemia. In mastocytoma-bearing DBA/2 mice Kamo and Friedman (1977)

noted a selective depression of IgM antibody forming cells with no significant changes in IgG levels.

Measurement of complement activity in tumor bearing animals has also yielded divergent results. Studies with seven transplantable murine tumors indicated that host complement activity is depressed in all cases after tumor transfer, but the rate and extent of depression was variable (Hartveit, 1965; Drake et al., 1973). The results of these experiments suggested that different complement components may be affected in each case as there was no correlation between immune adherence and hemolytic complement activities. Furthermore, there appeared to be no correlation between the mean survival time of tumor bearing animals and depression of complement activity. In contrast, Thunold, et al. (1973) reported normal C_{1q} and C_3 levels in mice bearing Ehrlich carcinoma while Weimer et al. (1964) noted elevated serum complement levels in tumor-bearing rats and rabbits. Whether these differences are due to enhancement or suppression of antibody fixation has not yet been clarified.

3. Alterations in cell mediated immunity

Depressed cell mediated immunity has been reported for a variety of murine tumor systems. The presence of a growing tumor has been documented to cause significant curtailment of graft-versus-host reactions in vivo (Sidky and Auerbach, 1976; Medzihradsky et al., 1972) as well as suppressed blastogenic responses to phytohemagglutinin (PHA) and other mitogens in vitro (Levy et al., 1974; Padarathsingh et al.,

1977; Kilburn et al., 1974; Gillette and Boone, 1975). Other investigations have revealed a correlation between tumor growth and loss of T-helper cell activity (Takatsu et al., 1974). The work of Gillette and Boone (1973) utilizing five different murine tumor lines suggested that the T-cell depression is a generalized phenomenon independent of tumorogenicity, immunogenicity or tissue of origin of the tumor.

4. Proposed mechanisms for tumor induced immunologic deficiencies

Underlying the diminished immunologic competence of tumor bearing animals are hematological changes and alterations in lymphoid cell populations. In C57BL/6 mice bearing the LLC a marked hemolytic anemia has been observed in conjunction with the appearance of thrombocytopenia (Poggi et al., 1976). In subsequent studies with the same tumor line, bone marrow smears have revealed a moderate normoblastic hyperplasia (Poggi et al., 1977). The work of other investigators have suggested that a T cell deficiency in the regional lymph nodes accompanies progressive tumor growth (Loring and Schlesinger, 1970; Rowland et al., 1974).

Splenomegaly, which often accompanies tumor growth, has been attributed to the existence of a consistant antigenic stimulus (Kamo and Friedman, 1977), an increase in erythropoietic activity (Gillette and Boone, 1975), the presence of metastasized tumor cells (Kamo et al., 1976) or an increase in the number of trapped lymphocytes (Fightlin et al., 1975). Yet despite this splenic enlargement, a

histologic loss of thymus dependent areas of the spleen has been noted by several investigators (Gillette and Boone, 1974; Takatsu et al., 1972; Gillette and Boone, 1975). Data published by Gillette and Boone (1975) indicated that the lymphocyte content of spleen cell preparations from tumor-bearing animals drops from 80 to less than 25% with a concomitant increase in myeloid elements.

In contrast, the work of Trainin and co-workers with the LLC suggests that the spleen is comprised of lymphoid subpopulations with opposing reactivities. Fractionation of sensitized lymphoid cells by velocity sedimentation yielded a subpopulation of lymphocytes in a blast stage capable of enhancing tumor growth and of nonspecifically suppressing the antigenic response elicited by a fraction of smaller lymphocytes (Small and Trainin, 1976). The occurrence of the splenic subpopulations in normal mice indicated that the difference between normal spleens and the spleens of tumor-bearing animals was quantitative (Treaves et al., 1976). As tumor growth proceeds, there was an increase in suppressor cell number and activity. The inhibitory activity of the larger blast cells predominated at the time of visible lung metastasis (Treaves et al., 1974). Adoptive transfer experiments utilizing lymphoid cells from the spleen, thymus and bone marrow indicated that thymus derived spleen lymphocytes are the cells which mediate the impairment of immune reactivity and enhancement of tumor growth (Umiel and Trainin, 1974; Carnaud et al., 1974).

However, it has not yet been demonstrated whether the same T-lymphocyte can exhibit both suppressive and active properties under

changing conditions or whether distinct splenic subpopulations exist. It is also unclear as to whether the same subpopulation of suppressor cells can suppress an antigenic response and also be involved in tumor growth enhancement, although both types of suppression have been eliminated by identical procedures (Rotter and Trainin, 1975).

Further investigations have revealed that a soluble factor found in the media of cultured spleen cells from LLC bearing animals has similar suppressive activity (Treaves et al., 1976). Removal of T-lymphocytes prevented the appearance of this factor in the culture media whereas removal of B-lymphocytes and macrophages was without effect. This suppressor activity, however, is believed to be nonspecific because non-related immune activities such as PHA responsiveness in tumor bearing animals were also suppressed by such factors (Adler et al., 1971). In addition, this suppressor factor did not require strain compatibility between the factor-producing cells and the recipients. The suppressor factor produced in LLC bearing animals could also enhance the syngeneic growth of tumors in C3H mice. Parallel investigations in other tumor lines by the same investigators imply that the appearance of suppressor factors is a generalized phenomenon in tumor bearing mice (Treaves et al., 1976).

Studies aimed at elucidating the mode of action of this suppressor substance suggested that this soluble factor suppresses the immune competence of the host, which leads indirectly to enhancement of tumor growth. Previous studies by Carnaud et al. (1974) which have documented an increased number of lung metastases in immunologically impaired LLC

animals support this concept. The cell types and immune reactivities affected by this suppressor factor are yet to be determined.

A number of other possible mechanisms for the decreased immunologic capabilities of tumor-bearing animals also appear in the literature. All the proposed mechanisms require continued presence of the tumor for immune suppression. This dependency is evidenced by the reversibility of immunosuppression after surgical removal of the tumor burden (Bray and Keast, 1975; Gillette and Boone, 1975). Additional support for this concept is derived from transfer experiments which demonstrated that spleen cells from immunologically impaired tumor bearing animals could still express immune competence when transferred to irradiated syngeneic recipients (Mocarelli et al., 1973; James et al., 1974b; Hrsak and Marotti, 1975).

Several reports have proposed that the immunosuppression results from the recruitment of reactive T and B cells from the spleen in response to the tumor (Gillette and Boone, 1975). Alternatively, in studies which have shown no change in lymphoid populations the possibility exists that the reactive lymphoid cells may have been replaced in the spleen by more immature, less reactive cells (Padarathsingh et al., 1977). The work of Frost and Lance (1973) suggested that the presence of a tumor suppresses lymphocyte trapping, i.e. the migration of lymphocytes to the spleen to promote the antigen, macrophage and lymphocyte interaction necessary for the generation of an immune response.

Attuned to the observation of Webb (1970) that serum "recognition factors" are important for macrophage function, Saba and Antikatzides

(1975) have reported a correlation between decreased levels of opsonic serum protein and impaired phagocytosis in tumor-bearing animals. In addition, North et al. (1976) have recently discovered a dializable phagocytosis depressing factor in tumor bearing mice. These findings, taken together with the observations of depressed phagocytosis in tumor-bearing animals suggests that decreased levels of a recognition factor and increased levels of a phagocytosis depressing factor may affect macrophage function, which, in turn, may suppress the normal immune responsiveness of a tumor bearing animal. Furthermore, as the addition of peritoneal exudate cells effectively restores normal immunocompetence to spleen cells from tumor bearing animals, antigenic competition for specific sites on macrophages has also been considered the possible mechanism of immunologic suppression (Pross and Eidinger, 1974; Feldman and Schrader, 1974).

Elevated levels of a normal serum component, $alpha_2$-globulin, during progressive tumor growth has also been reported to affect general immune competence. Although the actual mechanism of suppression by this serum factor is still unclear, investigations by Menzoian et al. (1974) have suggested that the target cells for $alpha_2$-globulin are T cells.

Soluble factors released by tumor cells also appear to be able to inhibit or regulate lymphoproliferative responses. Prostaglandins, RNA, immune complexes, along with peptides and proteins of various molecular weights have all been implicated in suppressing the host resistance in tumor-bearing mice. A review of this subject has been recently published by Kamo and Friedman (1977).

Lastly, the well documented effects of nutritional deficiencies and microbial contamination must also be considered as contributing factors to the suppressed immune status of tumor-bearing animals. The work of Jose and Good (1973) has revealed that mice maintained on diets deficient in essential amino acids exhibit markedly suppressed humoral and cellular responses. Similarly, chronic infection with lactic dehydrogenase virus and other nononcogenic viruses has also been shown to induce immunosuppression in tumor-bearing animals (Iorio et al., 1974; Riley, 1974).

In summary, the ability to manifest normal immune responsiveness is usually compromised in tumor-bearing hosts. A number of possible mechanisms have been cited for the tumor-induced innumosuppression which implicate different aspects of the host defense system. The relevance of this work is that the selection of a particular immunopotentiator to be used in tumor-immunotherapy may, in part, depend on whether the tumor-bearing host is suppressed or not and, if so, what the nature of the immunodeficiency is, i.e. does it reside at the T cell, B cell, or macrophage level?

C. Immunomodulators as Antitumor Agents

At present our ability to control tumor growth with nonspecific immunotherapy is modest but progress has been made toward defining conditions for optimal immunotherapy. Tumor size has constituted a critical limitation of therapy with most immunomodulators Mathe et al., 1969; Zbar et al., 1972). In general, larger tumors are less suscepti-

ble to immunotherapy. Direct contact between the tumor cells and the immunomodulator favors successful immunotherapy (Likhite, 1974; et al., 1976) yet even when contact between immunomodulators and tumor cells is assured, some tumors have resisted treatment (Sparks et al., 1974). The ability of the host to develop an immune response to tumor associated antigens also appears to contribute to successful immunotherapy. The more strongly immunogenic tumors have, in general, been more susceptable to treatment (Parr, 1972; Baldwin and Pimm, 1973). The immune status of the tumor-bearing host is another contributing factor which influences the efficacy of immunotherapy. The tumor growth retarding properties of many immunomodulators are less effective in immunosuppressed animals (Hanna et al., 1973; Scott, 1975). Furthermore, the administration of immunomodulators to animals with low levels of immune responsiveness has been reported to enhance tumor growth (Prehn, 1971; Sjogren et al., 1972). These findings indicate the possible dangers involved with immunotherapy and emphasize the necessity for delineating the mode of action and parameters of application before rational immunotherapeutic regimens can be designed. Of all the immunomodulators shown to inhibit growth of syngeneic tumors two immunomodulators were selected for study in these investigations: the synthetic polyanion pyran and the biological vaccine C. parvum.

1. Pyran copolymer

Pyran copolymer (4, 5-dicarboxy tetrahydro-6-methylene pyran-2-succinic acid, dianhydride polymer), is one of the most widely studied polyanion polymers. The copolymer was first synthesized by Butler (1960) by polymerization of divinyl ether (DIVE) and maleic anhydride (MA) in a 1:2 copolymer ratio and is therefore often referred to in the literature as DIVEMA. Yet, since the tetrahydropyran ring is an integral part of the polymer chain, the polymer became widely known as pyran copolymer and is indexed in the medical literature as such.

Pyran copolymer is a typical anhydride which under physiologic conditions becomes hydrolyzed to the corresponding carboxylic acid. The hydrolysate of pyran has been found to be fairly stable and not readily biodegradable, which may account for its prolonged activity (Ottenbrite and Regelson, 1977). Structure activity studies have indicated that the biological activity of pyran is dependent on a high density of free carboxylate groups situated along the backbone of the polymer. Pyran polymers with carboxylate functions bound by amidation of noncarboxylated polyethylenes are inactive at comparable doses (Breslow, 1976).

Synthetic polyanions are known to produce a wide spectrum of effects on immune reactivity. Pyran copolymer has been shown to induce the production of interferon (Merigan and Regelson, 1967; Merigan and Finkelstein, 1968), modify RES function (Munson et al., 1970), and to have immunoadjuvant (Brown et al., 1970; Baird and

Kaplan, 1975) and antiviral activity (Hirsch et al., 1972; Morahan et al., 1975; Morahan and Kaplan, 1976a). The antiviral activity of pyran has been studied in a number of systems. Pyran treatment protects mice from mortality following a lethal infection with both RNA or DNA cytopathic viruses (Came et al., 1969; Billiau et al., 1970; Morahan et al., 1972). Treatment of mice with pyran also inhibits tumor formation and delays mortality after infection with RNA or DNA tumor viruses (Hirsh et al., 1973; Regelson, 1967). The greatest protection is observed with prophylactic treatment of pyran. Therapeutic treatment often does not alter the course of disease particularly with rapidly fatal infections.

These results suggest that pyran may act very early during the viral infection, the drugs may need to be activated by the animal, and/or the drugs may act through modulation of host responses. Considerable effort has been directed toward defining the mechanism of antiviral action. Investigations have failed to demonstrate a correlation between interferon induction and antiviral activity (Regelson et al., 1970) and mounting experimental evidence suggests that activated macrophages are involved in the antiviral action of pyran (Billiau et al., 1971; Morahan et al., 1972; Morahan et al., 1977).

Pyran copolymer has also been shown to have immunoadjuvant activity in several animal model systems. Several investigators have demonstrated the ability of pyran to enhance the primary antibody response to the T-dependent antigen sheep erythrocytes (SRBC) in vivo (Braun et al., 1970; Morahan et al., 1972; Baird and Kaplan,

1975). In contrast, pyran was unable to enhance antibody formation to SRBC in thymectomized, irradiated, bone marrow reconstituted mice, or to a thymic independent antigen E. coli lipopolysaccharide (LPS) in normal mice (Baird and Kaplan, 1975). With regard to T-cell reactivity as measured by cell mediated cytotoxicity, pyran treatment prior to allogeneic tumor cell challenge caused a decrease in spleen cell cytolytic activity in addition to delaying the time of the peak response (Baird and Kaplan, 1975). A single intravenous injection of pyran has also been shown to significantly depress the in vitro blastogenic response of spleen cells to PHA as measured by counts per minute of tritiated thymidine into DNA at various times 2-14 days after in vivo administration (Baird and Kaplan, 1975).

These results are consistent with a dual action of pyran on T-lymphocytes and macrophages in the modulation of the immune response. The enhancement of the antibody response to SRBC and the inability to obtain comparable enhancement of the antibody response to a T-independent antigen (LPS) or a T-dependent antigen (SRBC) in T-depleted mice is compatible with a direct action on thymus-derived lymphocytes. The inhibitory effect of pyran on cell-mediated cytotoxicity could be due to a direct action on T cells, or an indirect effect mediated by macrophages. The inhibitory effects of pyran on PHA blastogenesis may be macrophage mediated, as removal of glass adherent cells from the spleens of pyran treated mice restored the PHA response (Baird and Kaplan, 1977).

The antineoplastic activity of pyran has been well documented in various tumor lines including the Rauscher (Chirigos, 1970), L1210 (Mohr et al., 1976a), LSTRA (Mohr et al., 1975) and MBL-2 (Mohr et al., 1976b) leukemias as well as the Madison 109 carcinoma (Marks et al., 1977), B-16 melanoma (Hirch et al., 1973), a methyl cholanthrene induced fibrosarcoma (Morahan and Kaplan, 1976a) and the Lewis lung carcinoma (Morahan et al., 1974; Snodgrass et al., 1975). Studies by Marks et al. (1977) with the M109 tumor indicate that daily systemic i.p. treatment with pyran (50 mg/kg) initiated 24 hours after tumor inoculation caused a slight reduction in tumor volume and produced an average increased life span (ILS) of 53% (range 33% - 72% in 12 experiments). Daily i.p. treatment with pyran (50 mg/kg) on days 1-8 after tumor inoculation has also been shown to inhibit the growth of the LLC and increase the mean survival time (MST) from 31.9 days to 50.4 days (Snodgrass et al., 1975). Intralesional treatment with pyran (50 mg/kg) on days 3, 5 and 7 proved to be more efficacious against the LLC. Two mice of 15 remained tumor free for 60 days after tumor inoculation and growth was markedly retarded in the others (Morahan and Kaplan, 1977).

Experiments designed to determine the ability of pyran to synergize with conventional modes of therapy have yielded divergent results. Surgical excision of the primary LLC tumor on day 14 combined with i.p. pyran treatment twice a week thereafter resulted in a significant number of long-term survivors ($>$100 days) which showed no signs of pulmonary metastasis. Similar experiments utilizing CTX as a cyto-

reductive agent failed to prolong the MST over that observed with CTX alone (Dr. Page S. Morahan, Dept. of Microbiology, MCV; unpublished observations).

The relative success of pyran against the M109 and LLC has to be qualified, however. The treatment regimens utilized in these experiments do not offer a realistic approach to the management of cancer with immunomodulators, as the tumor burden of the mice at the start of systemic or intralesional treatment was below the point of detectability (Snodgrass et al., 1975). Moreover, the metastatic ability of the LLC tumor line utilized in the combined modality studies has to be questioned as only 80% of the primary tumors had metastasized by day 14, as evidenced by a 20% survival rate in the surgery controls.

Paradoxically, certain virally induced tumors are stimulated by pyran (Gazdar et al., 1972; Schuller et al., 1975). This stimulation may be related to the ability of pyran to induce hepatosplenomegaly or splenomegaly in normal mice. Pyran has also been shown to cause accelerated development of benzo (a)-pyrene induced skin tumors in mice (Kripke and Borsas, 1974) and this raises the important point that immunomodulators can often inhibit or enhance tumor growth depending on a variety of factors including the route of drug administration (Schuller et at., 1975) and immune status of the tumor-bearing host (Prehn, 1971; Sjogren et al., 1972).

The antitumor activity of pyran is apparently not due to direct cytotoxicity. Studies of in vitro cytotoxicity have shown that greater than 1 mg/ml of pyran is required to destroy 50% of either tumor cells or normal cells, in contrast to nitrogen mustard which

at concentrations of 9.5 ug/ml destroyed 50% of the cells (Morahan et al., 1974). Several lines of evidence suggest that the mechanism of antitumor activity is mediated by macrophages. When several preparations of pyran and polyacrylic acid were tested for antitumor activity against the LLC, only those preparations which activated macrophages had antitumor activity (Morahan and Kaplan, 1976a). Moreover, peritoneal macrophages harvested from mice treated with pyran (50 mg/kg) intraperitoneally were cytotoxic for LLC tumor cells in vitro, whereas normal macrophages were without effect (Kaplan et al., 1974; Regelson et al., 1974). Pyran activated peritoneal macrophages mixed with LLC tumor cells in vitro and transplanted into syngeneic recipients has also been shown to inhibit tumor growth in vivo (Kaplan and Morahan, 1976). Morphologically, Snodgrass et al (1975) have noted an increased infiltration of histiocytes into the primary tumor bed in pyran (i.p.) treated mice. Although these data indicate that pyran activated macrophages have antineoplastic activity, proof is still needed that these cells are pyran's major effector cell in vivo.

Both the biologic activity and toxicity of pyran has been shown to be closely related to its molecular weight (Regelson et al., 1975), although this relationship does not appear to be a linear one. Most of the experimental research with pyran has been done with the polydisperse, broad molecular weight range polymer Hercules XA 124-177 which has an average molecular weight of 30,000 daltons. This compound has been reported to cause sensitization to endotoxin, inhibition of microsomal enzymes and hepatosplenomegaly in animal models

(Ottenbrite et al., 1978). Clinically, pyran was found to induce thrombocytopenia, fever, hypotension, seizures, and complete loss of vision (Regelson et al, 1977). Other toxic side effects included facial edema, onset of angina with myocardial infarction, confusion and somnolence.

In an effort to improve the therapeutic index of pyran, Dr. David Breslow (Hercules Inc., Wilmington, Del.) has prepared pyran at low temperatures in acetone using a free-radical initiator and tetrahydrofuran as a chain-transfer agent (Breslow, 1976). This method has yielded controlled molecular weight polymers with narrow polydispersity which, at the request of Hercules Inc., have been renamed maleic vinyl ether (MVE). Recent studies by Morahan et al., (1978) have shown that the lower molecular weight MVE fractions possess lower toxicity as evidenced by higher LD_{50}'s as well as decreased abilities to sensitize to endotoxin, inhibit microsomal enzymes or induce hepatosplenomegaly. The antitumor activity of two of these fractions, MVE·2 (M.W. 15,500) and MVE·3 (M.W. 21,300) were investigated in the course of our work.

2. Corynebacterium parvum

A number of anaerobic coryneforms have been shown to possess antitumor activity (Halpern et al., 1966; Woodruff and Boak, 1966), yet until recently, the taxonomic classification of these organisms has been confusing. In 1972 Johnson and Cummins attempted to clarify the taxonomy of the anaerobic coryneforms using serology, cell wall

composition and DNA homology. Their conclusions were that they should be called Propionibacteria, since they produce propionic acid by fermentation, and should be divided into three groups; P. acnes, P. granulosum and P. avidum. Studies by O'Neill et al. (1973) found antitumor activity among representative organisms from all three groups, with no single species showing outright superiority. According to this scheme, the Burroughs Wellcome Corynebacterium parvum, utilized in the present studies is classified as P. acnes. However, the original terminology of C. parvum has been retained by most investigators and will be referred to as such in these studies.

C. parvum, in the form of heat-killed or formalin-killed vaccines, produces a wide spectrum of effects on immune reactivity. Pretreatment of mice with C. parvum has protected them against subsequent infection with a variety of pathogens. The effects of C. parvum against Staphylococcus aureus, Bordetella pertussis, Salmonella enteritidis and Listeria monocytogenes have been evident both from prolonged survival or permanent protection against infection and from decreased bacterial counts in the liver and spleen (Adlam et al., 1972; Ruitenberg and van Noorle Jansen, 1975). C. parvum has also been shown to protect against protozoal infections. An increased resistance to malaria was caused by C. parvum injected intravenously 6-19 days before intravenous injection of Plasmodium berghei sporozoites (Nussenzweig, 1967). A similar protection against P. vinckei, P. chabaudi, and Toxoplasma gondii has been reported (Clark et al., 1977; Swartzberg et al., 1975). The antiviral activity of C. parvum has

been demonstrated with encephalomyocarditis virus. Intraperitoneal and intravenous inoculations of encephalomyocarditis virus caused the death of 95% of normal Swiss mice, but less than 50% of those treated intraperitoneally with C. parvum (0.5 mg) two to seven days earlier (Cerutti, 1975).

A macrophage-mediated resistance to infection has been implicated by several investigators. Protection against S. enteritidis did not correlate with any augmentation of specific anti-salmonella immunity (Collins and Scott, 1974), and protection against L. monocytogenes still occurred in the athymic nude mouse (Ruitenberg and van Noorle Jansen, 1975). Moreover, Swartzberg et al. (1975) have reported that peritoneal macrophages from C. parvum pretreated mice infected with T. gondii were capable of killing Toxoplasma in vitro. Similarly, peritoneal exudates of C. parvum pretreated mice infected with encephalomyocarditis virus contained a factor which inhibited multiplication of the virus in vitro (Cerutti, 1975). The inhibitor was resistant to heating at 56°C for 30 minutes, but did not require the integrity of cell-protein synthesis, a characteristic of the biological activity of interferon. Moreover, C. parvum, in contrast to pyran, was incapable of inducing interferon in an in vitro model (Cerutti, 1975).

C. parvum has also been shown to modify immunologic responses to unrelated antigens. Systemic pretreatment of mice with C. parvum has been shown to amplify the antibody response to the thymus-dependent antigens SRBC (Biozzi et al., 1968) and keyhole limpet

hemocyanin (Wiener and Bandieri, 1975) and to the thymus-independent antigens dinitrophenol hapten coupled to a levan carrier (del Guercio, 1972) and pneumococcal polysaccharide SIII (James et al., 1974a). Howard et al., (1973) have reported that the predomonantly T-cell dependent IgG response to SRBC in mice was amplified by C. parvum in T-cell deprived mice, which suggests that C. parvum may be operating as a T-cell bypass mechanism, possibly through direct stimulation of B cells. Moreover, Zola (1975) has demonstrated that C. parvum was mitogenic for B lymphocytes in vitro.

Conversely, C. parvum treatment has been associated with depression of cell-mediated immune responses to unrelated antigens. Systemic pretreatment with C. parvum has been shown to markedly depress the DTH response to picryl chloride (Asherson and Allwood, 1971) and SRBC (Scott, 1974a), prolong the survival of skin allografts (Castro, 1974) and protect F_1 recipient mice against the lethal effects of graft versus host resulting from an injection of parental spleen cells (Biozzi et al., 1965). In vitro studies of spleen cells from C. parvum treated mice have shown a depressed responsiveness in a mixed lymphocyte reaction or to the T-cell mitogen PHA (Scott, 1972).

The activated macrophage has been alluded to by several investigators as the cell type instrumental in C. parvum mediated alterations in immune responsiveness. Wiener (1975) suggested that the stimulatory effects of C. parvum on antibody production are most probably mediated by activated macrophages, as macrophages from C. parvum treated mice were more effective than normal macrophages at promoting

an *in vitro* primary response to SRBC. Watson and Sljivic (1976) confirmed these data and further demonstrated that antibody responses to macrophage independent antigens were not affected by *C. parvum*. Moreover, Wiener and Bandieri (1975) have shown that *C. parvum* activated macrophages retain large amounts of antigens on their surface and suggest that their intensified presentation of antigen to lymphocytes may be a causal factor in the adjuvant activity of *C. parvum*. Scott (1974b) attempted to analyze the mechanism underlying the depressed cell-mediated reactivity and has shown that the DTH depression did not occur in splenectomized mice and removal of macrophages from *C. parvum* stimulated spleen cells completely restored PHA reactivity.

The efficacy of *C. parvum* in animal tumor models has been highly variable. As with pyran, this variability may be related to the immunogenicity of the tumor under study (Smith and Scott, 1972), the size of the tumor mass at the start of therapy (Fisher *et al.*, 1970; Scott, 1975a), the immune competence of the tumor bearing host (Scott, 1975b) and the route of *C. parvum* administration (Scott, 1974c).

Several laboratories have examined the antitumor activity of *C. parvum* against the LLC. Oral administration of *C. parvum* has been shown to be ineffective against the LLC. Conflicting results have been reported for i.p. and i.v. *C. parvum* treatment against the LLC. Sadler and Castro (1976) have reported that C. parvum (0.4 mg) administered i.v. or i.p. 7 days after tumor inoculation resulted in significant reductions in the growth of the primary tumor and the number of metastatic nodules observed on day 21, yet no life span data were

presented. In contrast, Morahan and Kaplan (1976a) have reported that systemic i.p. or i.v. administration of C. parvum was ineffective in retarding the growth of the LLC or extending the MST of the tumor-bearing mice. Direct intralesional treatment with C. parvum (70 mg/kg) on day 3 post tumor inoculation has produced complete regressions in 17% of mice bearing the LLC, yet the remaining 83% displayed an accelerated tumor growth rate (Morahan and Kaplan, 1977). These data indicate some of the dangers involved with C. parvum therapy and suggest that there may be a balance between the beneficial antitumor effects of C. parvum and its immunosuppressive effects.

