Induction of Immunological Tolerance to Tissue Allografts with Antilymphocyte Serum*

ANTHONY P. MONACO And MARK A. HARDY

Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115

Introduction

At the present time successful clinical organ transplantation depends on chronic administration of immunosuppressive agents to prevent the persistent tendency of the recipient to reject the graft. Chronic immunosuppression—although often successful in maintaining graft function and viability—is frequently associated with numerous and often fatal complications. These associated problems include: 1) inherent toxicity of immunosuppressive drugs per se, e.g., Imuran (hepatitis), steroids (osteoporosis, gastrointestinal bleeding, etc.), Cytoxan and actinomycin C (thrombocytopenia, leukopenia); 2) occurrence and persistence of low-grade rejection in the graft, e.g., endarteritis in renal and cardiac allografts, while function is maintained; 3) occurrence of severe and bizarre infections, usually those associated with cellular immunity (particularly of the viral, protozoan and fungal varieties); and, finally, 4) spontaneous appearance of malignancy, presumably due to the abolition of the immunological surveillance mechanism which is probably operative in preventing the *de novo* appearance of malignancy in most normal human beings. For these reasons specific immunological tolerance remains the ultimate and eventual solution to the widespread application of clinical organ transplantation.

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Immunological Tolerance

The phenomenon of actively *acquired immunological tolerance* was discovered during studies of tissue transplantation. This term was used to describe a specific state of unresponsiveness to an antigen or antigens in adult life as a consequence of exposure to antigen in utero or in the neonatal period. In a study of the immunogenetic consequences of vascular anastomoses between cattle twins in utero, Owen (1945) showed that most cattle twins at birth are erythrocyte chimeras as they possess erythrocytes of their own genotype as well as those of the opposite twin. This chimeric state was shown to persist beyond the life span of the erythrocyte, suggesting that erythropoietic cells (stem cells) were exchanged in fetal life and continued to produce erythrocytes of characteristic serological type throughout adult life.

The wide significance of Owen's observation was emphasized by Burnet and Fenner (1948) in a general theory of the immune response which predicted the phenomenon of tolerance. To explain the failure of adult animals to react immunologically to their own tissues, these authors suggested that the body cells possess some type of self-marker component, and that the capacity to recognize this self pattern develops during embryonic

or early postnatal life. They predicted that exposure to an antigen in embryonic life would cause that antigen to be recognized as self in later life, and, consequently, no immune response would be made to it.

In future studies, it became apparent that erythrocyte chimerism was not the only manifestation of tolerance in dizygotic cattle twins which appeared after mixture of placental circulations. Dizygotic cattle twins were shown to accept skin grafts from each other, and this mutual tolerance was specific since skin grafts transplanted from third parties were quickly rejected (Anderson et al., 1951). These observations in nature set the stage for the production of actively acquired tolerance by Medawar and colleagues in the laboratory. These investigators injected mouse embryos of the CBA strain in utero through the anterior abdominal wall with a suspension of living spleen cell lymphocytes of the A strain (Billingham, Brent and Medawar, 1953). The CBA recipients, regularly accepted A-strain skin grafts when grafted in adult life. This tolerance of A-strain grafts was specific, since CBA mice tolerant of A-strain tissues easily rejected allografts from the unrelated AU strain. At the same time, Hašek reproduced Owen's observations in chickens by the ingenious method of making a deliberate synchorial parabiosis of embryos via the chorioallantoic membrane (Hašek, 1953). At hatching, the parabiots were separated and were found to be tolerant not only of each other's red cells, but skin allografts. Subsequently, it was found that in utero injection was unnecessary, and that intravenous injection during the early neonatal period also produced tolerance. In these experiments tolerance could be abolished by the injection of tolerant animals with lymphoid cells from normal adult members of the same syngeneic strain. Furthermore, careful testing showed that the tolerant mice were, in fact, chimeras insofar as their lymphoid cell populations were concerned (Billingham, 1958). Tolerance induced in the neonatal period was frequently permanent. This was in sharp contrast to the transient nature of the tolerance to chemical antigens observed by a number of other investigators when non-living, non-replicating antigens, such as proteins, were injected into neonatal animals for varying periods from birth to adulthood.

The demonstration by Smith and Bridges (1958) and Mitchison (1959) of the necessity of repeated injections of non-replicating antigens to maintain tolerance toward these antigens, coupled with the observation of chimerism in tolerant mice in Medawar's experiment, emphasized the concept that persistence of antigen was necessary for the maintenance of tolerance.

