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EXTRACTION OF XENOTRANSPLANTATION ANTIGENS
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AND STUDIES OF XENOGRAFT REJECTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy
in the Graduate School of the Ohio State University

By
Jacqueline Bowers Rice, B. A., M. T. (ASCP)

The Ohio State University
1977

Reading Committee:  Approved By
Raymond W. Lang, Ph.D.
Bernard U. Bowman, Ph.D.
Frank A. Kapral, Ph.D.
J. Dennis Pollack, Ph.D.

Adviser
Department of
Medical Microbiology
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VITA

June 10, 1938 .............. Born - Pittsburgh, Pennsylvania

1960 ..................... B. A., University of Wisconsin - Milwaukee, Milwaukee, Wisconsin

1967 - 1970 .............. NDEA Title IV Fellowship and NIH Traineeship, Marquette University, Milwaukee, Wisconsin

1973, 1974, 1975 ........ Graduate Research Associate, Department of Medical Microbiology, The Ohio State University, Columbus, Ohio

PUBLICATIONS


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INTRODUCTION

Clinical organ transplantation has achieved success in the past two decades. Transfer of a kidney from either a living related donor or a cadaver to a patient with terminal renal disease has now become an accepted therapeutic measure. The initial increasing success in human transplantation was due to the solving of surgical procedures as well as the use of improved immunosuppressive agents and methods of tissue typing. However, in recent years a decline in the number of successful transplants based on yearly kidney transplant survival rates has occurred. This is true despite immunosuppressive therapy and improved methods of tissue typing. Clearly, the immune response of a patient to a foreign graft persists as the major obstacle preventing widespread application of transplantation therapy for a variety of diseases.

To investigate means to increase graft survival, much work has been done in animal models using donor antigens, often in combination with immunosuppressive agents, to induce specific unresponsiveness to transplantation antigens. Most workers have found that greater graft prolongation is attained by the use of donor cells
or antigen extract before the time of grafting rather than afterwards. This pre-grafting antigen treatment method presents a major obstacle when transposing the techniques to the human situation. Because of the limited supply of suitable organs from living related donors, more than half of the kidneys for renal transplants come from cadaver donors and, necessarily, in the case of unpaired vital organs, all come from cadaver donors. One solution to this dilemma would be the use of animal organs for human subjects. A pool of selectively bred primate donors would offer the opportunity and time for appropriate donor-recipient matching. Any manipulations of donor or recipient, in addition to the preparation of donor specific antigen, could be performed well in advance of the anticipated transplant date.

In the first couple decades of this century, before the importance of immunological mechanisms in transplantation were recognized, several clinical renal xenografts were attempted with little success. Nevertheless, it was established that an organ from a non-human species could be anastomosed to man and function (Neuhof, 1923). The modern era of xenotransplantation began in 1963 after it was shown that immunosuppressive agents were successful in prolonging allografts. Kidneys from the chimpanzee
(Reemtsma et al., 1964a), baboon (Starzl et al., 1964) and monkey (Reemtsma et al., 1964b) were transplanted into man. The clinical results generally coincided with the genetic similarity of donor to man. Thus, monkey kidneys functioned for only 10 days, baboon kidneys for 2 months, while transplanted chimpanzee kidneys survived longer, with one lasting 9 months. These early clinical trials indicated the major barrier to successful xenotransplantation was immunological in nature.

Future success in clinical xenotransplantation will depend on gains in knowledge of the mechanisms of xenograft rejection, introduction of better immunosuppressive agents and regimens, improved methods of tissue typing and detection of pre-existing immunity, and most importantly, ability to induce specific tolerance or enhancement to xenotransplantation antigens. These areas can best be investigated by the study of an animal xenograft model. Although a primate model would offer information more directly applicable to man, the number of animals required and the cost make it prohibitive for basic studies. A rodent model offers the advantage of minimal cost and the availability of genetically inbred animals to simplify the study of mechanisms of graft rejection and tolerance/enhancement. In addition, there exists a wealth of useful
information on allografting and allotransplantation antigens in rodents. For these reasons we believe it is advantageous to conduct initial experiments in a rodent model and then apply the results to a primate model for further adaptation before attempting their use in man.

Research in xenotransplantation has been discouraged because of 1) adherence to the immunogenetic rule that the greater the genetic disparity, the faster the graft is rejected; 2) induction of tolerance with the aid of anti-lymphocyte serum (ALS) is difficult in proportion to this genetic disparity (Lance and Medawar, 1969, and Stuart, 1973); and 3) the weaker the histoincompatibility, the greater the effectiveness of specific immunoblocking antibodies (Hildemann, 1973). There is, however, an abundance of evidence which indicates that first-set xenograft rejection between closely related species is associated with the kind of rejection usually assigned to allografts, e.g. cell-mediated rejection. Pathologic studies of primate kidneys transplanted to man demonstrated changes indistinguishable from those seen in allografted kidneys (Porter, 1964). Perper and Najarian (Reemtsma, 1971) found that transplants between sheep and goats survived as long as did allografts. Baldamus et al. (1973) described the rejection of rat skin grafted onto
untreated adult mice. The xenografts behaved in most respects as allografts but were rejected sooner. Histologic examination at the onset of rejection revealed an early infiltration of leukocytes, followed by edema, interstitial hemorrhage and necrosis of donor cells. Cellular infiltration was more pronounced than that seen in allograft rejection, but in both situations it consisted primarily of mononuclear cells with polymorphonuclear leukocytes (PMN) appearing only after necrosis had developed. Lance (1970) reasoned that since antibody was detected in mice bearing intact rat or guinea pig skin and receiving ALS (which interferes mainly with cell-mediated immunity), cellular mechanisms were primarily responsible for skin xenograft rejection. He demonstrated that high-titered anti-rat antibody could accelerate breakdown of a rejecting graft and concluded that humoral antibody probably plays a more important role in rejection of xenografts than of allografts but that cellular factors are still the more important. Hamilton and Gaugas (1972) arrived at a similar conclusion through their experiments. They showed that either 0.1 ml of high-titered anti-graft serum obtained from mice which had undergone a second-set rejection of hamster skin or $10^5$ sensitized lymph node cells could initiate breakdown of established hamster skin grafts on thymectomized, irradiated mice. In contrast,
even large (0.4 ml) doses of antiserum collected after first-set rejection failed to cause any damage to the graft. It also had little complement-dependent cytotoxic activity for target hamster lymph node cells. Thus, second-set but not first-set skin xenograft rejection may be mediated by antibody alone. Histologic examination of graft sites confirmed which effector limb of the immune response caused rejection, i.e. an Arthus reaction was observed when antibody was introduced and lymphocytic infiltration when sensitized cells were injected. Further clarification of the mechanism responsible for first-set xenograft rejection was provided by Hines et al. (1976). They showed that while mouse spleen and lymph node cells caused cell-mediated cytotoxicity against donor rat lymphocytes after primary skin xenografting, it was significantly less than that demonstrated in the corresponding rat and mouse allogeneic systems. It was suggested that the more vigorous xenograft rejection was due to a larger participation of humoral antibody or perhaps to antibody-dependent cell cytotoxicity.

That hyperacute rejection of vascularized allografts and xenografts is mediated mainly by preformed humoral antibody to graft antigens is generally accepted. There is also abundant evidence that acute and chronic rejection
of human renal allografts is associated with humoral antibodies against donor tissues (Winn, 1970). This association was supported by the observation, on microscopic examination, of vascular obliterative lesions containing immunoglobulin and complement.