In combination with surgery, Sadler and Castro (1976) have reported that single i.v. or i.p., but not s.c. injections of C. parvum (0.4 mg) within 4 days before surgery reduced the incidence of metastatic nodules in the LLC tumor model. Yet, the significance of these results must be questioned as again no life span data were presented.

Similar to pyran, the antitumor activity of C. parvum is apparently not due to direct cytotoxicity. C. parvum added to mouse sarcoma cells in vitro did not inhibit their DNA synthesis or alter their ability to form colonies in vitro (Milas and Scott, 1978). The results of numerous investigations, however, are compatible with a macrophage mediated antitumor response. Scott (1974b) has demonstraed that peritoneal macrophages from mice treated with C. parvum inhibited the in vitro growth and DNA synthesis of syngeneic mastocytoma cells, and a direct cytotoxic effect of C. parvum activated macrophages on LLC cells has been described by Morahan and Kaplan (1976a). Non-

adherent peritoneal cells from C. parvum stimulated mice did not inhibit tumor cell growth in vitro and procedures for removing lymphocytes from peritoneal cells such as extensive washing, trypsinization or irradiation did not impair the antitumor activity of the peritoneal cells (Scott, 1974b). Similarly, the cytotoxic activity of C. parvum treated spleen cells was found to be unaffected by treatment with anti-theta-serum and complement, whereas macrophage removal using rayon adherence olumns or iron magnetic techniques almost entirely abolished the response (Kirchner et al., 1975).

The involvement of activated macrophages in vivo is implied by the findings of McBride et al. (1975). They demonstrated that gold salts, which inhibit macrophage lysosomal enzyme activity, suppressed the in vivo protective action of C. parvum against murine fibrosarcoma cells. Moreover, Morahan and Kaplan (1977) have shown that the addition of peritoneal macrophages from C. parvum stimulted mice to LLC cells in vitro and transplanted into syngeneic recipients resulted in suppression of tumor growth in vivo. Morphologically, Milas et al. (1974b) have noted that mouse tumors regressing after C. parvum treatment were heavily infiltrated with macrophages. Again, as with pyran, these data indicate that C. parvum activated macrophages have antineoplastic activity, yet direct evidence is still needed that macrophages are C. parvum's major effector cell in vivo.

D. Radiation and The Immune Response

The efficacy of radiotherapy in cancer treatment has been reasonably well defined since its first therapeutic application

against a basal cell epithelioma in 1899 (Kaplan, H. S., 1977). Yet, radiotherapy like surgery and chemotherapy has been shown to be immunosuppressive which could constitute a critical limitation to the combined use of radiotherapy with immunomodulators in the treatment of cancer.

The suppressive effects of radiation on the humoral antibody response have been recognized for many years (Hektoen, 1915). Dixon et al. (1952) demonstrated the dose dependent nature of this suppression by irradiating rabbits two days prior to the injection of bovine gamma-globulin. A slight inhibition of serum antibody levels was observed with 75 rads (r), whereas 200r was almost totally immunosuppressive. Cellular immune reactions have also been shown to be inhibited as evidenced by prolongation of allogeneic graft survival (Tyan and Cole, 1963), and suppression of DTH (Uhr and Scharff, 1960) and graft versus host reactions (Sprent et al., 1974). Yet, perhaps the best illustrations of the suppressive effects of ionizing radiation have been demonstrated in properly controlled studies showing a decreased resistance to infection with specific pathogens. An increased susceptibility to viral infection has been frequently observed in irradiated mice with many types of viral diseases including influenza (Smorodintsev, 1957), encephalomyelitis (Talmage, 1955) and hepatitis (Smith, 1963). Studies with fungal (Troitsky, 1962), rickettsial (Stoner et al., 1965) and bacterial pathogens (Schechmeister et al., 1952) have documented similar findings. Collectively, these studies amply demonstrate the immunosuppressive nature of ionizing radiation.

However, closer examination of the literature reveals that radiation has divergent effects on the immune response. Depending on a number of variables, including the cell types involved in the immune response and the time of radiation relative to antigen injection, immune responsiveness can be suppressed, unchanged or augmented.

1. Radiation and macrophage activity

It is generally considered that macrophages are quite resistant to radiation. Direct histologic studies have not shown evidence of damage (Brecher et al., 1948) and macrophages in irradiated lymphoid tissues have been noted to be very active in phagocytizing the debris of dead cells (Smith et al, 1967). The migratory activity of macrophages has also been shown to be quite radioresistant (Muramatsu et al., 1966) although Gradeburg et al. (1975) have reported that the capacity of phagocytes to replicate is as radiosensitive as any other cell population. The in vivo ability of macrophages to phagocytize and clear RE particles from the bloodstream (Geiger and Gallily, 1974) and the in vitro capacity of mouse peritoneal cells to engulf SRBC (Perkins et al., 1966) has also been shown to be unaffected by doses of irradiation up to 10,000r.

Analysis of various biochemical parameters however, suggests that radiation may enhance macrophage activity. Geiger and Gallily (1974) have reported that DNA synthesis and choline uptake in irradiated macrophages were slightly higher and RNA synthesis was 6 times higher than in nonirradiated controls. Macrophages irradiated in vivo also exhi-

bited an enhanced capacity to release lymphocyte activating factors (Geiger et al., 1973), a measure of macrophage activation (Gery et al., 1972). Further evidence of increased activation is derived from studies which have demonstrated that levels of lysosomal enzymes such as acid phosphatase, cathepsin and beta-glucuronidase were elevated in macrophages derived from heavily irradiated animals (Schmidke and Dixon, 1973).

2. Radiation and humoral immunity

As previously mentioned, radiation has a pronounced detrimental effect on antibody formation when it is given before antigen. However, numerous investigators using various antigens in rabbits (Keuning et al., 1963), guinea pigs (Vlahovic and Stankovic, 1961) and mice (Hoffstein and Dixon, 1974) have confirmed the initial findings of Taliaferro and Taliaferro (1954) that radiation given after antigenic challenge can result in enhancement of antibody production. The degree of enhancement appears to be dependent on the antigen, the time interval between antigen challenge and radiation, and the dose of radiation.

Studies by Schmidke and Dixon (1973) have indicated that the increased responsiveness in plaque forming ability induced by radiation was not only dependent on the priming dose of antigen but also the time interval between SRBC challenge and irradiation. Whole body irradiation with 440r at times other than 4 days after SRBC injection (i.e. 3, 5 or 6 days) did not result in enhancement, but in fact suppressed the PFC response in vitro. Similar findings have been shown after

local irradiation with 920r of murine lymph nodes draining the site of antigen injection. Irradiation 3 or 4 days after antigen challenge resulted in augmented levels of serum antibody, whereas irradiation on days 1 or 2 resulted in immunosuppression (Eltringham and Weissman, 1977). Although the proper comparisons could not be made in the above studies, the work of Fitch et al. (1956) suggests that in situations where irradiation after antigenic challenge results in immunosuppression, the degree of immunosuppression may not be as great as when irradiation precedes antigen. The dose of radiation also appears to be a critical limitation as 175r and 125r whole body exposure enhanced primary antihemocyanin antibody responses in SWR/J and C3H mice respectively, whereas 300r and 150r were immunosuppressive (Hoffstein and Dixon, 1974).

3. Radiation and cell mediated immunity

Generally, the effects of radiation on cell-mediated immune responses have paralleled those observed for humoral immunity. Radiation has been reported to suppress DTH reactions when radiation has been given before antigen sensitization (Tripathy and Mackaness, 1969), whereas enhancement of delayed hypersensitivity has been shown when rats where immunized with bovine serum albumin 10 days before receiving 800r of whole body irradiation (Visakorpi, 1972). Models for measuring the effects of radiation on cellular immunity utilizing Besnoitia jellisoni have shown that 600r administered up to 22 days before infection inhibited the primary immune response as measured by the

infected animal's survival time whereas 1,800r of fractionated whole body exposure were required to inhibit an established cellular immunity to this pathogen (Frenkel and Wilson, 1972).

4. Proposed mechanisms for radiation induced immunologic enhancement

These results suggest that cell-mediated immunity, like humoral immunity, can be suppressed, unchanged, or augmented by irradiation. Yet, the *in vivo* expression of immune responses is dependent upon the interaction of various subpopulations of T and B cells as well as the participation of other nonlymphoid cells. Direct studies attempting to assess the relative radiosensitivity of lymphocyte subpopulations and ascribe potential mechanisms for the above findings have revealed that the sensitivity of lymphocytes is not only a function of the dose of irradiation, but is also dependent on the lymphocyte subpopulation(s) under investigation, their metabolic rate, and their state of differentiation.

A variety of approaches have been utilized to demonstrate that B cells are more radiosensitive than T cells. Anderson *et al* (1974) have reported that irradiated B cells survived considerably less well than irradiated T cells when placed in tissue culture and followed for viable counts. Similarly, after whole body irradiation, a much greater reduction in numbers of splenic B cells than T cells was observed when cell suspensions were examined by immunofluorescence (Nossal and Pike, 1973).

Subpopulations of T and B cells have also been shown to differ in radiosensitivity. The differential responsiveness of T cells to PHA and concanavalin A (Con A) has been employed to define two subpopulations of T cells, originally termed T_1 and T_2 (Greaves et al., 1974). T_2 cells are preferentially responsive to Con A and T_1 cells equally response to PHA and Con A. The work of Stobo and Paul (1973) utilizing tritiated thymidine uptake as a measure of T cell responsiveness indicated that T_1 cells were more sensitive to immunosuppression by irradiation than the T_2 population.. Of perhaps more relevance to this thesis project are the findings of Tada et al. (1971) with suppressor T cells. Their work indicated that suppressor T cells were particularly sensitive to low doses of irradiation (25-200r) in relation to other T cell subpopulations, and that low doses of irradiation resulted in augmented levels of immune responsiveness to T-dependent antigens. B cell heterogeneity with regards to radiosensitivity has also been implicated by several investigators using cultured B lymphoid cell lines (Drewinko et al., 1972; Han et al., 1974).

The apparent differences in the radiosensitivity of resting lymphocyte subpopulations have not been ascribed to biochemical dissimilarities. Ikada (1969) and Altman and Gerber (1970) have studied the biochemical consequences of irradiated lymphocytes in detail and both groups concluded that the alterations described in their respective manuscripts appeared to be a secondary phenomena occurring subsequently to some undefined primary event. More recent investigations by

Anderson and Warner (1976) have suggested that the lymphocyte heterogeneity with regards to irradiation was related to subtle differences in the lymphocyte plasma membranes.

The metabolic rate of lymphocytes has also been shown to influence radiosensitivity in that the process of lymphocyte stimulation has been characterized by a descreased radiosensitivity of the responding population. Vaughan-Smith and Ling (1974), using tritiated thymidine to assess the response of porcine lymphocytes to Con A, found a roughly exponential decrease in the responsiveness of T cells as the time was increased between irradiation and stimulation. After a 48-hour separation, the response was about 1% of the non-irradiated control culture. However, when Con A was given before irradiation, there was a marked decrease in lymphocyte radiosensitivity as reflected by a 150r shoulder on the dose survival curve. Similar findings have been reported for murine lymphocytes using antigens or another T cell mitogen, PHA (Conrad, 1969; Sprent et al., 1974).

It has been postulated by several investigators that stimulated lymphocytes are more radioresistant because of increased repair mechanisms. Prempree and Merz (1969) documented an increased ability of PHA stimulated murine lymphocytes to repair chromosome breaks as compared to nonstimulated controls. Similar data has been reported for human lymphoid cell lines (Drewinko and Humphrey, 1971) Stefanescu et al. (1972) confirmed that above findings and noted that the tran-

sition from the resting to the proliferating state was accompanied by the development of break-repair competence. Further evidence for this theory has been derived from the studies of Lindahl and Edelman (1968) which noted that PHA stimulated peripheral blood lymphocytes contained markedly elevated DNA polymerase and ligase activity, two of the enzymes implicated in repair of DNA breaks (Regan et al., 1971).

The implications from the above studies are that radiation not only reduces the tumor burden, but under specific circumstances, may enhance immune responsiveness. Although all the reports cited in this thesis deal with nontumor antigens, the possibility exists that radiation can augment immune responsiveness toward tumor antigens as well. The high radiosensitivity of B cells and suppressor T cells, implicated as detrimental aspects of antitumor immune responses, coupled with the radioresistance of macrophages and proliferative T cells suggests that antitumor immunity could possibly be selectively preserved or augmented after exposure to radiation. Irradiation has also been shown to produce changes in the antigenic structure of tissues (Alekeoff, 1970) which could possibly enhance antitumor immunity by an alternative mode, i.e. increasing tumor cell antigenicity.

II. MATERIALS AND METHODS

A. Experimental Animals

C57BL/6 and BDF_1 (C57BL/6 x DBA/2) mice were used for experimentation in the LLC studies whereas Balb/c and CDF_1 (Balb/c x DBA/2) mice were utilized in studies involving the M109 tumor system. These mice were obtained through the courtesy of the National Cancer Institute from Simonsen Laboratories (Gilroy, Calif.).

Upon arrival, the mice were randomized and acclimated for at least one week prior to experimental use. The mice were maintained on Purina Laboratory Chow and tap water ad libitum in temperature controlled quarters with a 12 hr light - 12 hr dark cycle.

Routinely six to eight week old male or female mice, weighing from 18 to 25 gm, were used for experimentation. In studies designed to measure the DTH response to SRBC, 10 week old mice were used to optimize the DTH response. Within a single experiment however, all mice were matched for age and sex.

Mouse sera were periodically tested for the presence of lactic dehydrogenase virus by Microbiological Associates (Bethesda, Md.) and found to be negative.

B. Enumeration of Cells

Concentrations of spleen cells, red blood cells, white blood cells and trypsinized tumor cells were determined on a Coulter Counter model ZBI (Coulter Electronics; Hialeah, Fla.). Prior to use with a

specific cell type, the Coulter Counter was calibrated by modulating the amperage, aperature and threshold settings according to the specifications set forth in the instruction manual. To insure consistency in counting, a known concentration of 5 micron Latex particles (Dow Chemical Co.; Indianapolis, Ind.) was counted each time as an internal standard. When inconsistencies in counting arose due to brief periods of electrical interference, cell samples were maintained at 4°C until the interference subsided.

C. Determination of Cell Viability

Cell viability was determined by the trypan blue exclusion test (Merchant et al., 1964). To approximately $1x10^5$ cells in a volume of 0.5 ml was added 0.1 ml of 0.5% trypan blue (Gibco; Grand Island, NY) in a 12x75 mm test tube. The resulting solution was mixed and allowed to stand at room temperature for 5 minutes before counting on a hemocytometer (American Optical Co.; Buffalo, NY). The percentage of viable cells excluding the dye was determined by counting approximately 100 cells with the aid of a Nikon binocular light microscope (Nippon Kogaku K.K., Japan).

D. Maintenance of Tumor Lines

1. Lewis lung carcinoma

The LLC was maintained by inoculation of $1x10^6$ LLC cells into the gluteus maximus of C57BL/6 mice. At biweekly intervals the tumor bearing hosts were sacrificed by cervical dislocation and LLC cell suspensions prepared by trypsinizing sterile non-necrotic tumor sections (volume of sections approximately 1 cc) for 45 min.

at room temperature in 0.25% sterile trypsin (Difco., Detroit, Mich.) in EMEM (Gibco; Grand Island, NY) containing Earle's balanced salt solution and 100 u/ml penicillin and 100 ug/ml streptomycin. Sterile fetal calf serum (Gibco; Grand Island, NY) at a concentration of 6 ml/100 ml of medium was added after the 45 minute trypsinization period to stop enzymatic activity. The tumor cells were washed and centrifuged at 200xg for 10 minutes in an International CRU-5000 centrifuge (Damon, IEC Division; Needham, Mass.), resuspended in EMEM and counted on a Coulter Counter model ZBI (Coulter Electronics; Hialeah, Fla.). Tumor cell viability, as determined by trypan blue exclusion, was normally better than 98%.

Bacterial contamination was monitored by incubating $5x10^5$ cells with 2 ml of 50% Brain Heart Infusion (BHI) broth (Difco; Detroit, Mich.) for 72 hrs at 37°C, 5% CO_2, 95% air. Bacterial contamination was detected by a visual increase in the turbidity of the BHI broth. Viral contamination was monitored by periodically sending serum samples from the tumor bearing hosts to Microbiological Associates (Bethesda, Md.).

2. Madison 109 lung carcinoma

The M109 was maintained in Balb/c mice by intramuscular inoculation of $5x10^5$ trypsinized tumor cells at biweekly intervals. The tumor cell suspensions were prepared in a slightly different fashion from the procedures used with the LLC in that non-necrotic M109 tumor sections underwent a series of five 10 minute trypsinization (0.25%) periods. After each 10 minute period, the tumor cell suspen-

sions were decanted and exposed to 6% sterile fetal calf serum (Gibco., Grand Island, NY) in EMEM. Cell number and viability were determined for each fraction as previously described. Fractions which yielded tumor cells of greater than 85% viability were then pooled and used for experimentation. This procedure was utilized in order to maximize the yield and viability of the tumor cell preparation, as the M109 appeared to be more sensitive to the lethal actions of trypsin than the LLC. Bacterial contamination was monitored by incubating $5x10^5$ M109 cells with 2 ml of BHI broth for 72 hrs at 37°C, 5% CO_2, 95% air.

3. P815 mastocytoma

The P815 mastocytoma, utilized in the M109 concomitant immunity studies, was maintained in DBA/2 mice by serial i.p. passage of 10^4 cells every 10 days. The ascitic tumor cells were harvested aseptically in RPMI 1640 medium, washed 2x by centrifugation at 200xg, and enumerated on a Coulter Counter. Viability and bacterial contamination were monitored as before.

E. Flow Microfluorometric Analysis of Cells Residing in the Primary Tumor Bed

To determine the types and relative number of cells residing in the LLC primary tumor bed trypsinized suspensions of "tumor cells" were analyzed in a Coulter Electronics TPS-1 flow microfluoremeter (Hialeah, Fla.). The procedure for preparing the tumor cell suspension for flow microfluorometric analysis was according to the method of Crissman and Steinkamp (1973). Tumor cell fragments obtained

from 14 day old i.m. LLC tumors were trypsinized, washed in calcium free PBS and fixed on ice in 70% ethanol for 30 min. The cell suspension was centrifuged at 2000xg for 5 minutes at 4°C followed by digestion in RNase (10 ug/ml; Sigma, lot no. R5875) at 37°C for 30 minutes. After incubation the RNase was hydrolyzed in distilled water for 30 minutes at 37°C. The cells were subsequently washed twice with calcium free PBS and stained with a 6.9×10^{-5} M solution of propidium iodide (PI) monohydrate in 1.1% sodium citrate (Calbiochem, lot no. 537059) for 30 minutes at room temperature. The cells were then washed twice with calcium free PBS at 2000xg for 5 minutes and resuspended to a final concentration of 1×10^{6} cells/ml.

A single cell suspension of the stained sample was exposed in the flow chamber of the flow microfluoremeter to a focused argon ion laser beam (488 nm). A reddish-orange fluorescence resulting from the laser excitation of the PI-stained cells was collected and quantitated through a combination of appropriate mirrors, filters and photomultipliers. The resulting electrical pulses based on the excitation of the DNA-PI fluorescence in each cell were collected and stored in a pulse-height distribution analyzer. Histograms generated from analysis of 10,000 cells were displayed on the oscilloscope and simultaneously printed.

For comparative purposes, LLC cells grown in tissue culture (obtained courtesy of J.A. Munson, Dept. of Microbiology, MCV) were stained with PI and analyzed as above.

The relative DNA content of the stained cells was determined by interpretation of the DNA distribution histograms. The abscissa

of the histograms was divided into 127 channels of increasing linear value of DNA content while the ordinate represented relative cell number.

The flow microfluoremeter was also utilized to separate cell populations according to cell size. Non-stained "tumor" cells were suspended in calcium free PBS and introduced into the flow chamber as previously described. As the cells transversed a 75 um diameter orifice, an electronic signal was produced which was proportional to cell volume. The pattern displayed on the oscilloscope was used to select appropriate channels for separation and collection of cell populations of different volumes. The cells were subsequently centrifuged at 2000xg for 10 min, washed and resuspended in 10% fetal calf serum. Smears were fixed with methanol, stained with Giemsa Stain (Fischer Scientific Co.; Fair Lawn, N.J.) and observed under a Nikon binocular light microscope (Nippon Kogaku K.K., Japan).

F. Chromosome Analysis of Lewis Lung Cells

LLC chromosomal analysis was performed according to a modification of the method of Hungerford (1965). Approximately 10^7 trypsinized LLC cells were suspended in a 1% colcemid (Gibco; Grand Island, NY): saline solution and allowed to sit at room temperature for 3 hours. The cells were centrifuged at 200xg, resuspended in a 0.075 M KCl hypotonic solution and incubated at 37°C for 1 hour. Following incubation smears were fixed with Carnoy's fixative (acetic acid: methanol 1:3) and stained with Giemsa. The number of chromosomes per cell was enumerated at 100 x magnification employing a Zeiss binocular light microscope.

G. Employment of Footpad Tumors in Experimental Studies

For in vivo testing LLC footpad tumors were established in C57BL/6 or BDF_1 mice by inoculating $1x10^6$ trypsinized tumor cells into the left hind footpad in a volume of 0.02 ml utilizing a micro-syringe pipette (Hamilton Co.; Reno, Nev.). The M109 and P815 footpad tumors were established under similar conditions by inoculation of $5x10^5$ M109 cells into Balb/c and CDF_1 mice and $5x10^5$ P815 cells into CDF_1 mice. The footpad tumor models facilitated measurement of primary tumor size and provided flexibility as the primary tumors could be locally irradiated or excised by surgical amputation without problems of local tumor recurrence.

1. Measurement of primary tumor size

Primary tumor size was evaluated by measuring the tumor mass along two dimensions in mm with an engineer's micrometer (Combimike, Mitutoyo Mfg. Co.; Japan). The tumor volume (mm^3) was calculated by the method of Attia and Weiss (1966) in which the tumor volume $(V) = 0.4\ ab^2$, where a = longer axis and b = smaller axis.

2. Surgical excision of primary tumors

For studies on the metastatic LLC and M109, the tumor bearing mice and appropriate non tumor controls were anesthetized i.p. with 60 mg/kg pentobarbital (Diabutal, Diamond Laboratories; Des Moines, Iowa). The tumor bearing or control legs were then excised above the knee joint under aseptic conditions with a pair of 140 mm

scissors and cauterized using a Thermo Cautery Unit (Statham Inc.; Oxnard, Calif.). The incisions were closed with stainless steel suture clips (Clay Adams; Parsippany, N.J.) which were removed seven days after surgery.

With the LLC surgery was normally performed on days 13-15, a time at which 100% of the animals had lethal pulmonary metastasis. With the M109, studies were done in order to determine the metastatic rate of the tumor.

The mortality rate associated with this surgical procedure was normally less than 2%.

3. Local irradiation of primary tumors

Local irradiation of the primary LLC and M109 tumors was performed using a cobalt-60 (^{60}Co) teletherapy unit (Picker X-ray Corp.; Clevaland, Ohio). Prior to irradiation the mice were anesthetized i.p. with 60 mg/kg pentobarbital and the tumor bearing legs secured to a specifically designed lucite plate with micropore surgical tape (3M Co.; Saint Paul, Minn.). An 8 cm thick cerrobend doughnut was then placed on top of the lucite plate to reduce the whole body exposure to less than 1% of the tumor dose (personal communication, Dr. John Wilson; Radiation Biology Division, MCV).

To determine the actual exposure rate of the teletherapy unit, four one minute exposure readings on a Farmer Secondary Standard Dosemeter (Capintec Nuclear and Baldwin Instrument Co.; London, England) were taken at the start of each experiment. The dose rate,

and thus the total tumor dose, was calculated by the method of Johns and Cunningham (1971) in which the dose rate (rads/min) = average dosemeter reading x 3.81 x 0.96, where 3.81 is a chamber factor calibrated yearly for this particular unit by the National Bureau of Standards and 0.96 is the conversion factor from Roentgens to rads. The dose rate ranged between 185-160 rads/min during the course of experiments.

To insure that the mice were receiving a uniform exposure of local irradiation the lucite plate containing the test animals and the cerrobend doughnut were slowly rotated throughout the exposure period. A 1/4 inch lucite "build up" plate, located in the center of the cerrobend doughnut, was also employed to create a more homogeneous dose distribution throughout the tumor mass.

The mortality rate associated with this procedure was 0% at all times.

4. Assessment of concomitant immunity

For assessment of concomitant tumor immunity in the M109, Balb/c or CDF_1 mice were inoculated in the left hind footpad with $5x10^5$ M109 cells. Eleven days later these tumor bearing and normal mice were challenged in the right footpad with varying amounts of M109 tumor cells ranging from $1x10^4$ to $1x10^6$ M109 cells, or $1x10^5$ P815 tumor cells. Concomitant immunity was measured by the ability of mice immunized with a primary M109 tumor to suppress the growth of a secondary M109 challenge. The specificity of the concomitant immune response was evaluated by examining the ability of CDF_1 mice bearing a primary M109 tumor to suppress the growth of a P815 mastocytoma footpad tumor.

H. Quantification of Pulmonary Metastasis in Tumor Bearing Mice

The extent of pulmonary metastasis in LLC bearing animals was evaluated by enumerating the number of surface tumor nodules according to the method of Wexler (1966). The tumor bearing mice were sacrificed by chloroform and the trachea and chest cavity exposed by a midline chest incision. The trachea was transected well above the carina into which a #18 blunt needle and syringe, containing 2 ml of contrast medium, was fitted tightly with surgical suture. As the contrast medium was injected, the lungs became distended and stained deep black. The lungs were then dissected from the thoracic cage, rinsed in tap water to remove excell ink, and placed in Fekete's solution (Fekete, 1938) to bleach the tumor white and to preserve the lung. The extent of metastasis was quantitated by counting the number of surface tumor nodules under 2 1/2 x magnification. This procedure was adequate when the number of tumor nodules was less than 25, but became unsatisfactory with lungs heavily laden with tumor.

The contrast medium was prepared by mixing 85 ml of distilled water with 15 ml of India ink (Gunther Wagner; NYC, N.Y.). To facilitate uniform dispersion of the ink particles 0.5 ml of ammonia water was added to this solution.