Another important aspect of tolerance induction involves the genetic relationship between the donor and the immature host. It was found that the more distant the histocompatibility relationships between donor and host, the more difficult it was to induce tolerance. When strong histocompatibility barriers existed, large innoculums of cells were required, the intravenous route was obligatory, and the tolerance-responsive period extended only to a short time after birth. On the other hand, when the genetic and, therefore, antigenic disparity between donor and host was relatively weak, such stringent requirements disappeared. In fact, when mice of very weak histocompatibility differences were used, the tolerance-responsive period extended even into adulthood (Shapiro et al., 1961).

Graft-Versus-Host Reaction

Just as the phenomenon of actively acquired tolerance was discovered in tissue transplantation research, so also was the *graft-versus-host reaction* (hereafter, GVH reaction) basically an outcome of research in immunological tolerance. Simonsen (1957) found that adult splenic cells, when injected into newborn or embryo chickens and mice, produced a disease that could only be interpreted as an outcome of an immunological response by these grafted cells against the host. Simultaneously, Billingham and Brent (1957), in their attempts to induce tolerance in newborn mice, found that, in certain strain combinations, all injected mice died from a peculiar wasting disease. They concluded that a GVH reaction was the likely explanation of this pathological condition which they named runt disease. GVH reactions result when a recipient of a graft comprised of immunologically competent cells is incapable of rejecting the cellular graft. This situation typically arises in very young individuals (embryos and newborn recipients). It also occurs in adults when a graft from a homozygous donor, i.e., genetically AA, is given to a host that is an F_1 hybrid between the donor strain and a dissimilar strain, i.e., genetically AB. A graft A transplanted to an AB host represents a situation in which the hybrid cannot reject the graft by immunological means even though the host may be entirely competent immunologically. This is true because the graft does not possess any antigens that are foreign to the host. The graft, however, can react against the host, since the latter contains foreign antigens derived from the dissimilar parent of the hybrid.

The mouse is the classic animal for the production of GVH reactions and runt disease. In severe reactions, mice grow normally for about a week, but thereafter growth ceases, and there is subsequent weight loss, associated with diarrhea, alopecia, dermatitis and eventual death. This pathological condition has been demonstrated in numerous other species. It is of great clinical significance that human recipients of bone marrow

transplants, especially after immunosuppressive treatment with whole body irradiation or cytotoxic drugs, have shown all the symptoms of classical GVH reactions similar to those observed in lower species. The most important parameters which determine severity of GVH reactions are: 1) the immune competence of the cells utilized; 2) the dose of the cellular innoculum; and 3) the degree of histocompatibility difference between the donor and the recipient. The pathological symptoms and the mortality rate increase as these parameters increase.

Immunological Tolerance and Antilymphocyte Serum

Induction of specific immunological tolerance to either tissue allografts or non-replicating, chemically defined antigens, such as proteins, is by no means limited to neonatal or immunologically incompetent young animals. Tolerance can be induced in adult, immunologically competent animals under a number of special circumstances. For example, it may be induced by the injection of massive amounts of antigen (so-called immunological paralysis or antigen overloading) or by the injection of antigen during certain times of immunosuppression or lymphocyte depletion. Immunological depression in adult animals to abet the induction of tolerance has been achieved in a number of different ways, i.e., irradiation, cytotoxic drugs, steroids or a combination of these (Russell and Monaco, 1965). In our laboratory, heterologous antilymphocyte serum (ALS) has been used as an immunosuppressive agent to facilitate the induction of tolerance to tissue allografts in adult animals. It is the purpose of this report to review our experiments in this particular area.

Heterologous ALS is an extraordinary immunosuppressive agent. Mice treated with this reagent show a dramatic inability to reject tissue allografts and xenografts (Gray et al., 1966; Monaco et al., 1966). Recent evidence indicates that ALS acts mainly on peripheral circulating lymphocytes (Taub and Lance, 1968). The mechanism of action of ALS continues to be uncertain, but evidence suggests (Monaco, Wood and Russell, 1965a) that its immunosuppressive property depends on its effects on thymus-dependent, immunologically competent lymphocytes. ALS frequently induces peripheral lymphopenia and tissue lymphocyte depletion, and the treated animals are nonspecifically immunosuppressed. Some serums, equally immunosuppressive, do not necessarily produce a dramatic and sustained lymphopenia. It appears, therefore, that for ALS to be immunosuppressive, it must act on a select group of peripheral lymphocytes which is probably quite small in number, and its effectiveness is not dependent on the lymphopenia per se, but rather on the depletion or destruction of a certain population of immunocompetent, thymus-dependent cells. Recent work suggests that ALS is highly effective in depressing cellular-type immunities, as typified by reaction of tissue allografts, whereas the humoral antibody response to certain antigens, especially bacterial ones, is much less affected.