Because organ grafts with vascular endothelium are more susceptible to the effects of humoral antibody than are skin grafts (Winn, 1970), there is a controversy over the role played by antibody in skin allografts and xenografts and whether it alone can mediate rejection. In fowl Hasek et al. (1962) and Haskova et al. (1962) working with skin xenografts and Hasek et al. (1969) working with skin allografts clearly showed that humoral antibody could cause rejection of skin grafts on "tolerant" recipients.

In the rodent skin xenograft model besides the evidence of Hamilton and Gaugas (1972) discussed above, acute destruction by antibody of rat skin grafted onto mice was demonstrated by Jooste et al. (1973). Mice were immunosuppressed by adult thymectomy 1 to 2 weeks before grafting and a short course of ALS at time of grafting. Sensitivity of the grafts to 1.0 ml of high-titered mouse anti-rat antibody was sharply restricted in time. Administration of antiserum between the first and 6th day after grafting did not prevent healing of the graft nor
after grafting did not prevent healing of the graft nor did it lead to acute rejection. The most sensitive period was 12-18 days after grafting when antibody led to destruction of 85 percent of the grafts. Signs of inflammation were evident within 10 minutes after intravenous administration of antiserum and the over-all picture resembled an Arthus reaction (Baldamus et al., 1973). Long-standing grafts (36-66 days) were completely insensitive to antibody which may explain the failure of Lance et al. (1969) and Shaffer (1969) to demonstrate antibody-mediated rejection of skin xenografts.

Very similar results were obtained by Gerlag et al. (1975) in the rodent skin allograft model. Injection of 0.5 ml high-titered alloantiserum with 0.25 ml rabbit complement I.V. into normal or immunosuppressed mice resulted in hyperacute rejection of grafts. As in the experiments of Jooste et al. (1973), grafts were susceptible to rejection by antiserum in a limited period: 5 to 10 days after grafting in this case. Antiserum given between days 1 and 4 resulted in enhancement instead of hyperacute rejection. Both groups of authors arrived at similar conclusions, though one worked with a xenograft and the other with an allograft model. They demonstrated that skin grafts are susceptible to hyperacute rejection by humoral antibody but only after grafts have healed in and become vascularized. They found that ingrowing
grafts were completely resistant to the destructive effects of antibody.

These studies serve to illustrate the similarities between xenografts and allografts and to point out, therefore, that immunological manipulations aimed at prolonging graft survival should be possible in a xenogeneic as well as in an allogeneic system.

Immunosuppressive agents currently in use are non-selective and seriously impair host resistance to the microbial environment, suppress immunologic surveillance mechanisms and are associated with toxicity. The induction of specific graft directed tolerance or enhancement is one of the major goals of transplantation research today. Full tolerance is defined as a complete lack of peripheral response to donor antigens, i.e. the absence of donor-reactive cells (Simpson, 1973). Since the difficulty of inducing tolerance is proportional to the antigenic disparity between donor and host (Lance and Medawar, 1969; Stuart, 1973), it is doubtful that full tolerance can ever be achieved in an adult xenograft (or allograft) system without thymectomy. Reliance, therefore, must be placed on enhancement for effecting increased graft survival.

Lance et al. (1969) attempted rat skin graft tolerance by short intensive treatment of mice with ALS.
followed by massive \(10^8\) injections of rat lymphoid cells. A modest graft prolongation of 4 days was achieved over mice treated with ALS alone. However, thymectomy greatly prolonged graft survival up to 200 days and was accompanied by lymphoid cell chimerism, a requirement for full tolerance when whole cells are used. These authors found low doses of donor lymphoid cells \((20 \times 10^6)\) ineffective whereas in an allograft system, graft prolongation is easily achieved by those doses. In another xenograft study using whole donor cells, Cerilli et al. (1970) found that rat skin survival on mice chronically immunosuppressed with Natulan and ALS was significantly increased by injection of \(50 \times 10^6\) lymphocytes on day \(-14\), \(-11\) and \(-8\) (150 days for experimental mice vs. 103 days for controls). Tolerance was not achieved in these experiments, however, and to our knowledge has not been achieved in an adult xenograft model without thymectomy.

To avoid the serious consequences associated with graft vs. host reactions (GVHR), many authors have turned to cell-free antigen extracts as a means to induce specific unresponsiveness. Numerous studies have shown that active enhancement of dog and rat renal allografts with antigen extract alone is possible (reviewed by Stuart, 1973). Enhancement of renal allografts can also be induced passively in rats by administration of antibody alone (Fabre and
Enhancement/tolerance in the skin allograft system has been more difficult to demonstrate without the adjunct of concomitant immunosuppression. Law et al. (1972) in an effort to induce tolerance to H-2 antigens injected a purified papain extract of spleen cells into newborn mice daily for 4-5 weeks. Skin allografts were rejected at the same time by experimental and untreated mice although antibody tolerance was induced. Other workers found skin allograft survival could be moderately extended (16 d vs. 12 d) by use of antiserum alone, but it could be further prolonged by combination with nonspecific immunosuppression such as ALS (25 d vs. 16 d with ALS only) (Koene et al., 1975). Brent et al. (1973) obtained similar results in their experiments with cell-free antigen. A single injection of insoluble spleen or liver extract on day -16 (without immunosuppression) caused some prolongation of skin graft survival in H-2 compatible but not in H-2 incompatible recipients. In contrast, when the antigen injection was followed by a short course of ALS treatment at time of grafting, graft prolongation was extended by a factor of 2 to 4 over that of ALS controls. Thus, even with skin allografts, graft prolongation by injection of antigen or antiserum alone was shown to be difficult to obtain.
Owen (1971) has been able to induce significant xenograft prolongation by administration of antigen alone. He injected 1 ug/Kg of sonicated liver extract daily for five weeks before transplantation and daily afterwards resulting in increased guinea pig kidney survival in rabbits from a few hours to 14 days.

Rodent skin xenograft prolongation by administration of cell-free antigen has been more difficult to demonstrate than prolongation of skin allografts. Cerilli and Hattan (1972) were unable to prolong rat skin survival on mice by continuous injection of large doses of cell-free antigen obtained by freezing and thawing rat lymphocytes. But when antigen administration, which began on day -14 and continued daily, was combined with almost daily injection of 3 immunosuppressive agents, significant skin graft prolongation was achieved (169 days vs. 72 days with immunosuppressive agents alone). In another paper Cerilli et al. (1974) reported no effect on skin xenograft rejection when a single antigen injection of spleen or liver homogenate was given on day -16 combined with either a short course of ALS or a short or prolonged course of ALS and procarbazine. Brent et al. (1973) were also unable to demonstrate rat skin xenograft prolongation on mice after a single antigen injection on day -16 and a short course of ALS on days 2, 4 and 6. With this same regimen significant
skin allograft prolongation was obtained.

In a separate study mouse skin survival on rats was prolonged (13.9 days vs. 6.7 days) by injection of rabbit anti-mouse serum (0.4 ml) every other day from day -1 (Jeekel et al., 1974). This prolonged survival was increased further by a short course of ALS. The prolonged graft survival was attributed to enhancing antibodies in the anti-donor serum. However, when this same antiserum was administered to heart recipients, it caused hyperacute rejection.

Methods employed to extract rodent transplantation antigens should be useful in primates as well because of homology of the products of major histocompatibility loci (MHL) (Götze et al., 1973; Sachs et al., 1971; Staines, 1974; Kano et al., 1973; reviewed by Davies, 1973). The fact that these antigens are an integral part of cell membranes presents immense difficulty when attempting to isolate them in soluble form. This has given rise to numerous techniques in hopes of obtaining a potent extract for use in biological studies or for purification to study products of the MHL. However, it appears that purification of crude extracts is counterproductive when the goal is immunization or induction of specific unresponsiveness. The poor biological activity of purified antigen
may be due to loss of some specificities during purification, removal of adjuvanticity or loss of carrier effects (Davies, 1973). Moreover, it has been shown that the yield of purified, soluble antigen is rarely more than a few per cent of the crude extract (Davies, 1973).