I. Preparation of Drugs, Antigens and RE Particles

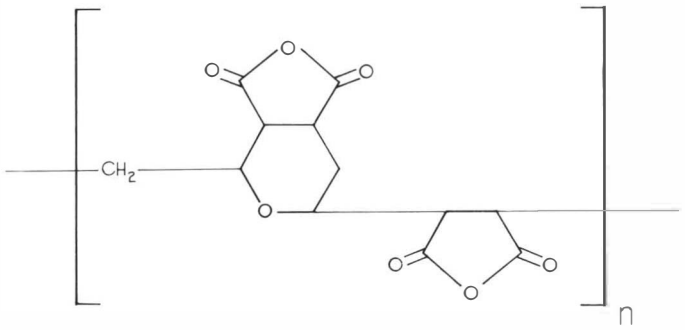
1. Maleic vinyl ether copolymers (MVE)

MVE·2 (MW 15,500) and MVE·3 (MW 21,300) which have the structure shown below, were kindly supplied by Dr. David Breslow of Hercules Research Center; Wilmington, Delaware. The drugs were dissolved in

Figure 1

Maleic vinyl ether (4, 5-dicarboxytetrahydro-6-methylene pryan-2-succinic acid, dianhydride polymer) was prepared at low temperatures in acetone using a free radical initiator and tetrahydrofuran as a chain-transfer agent, which yielded controlled molecular weight polymers with narrow polydispersity.

MALEIC **V**INYL **E**THER



physiologic saline (0.9%NaCl) at room temperature and adjusted to pH7.4 by the addition of IN NaoH. MVE injected i.p. or i.v. was administered in a volume of 0.1 ml/10g body weight whereas intratumor (i.t.) and intrapleural injections of MVE were delivered in 0.01 ml/10g body weight utilizing a microsyringe pipette (Hamilton Co.; Reno, Nevada). The MVE compounds were prepared on a weekly basis and stored at 4°C.

2. Corynebacterium parvum

C. parvum (Lot no. CA 380) was kindly provided by Dr. Richard L. Tuttle of Burroughs Wellcome Co. (Research Triangle Park, N.C.) as a formalin-killed suspension containing 7.0 mg dry weight of organisms per ml. The preparations were stored at 4°C until use. For i.v. administration the C. parvum suspensions were diluted with physiologic saline and injected in a volume of 0.1 ml/10g body weight. For intratumor injections the C. parvum suspensions were concentrated by centrifuging the preparations at 800xg and resuspending the pellets in lesser volumes of physiologic saline. Twenty microliters of these concentrated preparations were then delivered to the test animals using a microsyringe pipette.

3. Cyclophosphamide

Cyclophosphamide (NSC-26271) was supplied by the National Cancer Institute. The compound was dissolved in sterile saline within 30 minutes of use and administered i.v. in a volume of 0.1 ml/10g body weight.

4. Carrageenan

Carrageenan (Lot no. RE7228), used to induce an inflammatory response in LLC animals was obtained from Marine Colloids Inc. (Springfield, NJ) and prepared as a 1% solution in sterile saline.

5. Sheep erythrocytes

Sheep red blood cells, used to assess the immune status of LLC animals, were obtained from the MCV central animal facilities. The sheep blood was drawn sterily by venipuncture from the same sheep (#127) once per week. Fifty ml of freshly drawn blood were immediately mixed with 60 ml sterile Alsevers solution. The sheep blood was prepared for experimental use by centrifugation at 900xg for 10 minutes at 4°C. The buffy coat of lymphocytes at the interface of the SRBC and the Alsevers solution was removed by aspiration and the cells washed an additional two times with Alsevers solution by centrifugation at 900xg for 10 minutes at 4°C. The cell concentration was determined on a Coulter Counter and the suspension adjusted to a final concentration of $5x10^9$ SRBC/ml. Appropriate dilutions of SRBC for experimental use were done with Alsevers solution.

Preparation of sterile Alsevers solution was performed in the following manner. To each 1 liter solution 20.50g dextrose (Sigma Chemical Co.; St. Louis, Mo.), 8.00g sodium citrate dehydrate (Mallinckrodt; St. Louis, Mo.), 0.55g citrate acid monohydrate (Sigma) and 4.20g sodium chloride (J.T. Baker Chemical Co.; Phillipsburg, NJ) were dissolved in distilled water. The Alsevers solution was sterilized in a Castle autoclave (Wilmot Castle Co.; Rochester, NY) by autoclaving for 15 minutes.

6. ^{51}Chromium labeled sheep erythrocytes

^{51}Chromium labeled SRBC, used to evaluate the functional activity of the RES, were prepared by the methods of Greaves et al. (1969). The labeling procedure consisted of incubating 10 ml of $5x10^9$ SRBC/ml with one milliCurie of sodium ^{51}chromate (specific activity, 289-368 milliCurie/mg; New England Nuclear; Boston, Mass.) at 37°C for 30 minutes in a Dubnoff shaker bath (Precision Scientific Co.; Chicago, Ill.). The chromated erythrocytes were then repeatedly washed with equal volumes of Alsevers solution and centrifuged at 900xg until a 0.1 ml aliquot of the supernatant was no more than 500 cpms above background, as measured in a Beckman model 300 gamma counter (Beckman Instruments; Fullerton, Calif.). The cell suspension was then diluted with nonradioactive SRBC and Alsever's solution to yield a 10% solution (hematocrit value) with approximately 200,000 cpm per 0.1 ml. The chromated SRBC were stored at 4°C for up to one week. Prior to i.v. administration into BDF_1 mice the labeled SRBC were washed and centrifuged at 900xg and resuspended in sterile phosphate buffered saline.

7. Listeria monocytogenes

Listeria monocytogenes (strain 19303) used in host resistance studies with the LLC, was cultured in the following manner. To 3 ml of frozen Listeria stock obtained courtesy of Dr. H. J. Welshimer (Dept. of Microbiology, MCV) was added 250 ml of sterile BHI broth. The culture was incubated in a Dubnoff shaker bath at 37°C for approximately 6 hours or until the spectrophotometric absorbance value was between

0.40 and 0.45 at 610 nm (Varian Tectron model 635 spectrophotometer; Sunnyvale, Calif.). Previous studies from our laboratory have indicated that an absorbance value of 0.40 yields a concentration of $2.5 - 3.0 \times 10^8$ bacteria/ml (Ms. Ginny Sanders, personal communication). The incubation mixture was then aliquoted into 2 ml sterile vials and stored at -70°C (Kelvinator deep freeze series 100; Manitowoc, Wis.) until use.

To determine the actual titer of Listeria, plate counts were performed on the days of experimentation by serial 1:10 dilutions of the Listeria in sterile distilled water. Dilutions from 10^{-6} to 10^{-8} were plated out on tryptone glucose yeast agar plates (Difco; Detroit, Mich.) and incubated for 48 hours at 37°C (Forma Scientific CO_2 incubator model 3028; Marietta, Ohio). The number of colony forming units (CFU) were then enumerated with the aid of a darkfield Quebec colony counter (American Optical Co.; Buffalo, NY) and the titer calculated by multiplying the average number of colonies per plate by 10^6 to 10^8 respectively.

8. ^{125}I-Listeria monocytogenes

I^{125}-Listeria monocytogenes, used as a RE particle to evaluate the phagocytic activity of the fixed macrophage system, was radiolabeled as follows. To a culture of $2.5 - 3.0 \times 10^8$ Listeria/ml were added 750 uCi of ^{125}I iododeoxyuridine (specific activity $>$ 5Ci/mg, New England Nuclear; Boston, Mass.) and 12 ml of 5×10^{-4} M flourodeoxyuridine (Sigma Chemical Co.; St. Louis, Mo.). The flourodeoxyuridine was added to inhibit thymidylate synthetase (Heidelberger, 1965) and

thus allow for a greater incorporation of iododeoxyuridine into the DNA of the Listeria. The mixture was incubated for 60 minutes at 37°C followed by the addition of 1.25 ml formaldehyde (J.T. Baker Chemical Co.; Phillipsburg, N.J.) to inactivate the Listeria. The culture was incubated for an additional 30 minutes under the same conditions and then centrifuged at 1750xg for 30 minutes at room temperature. The pellet was resuspended in 125 ml EMEM, aliquoted into sterile injection vials and stored at -70°C. The resulting ^{125}I-labeled Listeria yielded approximately 200,000 cpm/0.1 ml.

J. LD_{50} Determinations

To obtain acute toxicity data on the five MVE fractions and C. parvum, groups of mice were inoculated i.v. at a constant rate with 5 doses of the respective compounds in 0.1 ml/10g body weight. The mice were observed for mortality for 14 days and the LD_{50} and slope determinations calculated by the method of Litchfield and Wilcoxon (1949).

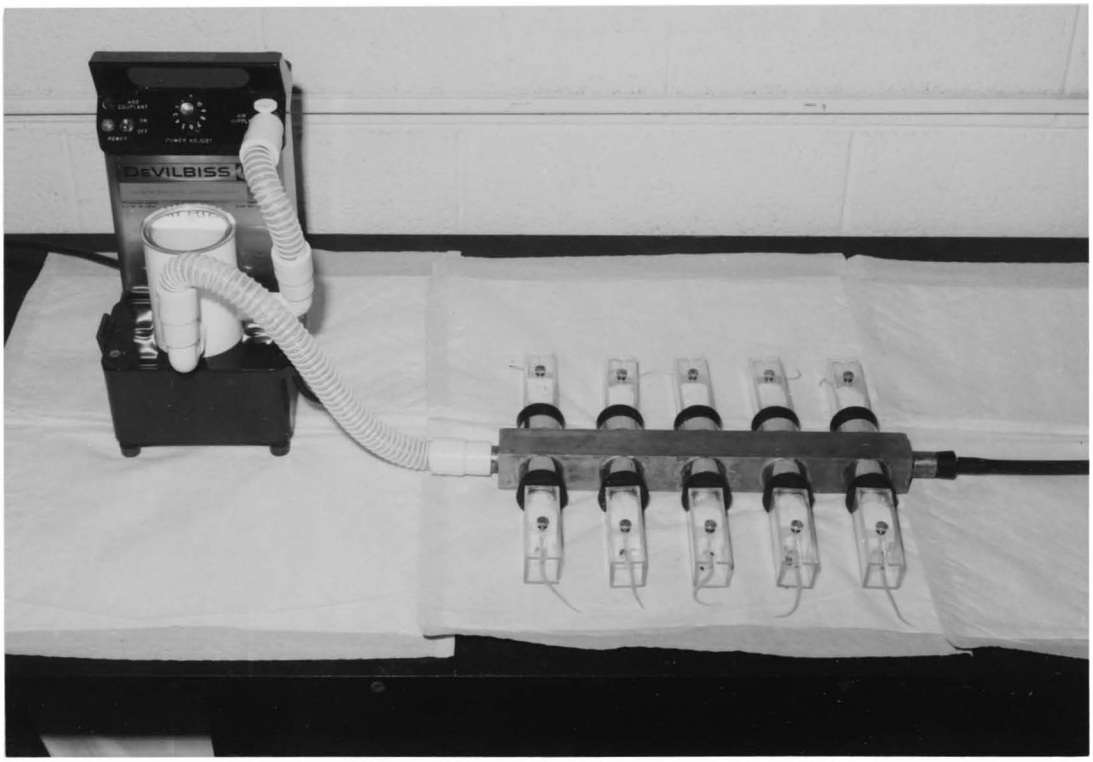
K. Inhalation Procedures

To increase the effective concentration of MVE-2 in the metastatic tumor bed, the noses and mouths of the test animals were exposed to an atomized mist of MVE-2 generated by a DeVilbiss model 65 nebulizer (Somerset, Pa.). As shown in Figure 2 the nebulizer unit consists of a transducer that generated vibrational energy which nebulized or atomized the MVE solution into 5 micron particles. A filtered air supply carried the

Figure 2

Inhalation apparatus

The nebulizer generated vibrational energy which atomized the MVE solution into 5 micron particles. A filtered air supply carried the aerosolized mist from the nebulizer chamber to an exposure chamber where the noses and mouths of test mice were exposed to the mist.


DEVILBISS

aerosolized mist from the nebulizer chamber to an exposure chamber where the noses and mouths of the restrained test animals were exposed to the mist (exposure chamber and mouse restrainers designed and constructed by Misters E. Talley and E. M. Dimen, Dept. of Pharmacology). The mist was vented through a carrier hose attached to an aspirator and discharged into a sink.

As the nebulizer unit has not yet been calibrated, the dosing regimens consisted of exposing each group of mice on a biweekly basis to 10 ml of physiologic saline or MVE-2 ranging in concentration from 0.1 mg/ml to 100 mg/ml. The exposure time was largely dependent upon the viscosity of the test solution but normally ranged from 10 to 15 minutes. Studies are now being conducted by Mr. Kimber White (Dept. of Pharmacology) to calibrate the inhalation procedure.

L. Assays for Immunologic Responsiveness

1. Hemolytic plaque assay

The Cunningham modification (Cummingham and Szenberg, 1968) of the Jerne plaque assay (Jerne and Nordin, 1963) was used to measure the primary IgM antibody response to SRBC in mice bearing the primary or metastatic LLC. Three days after tumor inoculation and every fourth day thereafter groups of tumor bearing and control mice were immunized with 4×10^8 SRBC by the intraperitoneal route. Four days after immunization the mice were sacrificed by cervical dislocation and their spleens removed, weighed and

placed in cold (4°C) RPMI-1640 (Gibco; Grand Island, N.Y.). Spleen cell suspensions were prepared by gently pressing the splenic contents through a 100 mesh stainless steel screen with a rubber-end plunger from a 10 c.c. syringe. The cells were washed twice at 200xg for 10 minutes at 4°C, counted on a Coulter Counter and adjusted to a constant cell concentration of $2x10^6$ cells/ml by the addition of RPMI-1640 medium. Cell viability was normally greater than 99%.

The spleen cell suspensions were added in volumes of 0.1, 0.05 and 0.025 ml to three separate wells of a Microtiter II test plate (Falcon Plastics; Oxnard, Calif.) placed on ice. To each well 0.01 ml guinea pig complement (Flow Labs; Rockville, Md.), 0.01 ml of $6x10^9$ SRBC/ml and enough medium were added to make a total volume of 0.15 ml. The contents of each well were mixed with a Pasteur pipette and applied to a two-sided chamber formed by joining two glass microscope slides (Scientific Products No. M6130; McGraw Park, Ill.) with double-sided tape (Scotch Brand No. 410; 3MCo.; St. Paul, Minn.). The two chambered slides were sealed with a warmed 1:1 mixture of petroleum jelly (Vaseline; Chesebrough Ponds, N.Y.) and parafin wax (Fulfwax; Fort Worth, Tex.) and incubated for 45 minutes at 37°C.

Studies by Harris et al. (1966) have shown that during the incubation period plasma cells synthesize and secrete antibodies, which along with complement, lyse surrounding SRBC. The lysed SRBC leave clear plaques with an antibody secreting plasma cell in the center of each plaque. Accordingly, IgM plaque forming cells (PFC) were enumerated by counting plaques with the aid of a colony counter

at 2 1/2 x magnification. All data were evaluated as PFC/10^6 nucleated spleen cells (specific activity) and total PFC/spleen.

2. Delayed type hypersensitivity assay

The DTH responsiveness of BDF_1 mice bearing the LLC was quantitated by modification of a radioisotope footpad assay originally developed by Paranjpe and Boone (1974). The rationale behind this technique is that the increase in vascular permeability which accompanies the DTH reaction (Crowle and Hu, 1967) allows for the extravasation of radiolabeled albumin from the blood stream into the extracellular space of the footpad.

Preliminary experiments designed to optimize the DTH assay with regards to the challenge dose, volume and time after sensitization as well as the duration of exposure of the test animals to the radiolabeled albumin are presented in the RESULTS section. The optimal protocol and thus the protocol used for experimentation consisted of sensitizing the test animals to SRBC by injection of $1x10^7$ cells in 0.02 ml into the front left footpad. Unsensitized controls received physiologic saline. A challenge dose of 10^8 SRBC was administered into the same footpad on day 4. Fourteen hours after challenge the mice were injected i.v. with one uCi ^{125}I-labeled human serum albumin (specific activity, 8.5 uCi/mg; Mallinckrodt Nuclear; St. Louis, Mo.) and four hours thereafter the test foot and contralateral foot were excised and radioassayed in a gamma counter (Beckman model 300).

DTH reactivity was calculated as a stimulation index (SI) as follows:

$$SI = \text{sensitized } \frac{\text{left ft pad}}{\text{right ft pad}} - \bar{x} \text{ unsensitized } \frac{\text{left ft pad}}{\text{right ft pad}}$$

in which the SI is equal to the difference in the ratios of the radioactivty in the test (left) foot divided by the radioactivity in the contralateral (right) foot between sensitized and unsensitized animals.

A reaction in which polymorphonuclear cells (PMNs) predominate among infiltrating cells is widely interpreted as an Arthrus reaction, whereas a predominantly mononuclear cell infiltrate is thought to signal a DTH reaction (Gell and Hinde, 1954). Yet studies by Collins and Mackaness (1968) and Cooper (1972) have indicated that if the challenging antigen is particulate and retained long enough at the challenge site, a mouse can make humoral antibodies at this site and develop a reaction which is delayed because of the time required for these antibodies to be manufactured and react, rather than because the reaction is a "true" DTH.

Therefore, to ascertain whether the above protocol resulted in a "true" DTH reaction footpads from sensitized and control animals were placed in a 10% formalin solution (J. T. Baker Chemical Co.; Phillipsburg, N.J.) and submitted to the Dept. of Oral Pathology (School of Dentistry, MCV) for routine histopathologic examination. The tissues were dehydrated with alcohol, cleared with xylene and embedded in paraffin. Seven micrometer thick sections were subsequently stained with hematoxylin and eosin and examined under a

light microscope for the presence of mononuclear cells, PMNs and plasma cells. To further confirm the absence of a humoral component in our assay system the draining brachial and axillary lymph nodes from animals sensitized with SRBC were aseptically removed, pooled and assayed for plaque forming ability as previously described.

3. Carrageenan induced inflammatory assay

A carrrageenan induced inflammation (Levy, 1969) was used to measure the capability of the LLC bearing mice to mount an inflammatory response. Twenty eight days after tumor inoculation naive BDF_1 mice or BDF_1 mice bearing a primary and/or metastatic LLC received a 0.02 ml injection of a 1% carrageenan solution or physiologic saline in the front left footpad in concert with an i.v. injection of one uCi ^{125}I-labeled human serum albumin (Mallinckrodt Nuclear; St. Louis, Mo.). Three hours thereafter the test foot and contralateral foot were excised and radioassayed in a gamma counter (Beckman model 300). The inflammatory response was calculated as a SI as previously described.

4. RES assay

The functional activity of the RES in LLC animals was evaluated by measuring the vascular clearance and organ distribution of two RE particles; ^{125}I-labeled Listeria ($3x10^8$ Listeria/ml) and ^{51}Cr-labeled SRBC (10% solution). The protocol consisted of injecting the RE particle intravenously into the lateral tail vein of the test animals in a volume of 0.1 ml/10g body weight. At selected times from 1 to 15 minutes after injection 0.01 ml blood samples were taken

from the tip of the tail, hemolyzed in 2 ml of distilled water and radioassayed in a gamma counter.

As the clearance of both of these RE particles have been shown to follow first order kinetics (Halpern, 1959) vascular clearance was determined by calculating the change in blood concentration of the RE particle with respect to time. The data was expressed as a phagocytic index (K) which is equal to the rate of clearance. To approximate the circulating blood volume in the test animals, the calculated number of cpms at time zero was divided by the number of injected counts/ml.

Organ uptake of the ^{51}Cr-SRBC remained constant from 30 to 90 minutes after i.v. injection (see RESULTS section). Therefore, sixty minutes after injection of the RE particle the mice were sacrificed by decapitation and allowed to exsanguinate. The liver, spleen, lung, thymus, kidneys and primary tumor were then excised, weighed and radioassayed. The data was calculated as percent organ uptake (% total cpms) and as cpms/mg tissue.

Sonicated LLC cells, used in the RES assay, were prepared by incubating 5×10^7 trypsinized LLC cells at room temperature for 30 minutes in a Cole-Parmer model 8845-4 sonicator (Chicago, Ill.). The sonicated tumor cell fragments were then centrifuged at 900xg for 15 minutes, washed 3x in EMEM and resuspended to their original volume.

5. Host resistance to Listeria monocytogenes

The effect of the LLC on resistance to Listeria monocytogenes, an infection in which macrophages and immune T cells have been shown to be involved in the elimination of the bacteria (Mackeness, 1970; North, 1973), was evaluated in the following manner. Naive and tumor bearing mice were challenged i.v. with 10^3 to 10^8 Listeria two days prior to a footpad inoculation of $1x10^6$ LLC cells, one day after LLC inoculation or 12 days after LLC inoculation. Mortality was recorded daily for 14 days and the fifty percent lethal endpoint (LD_{50}) calculated by the method of Litchfield and Wilcoxon (1949). The effect of the LLC on host resistance to Listeria monocytogenes was then determined by measuring changes in the LD_{50} of the pathogen in tumor bearing and control mice.

M. Statistical Analysis

The level of confidence for all experiments was set at ninety-five percent. A stated increase or decrease is understood to be statistically significant based upon this level of confidence. The student t test was employed to determine if a difference existed between a control and experimental group, and a one-way analysis of variance was used when one control group was compared to more than one experimental group. The method of Litchfield and Wilcoxon (1949) was used to determine LD_{50} and slopes of dose response curves. Linear regressions were evaluated by the method of least squares.

III. RESULTS

A. Characterization of the Lewis Lung Carcinoma

1. Primary tumor growth

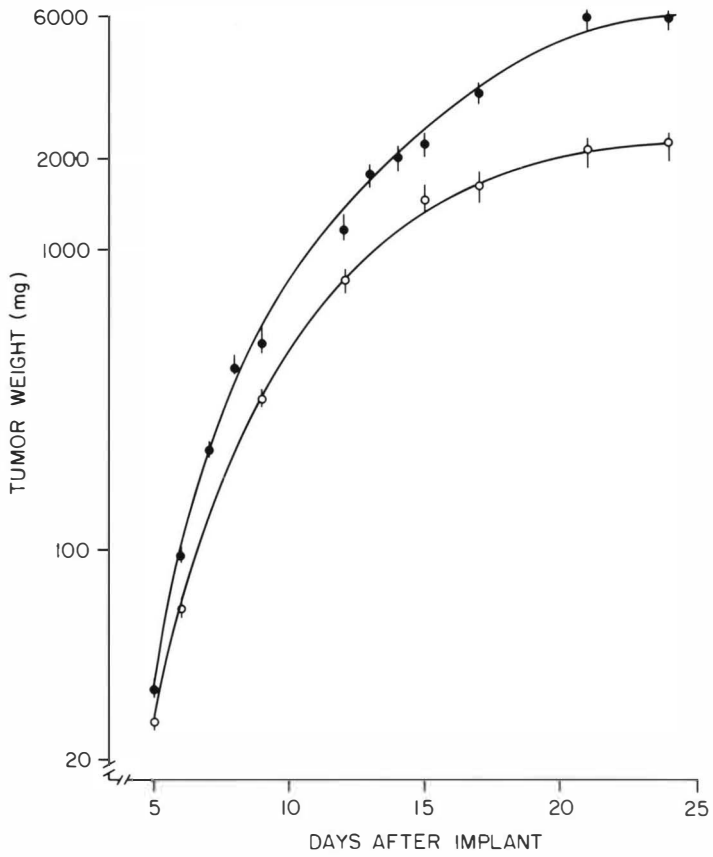
The growth characteristics of the LLC implanted intramuscularly have been described in detail by Mayo (1972a) and Simpson-Herren et al (1974). Yet, studies by Geran et al (1972) suggests that the location of tumor implant affects the primary tumor growth characteristics. Thus, initial experiments were conducted to compare the growth characteristics of the LLC implanted in the gluteus maximus and in the hind footpad (Figure 3). Examination of the data reveals that no difference exists between the growth rates with a correlation coefficient of 0.98, although the absolute values for the i.m. tumor are significantly higher on days 15 through 24. These data also indicate that both tumors follow Gompertzian growth with a decrease in growth rate as the tumor burden increases. Gross examination of the i.m. and footpad tumors revealed that the majority of tumors ulcerated by day 14 and by day 21 essentially all of the tumors had necrotic cores surrounded by a proliferating shell.

Preliminary experiments were also conducted to determine whether tumor volume measurements could be used to accurately reflect tumor weight. Presented in Figure 4 are the growth curves for the LLC footpad tumor as determined by tumor weight and tumor volume measurements. In accordance with the findings of Attia and Weiss (1966), the tumor volume measurements accurately reflected the primary tumor

Figure 3

Growth characteristics of LLC implanted in the gluteus maximus and hind footpad

BDF_1 mice were inoculated in the gluteus maximus (●) or hind footpad (○) with $1x10^6$ LLC cells. On days 5 through 24 groups of mice (n ranging from 8 to 23) were sacrificed, their primary tumors excised and weighed. Results are reported as mean tumor weights (mg) $\pm$ S.E.


6000
2000
1000
100
20
TUMOR WEIGHT (mg)
5
10
15
20
25
DAYS AFTER IMPLANT

burden of the test animals. A linear analysis revealed a positive correlation between the two curves with a correlation coefficient of 0.98. As tumor volume measurements could be made of living animals, the primary tumor burden of the test animals was normally determined by this method.

2. Metastic spread

In most therapeutic regimens for cancer the first avenue of defense is surgical removal of the primary tumor mass. Yet often times the tumor has metastasized beyond the primary tumor site prior to detection and surgical intervention. Thus, other treatment modalities are employed to reduce if not eliminate the metastatic foci. To mimic this clinical situation baseline experiments were conducted to determine what doses of LLC consistently produced lung metastasis after footpad injection. As shown in Table 1 doses of LLC cells from $3x10^5$ to $1x10^6$ produced 100% metastasis within 14 days of inoculation. The 50% metastasis producing cell number was $9.5x10^4$ cells ($4.8x10^4$-$1.9x10^5$, 95% confidence limits). A dose of $1x10^6$ LLC cells was selected for subsequent experimental studies.

To determine when 100% of the test animals had a metastatic tumor burden after injection of $1x10^6$ cells, the primary tumor was surgically excised on days 10, 13 or 15. As can be seen, metastasis occurred very early in LLC bearing mice with 100% deaths recorded when the primary tumors were removed 10 days after inoculation (Table 2). Surgical excision of the primary tumors on day 13 or thereafter had no effect on the mean survival time.

Figure 4

Correlation between tumor weight and tumor volume measurments in LLC bearing mice

BDF_1 mice were inoculated in the left hind footpad with $1x10^6$ LLC cells. On day 5 through 24 primary footpad tumors were excised and weighed (●), or measured with dial calipers to estimate tumor volume (○) . Results are expressed as mean tumor weights (mg) and volumes (mm^3) ± S.E. derived from 8-15 mice per group.