The depression of immune capability following treatment with ALS may be due to the decreased number of mature lymphocytes and/or to the immune incompetence of remaining lymphocytes. From our experiments it appears that the lymphoid cells exposed to ALS in vivo an din vitro do not live on and replicate (Van der Werf et al., 1968), since they do not induce GVH reactions in susceptible recipients or tolerance and chimerism in neonatally injected histoincompatible mice. This supports the concept that the cells remaining in lymph nodes after rabbit anti-mouse lymphocyte serum (RAMLS) treatment are immunologically incompetent. These remaining cells (or their descendents) eventually become immunologically competent, perhaps under the influence of the thymus or a thymic product.

Experiments have been done in mice and rats to determine whether or not tolerance is a property associated with the thymus or thymus-derived cells. Galton, Reed and Holt (1964) showed that allograft tolerance occurs with the achievement of thymic chimerism. This suggested that the presence of allogeneic cells within the thymus is essential for the induction and maintenance of a tolerant state and that the target cell for tolerance induction must be the lymphoid precursor cell within the thymus. The presence of the antigen within the thymus and the existence of tolerance may, however, be coincidental, as has been suggested by Miller and Osoba (1967). Other investigators (Follett, Battisto and Bloom, 1966) have shown that the lymphoid system in adult-thymectomized animals can be rendered specifically tolerant to defined antigens. Thus, the presence of the thymus is not necessary for tolerance induction. This does not exclude the lymphoid precursor cells as the target cells for tolerance induction. These may be present in the thymus-dependent areas of lymphoid organs (Holborrow, 1968) and be influenced by a thymic humoral factor (Hardy et al., 1968). The breakdown of tolerance occurs when immunologically competent cells are recruited from precursors which have not been exposed to the tolerance-inducing antigen. This recruitment appears to be dependent on the thymus or on a thymic humoral factor. Claman and Talmage (1963) showed that, in mice previously rendered tolerant to horse serum albumin, adult thymectomy delayed the spontaneous escape from tolerance. Argyris (1965) showed that the transplantation of C3H/He thymus tissue accelerated the breakdown of tolerance in C3H/He mice tolerant

to CBA skin grafts. Goldstein et al. have recently shown that tolerance to A/Jax skin in neonatally thymectomized CBA mice can be abolished by a thymic humoral factor (unpublished data). It appears, therefore, that the thymus or a thymic humoral factor plays an important role in the induction and the abolishment of a tolerant state, presumably by its influence on the development and the recruitment of immunologically competent cells.

Although ALS is highly effective in depressing cellular type immunities—as typified by rejection of tissue allografts—while preserving relatively intact the humoral antibody responses to certain bacterial antigens, it must be emphasized that ALS-induced suppression of tissue graft rejection is still nonspecific. Thus, third-party allografts placed on mice treated with ALS while an earlier test allograft is alive are rejected at the same time as the test allografts (Monaco et al., 1966).