A variety of methods have been employed to extract allograft antigens (reviewed by Reisfeld and Kahan, 1971). These include detergents, organic solvents, sonication, hypertonic (3 M KCl and 2 M KI) and hypotonic salt solutions, autolysis and enzymes (ficin and papain). All have disadvantages. With detergents, organic solvents and hypertonic salt solutions, the extraction procedures are time-consuming and complex, and the antigen is in nonphysiological conditions, making it difficult to work with in vitro and in vivo. Detergent extraction necessitates preparation of cell membranes which results in loss of antigen activity (Davies, 1973). Extraction by sonication and hypotonic and hypertonic salt solutions are actually extraction by autolysis (Davies, 1973). With this method only very low antigenic yields are obtained. Long incubation periods and the certainty that different specificities are destroyed at different rates implies destruction of some specificities (Davies, 1973). The exception is extraction of membrane antigens with 3 M KCl at 4°C where, probably due to restricted
destruction under conditions far from optimum, only a limited degree of autolysis occurs. The same drawback pertains when exogenous hydrolytic enzymes are added. Short incubation periods are required to prevent total destruction of activity with the resultant effect that some specificities remain in the insoluble fraction (are not released) while still others are destroyed (Davies, 1973).

Solubility of the antigen is a property generally regarded as advantageous in the production of unresponsiveness to tissue antigens (Medawar, 1963). Particulate antigens are eliminated rapidly from the circulation and may cause embolism. The activity of crude extracts of transplantation antigens is more often than not sedimented by an hour of high speed centrifugation (30,000 to 163,000 g used by different authors). Clearly, the need remains for a simple extraction-solubilization procedure for cell membrane antigens which results in high antigenic yields of all or nearly all specificities. Preferably, it should not require the preparation of cell membranes which is counter-productive. Nor should it require fractionation of cellular components since it has been shown by Albert and Davies (Davies, 1973) that H-2 and HL-A antigens are almost exclusively cell-surface
Two pairs of investigators have extracted xenoantigens with 3 M KCl. Stroehmann and Dewitt (1972) reported the extraction of a rat species antigen from lymphoid cells. The xenoantigen was identified by its ability to block the cytotoxic activity of rabbit anti-rat serum. It was of a molecular size that eluted at the front of the inner bed volume of a Sephadex G-200 column. Schwartz and Lang (1974) extracted baboon xenogeneic histocompatibility antigens also from lymphocytes. The antigens were identified by their ability to block cytotoxicity of chimpanzee anti-baboon serum. In dilute salt solution the antigens sedimented during centrifugation at 163,000 g, but when Triton X-100 was added before bringing the preparation to isotonicity, 4 active components could be isolated by polyacrylamide gel electrophoresis. Papain was ineffective in solubilizing baboon xenoantigens in this system (Schwartz, 1972).

This study was initiated to investigate several agents which could be used to solubilize cell surface membrane antigens simply and conveniently, to partially characterize the xenoantigen extract obtained and to investigate its usefulness in studies on the mechanisms of xenograft rejection and in prolonging skin xenografts. Lymphoid tissue was used as the source of material for
xenoantigen extraction because of its high content of major histocompatibility antigens.

Utilizing techniques involving intact cells as antigens, humoral immune responses of mice to rat lymphocyte antigen (RLA) were studied by hemagglutination and complement-dependent lymphocytotoxicity. Cellular immune responses of untreated and RLA-immunized mice were measured by 3 in vitro assays, e.g. antibody-independent and antibody-dependent cell cytotoxicity for rat lymphocyte target cells and lymphoblastic transformation of mouse splenocytes by RLA. The antigen preparation was utilized in attempts at skin xenograft prolongation by active enhancement (both alone and in conjunction with ALS) and by passive enhancement with antisera to antigen extract.
MATERIALS AND METHODS

A. Animals

Inbred C3H/HeJ female mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. All mice were adults between 10 and 25 weeks of age. Wistar-Furth (WF/Mai) female rats were obtained from Microbiological Associates, Bethesda, Maryland. Animals were stabilized in our colony for at least one week prior to use. New Zealand white rabbits were from local suppliers.

B. Skin Grafting Procedures

Skin grafting was performed by modification of the technique of Billingham and Medawar (1951) as described below.

1. Preparation of donor skin. Donor WF/Mai female rats were sacrificed by ether and cardiac exsanguination. The abdominal skin was shaved with clippers and disinfected with 70% ethanol. Pinch grafts, 1 cm. in diameter, were removed and scraped cautiously with a scalpel to remove adherent fat and small blood vessels, and placed on filter paper saturated with sterile saline. Grafts were kept at 4°C until used.

2. Grafting technique. Fur was removed with clippers from a dorsal area of recipient C3H mice and the skin surface was disinfected with 70% ethanol. The animals
were anesthetized with ether and the graft bed was prepared by cutting a pinch graft, 1 cm. in diameter, from the dorsal thoracic wall. The edges of the rat skin graft, slightly larger than the mouse skin defect, were tucked under the edges of the mouse skin in the graft bed. The grafts were covered by a piece of flexible plastic which was held in place by adhesive tape (Johnson & Johnson Dermiclear), and the tape was then covered with a plaster cast.

3. **Evaluation of graft survival.** The day of grafting was considered day 0 in all experiments. Condition of the grafts was judged daily by gross observation beginning on day 3. The grafts were observed for signs of erythema, edema, and destruction of the epidermis including hemorrhage and loss of pliability, and firmness of attachment to the graft bed. When 50 percent or more of the donor epithelium no longer appeared viable, the graft was considered rejected.

4. **Statistics.** A 2-tailed test for significance based on Student's t Test was used for all xenograft prolongation experiments.

C. **Collection of Mouse Serum and Ascites**

Following the initial immunization 0.1 ml of blood was collected from the tail vein of each mouse by cutting off the tip of the tail. The blood was allowed to flow into glass capillary tubes (two per mouse) which were
then sealed. After the blood clotted at 25°C the capillary tubes were placed at 4°C overnight to allow clot retraction. The capillary tubes were then broken and all serum obtained from a group of animals was pooled. After erythrocytes were removed by centrifugation the serum was heated at 56°C for 30 minutes and then stored frozen at -20°C.

Following the second and third immunizations, ascites fluid could be collected. A 20 ga. needle was inserted low in the peritoneal cavity and fluid was allowed to drip into a tube or gently pulled out with a 2.5 ml syringe and then placed into a tube containing a few drops of heparin. After centrifugation to remove cells, the ascites fluid obtained from a group of animals was pooled, heated at 56°C for 30 minutes and then frozen at -20°C until used.

D. Preparation of Mouse Anti-Rat Lymphocyte Ascites (MARLA)

Mouse anti-rat lymphocyte antibody for use in the serological detection of extracted rat lymphocyte xenantigens was prepared by the induction of extensive abdominal ascites in immunized mice using a modification of the technique described by Munoz (1957). Spleens, thymuses, and mesenteric lymph nodes from WF/Mai rats were removed aseptically and placed in Seligman's balanced salt solution (SBSS). A cell suspension was made by pressing the whole organs through a 60 gauze wire screen followed by extensive
washing of the screen and agitation of the resulting suspension with a Pasteur pipette. In order to remove as much serum protein from the cell suspension as possible the cells were washed four times at 4°C by suspension in 20 volumes of SBSS, followed by centrifugation at 500 g for 10 minutes. The final cell pellet was diluted to $2.5 \times 10^8$ cells per ml with SBSS and then emulsified with an equal volume of complete Freund's adjuvant (CFA, Difco). Fifty C3H/HeJ mice were injected intraperitoneally with 0.4 ml of the cell-CFA suspension ($5 \times 10^7$ cells per animal). All animals received an identical injection two weeks after the first, and half the mice received a third injection 3 1/2 weeks after the second. All animals developed noticeable abdominal distention within one to two weeks following the second immunization.