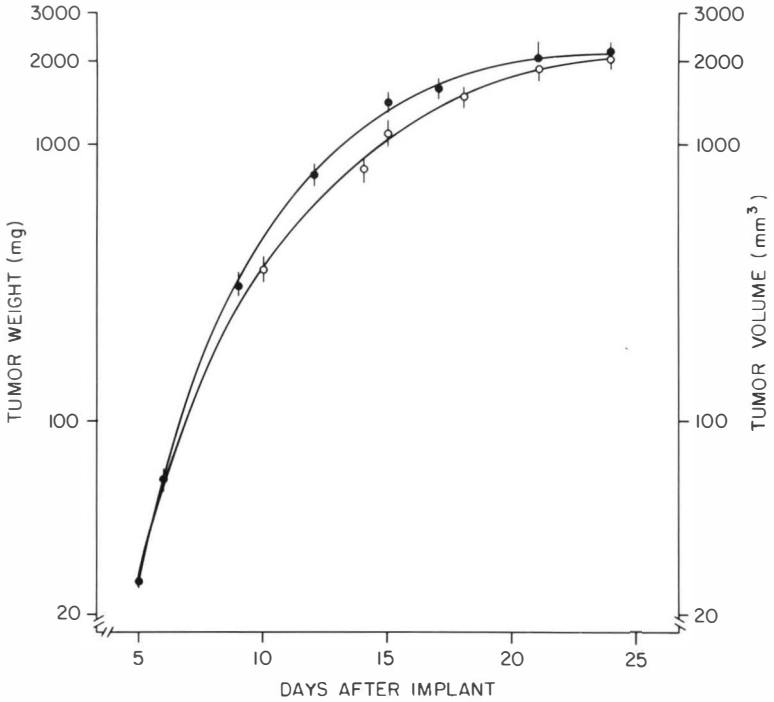

3000
2000
1000
100
20
TUMOR WEIGHT (mg)
3000
2000
1000
100
20
TUMOR VOLUME (mm³)
5
10
15
20
25
DAYS AFTER IMPLANT

Table 1

Degree of metastasis after injection of various doses of Lewis lung cells

BDF_1 mice were injected in the hind footpad with various doses of LLC cells. On day 13 the primary tumors were surgically excised and the mice followed for mortality. Mean survial times (MST) ± S.E. derived from 10 to 20 mice per group.

Dose of LLC (cell number)	Mortality (number dead/total)	MST (days) of mice that died
$1x10^6$	12/12	26.4±1.0
$3x10^5$	20/20	32.8±1.1
$1x10^5$	5/10	37.2±0.9
$3x10^4$	1/12	58
$1x10^4$	1/11	65

Table 2

Effect of primary tumor excision on survival of LLC bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the left hind footpad and the primary tumors removed on days 10, 13, 15. Mean survival time (MST) $\pm$ S.E. derived from 8 mice per group except for primary tumor controls where the number of mice was 16.

Day of Surgery	MST (days)
-	28.9±1.7
10	38.7±3.1[b]
13	31.3±2.6
15	30.5±2.3

[b] $p < 0.05$ as compared to nonsurgery group.

To quantitate the metastatic spread of the LLC to the lungs the method of Wexler (1966) was employed (Table 3). Surface tumor nodules became visible in two test animals (n=10) as early as day 9. Twelve days after LLC inoculation 100% of the mice had visible surface nodules and by day 15 the lungs were heavily laden with tumor.

Although the LLC metastasized primarily to the lungs subcutaneous implantation of liver, spleen and kidney from LLC bearing mice also resulted in the outgrowth of primary tumors (Table 4). The presence of tumor cells could not be detected in peripheral blood smears.

Underlying the progressive tumor growth and metastatic spread of the tumor bearing mice were hematological changes and alterations in lymphoreticular organ weights (Table 5). The erythrocyte count dropped progressively from control values of $9.3\pm0.2 \times 10^6/mm^3$ on day 9 to $2.6\pm0.3 \times 10^6/mm^3$ on day 21, whereas the leukocyte count increased 3 fold during the same time frame. Peripheral blood smears revealed a reticulocytosis with a slight elevation in PMN's and monocytes in the tumor bearing mice 21 days after tumor inoculation. An increase in lung and spleen weight accompanied tumor growth whereas thymic involution was noted in the terminal stages of disease. The alterations in liver weight, expressed as percent of total body weight reflect the interaction between an increasing tumor mass and decreasing total body weight. Although the alterations are statistically significant, no biological trends are evident.

Table 3

Quantification of pulmonary metastasis in LLC mice

BDF_1 mice were inoculated in the left hind footpad with $1X10^6$ LLC cells. On days 7 through 15 groups of mice (n ranging from 7 to 10) were sacrificed and their lungs insufflated with contrast medium. The extent of pulmonary metastasis was quantitated by counting surface tumor nodules and reported as means $\pm$ S.E.

Day after LLC inoculation	Number of surface tumor nodules
7	0
8	0
9	0.2±0.1
10	0.8±0.4
11	2.4±1.3
12	5.8±3.1
13	11.7±4.1
14	19.1±6.2
15	>25[a]

[a]Lungs were heavily laden with tumor nodules which were too numerous to count.

Table 4

Metastatic spread of the LLC of the spleen, liver and kidneys

BDF_1 mice were inoculated in the left hind footpad with $1X10^6$ LLC cells. Twenty four days after tumor inoculation the spleen, liver, kidneys and lung were aseptically removed and implanted s.c. into naive BDF_1 recipients. The liver and lungs were cut into halves to faciliate implantation. Metastatic spread of the LLC to these organs was determined by the outgrowth of primary tumors. The incidence of tumors and mean survival times (MST) $\pm$ S.E. derived from 3 mice per group.

Implanted organ	Tumor incidence (%)	MST (days)
lung	100	35.2±4.7
spleen	100	45.3±6.1
liver	100	49.2±6.7
kidney	100	58.6±9.0

Table 5

Effect of the Lewis lung carcinoma on organ weights and hematology

BDF_1 mice were inoculated in the hind footpad with $1x10^6$ LLC cells. At the indicated times after inoculation blood samples were taken from the tip of the tail and enumerated on a Coulter Counter. The liver, lung, spleen, thymus and kidneys were then excised and weighed. Results expressed as means ±S.E. derived from 5 mice per group except for the non-tumor controls (day 0) where N=70.

	Days Post Inoculation					
	0	1	3	9	15	21
Primary tumor wt. (mg)	-	14.6±3.7	21.0±8.2	315±36	1450±200	2140+440
Liver wt. (% body wt)	6.26±0.07	6.36±0.21	5.69±0.18[a]	6.80±0.18[a]	7.32±0.28[a]	5.98±0.06[a]
Lung wt. (% body wt)	1.14±0.02	1.17±0.13	1.22±0.05	1.27±0.10	1.34±0.07[a]	1.59±0.17[a]
Spleen wt. (% body wt)	0.43±0.01	0.53±0.03[a]	0.52±0.01[a]	0.63±0.03[a]	1.13±0.21[a]	2.20±0.18[a]
Thymus wt. (% body wt)	0.27±0.01	0.34±0.05	0.34±0.04	0.23±0.03	0.27±0.03	0.12±0.02[a]
Erythrocytes $x10^6/mm^3$	9.09±0.09	8.56±0.47	8.68±0.35	9.32±0.22	5.54±0.39[a]	2.63±0.29[a]
Leucocytes $x10^3/mm^3$	10.9±0.5	10.3±0.9	12.5±0.7	12.1±0.8	17.8±3.4[a]	37.3±4.2[a]

[a] $p < 0.05$ as compared to nontumor bearing controls.

3. Flow microfluorometric studies on the cells residing in the Lewis lung primary tumor bed

The efficacy of MVE and C. parvum as anti-tumor agents is believed to be dependent upon activation of host immune cells in close proximity to tumor cells. Yet, findings by Snyderman et al (1976) suggest that the chemotactive responsiveness of immune cells is markedly depressed in tumor bearing animals, which may result in a diminished capacity of host cells to be mobilized to the tumor bed. Thus, studies were conducted to determine whether macrophages and other immune cells resided in the LLC primary tumor bed.

Shown in Figure 5 is a DNA histogram prepared from a 14 day LLC implant. The histogram revealed that two populations of cells existed; a noncycling population of cells centered in channel 20, and a cycling population of cells whose G_1, S and G_2M peaks ranged from channel 32 to channel 80.

To investigate the possibility that the first peak of cells represented a host cell infiltrate histograms were generated from LLC cells grown in tissue culture. As can be seen in Figure 6 the peak centered in channel 20 was absent, suggesting that the noncycling cells were of host origin and that LLC cells were aneuploid.

To confirm the notion that LLC cells obtained from an i.m. implant didn't contain a mixture of diploid and aneuploid cells, a chromosome analysis from a 16 day LLC implant was performed. The analysis of 22 LLC preparations, an example of which can be seen in Figure 7, yielded a chromosome number between 66 and 77. The normal diploid number of chromosomes in mice is 40 (Makino, 1951).

Figure 5

DNA distribution histogram prepared from a 14 day Lewis lung implant

BDF_1 mice were inoculated in the gluteus maximus with $1x10^6$ LLC cells. Fourteen days after implant the primary tumors were excised, trypsinized and stained with propidium iodide. The histogram was generated by electrical pulses based on the DNA-propidium iodide fluorescence in 10,000 cells. G_1, S, G_2 and M refer to phases of the cell cycle.

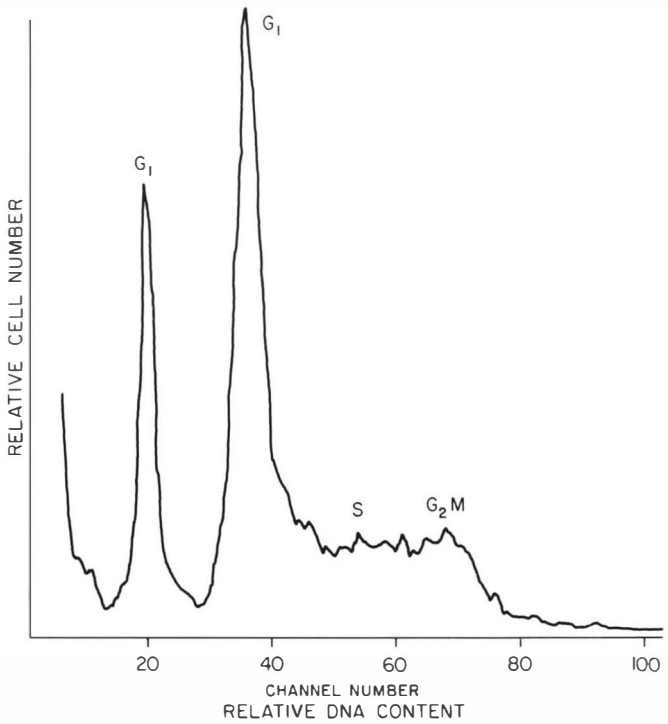

G1
G1
S
G2M
RELATIVE CELL NUMBER
20
40
60
80
100
CHANNEL NUMBER
RELATIVE DNA CONTENT

Figure 6

DNA distribution histogram prepared from Lewis lung cells grown in tissue culture

Lewis lung cells grown in tissue culture were trypsinized and stained with propidium iodide. The histogram was generated by electrical pulses based on the DNA-propidium iodide fluorescence in 10,000 cells. G_1, S, G_2 and M refer to phases of the cell cycle.

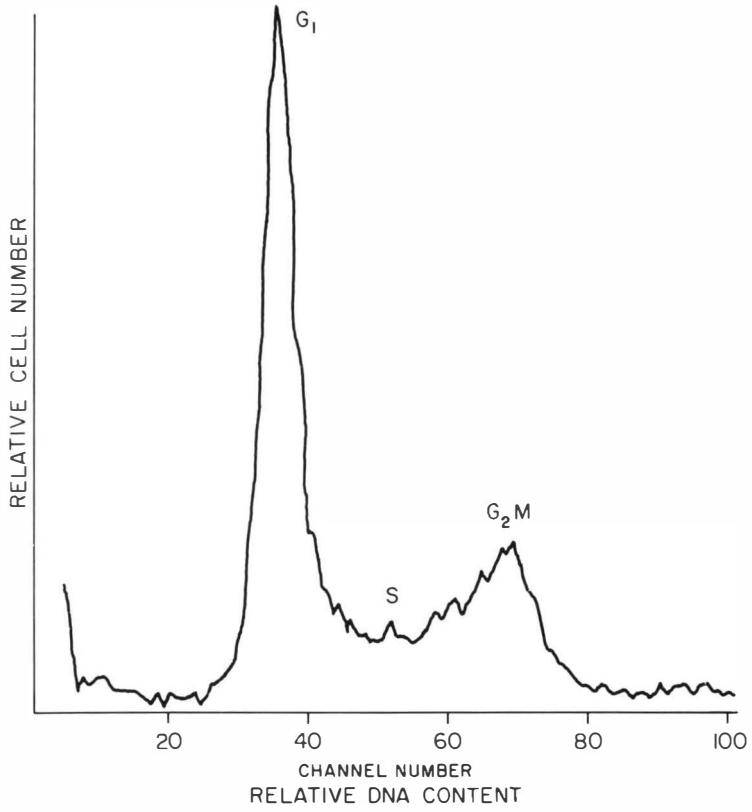
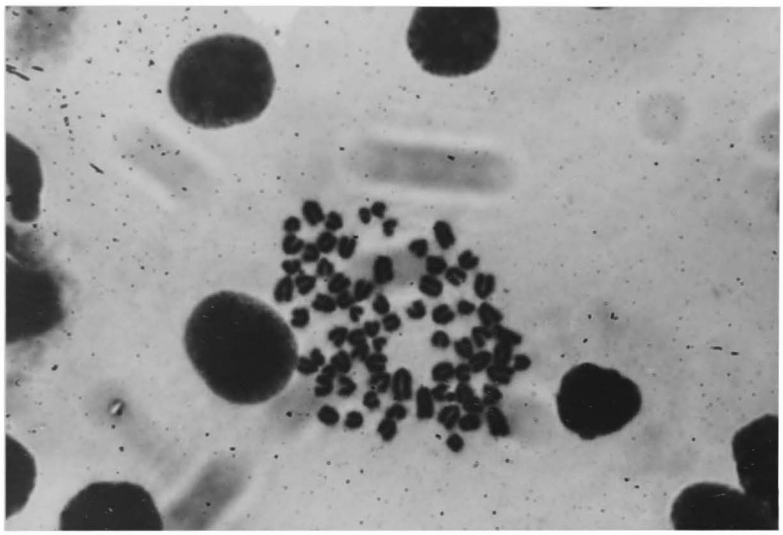

G1
G2M
S
RELATIVE CELL NUMBER
20
40
60
80
100
CHANNEL NUMBER
RELATIVE DNA CONTENT

Figure 7

Aneuploidy of Lewis lung cells

An example of aneuploidy in LLC cells. The basic diploid number in mice is 40 whereas the LLC exhibited a chromosome number between 66 and 77.



Analysis of the noncycling cells was accomplished by the ability of the flow microfluoremeter to sort out and collect various peaks obtained from a cell volume histogram (Figure 8). Cells collected from channels 39 to 53 contained host immune cells and tumor cells in a ratio of 5:1 (Table 6). Smears prepared from this center peak yielded the following differential: 42% granulocytes, 35% monocytes, 17% tumor cells and 6% lymphocytes.

B. Immunologic Status of Mice Bearing the Lewis Lung Carcinoma

The immune competence of the tumor bearing host is believed to constitute a critical limitation to the success of immunotherapy. Studies by Morahan et al (1974) and Scott (1975a) have shown that the tumor growth retarding properties of pyran and C. parvum are less effective in immunosuppressed animals. Moreover, the work of Prehn (1971) and Sjogren (1972) suggests that the administration of immunomodulators to animals with low levels of immune responsiveness can enhance tumor growth.

Thus, studies were undertaken to characterize the immune status of mice bearing the LLC in hopes of maximizing the cure rate. Assessment of antibody formation in LLC bearing animals was accomplished by determining the number of IgM antibody forming cells in the spleen. As an index of cell mediated immune responsiveness the DTH response to SRBC was employed. The function of fixed macrophages in the RES was evaluated by measuring vascular clearance and RES organ uptake of ^{51}Cr-labeled SRBC while host resistance to Listeria monocytogenes was determined by measuring changes in the LD_{50} of the pathogen in tumor bearing and control mice.

Figure 8

Cell volume histogram prepared from a 16 day Lewis lung implant

BDF_1 mice were inoculated in the gluteus maximus with $1x10^6$ cells. Sixteen days after implant the primary tumors were excised, trypsinized and suspended in calcium free PBS. The histogram was generated by electrical impulses based on the cell volumes of 10,000 cells. Shaded areas refer to peaks which were sorted, collected and analyzed under light microscopy.

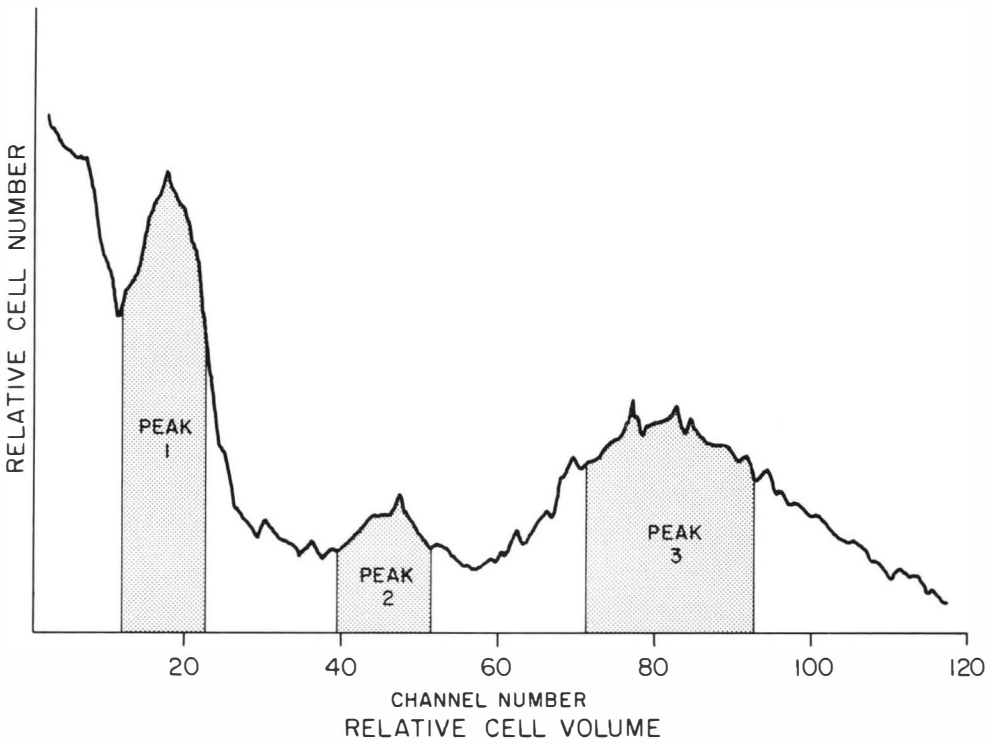

RELATIVE CELL NUMBER
PEAK 1
PEAK 2
PEAK 3
20
40
60
80
100
120
CHANNEL NUMBER
RELATIVE CELL VOLUME

Table 6

Differential analysis of cells residing the Lewis lung primary tumor bed

Cells residing in a 16 day LLC tumor were separated and collected in 3 parts corresponding to the 3 shaded peaks depicted in Figure 6. Differentials were performed on 3 slides per peak. Results expressed as mean percent of total cells ± S.E.

Peak 1 cell type	%	Peak 2 cell type	%	Peak 3 cell type	%
erythrocytes	89.0±2.3	granulocytes	42.3±1.9	tumor cells	95.0±1.2
epithedlial cells	11.0±2.3	monocytes	35.0±1.1	grandulocytes	4.3±0.9
		tumor cells	16.7±1.3	monocytes	0.7±0.3
		lymphocytes	6.0±0.6		

1. Alterations in humoral immunity

Preliminary experiments were conducted to assess the ability of C57BL/6 mice bearing the primary LLC to elicit a IgM response to SRBC. As shown in Figure 9B the number of PFC/spleen progressively decreased from non tumor bearing control values of 58.3±7.1 on day 9 to 3.5±2.1 30 days after inoculation. The data plotted as PFC/10^6 spleen cells (Figure 9A) exhibited a slightly different pattern due to the splenomegaly which accompanied tumor growth (Figure 9C). Total body weight (Figure 9D) remained at control levels of 22.8±0.3g until day 27 when a value of 18.2±0.6g was recorded for the tumor bearing mice.

It should be noted that the plaque forming ability of C57BL/6 mice to SRBC was quite variable in comparison to literature values. Standard errors of 12% of control were recorded for the PFC/10^6 and PFC/spleen responses which had an n of 40. Although the cause of the variability is not clear similar findings with C57BL/6 mice and SRBC have been noted by other investigators (Rector and Carter, 1973).

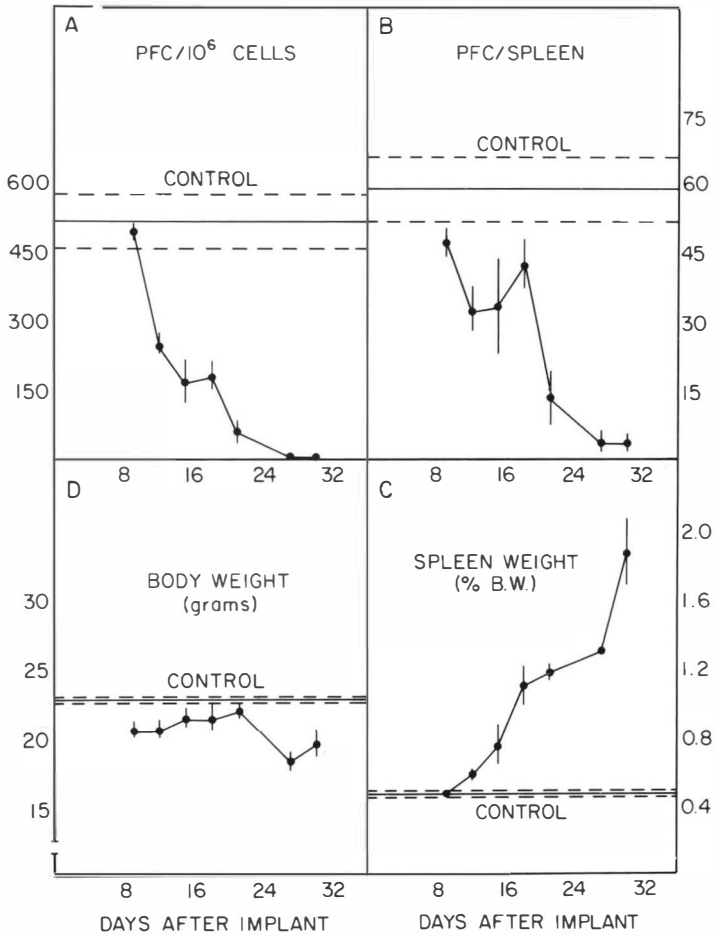
In an effort to minimize variability the plaque forming assay was conducted in BDF_1 mice (Figure 10). As can be seen not only was the plaque forming ability of control BDF_1 mice statistically greater with a control PFC/10^6 value of 828±39 and PFC/spleen value of 112±6, but the variability was significantly reduced.

The response of the BDF_1 mice bearing the LLC was slightly different however, with a reduction in PFC/spleen to 20±4 or 18% of control on the first day of measurement, day 7 (Figure 10B).

Figure 9

Ability of C57BL/6 mice bearing the Lewis lung carcinoma to elicit a primary IgM response

C57BL/6 mice were inoculated in the hind footpad with $1X10^6$ cells. Five days after tumor inoculation and every third day thereafter groups of tumor bearing and control mice were immunized with $4X10^8$ SRBC by the intraperitoneal route. Four days after immunization the mice were sacrificed and their spleens assayed for plaque forming cells (PFC). Panels A, B, C and D summarize changes in PFC/10^6 spleen cells, PFC/spleen, spleen weight expressed as percent body weight and total body weight respectively. Results are plotted as means $\pm$ S.E. derived from 5 mice per group except for non-tumor controls which contained 40 mice.


A
PFC/10^6 CELLS
CONTROL
600
450
300
150
B
PFC/SPLEEN
CONTROL
75
60
45
30
15
8
16
24
32
D
BODY WEIGHT
(grams)
CONTROL
30
25
20
15
C
SPLEEN WEIGHT
(% B.W.)
CONTROL
2.0
1.6
1.2
0.8
0.4
DAYS AFTER IMPLANT
DAYS AFTER IMPLANT

Yet, in agreement with the C57BL/6 studies, further immunologic impairment accompanied primary tumor growth. On day 18 the number of PFC/spleen was reduced to 11±7 and by day 24 no antibody response could be detected in mice bearing the primary tumor. Surgical excision of the primary tumor on day 13 had no effect on survival time yet resulted in a marked increase in the number of spleen cells with plaque forming ability. Fifteen days after tumor inoculation the number of PFC/spleen was 36±3 and by day 21 the plaque forming ability of mice bearing the metastatic LLC returned to control levels.

Spleen weights, calculated as percent body weight, increased 4 fold over control values of 0.58±0.01 in BDF_1 mice bearing the primary tumor (Figure 10C). In contrast, spleen weights in the amputated group were essentially half as large, reaching a peak value of 1.26±0.21 on day 15. The differences in the spleen indexes exhibited between the primary LLC group, metastatic LLC group and control group can't totally be attributed to body weight loss (Figure 10D). Body weight was significantly decreased to 83% of control on days 15-27 for mice bearing the primary LLC tumor whereas a significant loss of body weight in the amputated group only occurred on days 24-27.

Shown in Figure 11 are the results obtained from repeating the above experiment in BDF_1 mice. As a similar profile was generated with the primary and metastatic LLC mice these data suggest that strain differences may exist between the C57BL/6 and BDF_1 tumor bearing mice with regards to humoral responsiveness.

Figure 10

Ability of BDF_1 mice bearing the primary and metastatic Lewis lung carcinoma to elicit a IgM response

BDF_1 mice were inoculated in the hind footpad with $1X10^6$ cells. Thirteen days after inoculation half of the tumor bearing mice had their primary tumor excised by amputation. The ability of mice bearing the primary (●) and metastatic (▲) LLC to elicit a primary IgM antibody response to SRBC was quantitated by counting plaque forming cells (PFC) every third day beginning 7 days after tumor inoculation. Panels A, B, C and D summarize changes in PFC/10^6 spleen cells, PFC/spleen, spleen weight expressed as percent body eight and total body weight respectively. The arrow refers to the day of amputation, day 13. Results are plotted as means $\pm$ S.E. derived from 5 mice per tumor bearing group. Control group consisted of 24 naive and 24 sham operated mice.