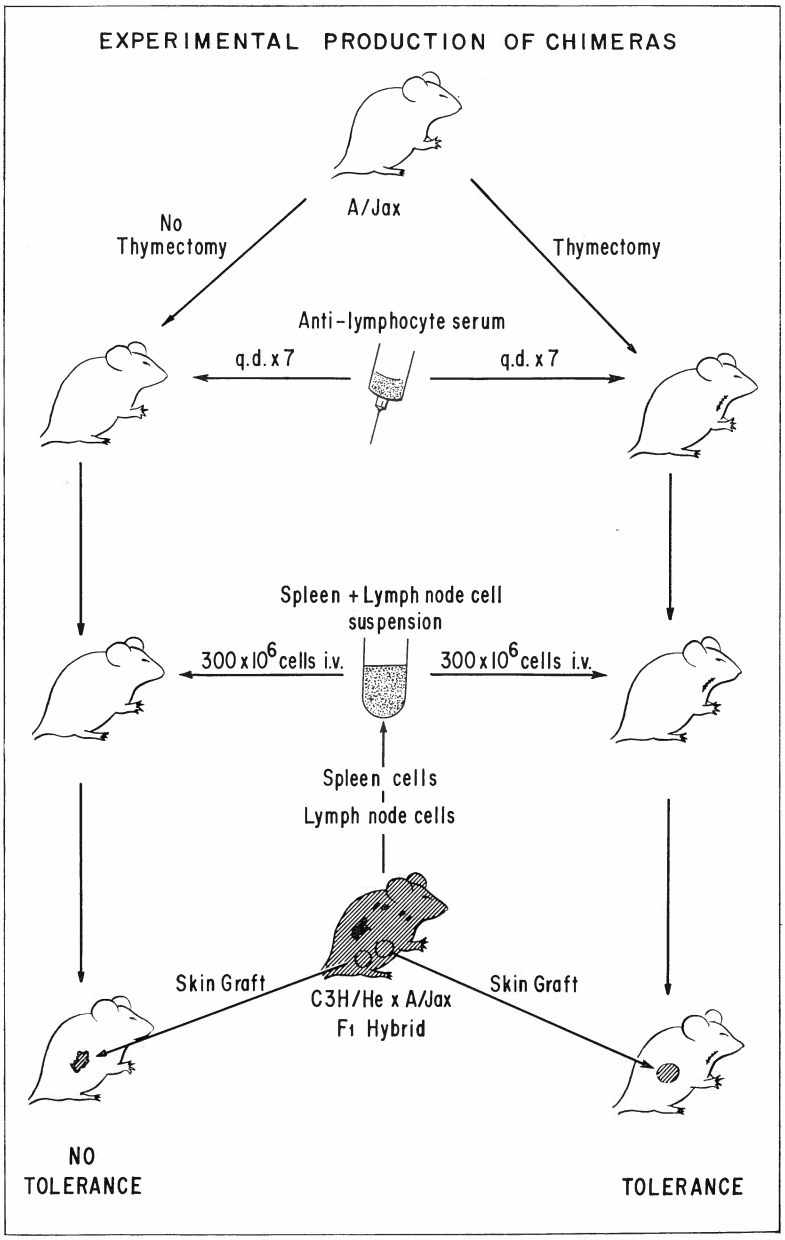


Fig. 1—The experimental production of chimeras and specific immunological tolerance with antilymphocyte serum. (Reprinted from Monaco, Wood and Russell, 1966.)

Furthermore, adult thymectomy and ALS treatment produce a prolonged period of nonspecific immune incompetence in the capacity to express cellular type immunities. Adult-thymectomized, ALS-treated mice frequently bear healthy allografts for over 100 days. Third-party grafts applied at that time also frequently show prolonged survival. This profound immune incompetence induced by adult thymectomy and ALS seemed analogous to the unresponsiveness seen in neonatally thymectomized mice. Because of this similarity, we felt it might be possible to induce tolerance in adult animals treated in this manner.

In the initial experiments (Monaco, Wood and Russell, 1966) adult-thymectomized and normal A/Jax mice were treated with ALS and infused with C3H/He × A/Jax F_1 hybrid cells intravenously. F_1 hybrid cells were used to avoid a GVH reaction (Fig. 1). C3H/He skin allografts in adult-thymectomized, ALS-treated, cell-infused mice showed a markedly prolonged survival, as compared to adult-thymectomized animals receiving ALS but no cell infusion. Furthermore, thymectomized, ALS-treated, cell-infused mice rejected third-party C57BL/6 J skin in a normal fashion while retaining their first and, even, second C3H/He skin allografts (Fig. 2). This emphasized the fact that they were specifically tolerant to C3H/He tissues. These tolerant mice were found to be lymphoid cell chimeras, containing lymphocytes of both A/Jax and C3H/He genotypes. The tolerance was abolished by injection of normal (syngeneic) A/Jax lymphocytes, a finding similar to that noted

by Medawar and colleagues in their tolerance experiments in neonatal animals. This acquired state of tolerance associated with a state of lymphoid cell chimerism was seen in all the thymectomized mice which received both ALS and a cell infusion.