Ascites fluid was collected weekly, from 3 to 5 weeks after the second injection and all fluid collected at one time was pooled. Simultaneously, a small serum sample was obtained by retroorbital sinus puncture. The ascites fluid did not clot but contained a large number of lymphocytes and macrophages which were removed by centrifugation at 600 g for 10 minutes at room temperature. The supernatant fluid was placed at 4°C for 18 hours and the lipid pellicle that formed was removed by filtration at 4°C through a single layer of cotton gauze. The resulting clear, straw-colored fluid was stored at -20°C.
Individual collections of ascites fluid were tested for serological activity using direct hemagglutination and lymphocytotoxicity tests. Batches of fluid with similar high antibody titers were pooled and stored in aliquots at -20°C. The final result was a total pool of about 150 ml of mouse anti-rat lymphocyte ascites.

E. Preparation of Rabbit Anti-mouse Lymphocyte Serum (ALS)

Rabbit anti-mouse lymphocyte serum was prepared by injection of $10 \times 10^6$ C3H/HeJ mouse thymocytes and lymphocytes into the footpads of New Zealand rabbits. Subsequent booster injections of $10 \times 10^6$ cells were given intravenously. Animals were bled 10 days after each booster injection. Pooled serum had a lymphocytotoxic titer of 4096 for mouse spleen lymphocytes and a direct hemagglutination titer of 512 for mouse erythrocytes.

F. Preparation of Cells for Use in Serological Tests

1. Viable rat blood lymphocytes. Rat blood lymphocytes were used as target cells in serological and cell-mediated cytotoxicity assays. Lymphocytes were separated from whole blood by a modification of the isopycnic centrifugation technique of Perper et al. (1968). One (1.0) ml of cardiac blood was defibrinated by swirling for 10 minutes with 10 to 15, 3 mm glass beads in a 25 ml Erlenmeyer flask. The defibrinated blood was then mixed with 4 volumes of SBSS, pH 6.7, modified from Nobel et al. (1968) to contain
500 mg of disodium EDTA per liter. The mixture was filtered through cotton gauze (to remove the glass beads and fibrin clot) into a 12 ml glass centrifuge tube. Two (2.0) ml of room temperature Ficoll-Hypaque solution (6.35 percent Ficoll, Pharmacia, and 10 percent diatrizoate sodium, Winthrop) was layered under the diluted blood. Centrifugation was then performed at 360 g (max.) for 25 minutes in a swinging bucket at room temperature.

The cloudy layer, just below the interface between the diluted serum and Ficoll-Hypaque, was aspirated and centrifuged at 600 g (max.) for 15 minutes. The supernatant fluid was discarded and the pellet of cells loosened by agitating the tube. Contaminating erythrocytes were destroyed by hypotonic lysis. One and a half (1.5) ml of distilled water was added to the cell button followed by brief agitation. Not more than 10 seconds later (longer periods caused clumping of leukocytes), 0.5 ml of 4 times concentrated Hanks' Balanced Salt Solution (HBSS) (Hanks and Wallace, 1949) and 0.25 ml of heat inactivated (56°C for 30 minutes) normal rat serum (NRtS) was added to the cell suspension. The NRtS was necessary to maintain good viability during subsequent centrifugation at 265 g (max.) for 10 minutes. The supernatant fluid was discarded and the cell button loosened by agitating the tube. The cells were then ready for dilution with appropriate media.

Total cell counts were obtained by use of a hemocytometer. A typical yield was about 2 x 10^6 cells per ml of blood. Differential cell counts were performed on glass slide preparations stained with Wright's Stain. They were based on observation of at least 100 cells
Typical differential cell counts were 85 percent lymphocytes, 5 percent monocytes and 10 percent neutrophils. Viability, as measured by ability to exclude 0.3 percent Trypan Blue, was usually greater than 95 percent.

2. Erythrocytes. Erythrocytes for direct hemagglutination were collected from rat or mouse cardiac blood. Erythrocytes to test for anti-complementariness of rat antigen preparations in the inhibition of lymphocytotoxicity assay were obtained from 80 percent sheep blood diluted in Alsever's solution (Laboratory Research, Columbus, Ohio). Rat blood was defibrinated for 10 minutes by swirling with 3 mm glass beads. The defibrinated blood was washed 3 times with 10 ml of 0.15 M NaCl, centrifuging at 650 g for 10 minutes. The washed cells were then used immediately. Mouse cardiac blood was collected in a 1.0 ml tuberculin syringe coated with heparin. The syringe was coated by drawing heparin into the 1.0 ml mark and emptying. Erythrocytes were washed 3 times with 10 ml of 0.15 M NaCl, centrifuging at 650 g for 10 minutes. The washed cells were used immediately. Sheep erythrocytes (0.5 ml) were washed 3 times with 10 ml of 0.15 M NaCl prior to sensitization (Levine, 1967) with sheep cell hemolysin (The Sylvana Co., Millburn, N.J.). Washed sheep erythrocytes diluted in Levine's buffer containing 1 percent bovine serum albumin (BSA) were adjusted to an absorbancy of 0.680 at 541 nm with 0.1 percent Na₂CO₃. An equal volume of hemolysin diluted 1:10000 in Levine's buffer with BSA was added dropwise while swirling. Sensitization was effected at 37°C in 15 minutes, after which the cells were further diluted 1:10 in buffer.
G. Serological Techniques

To titrate antibody levels following immunization, direct hemagglutination and lymphocytotoxicity tests were performed. Inhibition of lymphocytotoxicity, immunodiffusion and immunoelectrophoresis were used to test for antigen activity in lymphocyte antigen preparations.

1. Direct hemagglutination (HA). Serum to be tested had been heated at 56°C for 30 minutes. Serial two-fold dilutions were made in 0.1 ml quantities with 0.15 M NaCl. Fresh, washed, packed rat erythrocytes were diluted to a 2 percent suspension (vol/vol) in 0.15 M NaCl just prior to use. A 0.1 ml aliquot of the erythrocyte suspension was added to each 10 x 75 mm glass test tube containing 0.1 ml of a serum dilution. The mixtures were shaken until homogenous and then incubated at room temperature for 30 minutes after which they were centrifuged at 600 g for 3 minutes. The tubes were agitated individually to observe for agglutination. Readings were based upon how the erythrocytes resuspended as described below:

Positive 4+ Solid cell button with no fragmentation.
   3+ Some fragmentation of the cell button but no pinkness in the supernatant fluid.
   2+ Large clumps with a diffuse redness of the supernatant fluid.

Negative + Very few small particles.
   - No agglutination seen.
The last tube in the titration giving at least a 1+ was the endpoint. The serum titer was recorded as the reciprocal of that dilution. Controls consisted of known positive and negative sera and 0.15 M NaCl in place of antiserum. If the tests were repeated, final titers were expressed as the average of all tests.