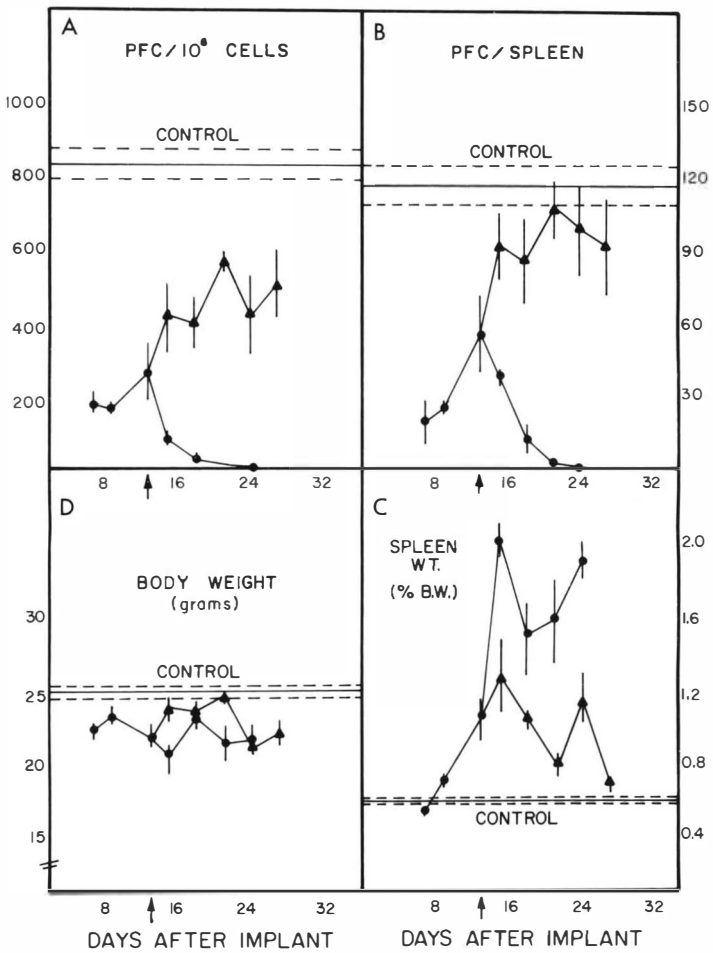
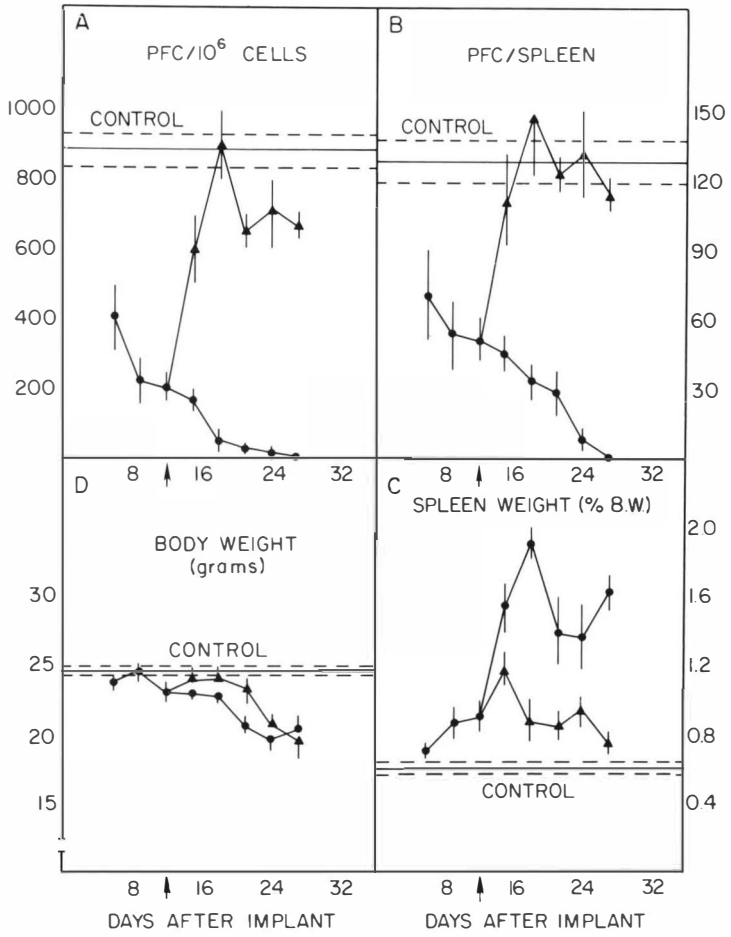

A
PFC/10⁶ CELLS
1000
800
600
400
200
CONTROL
B
PFC/SPLEEN
150
120
90
60
30
CONTROL
8
16
24
32
D
BODY WEIGHT
(grams)
30
25
20
15
CONTROL
C
SPLEEN
WT.
(% B.W.)
2.0
1.6
1.2
0.8
0.4
CONTROL
DAYS AFTER IMPLANT
DAYS AFTER IMPLANT

Figure 11

Ability of BDF_1 mice bearing the primary and metastatic Lewis lung carcinoma to elicit a IgM response

BDF_1 mice were inoculated in the hind footpad with $1X10^6$ cells. Thirteen days after inoculation half of the tumor bearing mice had their primary tumor excised by amputation. The ability of mice bearing the primary (●) and metastatic (▲) LLC to elicit a primary IgM antibody response to SRBC was quantitated by counting plaque forming cells (PFC) every third day beginning 7 days after tumor inoculation. Panels A, B, C and D summarize changes in PFC/10^6 spleen cells, PFC/spleen, spleen weight expressed as percent body eight and total body weight respectively. Results are plotted as means $\pm$ S.E. derived from 5 mice per tumor bearing group. Control group consisted of 24 naive and 24 sham operated mice.


A
PFC/10^6 CELLS
CONTROL
1000
800
600
400
200
B
PFC/SPLEEN
CONTROL
150
120
90
60
30
8
16
24
32
D
BODY WEIGHT
(grams)
CONTROL
30
25
20
15
C
SPLEEN WEIGHT (% B.W.)
CONTROL
2.0
1.6
1.2
0.8
0.4
DAYS AFTER IMPLANT
DAYS AFTER IMPLANT

2. Alterations in cell mediated immunity

The DTH responsiveness of BDF_1 mice bearing the LLC was quantitated by modification of a radioisotope footpad assay originally developed by Paranjpe and Boone (1974). The rationale behind this technique is that the increase in vascular permeability which accompanies the DTH reaction (Crowle and Hu, 1967) allows for the extravasation of radiolabeled albumin from the blood stream into the extracellular space of the footpad.

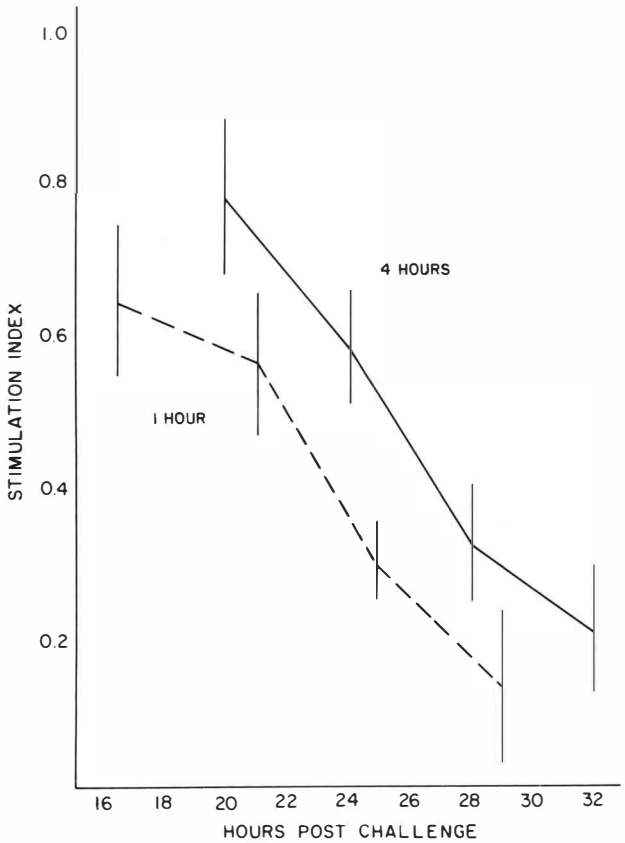
Presented in Figures 12-14 are the results obtained from pilot studies designed to optimize the DTH response with regards to the challenge dose, volume and time after sensitization as well as the duration of exposure to the radiolabeled albumin. Experiments were initially conducted to determine the optimal time of ^{125}I-human serum albumin (HSA) injection and the optimal duration of exposure to the isotope (Figure 12). Examination of the data reveals that test animals which were pulsed with ^{125}I-HSA for 4 hours recorded a consistently higher stimulation index (S.I.) than mice which were sacrificed one hour after injection. A maximum S.I. of 0.78 ± 0.14 was recorded at the first time point, 20 hours after challenge, suggesting that the optimal response occurred at or before 20 hours.

Thus, another time course experiment was conducted to ascertain the otpimal time of sacrifice using a 4 hour ^{125}I-HSA pulse. As shown in Figure 13 a maximum response of 1.03 ± 0.15 was recorded 18 hours after challenge with 1×10^8 SRBC in 20 microliters. Although

Figure 12

Characterization of the DTH assay: Determination of optimal istope exposure period

BDF_1 mice wre sensitized in the front footpad with 10^7 SRBC. A challenge dose of 10^8 SRBC in 20 microliters was administered into the same footpad on day 4. At various times after challenge the mice were injected i.v. with one Ci ^{125}I-labeled human serum albumin. One or four hours thereafter the test foot and contralateral foot were excised and radioassayed. DTH activity was calculated as a stimulation index (S.I.) and plotted at the time of footpad excision. Means $\pm$ SE were derived from 10 mice per group except for the unsensitized control group which had 15 mice.


1.0
0.8
0.6
0.4
0.2
STIMULATION INDEX
4 HOURS
1 HOUR
16
18
20
22
24
26
28
30
32
HOURS POST CHALLENGE

at the higher challenge dose of $2x10^8$ SRBC a S.I. of 1.04 ± 0.19 was recorded, the shape of the entire curve suggested that the DTH reaction was coupled with a delayed antibody response originally described by Collins and Mackaness (1968). Their findings indicated that if the challenging antigen is particulate and retained long enough at the challenge site a mouse can make humoral antibodies at this site and develop a reaction which is delayed because of the time required for these antibodies to be manufactured and react, rather than because the reaction is a true DTH. Therefore, a dose of $1x10^8$ SRBC was selected as the challenge dose for further experimentation.

To determine the peak day of response a third experiment was performed in which the mice where challenged with 10^8 SRBC on days 3, 4 or 5 after sensitization. The data in Figure 14 indicate that the peak DTH reacitivity occured on day 4, which is in agreement with the findings of Paranjpe and Boone (1974).

Collectively, the results of the foregoing experiments indicated that the optimum protocol for eliciting a DTH response was as follows. Mice were sensitized with 10^7 SRBC in the front footpad. A challenge dose of 10^8 SRBC was administered into the same footpad on day 4. Fourteen hours after challenge the mice were injected i.v. with one uCi of ^{125}I-HSA and four hours thereafter the test foot and contralateral foot were excised and radioassayed.

To ascertain whether the above protocol resulted in a true DTH reaction, footpads from sensitized mice were examined under a light

Figure 13

Characterization of the DTH assay: Determination of optimal challenge dose and volume

BDF_1 mice were sensitized in the front footpad with 10^7 SRBC. Four days after sensitization a challenge dose of 10^8 SRBC in 10 microliters (○), 10^8 SRBC in 20 microliters (●) or $2X10^8$ SRBC in 20 microliters (■) was administered into the same footpad. At various times after challenge the mice were injected i.v. with one Ci ^{125}I-human serum albumin and four hours thereafter the test foot and contralateral foot were excised and radio assayed. DTH reactivity was calculated as a stimulation index (S.I.) and plotted at the time of footpad excision. Results reported as means derived from 10 mice per group. Standard errors were within 15% of the means in all cases.

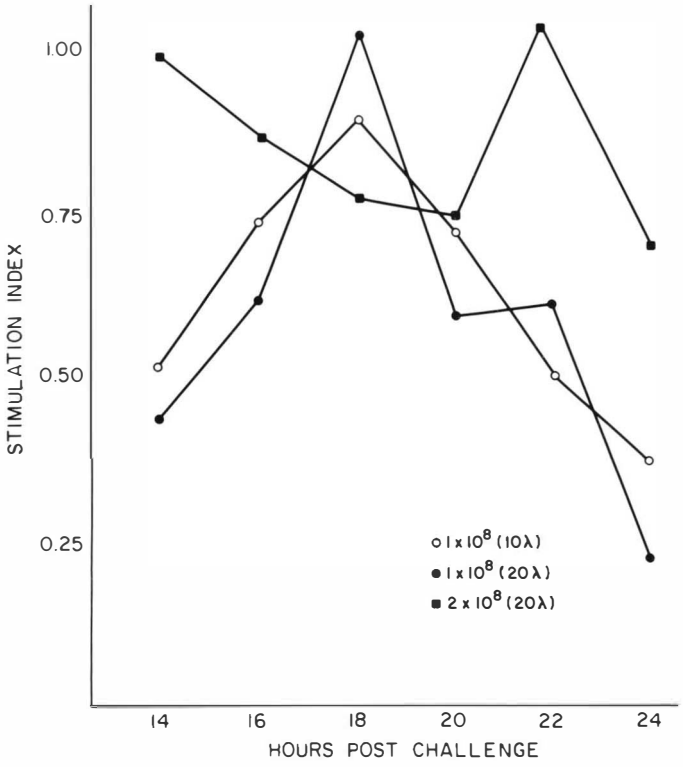
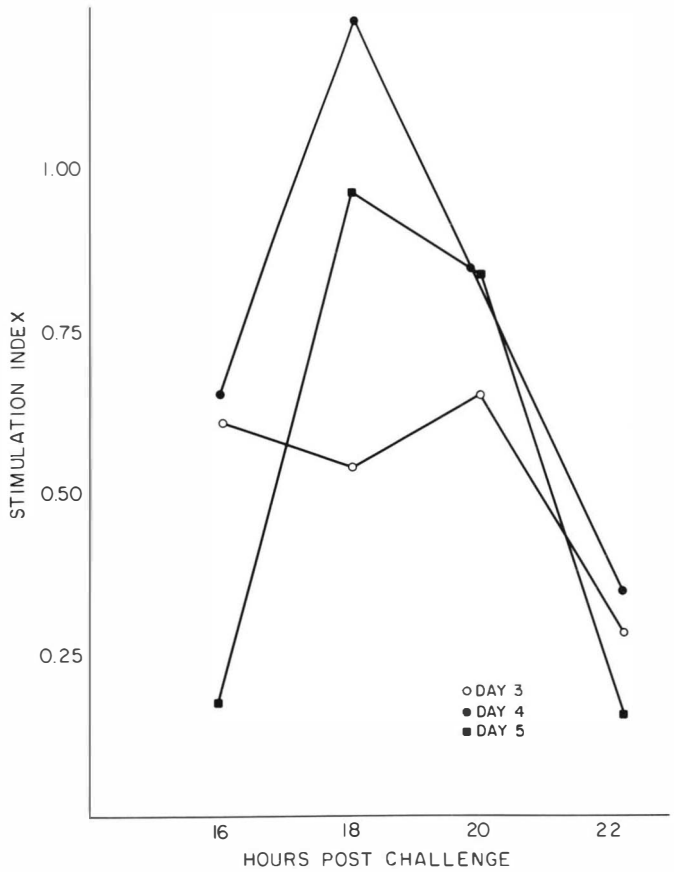

1.00
0.75
0.50
0.25
STIMULATION INDEX
14
16
18
20
22
24
HOURS POST CHALLENGE
○ 1 x 10^8 (10λ)
● 1 x 10^8 (20λ)
■ 2 x 10^8 (20λ)

Figure 14

Characterization of the DTH assay: Time course for DTH reactivity

BDF_1 mice were sensitized in the front footpad with 10^7 SRBC. A challenge dose of 10^8 SRBC in 20 microliters was administered into the same footpad on days 3 (○), 4 (●) or 5 (■). Fourteen hours after challenge the mice were injected i.v. with one Ci ^{125}I-human serum albumin and four hours thereafter the test foot and contralateral foot were excised and radioassayed. DTH reactivity was calculated as a stimulation index (S.I.) and plotted at the time of footpad excision. Results reported as means derived from 10 mice per group. Standard errors where within 15% of the means in all cases.


1.00
0.75
0.50
0.25
STIMULATION INDEX
16
18
20
22
HOURS POST CHALLENGE
DAY 3
DAY 4
DAY 5

microscope for the presence of host cells infiltrating the reaction site. Shown in Figure 15 are hematoxylin and eosin stained footpad sections from unsensitized (Panel A) and sensitized (Panel B) mice challenged 24 hours previously with 10^8 SRBC. Panel B reveals a heavy infiltration of mononuclear cells with an absence of polymorphonuclear and plasma cells suggesting that the DTH reaction was unaccompanied by an Arthrus or antibody response.

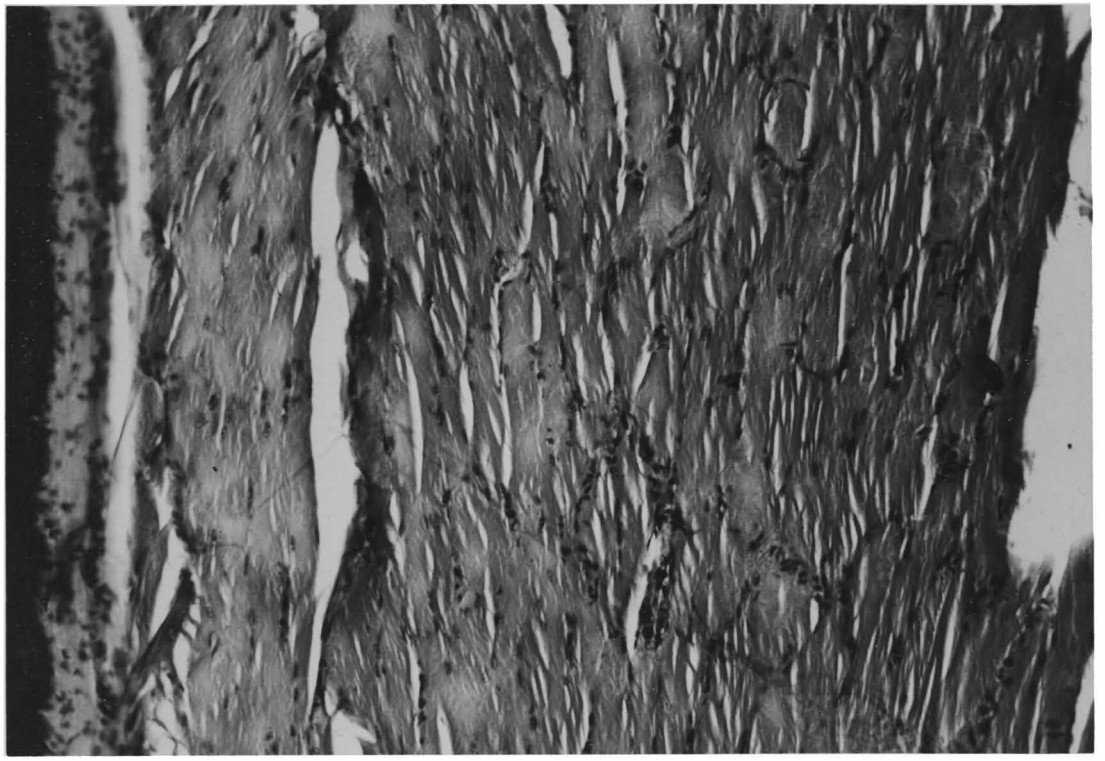
To further confirm the absence of a humoral component in our assay system the draining brachial and axillary lymph nodes from animals sensitized with SRBC were aseptically removed, pooled and assayed for plaque forming cells. No PFC were detected at a sensitizing dose of 10^7 SRBC, although at a higher sensitizing dose of 10^8 SRBC, 290 PFC/10^6 lymph node cells were noted.

The effect of the LLC on the DTH responsiveness of BDF_1 mice is summarized in Figure 16. In contrast to the early suppression in humoral responsiveness, DTH values in the tumor bearing mice remained at control levels of 0.78 ± 0.05 through day 13. Not until the mice had a primary tumor burden of 1.61 ± 0.28g on day 17 was there a significant suppression of DTH reactivity. Yet, in agreement with the humoral studies, excision of the primary tumor resulted in an increased responsiveness to SRBC. Mice which had their primary tumor excised on day 15 recorded a S.I. of 0.51 and 0.24 on days 21 and 25 respectively.

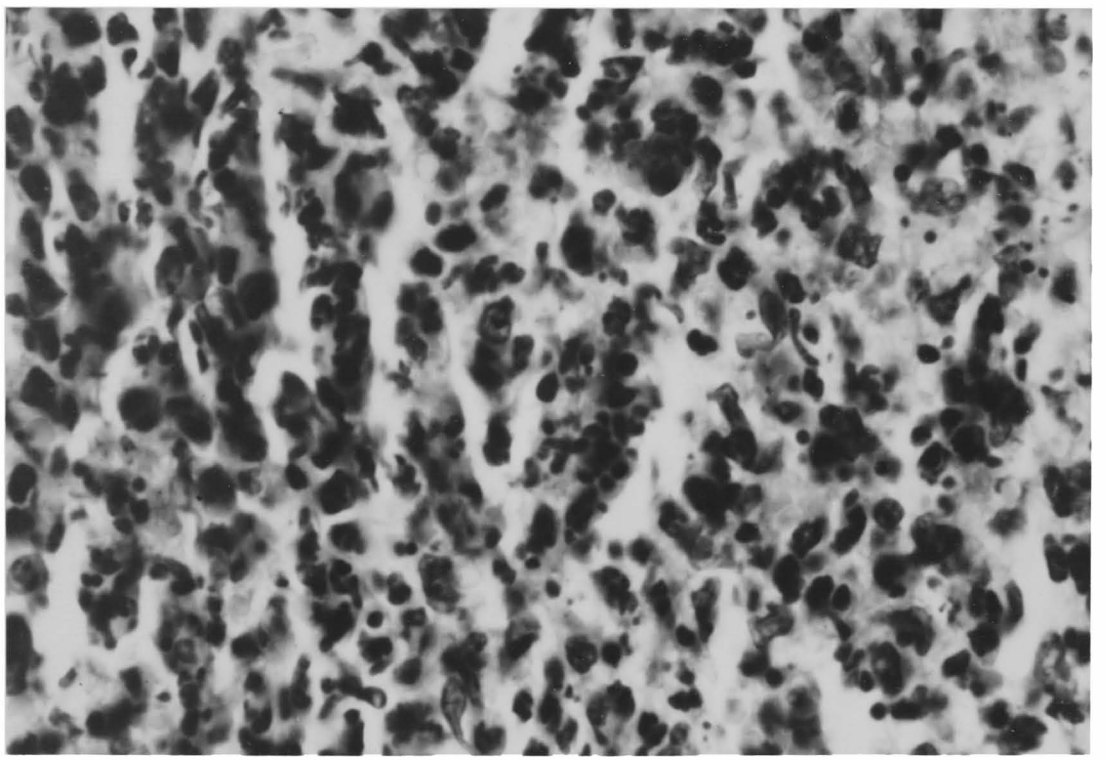
Figure 15

Mononuclear cell infiltration in DTH reaction

Photo micrographs (320 x magnification) of footpad sections from unsensitized (A) and sensitized (B) BDF_1 mice challenged 24 hours previously with 10^8 SRBC. Note presence of mononuclear cells and absence of PMN's and plasma cells in footpad undergoing a DTH reaction (B).



A

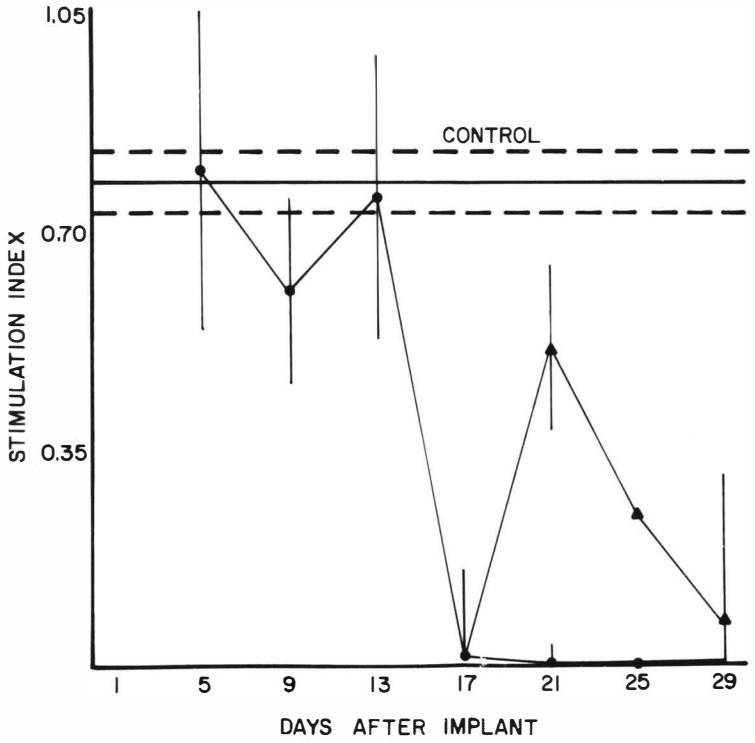


B

Figure 16

DTH responsiveness of BDF_1 mice bearing the primary and metastatic Lewis lung carinoma

BDF_1 mice were inoculated in the hind footpad with $1X10^6$ cells. Fifteen days after inoculation half of the tumor bearing mice had their primary tumor excised by amputation. The DTH responsiveness of mice bearing the primary (●) and metastatic (▲) LLC was quantitated by using a radioisotope footpad assay where a positive response was accompanied by an extravasation of ^{125}I-human serum albumin from the blood stream into the extracellular space of the footpad. DTH values were calculated as a stimulation index (S.I.) and plotted on the day of SRBC challenge. Means $\pm$ S.E. were derived from 5 mice per group except for the unsensitized control group which had 35 mice.


1.05
0.70
0.35
CONTROL
STIMULATION INDEX
1
5
9
13
17
21
25
29
DAYS AFTER IMPLANT

The data in Table 7 indicate that the lack of DTH responsiveness was not due to a decreased production of chemotactic factors such as C_{5a}, as PMN influx was not depressed in a carraggenan induced footpad swelling response. Groups of mice bearing 28 day old primary or metastatic tumors were still capable of eliciting an inflammatory response to carrogeenan similar to the control S.I. of 7.79.

3. Alterations in RES function

The functional activity of the RES in LLC animals was initially evaluated by measuring the vascular clearance and organ distribution of formalin killed ^{125}I-Listeria monocytogenes. Yet, the results generated from these experiments were eratic with no biological trends evident when organ uptake was plotted as % total cpms, cpms/mg tissue or normalized to the number of bacteria per organ. In retrospect a control time course experiment characterizing the organ distribution of ^{125}I-Listeria monocytogenes was performed. As shown in Figure 17 the percent uptake of Listeria in the liver, lung and thymus were not constant but decreased over time. Therefore tissues excised and radioassayed at any one given time point would not necessarily correlate with RES function, as alterations in phagocytic activity could also be reflected by changes in the rate of organ clearance. Thus, a second time course experiment was conducted using another RE particle, ^{51}Cr-labeled SRBC (Figure 18). Examination of the data reveals that the organ uptake of the ^{51}Cr-labeled SRBC remained constant from 30 to 90 minutes after i.v. injection.