Since hybrid donor lymphoid cells would never be available in clinical situations, attempts were made to reproduce the above experiment using non-hybrid (homozygous) allogeneic lymphoid cells. Severe GVH reactions resulted when high doses (300×10^6) of cells were used. To avoid the GVH reaction, we used allogeneic histocompatibility antigens in a cell-free form from the supernatant of mechanically disrupted spleen cells (Monaco, Wood and Russell, 1965b) of the skin graft donor. The addition of this cell-free antigen (CFA) to a course of ALS increased the prolongation of skin allografts slightly over that achieved with ALS alone. The addition of CFA to a course of ALS in adult-thymectomized mice almost doubled skin allograft survival as compared to that in ALS-treated thymectomized mice (Abbott, Monaco and Russell, 1969). Of major importance was the finding that third-party grafts were rejected relatively normally in the thymectomized animals receiving ALS and CFA, and, therefore, the prolonged survival of the original allograft was a form of specific acquired tolerance in the adult.

The tolerance induced with CFA in such lymphocyte-depleted animals may be long lasting but is not permanent. Furthermore, the number of animals in any group showing a great degree of tolerance is less than when replicating lymphoid cells are used as the donor antigen. Presumably this results from a regeneration or increase in the number of immunologically competent cells which can, again, react to the foreign antigen. Since these cells are probably thymus-dependent, as we indicated earlier in this article, one may justifiably question the reappearance of immunocompetent cells in an ALS-treated thymectomized mouse. It is probable that a small number of immunologically competent cells can regenerate from

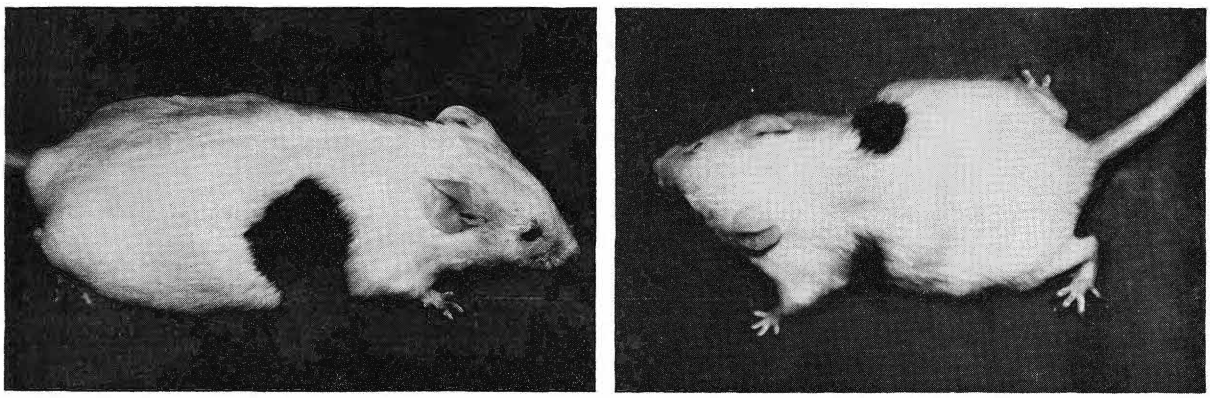
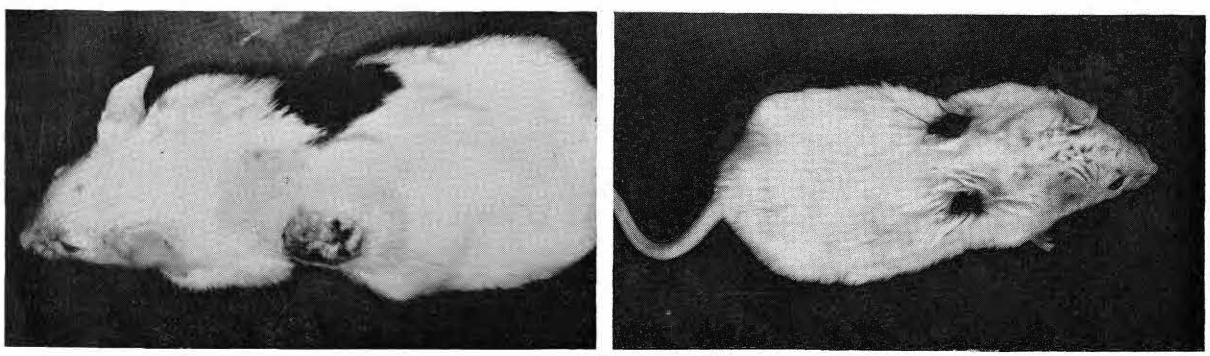


Fig. 2—A. An adult-thymectomized A/Jax mouse which received ALS for seven days followed by 300×10^6 C3H/He lymphoid cells, as in Figure 1. The mouse bears a C3H/He graft in perfect condition for 100 days.