2. Lymphocytotoxicity (LC). The LC activity of mouse serum and ascites was assayed by a modification of the micro-method of Terasaki (Terasaki and McClelland, 1964, Mittal et al., 1968). The serum to be tested had been previously heated at 56°C for 30 minutes and serial two-fold dilutions were made in 0.1 ml quantities with 0.15 M NaCl. All dilutions were tested in duplicate. Reagents were added to wells of microtest I trays (Falcon Plastics) under a drop of heavy mineral oil to prevent evaporation, using microliter syringes with a repeating dispenser (Hamilton Company, Whittier, Calif.). To 1 μl of diluted serum in the wells was added 1 μl of a suspension of lymphocytes in HESS containing 1000 cells and the mixtures incubated at room temperature for 1 hour. Then 4 μl of guinea pig complement (Microbiological Associates, Bethesda, Md.) diluted one to 10 with HESS was added to each well. After further incubation for 1 hour at room temperature 5 μl of 5 percent eosin Y dye in 0.15 M NaCl was added, followed in 3 to 5 minutes with 4 μl of 36 percent neutral formalin. A coverslip was placed on the tray and the
percentage of viable cells in each well estimated by the ability of viable cells to exclude eosin Y, as observed on an inverted phase microscope after settling overnight at 4°C. The reciprocal of the highest serum dilution causing 50 percent cell death was considered the endpoint (titer). Controls included diluted mouse ascites (MARLA) known to cause 95 percent rat target cell death, normal mouse or rat serum at this dilution without complement (cell control), and a complement control in which normal rat serum was substituted for MARLA.

3. Reactivity of MARLA with Rat Cell-Surface Antigens. MARLA was used to demonstrate that rat lymphocyte antigen (RLA) contained cell-surface antigens. MARLA had HA and LC antibody titers of 4096 and 256 respectively, while normal C3H/HeJ mouse serum and ascites (raised to CFA) were negative in both tests. Absorption with washed, pooled, viable rat spleen, thymus and mesenteric lymph node cells indicated 10 x 10⁶ cells (the AD₅₀ of MARLA) were necessary to reduce by 50 percent the LC titer of 1.0 ml of a dilution of MARLA causing 90 percent cell death of 1 x 10⁶ rat lymphocytes.

4. Reactivity of MARLA with Rat Serum. Since MARLA was used to detect rat cell-surface antigens in extracts of lymphocytes that contained rat serum components it was important to determine if MARLA contained antibodies
to rat serum. No inhibition of lymphocytotoxic activity was obtained using MARLA and normal rat serum as antigen; also no lines of precipitation were seen in immunodiffusion or immunoelectrophoresis using rat serum and MARLA. From this we concluded that MARLA did not contain sufficient antibody to rat serum components to interfere with interpretation of test results using these procedures for the detection of rat lymphocyte antigens.

5. Inhibition of lymphocytotoxicity (ILC). MARLA was used to detect the presence of rat lymphocyte surface antigens by their ability to inhibit LC activity. ILC tests were performed exactly as the LC tests with the modifications discussed below. When lymphocyte antigen extracts were tested for their ILC capacity, 1 μl of MARLA, diluted to cause 95 percent cell death of 1000 rat lymphocytes, was added to the well followed by 1 μl of antigen diluted in 0.15 M NaCl. The antigen-antibody mixtures were incubated overnight at 4°C, the trays warmed to room temperature, and the test completed. Controls were the same as for the LC test with the following additions: one lower and one higher two-fold dilution of MARLA, besides that which caused 95 percent cell death; an antigen control with NRTS at the appropriate dilution substituted for MARLA; a control for antigen anti-complementariness consisting of undiluted antigen, complement and 25,000 sensitized
sheep erythrocytes. When the level of delta toxin (used for antigen extraction) was sufficient to cause cytotoxicity by itself, a delta toxin cytotoxicity control was run consisting of 25,000 sheep erythrocytes without complement. A duplicate test (with duplicate wells) was performed with addition of serial dilutions of lecithin, a known inhibitor of delta toxin (Kapral, 1972), to the serial dilutions of antigen, such that undiluted antigen received 1 mg/ml lecithin. When the level of delta toxin used to extract rat membrane antigens was decreased to levels causing negligible cytotoxicity (5 percent), these controls were no longer necessary.

When whole cells were tested for their absorptive capacity, a cell suspension identical to that used for antigen extractions was prepared, except that it was suspended in HBSS. The cells were washed once in 10 ml of HBSS. A 1.0 ml aliquot of the cell suspension containing a known number of cells was placed in a 10 x 75 mm glass test tube and centrifuged at 385 g for 10 minutes. The supernatant fluid was carefully removed and discarded. The cell pellets were resuspended in 1.0 ml of MARLA, suitably diluted with HBSS to effect 95 percent cell death, and incubated overnight at 4°C with mixing several times. The suspensions were then centrifuged at 385 g for 10 minutes and 1 µl aliquots of the supernatant fluid were tested in quadruplicate.
for remaining LC activity. The number of absorbing cells in each tube (before addition of MARLA) was rechecked by counting with a Coulter Counter.

The dilution of antigen required to cause 50 percent inhibition of lymphocytotoxicity \( (\text{LCID}_50) \) was estimated by the method described by Gotze and Reisfeld (1974). The activity of each antigen dilution was calculated as percent of inhibition:

\[
\% \text{ inh.} = 100 - \frac{\% \text{ cells killed in presence of antigen}}{\% \text{ cells killed in absence of antigen}} - \frac{\% \text{ cells killed without antiserum}}{\% \text{ cells killed without antiserum}}
\]

The number of intact cells necessary to absorb 50 percent of the cytotoxic activity of a dilution of MARLA \( (\text{AD}_50) \) was estimated in a similar manner.

6. Immunodiffusion (ID). Double diffusion was carried out in 0.5 percent agarose gel (electrophoresis grade, General Biochemicals) in a solution of 0.15 M NaCl containing 0.02 percent sodium azide. Five ml of agar were poured into a 50 x 12 mm plastic petri dish (Falcon plastics) and wells were cut 6 mm in diameter, 4 mm apart. Following overnight refrigeration the wells were aspirated dry and reagents added. The plates were incubated at 25°C for one week and examined daily.

7. Immunoelectrophoresis (IEP). Immunoelectrophoresis was performed as described by Scheiddegger (1955) using
Gelman Immunoelectrophoresis equipment. Using 1 percent Noble agar (Difco Laboratories, Detroit, Mich.) barbital acetate buffer pH 8.6 (ionic strength 0.025) and 1 or 3 mm wells, electrophoresis was conducted at a constant voltage of 260 volts for 75 minutes. Following electrophoresis, antiserum was added to the 1 mm trough and the slide frames incubated in humidity chambers at 4°C for two weeks with periodic examination.

H. Techniques to Detect Cell-Mediated Immune Responses

As an in vitro assessment of cell-mediated immune responses following immunization, cytotoxicity of lymphoid cells for target cells, either alone or with antibody, and lymphoblastic transformation (LBT) were measured. The technique used to detect cell-mediated cytotoxicity (CMC) was a modification of the ⁵¹Cr release method described by Berke et al. (1972). Those of inhibition or potentiation of cell-mediated cytotoxicity and antibody-dependent cell cytotoxicity (ADCC) were modifications of the method described by Sonis et al. (1975). The LBT assay was a modification of the technique used by Strong et al. (1973).

1. Cell preparation. Mice were sacrificed by cerebrospinal dislocation. Effector spleen cell suspensions were prepared in cold phosphate buffered saline (Dulbecco, 1964) containing 25 percent fetal calf serum (Grand Island Biological Co.) heated at 56°C for 30 minutes with 100
units per ml Penicillin and 100 µg per ml Streptomycin, hereafter referred to as PBS-FCS. The cells were prepared by teasing the spleen with forceps in 12 ml PBS-FCS, followed by agitation of the cell suspension with a Pasteur pipette, filtration through a single layer of cotton gauze and centrifugation for 10 minutes at 380 g. The cell suspension was washed 2 times with 3 ml of PBS-FCS before 3 ml of 0.85 percent NH₄Cl was added 2 times to lyse erythrocytes. The cells were again washed with 3 ml of PBS-FCS 2 times, counted with a Coulter Counter, tested for viability with 0.3 percent Trypan Blue, and adjusted to 5.0 x 10⁷ viable cells per ml. The viability was always 90 percent or greater. Effector cells were kept at 4°C until used.