Table 7

Effect of the Lewis lung carcinoma on the carrageenan induced inflammatory response

BDF_1 mice were inoculated in the hind footpad with $1X10^6$ cells and fifteen days later half of the tumor bearing mice had their primary tumor excised by amputation. Twenty eight days after the tumor inoculation the tumor bearing and control mice received a 0.02 ml injection of a 1% carrageenan solution or physiologic saline in the front left footpad in concert with a i.v. injection of ^{125} I-human serum albumin. Three hours thereafter the test foot and contralateral foot were excised and radioassayed. Results reported as mean stimulation indices $\pm$ S.E. derived from 3 mice per group.

Group	Stimulation Index
Naive	7.79 + 0.64
LLC[a]	7.87 $\pm$ 0.56[c]
LLC[b]	6.94 $\pm$ 2.02[c]

[a]Mice bearing 28 day old LLC with primary tumor intact.

[b]Mice bearing 28 day old LLC with primary tumor excised on day 15.

[c]Stimulation index not stastistically different from control value.

Figure 17

Organ distribution of ^{125}I-*Listeria monocytogenes*

BDF_1 mice were injected i.v. with ^{125}I-*Listeria monocytogenes* in a volume of 0.1 ml/10g body weight. At selected times from 15 minutes to 24 hours after injection groups of mice were sacrificed by decapitation and allowed to exsanguinate. The liver, spleen, lung, thymus and kidneys were then excised and radioassayed. Data was calculated as percent organ uptake (% total cpms) and reported as means derived from 8 mice per group. Standard errors were within 12% of the means in all cases.

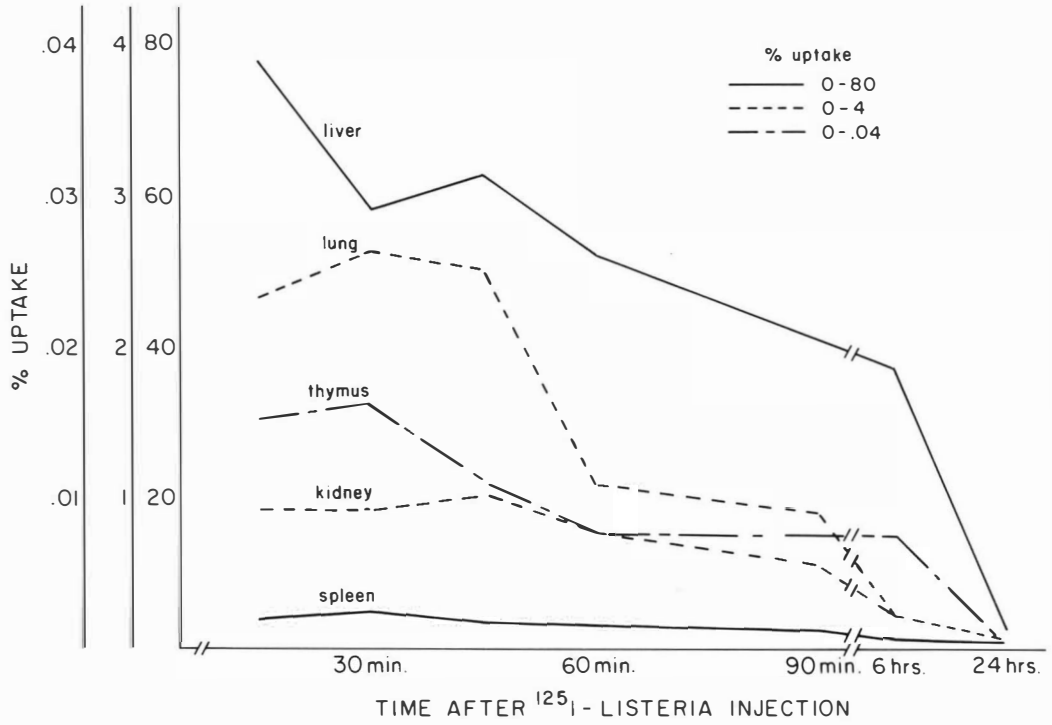
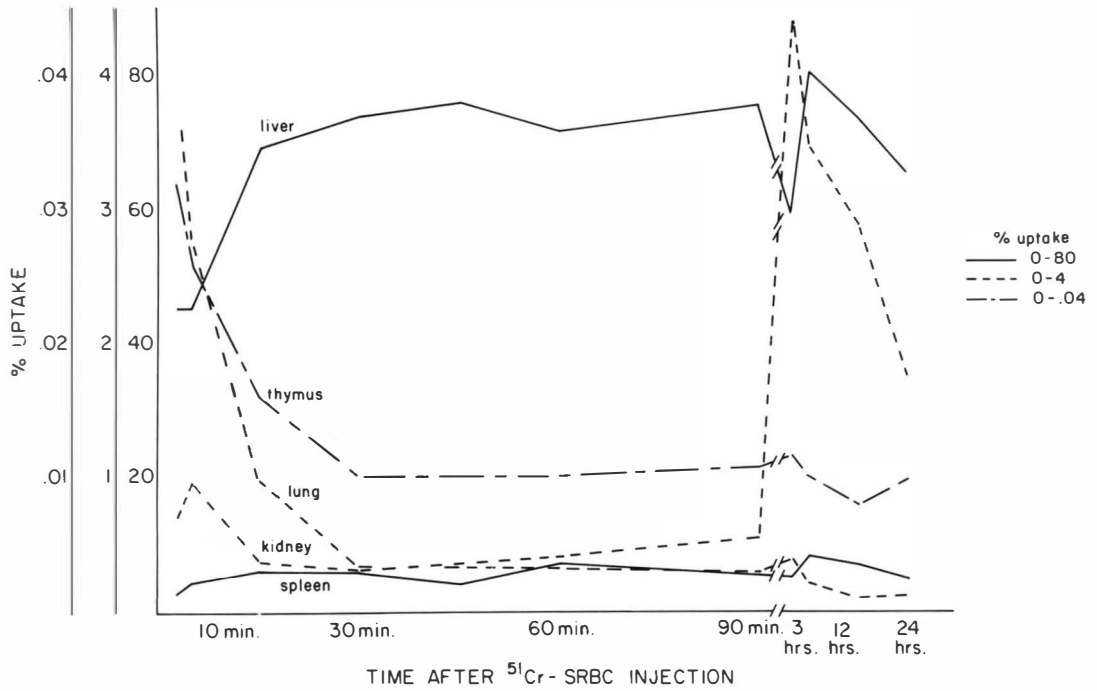

% uptake
0-80
0-4
0-.04
liver
lung
thymus
kidney
spleen
% UPTAKE
.04
.03
.02
.01
4
3
2
1
80
60
40
20
30 min.
60 min.
90 min.
6 hrs.
24 hrs.
TIME AFTER 125I - LISTERIA INJECTION

Figure 18

Organ distribution of ^{51}Cr-SRBC

BDF_1 mice were injected i.v. with a 10% solution of ^{51}Cr-labled SRBC in a volume of 0.1 ml/10g body weight. At selected times from 3 minutes to 24 hours after injection groups of mice were sacrificed by decapitation and allowed to exsanguinate. The liver, spleen, lung, thymus and kidneys were then excised and radioassayed. Data was calculated as percent organ uptake (% total cpms) and reported as means derived from 7 mice per group. Standard errors were within 12% of the means in all cases.


% UPTAKE
.04
.03
.02
.01
4
3
2
1
80
60
40
20
liver
thymus
lung
kidney
spleen
10 min.
30 min.
60 min.
90 min.
3 hrs.
12 hrs.
24 hrs.
TIME AFTER 51Cr - SRBC INJECTION
% uptake
0-80
0-4
0-.04

Therefore, tissues excised and radioassayed 60 minutes after injection provided information as to the phagocytic activity of the various RES organs.

Variations in RES activity as a function of tumor growth are shown in Figure 19. Figure 19 a summarizes the changes in the phagocytic index (K) and clearly shows that the vascular clearance of ^{51}Cr-labeled SRBC was markedly reduced 1 and 2 days after tumor cell inoculation. On day 1 a K value of 0.02, which corresponds to a vascular half life of 15 min. was recorded as compared to the control value of 0.04. Similarly, the phagocytic capacity of the liver was suppressed early on, with a 31% and 35% uptake recorded on days 1 and 2, as compared to 66% in the non tumor bearing controls (Figure 19b). Viable tumor cells appear to be necessary for this suppression as an equivalent injection of $1x10^6$ sonicated LLC cells were without effect RES function (Table 8). The initial decrease in vascular clearnace and liver uptake were followed by a phase of increased activity, which was maximal at 6 to 9 days, and a subsequent decrease to control values by day 15. These alterations could not be accounted for by uptake of the RE particle into the primary tumor bed which was less than 3% at all times, or by changes in circulating blood volume as determined by extrapolation of the slope of K back to zero time. Figures 19c and 19d depict the functional activity of splenic and alveolar macrophages in the tumor bearing mice. The only significant changes in phagocytic uptake occurred on days 1 and 2 which could simply reflect an increased

Figure 19

Variations in RES activity in mice bearing the primary and metastatic Lewis lung carcinoma

BDF_1 mice were inoculated in the hind footpad with $1X10^6$ cells. Fourteen days after inoculation half the tumor bearing mice had their primary tumors excised by amputation. The functional activity of the RES in mice bearing the primary (●) and metastatic (▲) LLC was evaluated by measuring the vascular clearance and organ distribution of a 10% solution of ^{51}Cr-labeled SRBC injected i.v. in a volume of 0.1 ml/10g body weight. Panel A summarizes changes in vascular clearance calculated as a phagocytic index (K). Panels B, C and D reflect changes in liver, spleen and lung uptake calculated as a percentage of injected radioactivity. Results are plotted as means $\pm$ S.E. derived from 5 mice per group except for the non-tumor bearing control group which had 45 mice.

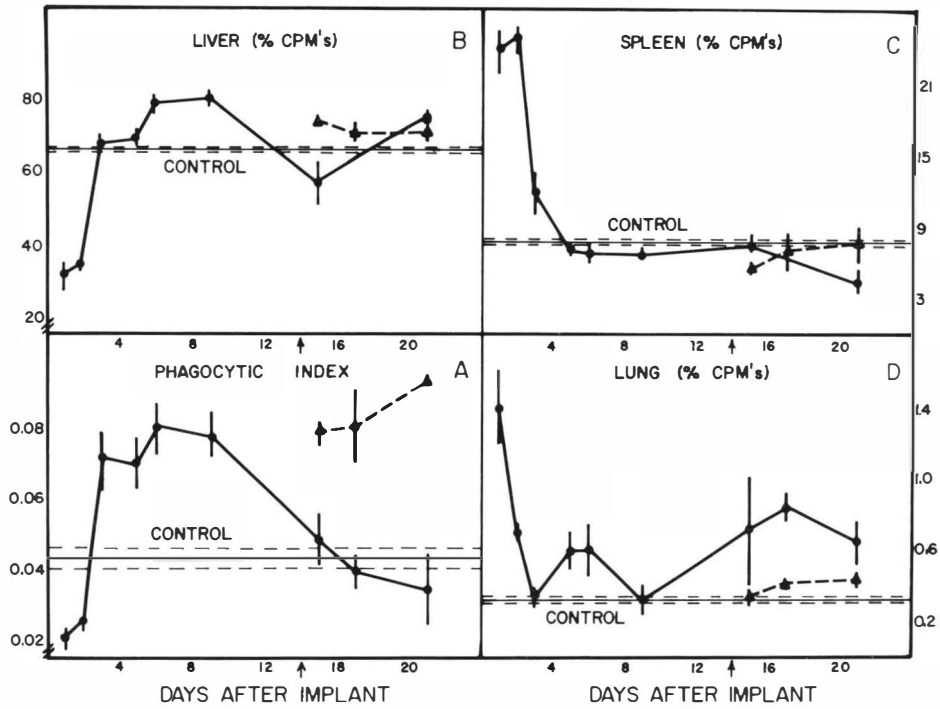

LIVER (% CPM's)
B
80
60
40
20
CONTROL
4
8
12
16
20
SPLEEN (% CPM's)
C
21
15
9
3
CONTROL
PHAGOCYTIC INDEX
A
0.08
0.06
0.04
0.02
CONTROL
LUNG (% CPM's)
D
1.4
1.0
0.6
0.2
CONTROL
DAYS AFTER IMPLANT
DAYS AFTER IMPLANT

Table 8

Necessity of viable tumor cells for RES suppression

BDF_1 mice were injected in the hind footpad with $1X10^6$ viable or sonicated LLC cells. RES function was assessed twenty four hours later by measuring the vascular clearance and organ distribution of a 10% solution of ^{51}Cr-labeled SRBC injected i.v. in a volume of 0.1 ml/10g body weight. Vascular clearance was calculated as a phagocytic index whereas organ uptake was calculated as a percentage of injected radioactivity. Results expressed as means ± S.E. derived from 5 mice per group.

Group	Phagocytic Index	Liver	Spleen	Liver	Thymus
Naive	0.05±0.01	67±3	7.6±1.2	0.19±0.02	0.014±0.004
Viable LLC cells	0.02±0.00[a]	31±4[a]	23.4±1.2	1.39±0.22[a]	0.018±0.004
Sonicated LLC cells	0.04±0.00	67±1	8.2±0.2	0.25±0.02	0.010±0.000

[a] $p < 0.05$ as compared to naive control.

exposure to the RE particle due to decrease hepatic uptake. The erratic uptake in the lungs on days 15 through 21 probably reflects the growth of the metastatic foci in the pulmonary bed. Thymus uptake remained at control levels throughout the study (data not shown). In contrast to the previous studies, excision of the primary tumor on day 14 resulted in no significant changes in organ uptake, although vascular clearance remained elevated on days 17 through 21.

4. Host resistance to Listeria monocytogenes

The foregoing results indicated that the LLC markedly suppressed RES function 24 and 48 hours after inoculation. The present studies were designed to determine whether the ability of the LLC to inhibit RES function would manifest itself by decreasing host resistance to Listeria monocytogenes, an infection in which macrophages and immune T cells have been shown to be involved in the elimination of the bacteria (Mackeness, 1970; North, 1973).

Presented in Table 9 are the results obtained from exposing tumor bearing and control BDF_1 mice to various dilutions of Listeria monocytogenes ranging from 10^3 to 10^8 bacteria. As can be seen the LD_{50}'s for the various LLC groups were not statistically different from the value of $\log_{10} 10^{7.18}$ CFU LD_{50} obtained for the non tumor bearing control group.

C. Responsiveness of Lewis Lung Carcinoma to MVE

1. LD_{50} determinations for the MVE polymers

Studies were initially conducted to obtain acute toxicity

Table 9

Effect of the Lewis lung carcinoma on resistance to Listeria monocytogenes

Naive and tumor bearing BDF_1 mice were challenged i.v. with 10^3 to 10^8 Listeria monocytogenes two days prior to a footpad inoculation of $1X10^6$ LLC cells, one day after LLC inoculation or 12 days after LLC inoculation. Mortality was recorded daily for 14 days. LD_{50}'s were calculated by the method of Litchfield and Wilcoxon and reported as the $\log_{10}$ colony forming units (CFU) that resulted in 50% mortality. Results were derived from 8 mice per group.

Group	$\frac{LD_{50}}{\log_{10}CFU}$	95% confidence limits
Naive	7.18	6.64 - 7.71
LLC[a]	6.54[d]	6.03 - 7.05
LLC[b]	6.19[d]	5.41 - 6.97
LLC[c]	7.18[d]	6.64 - 7.71

[a]Mice were challenged with Listeria monocytogenes two days prior to LLC inoculation.

[b]Mice were challenged with Listeria monocytogenes one day after LLC inoculation.

[c]Mice were challenged with Listeria monocytogenes twelve days after LLC inoculation.

[d]LD_{50} not stastistically different from naive control.

data on the five MVE fractions. As shown in Table 10 the LD_{50}'s of the MVE polymers increased with increasing molecular weight; MVE-1 was significantly less toxic than MVE 2, 3, 4 and 5, and MVE 2, 3 and 4 were significantly less toxic than MVE-5. Lethality occurred within 3 minutes of injection and the cause of death is at present unknown. Necropsy at the time of death showed no macroscopic pathology.

MVE-3 was selected for the Lewis lung studies as recent findings by Morahan et al (1978) have indicated that MVE-3 maintains the ability to activate macrophages and provides protection against infection with an improved therapeutic index.

2. Efficacy of MVE-3 alone or MVE-3 coupled with cytoreductive therapy

The results of the preceding section indicated that the immunologic capabilities of mice bearing the Lewis lung carcinoma are impaired. Yet, the patterm of immunodeficiency suggested that RES and T cell functions were intact from day 3 through the second week of tumor growth. Moreover, surgical excision of the primary tumor burden partially restored the suppressed immune functions. Accordingly, the immunotherapeutic regimens were designed to treat early in the disease state or after cytoreductive therapy in an effort to have immunocompetent animals with a "minimal" tumor burden.

Preliminary experiments were designed to ascertain whether MVE-3 was effective by itself against the LLC when administered early

Table 10

LD_{50} determinations for the MVE fractions

CDF_1 mice were administered the MVE polymers intravenously in various doses in 0.1 ml/10g body weight. Mice were observed for mortality for 14 days. LD_{50} and slope determinations were calculated by the method of Litchfield and Wilcoxon. Results derived from 8 mice per group.

Polymer	M.W.[a]	LD_{50} (mg/kg) (95% confidence limits)	slope (95% confidence limits)
pyran (NSC 46015)	30,000	78 (73-83)	1.112 (1.106-1.118)
MVE-1	12,500	112 (103-122)	1.103 (1.100-1.106)
MVE-2	15,500	98 (94-102)	1.047 (1.046-1.047)
MVE-3	21,300	94 (89-99)	1.061 (1.060-1.061)
MVE-4	32,000	95 (91-98)	1.040 (1.040-1.041)
MVE-5	52,600	86 (81-91)	1.120 (1.117-1.123)

[a]Molecular weights determined by gel permeation chromatography.

in the disease state. Table 11 represents 1 out of 3 experiments where weekly i.v. treatment with MVE-3 initiated on day 7 was not effective in increasing life span or inhibiting primary tumor growth.

Presented in Table 12 are the results obtained from combining MVE-3 with surgical excision of the primary tumor. Although surgery partially restores the immune competence of the test animals weekly MVE-3 treatment still proved to be ineffective in enhancing life span over the control MST of 29.7±1.2 days.

The ability of MVE-3 to synergize with conventional modes of therapy was first assessed by employing MVE-3 in combination with surgery and cylclophosphamide (CTX) (Figure 20). Acute i.v. injections of CTX significantly prolonged the MST in a dose response fashion. Doses of 50, 75, 100 and 150 mg/kg produced mean survival times of 33.2±1.0, 37.8±1.8, 41.6±2.2 and 42.6±1.4 days respectively as compared to 27.9±1.5 days for the surgery control group. However, weekly administration of MVE-3 coupled with CTX failed to enhance the life span of the tumor bearing mice over that observed with CTX alone. Multiple injections of CTX (100 mg/kg) further reduced the metastatic tumor burden of the mice as evidenced by the 81% increase in life span (Table 13). Yet, similar to the above experiment survival time for the MVE treated mice was not statistically different from the surgery-CTX control.

As MVE-3 was unable to synergize with surgery and chemotherapy experiments were conducted employing MVE-3 in combination with radiotherapy (Table 14). Local irradiation of the primary tumors on day

Table 11

Antitumor activity of MVE-3 against the Lewis lung carcinoma

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad. On days 7, 14 and 21 the mice were injected i.v. with physiologic saline or MVE-3. Means ± S.E. derived from 8 mice per group.

Treatment	MST[a] (days)	Tumor volume (mm^3) Days Post LLC inoculation				
		7	10	14	18	22
none	31.3±1.0	122±16	298±21	697±44	1421±121	2204±227
saline	30.4±2.0	96±14	280±29	714±68	1553±172	2318±287
MVE-3 (5mg/kg)	27.3±2.0	79±17	237±32	725±78	1632±185	2283±309
MVE-3 (25mg/kg)	29.8±1.7	136±27	354±45	822±90	1744±201	2474±413
MVE-3 (50mg/kg)	33.2±2.2	102±15	269±31	652±53	1459±157	2011±266

[a]Mean survival time.

Table 12

Effect of MVE-3 coupled with surgery on the survival time of Lewis lung bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0 followed by surgical excision (Sx) of the primary tumors on day 14. MVE-3 was administered intravenously on days 16 and 23. Mean survival times (MST) ± S.E. derived from 8 mice per group.

Treatment	MST (days)
none	29.7±1.2
Sx	32.4±1.4
Sx + MVE (5mg/kg)	30.7±1.2
Sx + MVE (25mg/kg)	31.9±1.0
Sx + MVE (50mg/kg)	31.8±0.9

Figure 20

Effect of MVE-3 coupled with surgery and cyclophosphamide on the survival time of Lewis lung bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0 followed by surgical excision (Sx) of the primary tumor on day 14. Sixteen days after inoculation cyclophosphamide (CTX) was administered i.v. in doses of 50 mg/kg (○), 75 mg/kg (●), 100 mg/kg (□) or 150 mg/kg (■). MVE-3 (25 mg/kg) was administered i.v. on days 18, 25, and 32. Survival curves were generated from 10 mice per group except for the Sx control group which had 34 mice.

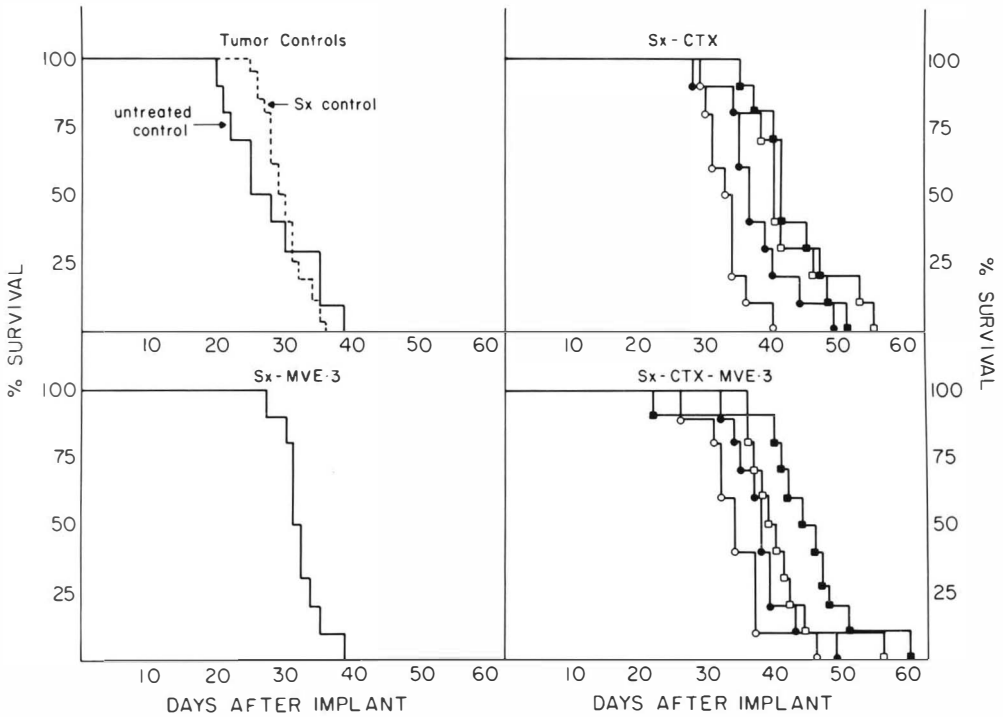

Tumor Controls
untreated control
Sx control
Sx-CTX
Sx-MVE-3
Sx-CTX-MVE-3
% SURVIVAL
100
75
50
25
10
20
30
40
50
60
DAYS AFTER IMPLANT

Table 13

Effect of MVE-3 coupled with surgery and multiple cyclophosphamide injections on the survival time of Lewis lung bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0 followed by surgical excision (Sx) of the primary tumor on day 14. Cyclophosphamide (CTX) was administered i.v. in doses of 100 mg/kg on days 16 and 23. MVE-3 (25 mg/kg) was administered i.v. on days 17 and 24. Mean survival times (MST) ± S.E. derived from 8 mice per group.

Treatment	MST (days)	ILS[a] (%)
none	27.1±1.2	
Sx control	29.4±1.1	-
Sx-MVE	29.9±0.9	1.7
Sx-CTX	53.1±1.8[b]	80.6[b]
Sx-CTX-MVE	49.4±2.5[b,c]	68.0[b,c]

[a]Increaded life span calculated by comparison of MST between drug treated and Sx control group.

[b]$p < 0.05$ as compared to Sx control.

[c]Survival time not stastistically different from Sx-CTX control.

7 with 2500, 3500 or 4500 rads increased life span by 51%, 66% and 80% respectively and inhibited primary tumor growth in dose response fashion up to 85%. Yet, four weekly injections of MVE-3 (25 mg/kg) initiated 2 days after radiotherapy still failed to enhance life span or inhibit tumor growth over that observed with radiation alone.

It should be noted that the mean survival times for the primary tumor control groups in this experiment and the following experiment were significantly decreased in comparison to previous LLC studies. The tumor passages were monitored for bacterial contamination and found to be negative.

As MVE-3 was unable to synergize with relatively high doses of radiation an attempt was made to minimize the immunosuppressive effects of radiation by lowering the doses of radiation and administering MVE-3 i.t. prior to radiotherapy. It was hoped that intralesional MVE-3 treatment before radiotherapy would not only display antitumor activity but stimulate host cells residing in the primary tumor bed to proliferate and become more radioresistant.

The results obtained from administering MVE-3 before and after radiotherapy are summarized in Table 15. As observed in earlier experiments local radiotherapy alone was effective and weekly i.v. MVE-3 therapy alone ineffective in prolonging life span. In contrast to systemic MVE-3 treatment, an acute intralesional injection of MVE-3 proved to be efficacious by increasing life span 35.9% over saline controls. Moreover, the combination of intralesional MVE-3 with 3000 rads significantly prolonged survival time as did MVE-3

Table 14

Effect of MVE-3 coupled with radiotherapy on the survival time of Lewis lung bearing mice

C57BL/6 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0. Seven days after inoculation the primary tumors were locally irradiated with 2500, 3500 or 4500 rads (r). Four weekly i.v. injections of MVE-3 (25 mg/kg) were initiated on day 9. Mean survival times (MST) ± S.E. were derived from 10 mice per group.