B. The same mouse bearing the same first C3H/He graft as well as a second C3H/He graft on day 150.



C. A similar adult-thymectomized, ALS-treated, cell-infused mouse bearing a perfect C3H/He graft over 100 days but simultaneously rejecting a third-party C57B1/6J skin allograft—i.e., indicating specific tolerance.

D. The mouse visualized in B after infusion with normal syngeneic A/Jax lymphoid cells, showing rejection of previously well-tolerated C3H/He grafts—i.e., the abolition of specific tolerance.

the bone marrow in the absence of the thymus. Another possibility is that residual stem cells in the peripheral lymphoid system (lymph nodes, Peyer's patches, etc.) may differentiate to immunologically competent cells under the influence of a thymic humoral factor which persists in such tissues after thymectomy. It may be possible to circumvent this regeneration by the addition of small doses of immunosuppressive agents (perhaps in association with specific antigen) to prevent the regeneration of new immunocompetent cells. Apparently, when a chimeric state is established, regeneration of competent cells and loss of the tolerant state is delayed or prevented by persisting donor antigen in the form of chimeric lymphoid cells.

Until recently we were unable to induce any form of specific tolerance in the non-thymectomized, ALS-treated adult mice. On the contrary, we found that such animals were sensitized after infusion with allogeneic F_1 hybrid lymphoid cells. These seemingly incompatible results may be explained by Mitchison's (1965) elegant demonstration that the antigen dose frequently determines whether tolerance or sensitization results from antigen introduction. This investigator showed that tolerance to bovine serum albumin in mice resulted when the protein was administered in two dose zones, i.e., in high and low ranges. Doses in other ranges either produced sensitization or no effect. The concept of high zone and low zone tolerance was thus introduced. We tested the effects of various doses (0.01, 0.1, 1.0, 10 and 100 $\times$ 10^6) of homozygous allogeneic C3H/He lymphoid cells in five groups of ALS-pretreated (non-thymectomized) A/Jax mice. Controls received ALS but no cells. Figure 3 shows the survival curves of the test allografts in the various groups. C3H/He skin allografts survived longest in A/Jax mice which received 10 $\times$ 10^6 cells, thus indicating a potentiation of ALS immunosuppression by cell infusion, i.e., partial tolerance. The grafts in the groups receiving 0.1 $\times$ 10^6 or 100 $\times$ 10^6 had a shorter survival time than the ALS controls, indicating a partial sensitization by this dose of cell infusion. Mice receiving .01 $\times$ 10^6 or 1 $\times$ 10^6 cells rejected their grafts at the same time as the controls treated with ALS alone, showing that there was no effect of doses of cells in these ranges. These observations in ALS-treated mice receiving cell infusions are analogous to those of Mitchison on various protein antigens in which the resulting reactions of sensitization and/or tolerance are probably dependent on the dose of antigen utilized. Of importance in these experiments, from the point of view of potential clinical application, was the observation that in these lower dose ranges where partial tolerance resulted, there was little if any observed GVH reaction.