Target cells were prepared from rat cardiac blood by the Ficoll-Hypaque technique described previously, and adjusted to 1 x 10⁷ cells per ml. They were labeled with 250 µCi of sodium chromate (⁵¹Cr) solution (New England Nuclear Corp., Boston, Mass.) by incubation at 37°C for 30 minutes with occasional shaking. Labeled target cells were then washed 2 times with 40 ml cold PBS-FCS, centrifuging the cells at 380 g for 10 minutes and allowing the cells to stand at 4°C for 30 minutes during the second washing. Labeled target cells were adjusted to a concentration of 2.5 x 10⁵ viable cells per ml. The viability was always 90 percent or greater.
2. **CMC test procedure.** 5.0 x 10^6 viable effector cells and 2.5 x 10^4 viable target cells, effector to target cell ratio of 200 to 1, were placed in a total volume of 1.0 ml of PBS-FCS in 35 mm tissue culture dishes (Falcon plastics). The plates were incubated on a Rocker Platform (Bellco Glass, Inc., Vineland, N.Y.) at 6 cycles per minute at 37°C for 4 hours.

At the end of the incubation period 1.0 ml cold PBS-FCS was added to each plate and the contents transferred to 12 x 75 mm glass tubes. The tubes were centrifuged at room temperature at 415 g for 7 minutes and 1.0 ml of the supernatant fluid was transferred to 12 x 75 mm glass tubes for counting. Radioactivity was assayed in a Gamma scintillation spectrometer (Packard Model 578).

To determine the maximum amount of releasable radioactivity, labeled target cells were incubated with normal spleen cells for 30 minutes and treated as above except the cells were frozen and thawed 3 times before centrifugation. Effector cell suspensions from each animal were assayed for CMC activity in triplicate. Results were expressed as the percentage of maximum ^51^Cr release, given as percent CMC, according to the equation (Cerottini and Brunner, 1971):

\[
\% \text{ CMC} = 100 \times \frac{\text{CPM} \overline{\epsilon} \text{ImSpC} - \text{CPM} \overline{\epsilon} \text{NSpC}}{\text{Max. CPM} - \text{CPM} \overline{\epsilon} \text{NSpC}}
\]
CPM \( \tilde{\circ} \) ImSpC = Counts per minute released in presence of immune spleen cells.

CPM \( \tilde{\circ} \) NSpC = CPM in presence of normal spleen cells.

Max. CPM = Maximum releasable CPM by freeze-thawed cells.

Radioactivity released from target cells in the presence of normal cells was 7 to 8 percent less than that released from target cells incubated alone. Maximum release represented 80 to 90 percent (ave. 84 percent) of total radioactivity incorporated into the target cells. Data was used only when release of radioactivity in the presence of normal cells was less than 33 percent.

3. Inhibition or Potentiation of CMC (ADICC). The method was the same as that for CMC except that 0.1 ml of antiserum in the appropriate dilution of normal serum or ascites in the same dilution was incubated for 30 minutes with target cells before addition of immune effector cells. Results were expressed for each antiserum dilution tested as percent specific \(^{51}\text{Cr} \) release.

\[
\% \text{ Specific } ^{51}\text{Cr Release} = \frac{100 \times \frac{\text{CPM } \tilde{\circ} \text{ ISpC + AS} - \text{CPM } \tilde{\circ} \text{ ISpC + NS}}{\text{Max. CPM} - \text{CPM } \tilde{\circ} \text{ ISpC + NS}}}{\text{Max. CPM} - \text{CPM } \tilde{\circ} \text{ ISpC + NS}}
\]

CPM \( \tilde{\circ} \) ISpC + AS = CPM with immune spleen cells plus antiserum.

NS = Normal serum.
4. **ADCC.** The method was the same as that for ADICC except that normal spleen cells (usually pooled from 2 mice) were used as effector cells instead of immune spleen cells. Results were expressed for each antiserum dilution tested as percent specific $^{51}$Cr release:

$$\% \text{ Specific } ^{51}\text{Cr Release} = \frac{100 \times (\text{CPM} \text{ effector cells + antiserum} - \text{CPM} \text{ effector cells + normal serum})}{\text{Max. CPM} - \text{CPM} \text{ effector cells + normal serum}}$$

The intragroup variance for a given experiment was computed from the difference between CPM with effector cells and antiserum and CPM with effector cells and normal serum for all dishes. Student's $t$ Test was used to calculate significance between ADCC and ADICC at a given antiserum dilution.

5. **LBT.** A modification of the method of Strong et al. (1973) was used. Mice were sacrificed by cerebrospinal dislocation and spleen cell suspensions were prepared like those for CMC except that RPMI 1640 (Gibco) with bicarbonate + 2 mM L-glutamate containing 100 units of penicillin, 100 μg streptomycin per ml and 5 percent FCS was used instead of PBS-FCS. Spleen cells were counted with a Coulter Counter and adjusted to $6 \times 10^6$ viable cells per ml. One tenth (0.1) ml of spleen cells was added to medium or 0.5 percent or 1 percent PHA-M (phytohemagglutinin-M, Difco) or to various rat xenotransplantation antigen dilutions in microtest II tissue culture plates (Falcon Plastics) with a loose fitting lid. Each test was run in triplicate. After incubation for 48 hours in 10 percent CO$_2$, 90 percent humidity at 37°C, 1.0 μCi of $^3$H-thymidine (New England Nuclear) was added to each well. Following an additional 18 hour incubation, the
cells were harvested in a multiple automated sample harvester (M.A.S.H. II) and the dried circle of filter paper, containing cells from one well, placed in a vial containing 10 ml of scintillation fluid (5 grams of Permablend II (98 percent PPO, 2 percent POPOP) per liter toluene). Radioactivity was counted for 5 minutes in a beta liquid scintillation counter (Searle Delta 300). Stimulation Index (S.I.) was calculated as average CPM obtained with antigen or PHA-M (3 wells each) divided by average CPM obtained with medium only (4 wells).

I. Extraction of Rat Lymphocyte Antigen (RLA)

The spleen, thymus, and mesenteric lymph nodes were removed aseptically from rats, sacrificed by cardiac exsanguination under ether anesthesia, and placed in 0.25 M sucrose. Cell suspensions were prepared by teasing the organs with forceps and needles, followed by 5 minutes' agitation of the mixture with a Pasteur pipette. The cell suspension was filtered through a single layer of cotton gauze to remove large particles and the volume adjusted to approximately 20 ml per animal sacrificed. The volume was then measured and cells counted and adjusted to 40 x 10^6 leukocytes per ml. The appropriate amount of Staphylococcus aureus delta toxin (DT) was added directly to the cell suspension, which was then incubated at 37°C for 30 minutes. Following incubation the suspension was
centrifuged at 1000 g (max.) for 15 minutes to remove whole cells (see Fig. 1). The pellet was either discarded or resuspended in an equal volume of extracting solution. The supernatant fraction was centrifuged at 10,000 g (max.) for 15 minutes at 4°C using a Sorvall RC2B centrifuge with an SS-34 rotor. The resulting pellet was either discarded or resuspended in an equal volume of extracting solution. The supernatant fraction was then centrifuged at 200,000 g (ave.) for 3 hours at 4°C in an IEC B-60 ultracentrifuge using an SB-283 rotor. The pellet was either discarded or resuspended in a volume of extracting fluid equal to that of the supernatant.

Delta toxin was kindly supplied by Dr. Frank A. Kapral. For RLA extraction experiments 1-20, Lot 51 of S. aureus soluble DT was used. Its activity was 99 HD50 per mg. For extraction experiments 21-35, Lot 49 of soluble DT containing 230 HD50 per mg was used.