Treatment	MST (days)	ILS[a] (%)
none	17.9±0.9	-
MVE (5mg/kg)	18.2±2.1	1.7
MVE (25mg/kg)	19.6±2.4	9.5
MVE (50mg/kg)	20.0±3.3	11.7
2500r	27.0±3.0[b]	50.8[b]
3500r	29.7±2.6[b]	65.9[b]
4500r	32.3±2.6[b]	80.4[b]
2500r + MVE (25mg/kg)	26.0±3.0[b,c]	45.3[b,c]
3500r + MVE (25mg/kg)	27.6±3.7[b,c]	54.2[b,c]
4500r + MVE (25mg/kg)	31.6±2.3[b,c]	76.5[b,c]

[a]Increased life span.

[b]$p < 0.05$ as compared to primary tumor control.

[c]Survival time not stastistically different from appropriate radiation control.

Table 15

Efficacy of MVE-3 administered before and after radiotherapy of the Lewis lung carcinoma

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0. Intratumor (i.t.) injections of physiologic saline or MVE-3 (50 mg/kg) were administered 5 days after tumor inoculation followed by local irradiation of the primary tumors on day 9. Weekly i.v. injections of MVE (25 mg/kg) were initiated on day 11. Mean survival times (MST) ± S.E. were derived from 10 mice per group.

Drug	route	MST at radiation doses of			
		none	1000r	2000r	3000r
saline	i.t.	21.7±1.5	32.5±2.9[a]	37.5±2.4[a]	37.4±2.4[a]
MVE	i.t.	29.5±3.8[a]	35.8±2.1[a]	38.3±3.1[a]	47.0±1.8[a,b]
MVE	i.v.	21.5±2.5	33.9±2.7[a]	40.1±2.4[a]	43.7±2.7[a]
MVE	i.t.+i.v.	ND[c]	40.5±3.2[a]	53.2±1.4[a,b]	46.4±2.2[a]

[a] $p < 0.05$ as compared to non irradiated saline control.

[b] $p < 0.05$ as compared to appropriate irradiated control.

[c] Not done.

therapy before and after 2000 rads of radiation. The relative success of these combined treatment modalities has to be qualified however as the appropriate MVE-3 i.t.+i.v. control was omitted.

With the last available group of BDF_1 mice, the temporal relationship between intralesional MVE-3 treatment and radiotherapy was investigated (Table 16). In accordance with the previous study MVE-3 administered intralesionally significantly enhanced life span by 28.1% and inhibited primary tumor growth up to 37%. Systemic MVE-3 therapy coupled with intralesional MVE-3 (control group omitted in the previous study) did not increase the antitumor effect over that observed with intralesional MVE-3 alone. Local irradiation of the primary tumor with 2000 rads inhibited primary tumor growth and prolonged life span, while the combination of MVE-3 with day 8 radiation marginally enhanced life span by 18.8% over radiation controls.

3. Efficacy of MVE-2 administered by inhalation

Based on the relative success of MVE-2 administered by inhalation to mice bearing the Madison 109 carcinoma (see M109 result section) an attempt was made to increase the efficacy of MVE against the LLC by localizing the compound in the metastatic tumor bed. Yet, as shown in Table 17, concentrations of MVE-2 ranging from 0.1 mg/ml to 5 mg/ml were ineffective in prolonging the life span of mice bearing the metastatic LLC. It should be noted that C57BL/6 mice and a lower molecular weight MVE fraction, MVE-2, were employed in this study due to the unavailability of BDF_1 mice and MVE-3.

Table 16

Determination of the temporal relationship between intralesional MVE-3 treatment and radiotherapy

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0. Intratumor (i.t.) injections of physiologic saline or MVE-3 (50 mg/kg) were administered 5 days after tumor inoculation followed by local irradiation (2000 rads) of the primary tumors on days 7, 8, 9, 10 or 11. Weekly i.v. injections of MVE-3 (25 mg/kg) were initiated 2 days after radiotherapy. Mean survival times (MST) ± S.E. derived from 10 mice per group.

Treatment		MST	ILS[a]
Drugs	Radiation on day	(days)	(%)
saline i.t.+i.v.	-	28.8±2.1	-
MVE i.t.	-	36.9±1.5	28.1[b]
MVE i.t + i.v.	-	36.6±1.9	27.1[b]
saline i.t.	9	39.4±1.4	-
MVE i.t.	9	39.7±2.3	0.8
MVE i.v.	9	37.3±2.4	-5.3
MVE i.t.+i.v.	7	42.7±3.1	8.4
MVE i.t.+i.v.	8	46.8±3.0	18.8[c]
MVE i.t.+i.v.	9	42.6±3.3	8.1
MVE i.t.+i.v.	10	44.7±2.2	13.5
MVE i.t.+i.v.	11	44.2±2.6	12.2

[a] Increased life span as compared to appropriate control group.

[b] $p < 0.05$ as compared to non irradiated saline control.

[c] $p < 0.05$ as compared to appropriate drug treated or irradiated control.

Table 17

Efficacy of MVE-2 administered by inhalation to mice bearing the Lewis lung carcinoma

C57BL/6 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0 followed by surgical excision (Sx) of the primary tumors on day 14. Seventeen days after inoculation and twice a week thereafter the noses and mouths of the test animals were exposed to a nebulized mist of physiologic saline or MVE-2. Mean survival times (MST) ±S.E. derived from 7 to 10 mice per group.

Treatment	MST (days)
none	27.4±1.5
Sx	30.4±1.5
Sx-saline	29.6±1.5
Sx-MVE-2 (0.1 mg/ml)	33.9±1.2
Sx-MVE-2 (0.5 mg/ml)	32.9±1.3
Sx-MVE-2 (1.0 mg/ml)	32.9±2.3
Sx-MVE-2 (5.0 mg/ml)	29.0±1.5

D. Responsiveness of the Lewis Lung Carcinoma to Corynebacterium Parvum

1. LD_{50} determination for C.parvum

A preliminary LD_{50} study was conducted to determine the i.v. dosages of C.parvum for subsequent experiments. As shown in Table 18 the LD_{50} for C.parvum injected i.v. into BDF_1 mice was 53 mg/kg with 95% confidence limits of 37 to 71 mg/kg. Lethality occurred approximately 15 minutes after injection and the cause of death is at present unknown. Necropsy at the time of death showed no macroscopic pathology.

2. Efficacy of C.parvum alone or C.parvum coupled with cytoreductive therapy

Table 19 summarizes the results obtained from 2 separate experiments designed to ascertain the efficacy of C.parvum against the LLC. Weekly i.v. administration of C.parvum at doses of 14 mg/kg and 21 mg/kg marginally enhanced life span by 24.5% and 24.1% respectively. Similarly, intralesional C.parvum treatment produced a slight but significant prolongation in MST at doses of 7,21 and 70 mg/kg. Tumor growth was unaffected by intravenous C.parvum whereas intralesional C.parvum (70 mg/kg) treatment caused a 13.1% and 20.2% inhibition of primary tumor growth on days 9 and 12 respectively, returning to control values by day 15 (data not shown).

Presented in Table 20 are the results obtained from combining intravenous C.parvum with surgical excision of the primary tumor. Surgery on day 14, a time when the tumor had already metastasized

Table 18

LD_{50} determination of C.parvum

BDF_1 mice were administered C.parvum intravenously in various doses in 0.1 ml/10g body weight. Mice were observed for mortality for 14 days. LD_{50} and slope determinations were calculated by the method of Litchfield and Wilcoxon. Results derived from 6 or 7 mice per group.

Dose (mg/kg)	#dead / #mice	LD_{50} (95% confidence limits)	slope (95% confidence limits)
10	0/7		
25	0/7	52	1.520
50	3/6	(37-71)	(1.464-1.577)
75	5/7		
100	7/7		

Table 19

Antitumor activity of C.parvum against the Lewis lung carcinoma

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0. On days 7, 14 and 21 the mice were injected with physiologic saline or C.parvum by the indicated route. Mean survival times (MST) ± S.E. derived from 10 mice per group except for the saline control groups which had 20 mice per group.

Treatment Drug	route	MST (days)	ILS[a] (%)
saline	i.v.	26.1±1.5	-
C.parvum (7 mg/kg)	i.v.	29.8±2.3	14.2
C.parvum (14 mg/kg)	i.v.	32.5±2.1[b]	25.5[b]
C.parvum (21 mg/kg)	i.v.	32.4±1.8[b]	24.1[b]
saline	i.t.	28.7±1.5	-
C.parvum (2 mg.kg)	i.t.	26.3±2.4	-8.4
C.parvum (7 mg/kg)	i.t.	34.0±2.1[b]	18.5[b]
C.parvum (21 mg/kg)	i.t.	34.3±2.2[b]	19.5[b]
C.parvum (70 mg.kg)	i.t.	35.9±2.2[b]	25.1[b]

[a]Increased life span as compared to appropriate saline control.

[b]$p < 0.05$ as compared to appropriate saline control.

to the lung, had no effect on survival time. However, when C.parvum treatment was coupled with surgery a slight prolongation in MST occurred at the 10, 15 and 20 mg/kg doses.

The results obtained from combining systemic C.parvum treatment with radiotherapy are summarized in Table 21. As observed in earlier experiments irradiation of the tumors with 2500, 3500 or 4500 rads significantly prolonged the MST in a dose response fashion. Intravenous administration of C.parvum alone also enhanced life span by 20.3%. Moreover, the combination of C.parvum with 2500 rads or 3500 rads significantly increased life span by 23.2% and 33.1% respectively in comparison to the appropriate irradiated controls.

E. Characterization of the Madison 109 Lung Carcinoma

1. Primary tumor growth

Presented in Figure 21 are growth curves for the M109 inoculated in the hind footpad of Balb/c and CDF_1 mice. The results indicate that no detectable differences exist in the growth rates of the primary M109 tumor in the two strains of mice and that both tumor models follow Gompertzian growth with a decrease in growth rate as the tumor burden increases. Gross examination of the tumors revealed that the majority of tumors ulcerated by day 20 and by day 25 essentially all of the tumors had necrotic cores surrounded by a proliferating shell.

Table 20

Effect of C.parvum coupled with surgery
on the survival time of Lewis lung bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind foot on day 0 followed by surgical excision (Sx) of the primary tumors on day 13. C.parvum was administered intravenously on days 15 and 23. Mean survival times (MST) ±S.E. derived from 9 mice per group.

Treatment	MST (days)	ILS[a] (%)
none	30.7±1.1	
Sx	21.0±0.8	
Sx + C.parvum (5mg/kg)	32.0±0.8	3.2
Sx + C.parvum (10 mg/kg)	35.0±1.0[b]	12.9[b]
Sx + C.parvum (15 mg/kg)	34.8±0.7[b]	12.3[b]
Sx + C.parvum (20 mg/kg)	35.0±1.2[b]	12.9[b]

[a]Increased life span as compared to Sx control group.

[b]$p < 0.05$ as compared to Sx control group.

Table 21

Effect of C.parvum coupled with radiotherapy on the survival time of Lewis lung bearing mice

BDF_1 mice were inoculated with $1X10^6$ LLC cells in the hind footpad on day 0. Seven says after inoculation the primary tumors were locally irradiated with 2500, 3500, or 4500 rads (r). Four weekly i.v. injections of C.parvum (14 mg/kg) were initiated on day 9. Mean survival times (MST) ±S.E. were derived from 10 mice per group except for the primary tumor control group which had 20 mice.

Treatment	MST (days)	ILS[a] (%)
none	26.1±1.5	-
C.parvum	31.4±1.6[b]	20.3[b]
2500r	31.1±1.5[b]	19.2[b]
3500r	35.4±1.8[b]	35.6[b]
4500r	36.1±2.8[b]	38.3[b]
2500r + C.parvum	38.3±1.6[b,c]	46.7[b,c]
3500r + C.parvum	47.1±2.3[b,c]	80.5[b,c]
4500r + C.parvum	37.2±1.9[b]	42.5[b]

[a]Increased life span.

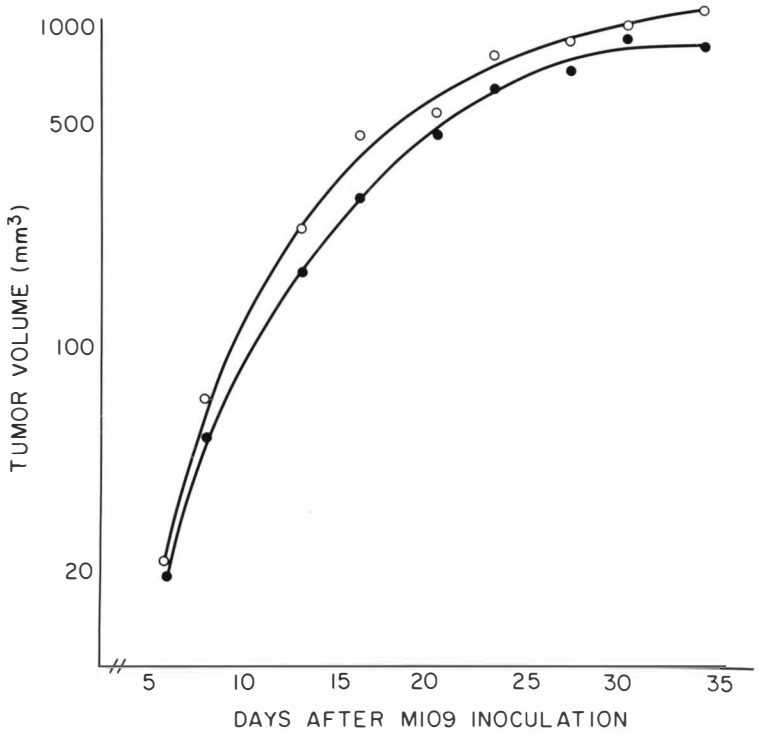
[b]$p < 0.05$ as compared to primary tumor control.

[c]$p < 0.05$ as compared to appropriate irradiated control.

Figure 21

Growth characteristics of the M109 footpad tumor

Balb/c (○) or CDF_1 (●) mice were inoculated in the hind footpad with $5X10^5$ M109 cells. The primary footpad tumor volumes were measured with dial calipers and expressed as mean tumor volumes (mm^3) derived from 7 to 10 mice per group. Standard errors were within 17% of the means in all cases.


1000
500
100
20
TUMOR VOLUME (mm3)
5
10
15
20
25
30
35
DAYS AFTER MI09 INOCULATION

2. Metastatic spread

Recent studies by Marks et al (1978) have indicated that the M109 tumor metastasizes primarily to the lung within 3 weeks and results in the death of the host about 35 days after tumor implant. The following experiment was designed to supplement these observations by determining the metastatic rate of the M109 tumor. As shown in Table 22 metastasis occurred very early in Balb/c mice with 100% deaths recorded when the primary tumors were removed 7 days after tumor inoculation. Surgical excision of the primary tumor on day 18 or thereafter had no effect on the survival time of the Balb/c mice. In the CDF_1 host the MST was less than in the syngeneic Balb/c mouse, yet the tumor appeared to metastasize slower as evidenced by the greater increases in life span afforded by surgical removal of the primary footpad tumor.

Necropsy at the time of death revealed that the M109 metastasizes primarily to the lungs. Yet, small metastatic foci were also observed in the liver, spleen and regional lymph nodes when viewed under a disecting microscope.

3. Immunogenicity

Presented in Figure 22 are growth curves for a secondary M109 tumor challenge in normal Balb/c recipients (Panel A) or in Balb/c mice bearing an established M109 tumor in the contralateral foot (Panel B). Mice bearing a primary M109 tumor clearly displayed concomitant tumor immunity, exhibiting the capacity to suppress the

Table 22

Effect of primary tumor excision on survival of M109 bearing mice

Balb/c or CDF_1 mice were inoculated with $5X10^5$ M109 cells in the hind footpad and the primary footpad tumors removed on days 7, 10, 14 or 18. Mean survival times (MST) ± S.E. derived from 8 mice per group except for the primary tumor controls which had 25 mice per group.

Mouse strain	Day of surgery	MST (days)	ILS[a] (%)
Balb/c	-	38.5±1.4	-
Balb/c	7	63.1±3.2[b]	63.9[b]
Balb/c	10	45.9±2.6[b]	19.2[b]
Balb/c	18	40.2±2.2	4.4
Balb/c	22	35.1±1.9	-8.8
CDF_1	-	31.9±1.9	-
CDF_1	10	57.0±7.5[b]	78.7[b]
CDF_1	18	37.0±1.5[b]	16.0[b]
CDF_1	22	34.4±1.9	7.8

[a]Increased life span calculated by comparision of MST between surgery groups and appropriate primary tumor control.

[b]$p < 0.05$ as compared to appropriate primary tumor control.

growth of a secondary M109 challenge. Inoculation of the secondary challenge however, had no effect on the growth of the primary tumor (data not shown).

The concomitant immune response was tumor specific (Figure 23). Mice bearing a primary M109 tumor in the left hind footpad suppressed the growth of a secondary M109 challenge, but were without effect on the growth of the P815 mastocytoma.

F. Responsiveness of the Madison 109 Lung Carcinoma to MVE-2

1. Efficacy of MVE-2 alone against the M109

Summarized in Table 23 are the results obtained from administering a single dose of MVE-2 to M109 bearing animals. While a marginal increase in life span was observed with a single i.v. injection of MVE-2 (50 mg/kg) on day 7, the data suggests that acute administration of MVE-2, given i.v. or intralesionally, does not markedly affect MST. However, multiple injections of MVE-2 (25 mg/kg) significantly enhanced life span by all three routes of administration tested; i.v., i.t. and i.p. (Table 24). The i.v. and i.t. routes of administration were more effective than MVE-2 delivered by the i.p. route. In accordance with the life span data i.v. and i.t. administration of MVE-2 also inhibited primary tumor growth up to 65%, whereas i.p. administration was without a significant effect on tumor volume.

Studies were next undertaken to compare the antitumor activity of MVE-2, the more toxic polyanion pyran-copolymer, and the biological vaccine C. parvum. As shown in Table 25 the activity of all the

Figure 22

Effect of an established M109 tumor on the growth of a second M109 tumor challenge

Balb/c mice were injected in the left hind footpad with $5X10^5$ M109 cells. Eleven days later (day 0 on the figure) these tumor bearing mice and normal mice were challenged in the right hind footpad with varying numbers of M109 tumor cells ranging from $1X10^4$ to $1X10^6$ M109 cells. Depicted are growth curves for the secondary tumor challenge in normal Balb/c recipients (Panel a) or in Balb/c mice bearing an established tumor in the contralateral foot (Panel b). Results are derived from 8 mice per group. Standard errors are within 14% of the means in all cases.

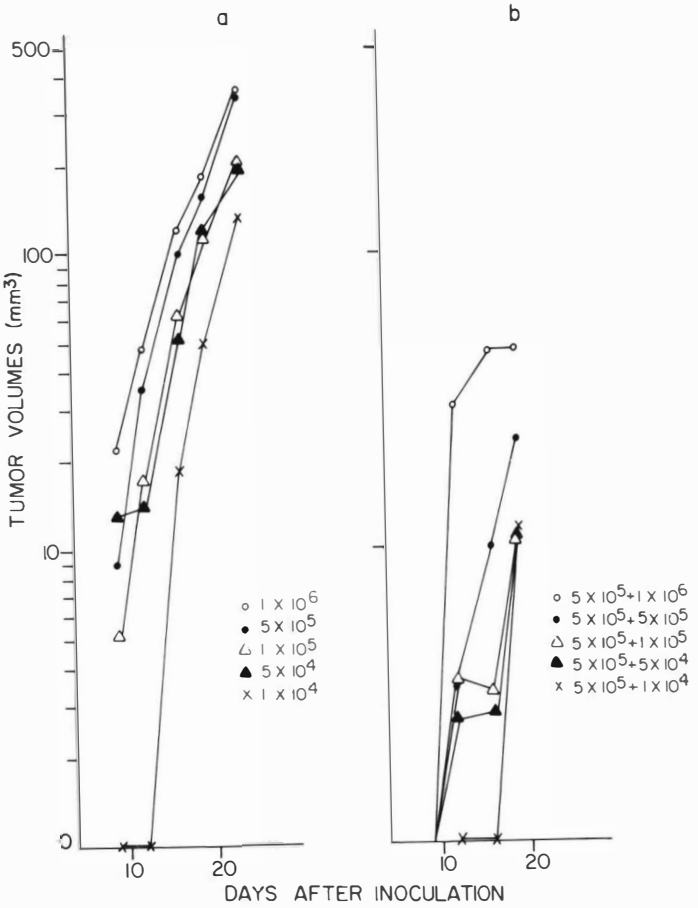

a
b
500
100
10
0
TUMOR VOLUMES (mm³)
10
20
DAYS AFTER INOCULATION
1 X 10⁶
5 X 10⁵
1 X 10⁵
5 X 10⁴
1 X 10⁴
5 X 10⁵+1 X 10⁶
5 X 10⁵+5X 10⁵
5 X 10⁵+1 X 10⁵
5 X 10⁵+5X 10⁴
5 X 10⁵+1 X 10⁴

Figure 23

Effect of an established M109 tumor on the growth of a second M109 or P815 tumor challenge

CDF_1 mice were injected in the left hind footpad with $5X10^5$ M109 cells. Eleven days later (day 0 on the figure) these tumor bearing mice and normal mice were challenged in the right hind footpad with $5X10^5$ M109 or 1X10 P815 tumor cells. Depicted are growth curves for the secondary M109 tumor challenge in normal CDF_1 recipients (●) or in CDF_1 mice bearing an established M109 tumor in the contralateral foot (○); and growth curves for the P815 footpad tumor in normal CDF_1 recipients (▲) or in CDF_1 mice bearing an established M109 tumor in the contralateral foot (△). Results are derived from 8 mice per group. Standard errors are within 13% of the means in all cases.

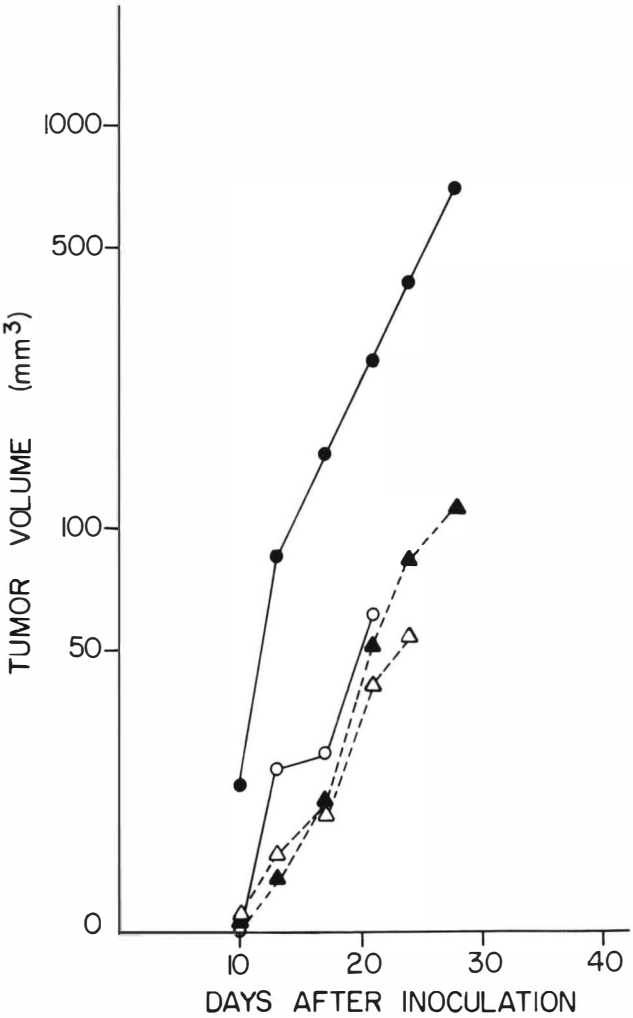

1000
500
100
50
0
TUMOR VOLUME (mm3)
10
20
30
40
DAYS AFTER INOCULATION

Table 23

Ineffectiveness of acute administration of MVE-2 against the M109

Balb/c mice were inoculated with $5X10^5$ M109 cells in the left hind footpad on day 0. A single injection of physiologic saline or MVE-2 (50 mg/kg) was administered on days 7, 8 or 12 by the indicated route. Mean survival time (MST) $\pm$ S.E. derived from 8 mice per group.

Treatment		MST (days)
Day 7	saline (i.t.)	45.8±1.5
Day 7	MVE-2 (i.t.)	40.2±3.0
Day 7	saline (i.v.)	36.2±1.7
Day 7	MVE-2 (i.v.)	42.2±1.3[a]
Day 8	saline (i.t.)	36.2±1.4
Day 8	MVE-2 (i.t.)	39.2±1.4
Day 12	saline (i.t.)	38.5±1.4
Day 12	MVE-2 (i.t.)	36.3±1.2
Day 12	saline (i.v.)	37.3±1.6
Day 12	MVE-2 (i.v.)	40.7±1.5

[a] $p < 0.05$ as compared to appropriate saline control group.

Table 24

Efficacy of multiple MVE-2 injections against the M109

CDF_1 mice were inoculated with $5X10^5$ M109 cells in the left hind footpad on day 0. Beginning on day 7 and then weekly, the mice were administered physiologic saline or MVE-2 (25 mg/kg) by the indicated route. Means ± S.E. derived from 16 mice per group and 9 mice per MVE-2 treated group.

Treatment	MST[a] (days)	Tumor volume (mm^3) Days post M109 inoculation				
		20	23	27	30	34
saline	39.4±2.0	163±33	231±32	395±64	616±50	842±81
MVE-2 (i.v.)	58.7±5.1[b]	26±9[b]	52±12[b]	116±31[b]	152±31[b]	351±73[b]
saline	37.0±2.2	176±23	316±37	499±70	665±55	888±91
MVE-2 (i.v.)	68.6±3.5[b]	26±10[b]	44±8[b]	46±9[b]	56±14[b]	119±21[b]
saline	42.9±3.9	46±10	88±15	165±19	234±37	619±116
MVE-2 (i.p.)	54.0±3.3[b]	59±13	66±18	136±29	218±73	660±213

[a]Mean survival time

[b]$p < 0.05$

immunomodulators was comparable at doses of 10 mg/kg and 25 mg/kg. MVE-2 lost activity at 5 mg/kg.

As tumor volume has been shown to constitute a critical limitation to the success of immunotherapy, experiments were also conducted in which MVE-2 therapy was initiated at different intervals after M109 inoculation. Summarized in Table 26 are the results obtained from 4 separate studies employing multiple i.v. injections of MVE-2 (50 mg/kg). The initiation date of systemic therapy markedly affected the efficacy of MVE-2. Weekly i.v. administration initiated on day 7 produced a 49% prolongation in survival time whereas treatment withheld until day 20 was without a significant effect on life span.

2. Efficacy of MVE-2 coupled with surgery or radiotherapy

The ability of MVE-2 to synergize with conventional modes of therapy was assessed by employing MVE-2 in combination with surgery or radiation. Surgical excision of the primary footpad tumor on days 10 or 14 significantly prolonged the MST (Table 27). Likewise, when the tumor bearing hosts were treated i.v. with MVE (50 mg/kg) an increased life span was observed. However, surgery combined with MVE-2 failed to prolong the MST over that observed with MVE-2 alone. Similar results were obtained when MVE-2 (50 mg/kg) was administered i.v. or i.t. 6 days prior to surgery, weekly (i.v.) starting 2 days after surgery, or both prior to and after surgery.