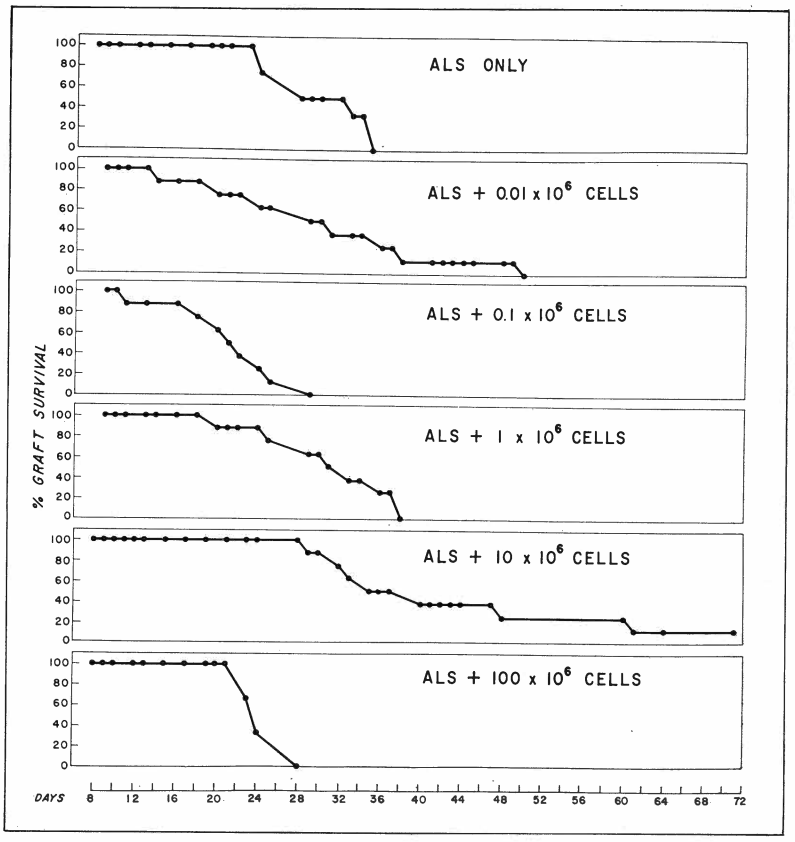


Fig. 3—Survival curves of C3H/He skin allografts on ALS-treated A/Jax mice infused with various doses of C3H/He allogeneic, non-F_1 hybrid lymphoid cells. (Monaco, in press, 1969.)

Lance and Medawar (1969), in a similar experiment, have emphasized the effect of the timing of injections of cells on the probability of their potentiating or sensitizing the recipient of the graft. Furthermore, these investigators demonstrated that GVH reactions can be avoided by using cells with decreased immune competence, i.e., thymocytes, bone marrow cells, and cells from ALS-treated donors.

That this phenomenon of acquired partial tolerance induced with donor antigen is not peculiar to skin allografts in mice is well established. Organ allografts appear to be less antigenic than skin allografts; hence, it will probably be easier to develop a tolerant state

to an organ allograft than to a skin allograft. We must further remember that organs are attached by direct vascular anastomosis and that the antigen is chronically released. This may be important in maintaining a tolerant state. Thus, a permanent state of chimerism might not be necessary to achieve a permanent state of tolerance. Seifert et al. (1966) obtained significant improvement in renal allografts by pretreating dogs with subcellular (cell-free) antigen and 6-mercaptopurine and adding methylprednisolone after transplantation. Dagher et al. (1967) used a two-week pretreatment course with soluble cytoplasmic antigen from viable donor spleen cells and demonstrated a prolonged renal allograft survival in dogs when minimal doses of azathioprine and methylprednisolone were given after renal transplantation. These studies of whole organ allograft survival suggest that the prolongation was augumented by the donor antigen. The use of specific donor antigen appears promising as an adjunct in immunosuppressive therapy of whole organ allografts.

Conclusion

Our interest in the problem of tolerance induction is directly concerned with clinical organ transplantation. ALS is highly effective in depressing cellular immunities. Since at least initial allograft rejection is predominantly a cellular phenomenon, one would expect ALS to be highly effective clinically. Our initial observations in this regard support this concept. However, non-specific depression of cellular immunity may also lead to an increased number of viral, fungal, and protozoan infections. Experiments, such as those presented, strongly suggest that a specific state of tolerance to organ grafts in man should be attainable with the aid of ALS followed by introduction of appropriate antigen. A number of avenues are open to increase the likelihood of success for clinical induction of tolerance. Improved methods of histocompatibility typing will aid in tolerance induction, since tolerance is more easily established when histocompatibility differences are minimized. Much effort is being expended in isolation of human cell-free histocompatibility antigens in a soluble form (Kahan and Reisfeld, 1969). Experimental evidence so far suggests that soluble antigens are less immunogenic and more tolerogenic. Development of optimal (non-sensitizing, tolerogenic) antigenic dose schedules—to be administered after immunosuppression has been achieved—should facilitate clinical tolerance induction. If antigens are utilized in a cellular form—as is our tendency at present—then replicating lymphoid cells from ALS-treated donors, replicating non-immunologically competent cells (thymocytes, bone marrow cells), or nonreplicating parenchymal cells of appropriate organs (liver, kidney, etc.) might, theoretically, be utilized. It is apparent that organ transplantation cannot be fully applied until the problem of specific allograft tolerance is solved. A practical solution to the problem may be closer at hand than we realize.

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