J. Concentration of Antigen Preparations

Concentration was accomplished by positive pressure (nitrogen) ultrafiltration at 4°C with magnetic stirring, using a Diaflo Chamber (Amicon) at 40 lbs. per square inch applied to a Diaflow-PM10 membrane which excludes molecules of molecular weight (m) 10,000 or greater. In some experiments 14 I.U. of heparin per ml was added before concentration to prevent aggregation of antigen.
Rat spleen, thymus and mesenteric lymph nodes

Preparation of cell suspensions in isotonic sucrose

Incubation 37°C 30 minutes with S. aureus delta toxin

1000 x g (max.) 15 minutes

Pellet | Supernatant

10,000 x g (max.) 15 minutes

Pellet | Supernatant

200,000 x g (ave.) 3 hours

Pellet | Supernatant

Figure 1

Procedure for preparation of rat lymphocyte antigen (RLA).
K. Estimation of Protein Concentration

The protein concentration of all antigen preparations was estimated by a modification of the method of Lowry et al. (1951), as described by Chase and Williams (1968). Samples were tested in duplicate with dilutions in 0.15 M NaCl and 0.25 N NaOH. Bovine plasma albumin (Armour) was used as a standard. A standard curve was determined with each protein estimation.

L. Ultrafiltration

Diaflow XM300 (which excludes molecules of 300,000 M or greater) and XM100A (which excludes molecules of 100,000 M or greater) membranes were used to determine the molecular size of some antigen preparations. Positive pressure (nitrogen) ultrafiltration was accomplished at room temperature with magnetic stirring using a Diaflow Chamber (Amicon) at 8 lbs. per square inch.

M. Sepharose 6B Column Chromatography

Sepharose 6B (Pharmacia Fine Chemicals) was prepared in 0.25 M sucrose and 0.02 M barbital buffer, pH 6.8, and poured into a 100 cm x 2.5 cm column. The column was run at 4°C with a peristaltic pump set at 10.0 ml per hour. It was equilibrated and eluted with 0.25 M sucrose and 0.02 M barbital buffer. 9.0 ml of RIA was applied and the eluant collected in 3.0 ml fractions. Ten ml of Blue Dextran 2000 (0.2 percent) were passed
through the column to calibrate the void volume, and 30 ml normal rat serum were applied to partially characterize the column.

N. **Polyacrylamide Gel Electrophoresis**

A modification of the method of Davis (1964) was used using the 7 percent gel alkaline system, pH 8.9, and a model no. 3-1750 Polyanalyst (Buchler Instruments, Inc., Fort Lee, N.J.). A small pore solution (pH 8.9) was overlaid with a stacking gel (pH 6.7). The RLA sample was incorporated into a large pore solution (pH 6.7) and polymerized or mixed with a heavy sucrose solution (40 percent). Electrophoresis was carried out at 3 milliamps per column, constant current, until the tracking dye, 0.1 percent bromphenol blue, had run through. After electrophoresis, the gels were extruded, stained and fixed for 1 hour in 1 percent Naphthol-Blue-Black in 7 percent acetic acid and destained in 7 percent acetic acid.
RESULTS

Antigen Preparation

*S. aureus* delta toxin (DT) was evaluated as a means for releasing cell membrane antigens from rat lymphocytes. The quantity of membrane antigens released was determined by measuring the amount of antigen activity remaining in the supernatant fraction following ultracentrifugation. (Failure of active components to sediment during centrifugation at 200,000 g for 3 hours was used as a criterion of solubility.) Antigen activity was measured by its ability to inhibit cytotoxic mouse antiserum directed against rat lymphocyte target cells. Thus, the antigens detected are predominantly, or perhaps entirely, cell membrane xenoantigens.

In initial experiments, antigen yield from two different types of rat cell preparations were compared. Cell suspensions recovered by simply disrupting whole organs (spleen, thymus and lymph node) in 0.25 M sucrose were compared with the lymphocyte suspension obtained from Ficoll-Hypaque centrifugation of the crude cell suspension. The purified lymphocyte and crude cell suspensions were treated with equal amounts of DT per leukocyte. There was an equivalent amount of antigen recovered per leukocyte in the ultracentrifuged supernatant in both the purified lymphocyte and crude
cell preparations, as measured by inhibition of lymphocytotoxicity (ILC). No ILC activity was recovered from a Ficoll-Hypaque purified RBC suspension treated in the same manner. Therefore, all subsequent antigen preparations were made from crude cell suspensions derived from rat spleen, thymus, and lymph nodes. The antigen yield in different preparations was estimated from the LCID₅₀ and the number of leukocytes in the cell suspension.

Preliminary experiments determined the optimum concentration of DT to use for antigen extraction. To aliquots of an arbitrarily selected concentration of leukocytes (13⁴ x 10⁶ per ml) in 0.25 M sucrose was added increasing amounts of DT (99 HD₅₀ per mg) ranging from 0.1 to 1.0 mg per ml (Exp. 1, Table 1). The amount of antigen activity recovered in the ultracentrifuged (200,000 g) supernatant increased from 1 to 16 LCID₅₀ with increasing amounts of DT. However, when higher levels of DT (2.0 and 5.0 mg per ml) were added, no additional soluble antigen was released (Exp. 2, Table 1). A pool of low speed pellets (1000 g) from cell suspensions initially treated with 1.0, 2.0, and 5.0 mg per ml DT was retreated with 1.0 mg per ml DT. No antigen activity was released from the pooled pellets. One (1.0) mg of DT, therefore, released all the antigen activity that DT could release from 13⁴ x 10⁶ WBC. In later experiments (Exp. 8-35, Table 1)
Table 1. Release of Soluble Rat Lymphocyte Antigens Using Various Concentrations of *S. aureus* Delta Toxin (DT)

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>No. Cells/ml</th>
<th>Amt. DT (mg/ml)</th>
<th>200,000 x g supern.</th>
<th>200,000 x g pellet</th>
<th>Membrane Filters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>134x10^6</td>
<td>None</td>
<td>1</td>
<td>6</td>
<td>XM 300 retentate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>8</td>
<td>16</td>
<td>XM 300 UF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3</td>
<td>12</td>
<td>16</td>
<td>XM 100 UF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>16</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>120x10^6</td>
<td>1.0</td>
<td>8</td>
<td>-</td>
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</tr>
<tr>
<td></td>
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<td>8</td>
<td>-</td>
<td>XM 300 UF</td>
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<tr>
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<td>-</td>
<td>XM 100 UF</td>
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<td>8.7±e</td>
<td>0.3±</td>
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<tr>
<td></td>
<td></td>
<td>HD50</td>
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<td>0.3±</td>
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<td>14.0±</td>
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<tr>
<td></td>
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<td>10^6 WBC</td>
<td>2.5</td>
<td>0.3±</td>
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Inhibition of Lymphocytotoxicity (LCID50)\(^a\)

<table>
<thead>
<tr>
<th>Membrane Filters</th>
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<tr>
<td>XM 300 retentate</td>
</tr>
<tr>
<td>XM 300 UF</td>
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<tr>
<td>XM 100A UF</td>
</tr>
</tbody>
</table>

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\(^a\) LCID50 is the inverse of the antigen dilution (1 μl) which inhibits 50% of the cytotoxicity of a dilution of MARLA which causes 95% death of 1000 rat lymphocytes.

\(^b\) UF = ultrafiltrate

\(^c\) The combined 1000 g pellets from cell suspensions initially treated with DT were treated with 1.0 mg/ml DT to determine the amount of releasable antigen remaining.