Presented in Table 28 are the results obtained from combining MVE-2 with local radiation of the primary footpad tumor. Local radiotherapy with 1000, 2000, or 3000 rads failed to prolong sur-

Table 25

Comparision of antitumor activity of MVE-2, Pyran and C.parvum against the M109

Balb/c mice were inoculated with $5X10^5$ M109 cells in the hind footpad on day 0. Weekly i.v. administration of MVE-2, pyran and C.parvum wre initiated on day 7. Mean survival times (MST) ± S.E. were derived from 10 mice per group.

Treatment		MST
Drug	Dose (mg/kg)	(days)
saline	-	31.8±1.5
MVE-2	5	32.9±1.9
MVE-2	10	43.5±2.5[a]
MVE-2	25	45.1±2.4[a]
MVE-2	50	43.6±2.0[a]
Pyran	5	41.0±2.4[a]
Pyran	10	43.9±2.9[a]
Pyran	25	49.0±3.8[a]
C. parvum	5	40.1±1.7[a]
C. parvum	10	41.4±2.0[a]
C. parvum	25	41.9±1.7[a]

[a] $p < 0.05$ as compared to primary tumor control group.

Table 26

Efficacy of multiple MVE-2 injections initiated at different intervals after M109 inoculation

Balb/c mice were inoculated with $5X10^5$ M109 cells in the left hind footpad. Weekly i.v. administration of MVE-2 (50 mg/kg) or physiologic saline was initiated on days 7, 13, 15 of 20 after tumor inoculation. Tumor volumes and means survival times (MST) ± S.E. were derived from 8 mice per group.

Treatment	Tumor volume[a] (mm^3)	MST (days)	ILS[b] (%)
Day 7 - saline	46±15	39.4±2.0	-
Day 7 - MVE-2	51±4	58.7±5.1	49.0[c]
Day 13 - saline	182±15	34.7±3.0	-
Day 13 - MVE-2	197±19	47.8±1.8[c]	37.8[c]
Day 15 - saline	306±24	36.2±1.4	-
Day 15 - MVE-2	284±21	45.0±2.3[c]	24.3[c]
Day 20 - saline	476±33	38.5±1.1	-
Day 20 - MVE-2	671±104	42.0±2.1	9.1

[a]Tumor volume on initial day of drug therapy.

[b]Increased life span calculated by comparison of MST between drug treated and appropriate saline control group.

[c]$p < 0.05$

Table 27

Effect of MVE-2 coupled with surgery on the survival time of M109 bearing mice

Balb/c mice were inoculated with $5X10^5$ M109 cells in the left hind footpad on day 0 followed by surgical excision of the primary tumors on days 10, 14 or 19. MVE-2 (50 mg/kg) was administered intravenously on days 7, 14 and 21. Mean survival times (MST) ± S.E. derived from 8 mice per group except for the primary tumor control group which had 25 mice.

Treatment		MST
Drug	Surgery on day	(days)
saline	-	38.5±1.4
MVE-2	-	50.5±2.6[a]
saline	10	45.9±2.6[a]
MVE-2	10	55.0±2.7[a,b]
saline	14	45.6±2.8[a]
MVE-2	14	50.6±2.8[a,b]
saline	19	39.1±2.1
MVE-2	19	46.5±2.2[a,b]

[a] $p < 0.05$ as compared to the primary tumor control.

[b] MST for surgery-MVE group was not stastically different from the group treated with MVE-2 alone.

vival time although primary tumor growth was inhibited up to 50%. Weekly i.v. administration of MVE-2 (50 mg/kg) to non-irradiated tumor bearing hosts, or a single i.t. injection coupled with the weekly i.v. injections were equally effective in prolonging survival time. Yet, similar to the surgery experiments, treatment with MVE-2 before or after radiotherapy did not increase the antitumor effect of MVE-2 over that observed with MVE-2 alone.

3. Efficacy of MVE-2 administered intrapleurally or by inhalation

As death of the animals bearing the M109 results from lung metastasis (Marks et al, 1978) an attempt was made to increase the efficacy of MVE-2 by administering the compound intrapleurally in close proximity to established metastatic tumor foci (Table 29). Intrapleural administration of MVE-2 not only allowed for greater contact between MVE-2, alveolar macrophages and tumor cells, but 5 fold higher concentrations of MVE-2 could be employed. The results indicate that intrapleural injections of MVE-2 (250 mg/kg) significantly prolonged life span by 30% over surgery controls, whereas i.v. administration of MVE-2 (25 mg/kg) at this late date was without effect on survival time.

To follow up on this observation experiments were designed to deliver MVE-2 to the tumor bearing mice by the inhalation route (Table 30). Biweekly exposure of the noses and mouths of the test animals to MVE-2 (1mg/ml) resulted in greater than a 50% increase in life span as compared to the surgery control group. Higher doses

Table 28

Effect of radiotherapy or radiotherapy coupled with MVE-2 on survival time of M109 bearing mice

Balb/c mice were inoculated with $5X10^5$ M109 cells in the hind footpad on day 0 followed by local irradiation of the primary tumors on day 13. Intratumor (i.t.) injections of physiologic saline of MVE-2 (50 mg/kg) were administered 8 days after tumor inoculation. Weekly i.v. injections of physiologic saline or MVE-2 (50 mg/kg) were initiated on day 15. Mean survial times (MST) ± S.E. were derived from 10 mice per group.

Treatment		MST at radiation doses of			
Drug	Route	none	1000r	2000r	3000r
saline	i.t. and i.v.	36.2±1.4	37.5±0.8[b]	41.7±11.3[b]	39.5±1.1[b]
MVE-2	i.v.	45.0±2.3[a]	44.7±2.4[a,b]	47.4±3.5[a,b]	48.6±3.5[a,b]
MVE-2	i.t. and i.v.	49.3±2.5[a]	56.4±4.0[a,b]	48.8±2.8[a,b]	53.8±4.3[a,b]

[a] $p < 0.05$ as compared to appropriate saline or saline-irradiation control.

[b] MST not significantly different from appropriate drug treated control.

Table 29

Antitumor activity of MVE-2 administered intrapleurally to mice bearing the metastatic M109

Balb/c mice were inoculated with $5X10^5$ M109 cells in the hind footpad on day 0 followed by surgical excision of the primary tumors on day 15. Intrapleural injections of physiologic saline or MVE-2 (250 mg.kg) were administered on days 17, 20 and 24 in a volume of 0.02 ml utilizing 30 gauge needles. No deaths were recorded over the 75 day observation period in non-tumor bearing control mice receiving intrapleural MVE-2 treatment. Mean survival times (MST) ± S.E. were derived from 8 mice per group.

Treatment		MST
Drug	Route	(days)
saline	i.v.	43.0±1.8
MVE-2	i.v.	41.7±1.3
saline	intrapleural	40.1±4.8
MVE-2	intrapleural	55.9±4.7[a]

[a] $p < 0.05$ as compared to the group receiving intrapleural saline treatment.

of MVE-2 were ineffective in prolonging the MST. Necropsy at the time of death indicated that the lungs of the test animals were only moderately laden with tumor. Moreover, metastatic foci were readily observable in the livers of several test mice.

Table 30

Antitumor activity of MVE-2 administered by inhalation to mice bearing the metastatic M109

Balb/c mice were inoculated with $5X10^5$ M109 cells in the hind footpad on day 0 followed by surgical excision (Sx) of the primary tumors on day 15. Seventeen days after inoculation a twice a week thereafter the noses and mouths of the test animals were exposed to a nebulized mist of physiologic saline or MVE-2. No deaths were recorded for non-tumor bearing control mice receiving MVE-2 by inhalation. Mean survival times (MST) $\pm$ S.E. derived from 10 mice per group.

Treatment	MST (days)
none	38.0±0.7
Sx-saline	47.0±2.9
Sx-MVE-2 (1 mg/ml)	>70[a]
Sx-Mve-2 (20 mg/ml)	47.8±3.7
Sx-MVE-2 (50 mg/ml)	47.6±3.5
Sx-MVE-2 (100 mg/ml)	46.0±3.4

[a] $p < 0.05$ as compared to Sx-saline control group.

IV. DISCUSSION

A successful outcome from any form of cancer treatment must depend on those features of a tumor which make it distinct in some way from surrounding normal tissue. When viewed in this way successful cancer surgery or radiotherapy can be seen to depend not only on the location of the tumor but also on the histopathology of the tumor which distinguishes the neoplastic growth from normal tissue. Treatment with irradiation or chemotherapy will also tend to rely on the abnormal growth rate and metabolism of the malignant cells to distinguish them from normal cells. Thus, in part, the current inadequacies of cancer treatments reflects their lack of selectivity; the absence of specific target sites on the malignant cell and the consequent inability to distinguish between normal and neoplastic tissue. It is in this context that immunotherapy provides such an attractive concept, as theoretically the immune response should be able to selectively destroy tumor cells not amenable to killing by conventional treatment modes. Yet, in practice, an optimal lethal interaction between the effector limbs of the immune response and tumor cells has been shown to be dependent on a minimal tumor burden and an intact lymphoid system.

Based on these findings the overall objectives of this thesis project were to (1) characterize the immune status of tumor bearing mice and (2) develop a rational protocol consisting of surgery or radiotherapy combined with immunotherapy to maximize their cure rate.

To carry out these objectives the Lewis lung carcinoma was initially selected. Data was presented which indicated that the LLC footpad tumor metastasizes primarily to the lungs. These results confirmed the findings of Mayo (1972a) with the i.m. LLC and extended his work by noting that metastatic foci could also be detected in the liver, spleen and kidneys. Moreover, in contrast to the published observations by Geran et al (1972) no differences could be detected in the growth rates of the i.m. and footpad LLC tumors which suggests that our data generated with the footpad LLC would be comparable with the results obtained by others using the i.m. LLC.

As BDF_1 mice became unavailable during the course of these investigations, experiments were also conducted with the M109 carcinoma in Balb/c and CDF_1 mice. Data was presented which indicated that the M109, like the LLC, is a solid murine tumor which metastasizes primarily to the lungs. Yet, in marked contrast to the findings of others with the LLC, (Otu et al., 1977; Morahan and Kaplan, 1977) the M109 clearly displayed concomitant tumor immunity, exhibiting the capacity to suppress the growth of a secondary M109 challenge.

Studies undertaken to characterize the immune competence of mice bearing the LLC indicated that the immunologic capabilities of the tumor bearing hosts were impaired. The LLC mice developed an anemia and leukocytosis and were deficient in their ability to elicit an antibody response, develop a DTH reaction, or phagocytize SRBC. It has been suggested that a poor response to SRBC may not be indicative of an immune deficiency, but rather may demonstrate the possibility that mice are undergoing an active immune response to

their tumors (Adler et al., 1971; Kerbel, 1974). However, investigations by Otu et al (1977) and Morahan and Kaplan (1977) have been unable to detect any concomitant immunity or transplantation resistance to the LLC. It could also be argued that the above results are related to the carriage of virus. Chronic infection with lactic dehydrogenase virus has been shown to compromise the immune status of tumor bearing animals (Iorio et al, 1974; Riley, 1974). Yet, the tumors utilized for these studies were periodically shown to be free from lactic dehydrogenase virus. Thus, we feel that the decreased immune responsiveness to SRBC in the tumor bearing mice is indicative of a defect in non-specific immunity induced by the tumor.

The pattern of immunodeficiency in the LLC animals appeared to be in contrast to most studies. Generally it has been found that, in man and animals bearing solid tumors, cell mediated immunity is moderately to severely depressed while antibody responsiveness remains intact (Adler et al, 1971; Biozzi et al., 1953; Fahey and Humphrey, 1962; Gatti et al., 1962; Solowey and Rapapert, 1965). However, in the present studies reduction in RES activity and the number of PFC were noted on the first days of measurement, days 1 and 7 respectively, while DTH reactivity remained intact until day 17. The impairment of humoral responsiveness and RES function cannot be attributed to the general debilatating effect of tumors, since the depression in plaque forming ability and phagocytosis were noted before the tumors became palpable and the mice showed any overt signs of illness. The possibility that the growing tumor releases an inhibitor of immune function, either directly or indirectly, seems therefore to be the most likely

explanation for these findings. This concept is supported by data which indicated that surgical removal of the primary tumor results in a rebound increase in antibody forming cells to control levels. Moreover, intact tumor cells appeared to be necessary for the impaired immune competence as sonicated tumor cells were without effect on RES function. An extensive review on the possible mechanisms for the decreased immunologic capabilities of tumor bearing animals has been presented in the Introduction.

Enhancement of vascular clearance and liver uptake were recorded in the second week of tumor growth. In contrast to the findings of Saba et al (1975) this activation cannot be attributed to liver hypertrophy, since liver weight and liver weight expressed as percent body weight in the tumor bearing animals did not significantly differ from control values. The enhanced RES function did coincide with rapid expansion of the tumor mass from 27 mg of tumor on day 5 to 316 mg 9 days after tumor inoculation, and it is tempting to speculate that the tumor products of rapidly dividing cells may be acting as a Kupffer cell stimulus.

In the terminal stages of disease, the systemic spread of the tumor coupled with the biological events secondary to the presence of the neoplasia appeared to create a generalized anergy in the LLC bearing mice. The thymic involution observed late in the disease state has been attributed by Begg (1953) and Ertl (1972) to elevated steroid levels. It follows that if seroid levels in tumor bearing hosts are sufficiently high to cause a marked and progressive thymus

atrophy, then such levels may also be high enough to contribute to the suppressed PFC and DTH responsiveness of the LLC mice.

The biological consequences of tumor induced immunosuppression have not yet been fully elucidated. Data was presented which indicated that the LLC does not suppress host resistance to Listeria monocytogenes, an infection in which macrophages and immune T cells are involved in the elimination of the microorganism. Yet, these findings may be related to the low sensitivity of the biological assay. From the data presented in Table 9 it would appear that greater than a log difference in the number of Listeria monocytogenes colony forming units is needed to demonstrate a significant reduction in host resistance, which would translate into greater than a 90% reduction in macrophage and/or T cell activity. The question also remains as to whether the depressed immune status of the tumor bearing host is directed at the tumor per se, permitting continued growth and expansion of the tumor, or only a nonspecific impairment directed at nontumor antigens. Based on the observations of Otu et al (1977) and the results presented herein, it is possible that the ability of the LLC to suppress immune function soon after inoculation is responsible for the lack of immunogenicity exhibited by this tumor. Another consequence of the suppressed immune status is that lesions may minimize the effectiveness of cancer therapy. Surgery, radiation, and chemotherapy have all been shown to be immunosuppressive (Harris and Bagai, 1972). The aggresiveness of therapy may therefore have to be minimized in immunosuppressed hosts. Moreover, the efficacy of immunotherapy

has been shown to be dependent on the immune status of the tumor bearing host (Hanna et al., 1973; Scott, 1975a).

Studies conducted with systemic MVE-3 against the LLC clearly indicated that i.v. treatment with MVE-3 alone was ineffective in prolonging life span or inhibiting primary tumor growth. Yet Snodgrass et al (1975) have reported that systemic treatment with pyran on days 1-8 after LLC inoculation inhibited the growth of the primary tumor up to 87% and increased the MST from 31.9 days to 50.4 days. The disparity between their results and ours may be related to differences in the molecular weight of the polymers and the dosing regimens employed.

The antitumor activity of polyanionic polymers has been shown to be closely related to molecular weight (Regelson et al, 1975). The experiments performed by Snodgrass et al were with a polydisperse broad molecular weight range pyran co-polymer, Hercules XA 124-177, which has an average molecular weight of 30,000 daltons (Regelson et al, 1975). Yet, pyran has been shown to be highly toxic in the clinic, producing thrombocytopenia, fever, hypotension, seizures and loss of vision in some patients (Regelson et al, 1977). In an effort to improve the therapeutic index of this class of compounds, our studies employed a lower molecular weight fraction of the parent copolymer, MVE-3, which has recently been shown by our laboratory to possess less antitumor activity against the LLC (Morahan et al., 1979).

Moreover, the treatment regimen employed by Snodgrass and co-workers differed from our protocols not only in the frequency of drug

administration, but in the initiation date of therapy. As studies by Florentin et al (1976) with BCG and Morahan (personal communication) with pyran have indicated that daily treatment appears to be no better than alternate day or weekly treatment, the relative success of their studies may largely be attributed to the early initiation date of therapy, i.e. day 1.

In contrast to systemic MVE-3 (25 mg/kg) treatment, an acute intralesional injection at 50 mg/kg proved to be efficacious by increasing life span 35.9% and 28.1% in two separate experiments. (Tables 15 and 16). It is unlikely that the efficacy of intralesional MVE-3 can be attributed to the 2-fold increase in dose as earlier experiments with i.v. MVE-3 treatment at 50 mg/kg was ineffective in prolonging MST. It is therefore our belief that the differences seen with the different routes of MVE-3 administration can be explained on the basis of drug distribution. Although synthetic polyanions are not directly cytotoxic for tumor cells (Morahan et al, 1974) localized concentrations of MVE-3 within the primary tumor bed may allow for activation of effector cells in close proximity to the tumor bed and thus inhibit the metastatic process. This concept is supported by the flow microflourimetric studies which indicated that host cells resided in the primary tumor bed. Moreover, Snodgrass et al (1975) have noted an increased infiltration of histiocytes into the primary tumor bed in pyran treated mice and recent studies by Kaplan and coworkers (unpublished observations) have shown that host cells derived from the primary tumor bed of pyran treated mice inhibited the growth of LLC cells both in vitro (^{125}IUDR incorporation) and in vivo (tumor growth).

The ability ot MVE-3 to synergize with conventional modes of treatment was assessed by employing MVE-3 in combination with surgery, chemotherapy, or radiotherapy. Although surgery and local radiotherapy markedly reduced the primary tumor burden and CTX administration substantially inhibited the metastatic growth of the LLC, systemic MVE-3 treatment still proved to be ineffective in further prolonging life span. One possible factor which may have contributed to the ineffectiveness of MVE-3 in these protocols is the immunosuppression induced by cytoreductive therapy. Surgery, radiation and chemotherapy have all been shown to be immunosuppressive (Harris and Bagai, 1972) and studies by Hanna et al (1973), Scott (1975a) and Morahan et al (1974) have shown that immunosuppression can abrogate the antitumor activity of BCG, C. parvum and pyran respectively. A second component which may have contributed to the inactivity of MVE-3 relates to the relative distribution of the polyanion. In the LLC tumor system the mice die of pulmonary insufficiency resulting from metastasis to the lung. Yet, the systemic administration of pyran to naive animals is known to distribute itself primarily to the liver and spleen, with less than 4% localizing in the lung (Regelson et al., 1970). Accordingly, systemic administration of MVE-3 probably results in "low" concentrations of the compound in the metastatic tumor bed which would not favor successful immunotherapy. This is supported by studies with BCG (Zbar et al, 1972), C. parvum (Scott, 1975b) and Bordetella pertussis (Bast et al, 1977) which have indicated that direct contact between tumor cells and the immunomodulator markedly enhances the antitumor activity of the immunomodulator.

The concepts of immunosuppression and drug distribution undermining the antitumor activity of MVE-3 are also supported by the results obtained from combining intralesional MVE-3 treatment with local radiotherapy. Intralesional therapy not only resulted in "high" concentrations of MVE-3 in the primary tumor bed early in the disease state, but may have minimized the immunosuppressive efffects of ionizing radiation (Lindahl and Edelman, 1968; Prempree and Merz, 1969; Schmidke and Dixon, 1973; Geiger et al, 1973). Alternatively, irradiation has also been shown to produce changes in the antigenic structure of tissues (Alekeoff, 1970) which may have enhanced the tumor cell antigenicity of the LLC.

Data was presented which indicated that the LLC quickly metastasizes to the lung where it is not amenable to surgery or local radiotherapy. Moreover, results obtained from intralesional MVE treatment indicated that localized concentrations of MVE-3 in the primary tumor bed were effective in prolonging life span, presumably by inhibiting the metastatic process. Thus, an attempt was made to localize MVE-2 in the lung by exposing the noses and mouths of the LLC bearing mice to an aerosolized mist of MVE-2. Preliminary studies with naive mice of different strains suggested that MVE-2 administered by inhalation becomes localized in the lungs as lung weights were significantly increased, but the hepatosplenomegaly which accompanies systemic MVE-2 treatment was not evident after 10 daily exposures to an aerosolized mist of MVE-2. Nevertheless, in the one pilot study conducted with LLC bearing mice, MVE-2 failed to prolong survival time. Necropsy at the time of death revealed lungs heavily laden with tumor suggesting

that death was not due to the metastatic spread of the LLC to other vital organs.

One possible reason why MVE-2 was ineffective in prolonging MST relates to the therapy regimen which was empirical in regards to dosimetry and schedule. As synthetic polyanions have been shown to inhibit as well as enhance tumor growth (Kripke and Borsas, 1974; Schuller et al, 1975) the efficacy of MVE-2 administered by inhalation to LLC mice should not be discounted until systematic studies have deliniated the optimum dose and schedule dependency of MVE-2 administered by this route of administration.

Another factor which most assuredly contributed to the ineffectiveness of MVE-2 is the lack of tumor cell antigenicity exhibited by the LLC (Otu et al, 1977; Morahan and Kaplan, 1977). As an underlying concept of tumor immunology is that there are characteristic antigens in or on tumor cells that distinguish them from normal host cells it is not surprising that weakly antigenic tumor systems have not been very responsive to treatment with immunotherapy (Parr, 1972; Baldwin and Pinn, 1973; Bast et al, 1976). Indirect evidence in support of this concept was derived from experiments performed in our laboratory with contaminated LLC cells. In two separate studies, systemic MVE-3 treatment resulted in a significant prolongation in survival time and inhibition of tumor growth when highly antigenic bacteria resided in the primary tumor bed.

The results obtained with systemic and intralesional C. parvum against the LLC were significantly better in comparison with MVE-3. Yet, the reasons or the increased efficacy of C. parvum are unknown.

Activated macrophages have been implicated as major effectors of tumor resistance induced by both of these drugs (Kaplan and Morahan, 1976), and comparable antitumor activity is usually displayed by these agents (Table 25). Moreover, the distribution and persistence of C. parvum as determined by ^{125}I-labeling (Scott and Milas, 1977) or ^{99m}technetium-labeling (Barth and Singla, 1978) parallels the results obtain with pyran (Regelson et al, 1970). However, the lack of a pronounced effect with C. parvum can probably be attributed to the same factors as previously mentioned; lack of LLC tumor cell antigenicity, low levels of C. parvum localized in the lung, and immunosuppression induced by the tumor and cytoreductive therapy.

In contrast to the LLC studies with MVE-3, the M109 was responsive to a less active lower molecular weight fraction of maleic vinyl ether, MVE-2. Although acute administration of MVE-2 did not markedly effect MST, multiple injections of MVE-2 significantly prolonged the life span of the host and inhibited the growth of the primary M109 tumor. MVE-2 proved to be effective when the hosts had a mean primary tumor burden of 284 mm^3 and established metastatic foci (Table 26). These data also confirmed the findings of the LLC studies in that the efficacy of MVE-2 appeared to be partially dependent on drug distribution. Early in the disease state intralesional injections proved to be most effective in inhibiting tumor growth and prolonging the MST. Yet, as the tumor metastasized to the lung, systemic administration of MVE-2 alone, or MVE-2 coupled with surgical excision or radiotherapy of the primary tumor became increasingly less effective.

Late in the disease state only MVE-2 introduced directly into the metastatic tumor bed by intrapleural injections or inhalation proved to be efficacious in prolonging life span.

In summary, data was presented which indicated that the ability to control growth of the LLC with nonspecific immunotherapy was modest. Early intralesional administration of MVE-3 marginally enhanced life span, presumably by inhibiting the metastatic process. Yet, as the tumor metastasized to the lung, systemic administration of MVE-3 alone, or MVE-3 coupled with surgery, chemotherapy, or local radiotherapy was ineffective. Conversely, the M109 tumor was shown to be relatively responsive to MVE treatment. Multiple injections of MVE-2 in doses ranging from 10 to 50 mg/kg significantly prolonged the life span of the host and inhibited the growth of the primary tumor. The efficacy of MVE therapy appeared to be dependent on drug distribution since routes of administration which localized MVE in the primary tumor bed early in the disease state or in the metastatic tumor bed late in the disease state were the most effective in prolonging life span. The divergent results obtained with the two tumor lines may be attributed to the immunosuppressive effects of the LLC and the tumor cell antigenicity exhibited by the M109.

V. PROPOSED FUTURE RESEARCH

Future research efforts with MVE should be directed towards optimizing the parameters for inhalation treatment and understanding the mechanism(s) of antitumor action. Initial experiments should be aimed at deliniating procedures that will maximize the effectiveness of MVE-2 against the metastatic M109. These studies would involve determining the schedule and dose dependency of MVE delivered by the inhalation route. Moreover, as preliminary inhalation experiments have suggested that death of the M109 bearing mice might be due to neoplastic growth in the liver, experiments should also be performed employing inhalation treatment in conjunction with systemic therapy (MVE and/or chemotherapy). Simultaneously with the foregoing studies experiments should be directed towards calibrating the inhalation procedure possibly by using quinine sulfate in a flourescence assay or by employing radiolabeled MVE under controlled conditions.

To adequately assess the efficacy of MVE delivered by the inhalation route it would be desirable to conduct parallel investigations in other metastasizing murine tumor models which display a different spectrum of activity towards immunotherapy, such as the LLC or B16 melanoma. Furthermore, as MVE has been shown to possess antiviral activity (Hirsch et al, 1972; Morahan et al, 1975), an interesting and clinically useful fallout from these studies might materialize by administering MVE by inhalation to mice infected with influenza virus.

Once the parameters required for maximum cures have been delineated the major effort should be directed towards determining the mechanism(s) of action. Transplantation resistance studies should be initially conducted as it would be of primary importance to determine whether the "cured" mice are immune to rechallenge, or whether tumor growth was inhibited by non-immunologic mechanisms. Briefly, mice that are cured would be rechallenged with graded doses of homologous and heterologous tumor cells, and the incidence, latency and growth of the tumors measured. If immunity is observed, the particular effector cells could be identified by means of a Winn neutralization assay or passive transfer of various cell types (alveolar macrophages, blood monocytes, etc.) into tumor bearing recipients. If it became apparent that the cured animals are not specifically immune, this would suggest a role for nonspecifically activated macrophages in eliciting the cures. Once the cell type(s) responsible for the tumor inhibition have been identified in vitro assays should be designed to characterize the host cell-tumor interaction.

The proposed investigations should allow immunotherapeutic protocols to be developed on more rational bases than empirical grounds. Understanding the optimum parameters of application and mechanism of action of MVE is required before consistent beneficial effects can be produced.

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