\(^d\) For experiments 3-35 the dose of DT used was based upon the hemolytic activity of the preparation.

\(^e\) LCID50 ± S.E.
both the number of cells and the amount of DT were decreased without significantly changing the quantity of antigen released.

During the 30 minute incubation (37°C) of the cell suspension with DT, there was a partial clearing of the turbid red-orange suspension. On microscopic examination Wright-stained cells appeared intact but aggregated into small clumps. There were fewer RBC present after treatment with DT than in untreated preparations and many were crenated. There was also debris present that was absent from control preparations. After treatment with DT there were few viable cells present (as measured by 0.3% Trypan Blue exclusion), while in control suspensions greater than 90 percent were viable. Centrifugation at 1000 g removed whole cells and after centrifugation at 10,000 g the supernatant was orange and slightly opalescent. Microscopic examination of the 10,000 g pellet revealed what appeared to be small circular membrane fragments and amorphous debris. After centrifugation for 3 hours at 200,000 g, the supernatant was clear, yellow-orange in color, and was designated rat lymphocyte antigen (RLA).
I. Characterization of Rat Lymphocyte Antigen (RLA)

A. Molecular Size

An attempt was made to estimate the size of the active component(s). For this purpose sedimentation, ultrafiltration (using Diaflow membranes of different molecular weight exclusion properties) and Sepharose 6B gel chromatography were used.

1. Sedimentation. The activity of the 1000 g supernatant represented the initial yield of DT extractable antigen. Centrifugation at 10,000 g did not remove any activity and ultracentrifugation at 200,000 g for 3 hours removed less than 4 percent of the initial activity (Exp. 3-35, Table 1).

2. Ultrafiltration. Most of the antigen activity (87%) was retained by an XM300 Diaflow membrane. No activity was recovered in the ultrafiltrate of PM10 or YM100A membranes, while 13% was recovered in the ultrafiltrate of an XM300 membrane (Exp. 1-7, Table 1). Therefore, the bulk of the activity as determined by ultrafiltration was associated with molecules greater than 300,000 M in size.

To determine if the active moiety which was less than 300,000 M aggregated on concentration, two separate antigen preparations were passed through XM300 membranes and the ultrafiltrates concentrated two-fold or three-fold on XM100A membranes. The three-fold concentrated
ultrafiltrate, when again passed through an XM300 membrane, lost 75% of its activity and the two-fold concentrated sample lost 50% when treated similarly. This demonstrated that the smaller M antigenic moiety did aggregate during concentration.

3. **Sepharose 6B column chromatography.** A 9.0 ml sample of RLA was applied to a Sepharose 6B column and eluted with 0.02 M barbital buffer in 0.25 M sucrose, pH 6.8. The 3 ml fractions were studied for their absorbancy at 280 and 413 nm (Figure 2). Four major peaks were obtained. The first peak eluted with the void volume, as determined by comparison with chromatography of 0.2% Blue Dextran, and thus contained components equal to, or greater than, $4 \times 10^6$ M. Fractions were combined into 15 pools, as shown, then concentrated 10-fold by positive pressure ultrafiltration. All 15 pools were tested for ILC activity. Only pools II and III had significant amounts of antigenic activity ($\text{LCID}_{50}$ of 8 and 32 respectively) while 10-fold-concentrated fraction 196 had a low level of activity ($\text{LCID}_{50}$ of 1). Normal rat serum (NRtS) was chromatographed to serve as a marker. The third protein peak of RIA eluted in the same position as the last protein peak of NRtS (albumin) and probably represents serum contamination of the RLA preparation.
RLA (9.0 ml) was applied to the column which was equilibrated and eluted with 0.02 M barbital buffer in 0.25 M sucrose. The column was run at 10 ml per hour at 4°C and fractions were collected in 3.0 ml volumes. Fractions were pooled as indicated, concentrated 10-fold, and tested for ILC activity. The symbol ▼ represents the position of the protein peaks of chromatographed normal rat serum.
B. Protein Content

The mean Lowry protein content (in mg per ml ± standard error of the mean) of 4 separately determined RLA preparations made from $40 \times 10^6$ WBC per ml was $2.3 ± 0.09$. The mean protein content of 4 separate RLA pools, also made from $40 \times 10^6$ WBC per ml, was $2.25 ± 0.15$.

Antigen, made with mouse (C3H) spleen, thymus and mesenteric lymph node cells in the same manner as RLA, contained 2.75 mg protein per ml. The protein concentration of an RLA preparation determined at the same time was 2.85 mg per ml.

C. Polyacrylamide Gel Electrophoresis (PAGE).

RLA samples from Extraction Exp. 2, where aliquots of a cell suspension were treated with 1, 2 or 5 mg of DT per ml, were passed through an XM300 Diaflow membrane, and the retentates and ultrafilters examined by PAGE (Fig. 3). Samples of the three ultrafilters (20 µl each) were incorporated into sample gels. (All 3 retentates had the same LCID$_{50}$ of 8. The 1, 2, and 5 mg DT ultrafiltrates contained 2 LCID$_{50}$, 1 LCID$_{50}$ and no activity, respectively.) At least 17 protein bands were apparent in the retentates and only three bands were seen in the ultrafiltrates. Conspicuously missing in the ultrafiltrates is the DT band which is normally found at the top of the gel (cathodal end) after PAGE in a basic, 7 percent gel
Figure 3

PAGE of Diaflow XM300 membrane retentates and ultrafiltrates of RLA (Exp. 2). A) 1 mg DT retentate; B) 1 mg DT ultrafiltrate; C) 2 mg DT ultrafiltrate; D) 2 mg DT retentate; E) 5 mg DT ultrafiltrate; F) 5 mg DT retentate. Anode is at bottom.
Figure 4

PAGE of Pooled RLA (Exps. 8 - 15). Anode is at bottom.
system (Kreger et al., 1971, and our observations).

Pooled RLA from Extraction Experiments 8-15 was examined by PAGE (Fig. 4). One hundred μl (220 μg protein) were incorporated into 40 percent sucrose which was layered on top of the stacking gel. At least 20 protein bands were visible in the running gel. Four mm cuts were made of two unstained gels and similar portions were pooled and pulverized. They were then treated for ILC activity by incubation of the fractions overnight at 4°C in a dilution of MARLA known to cause 95 percent rat lymphocyte death. The next day a titration of supernatant indicated the amount of ILC activity in each gel cut. (Control gels without RLA were electrophoresed and similarly treated. No inhibition was obtained with the gel cuts from controls.) Although there appeared to be some inhibition from the first (cathodal end) and second to last cuts of test gels, it was less than 50 percent (35 percent). A major problem when attempting to elute antigen activity from acrylamide gels was the concomitant elution of cytotoxic acrylamide particles. Elution of activity from gels with saline instead of MARLA was also tried, but if any activity was present it was abrogated by cytotoxicity due to acrylamide.

D. Immunodiffusion (ID)

Pooled RLA from Experiments 8-15 was concentrated 8-fold and tested with undiluted, 1:4 or 1:8 MARLA in
ID. It was also tested with AS 3, a mouse anti-RLA ascites, at the same dilutions. The lymphocytotoxic (LCT) and hemagglutination titers (HAT) of AS 3 were 512 and 3072, respectively. As a control for non-antibody precipitation of RLA with serum or mouse ascites components, 8-fold concentrated RLA was tested with undiluted, 1:4 and 1:8 normal rat serum (NRTS) and normal mouse ascites (raised to CPA). Since lines of precipitation are known to form between DT and serum lipoproteins (Kreger et al., 1971; Kantor et al., 1972), 0.8 mg DT per ml in 0.25 M sucrose was tested against undiluted, 1:4 and 1:8 MARLA, AS 3, NRTS and normal mouse ascites. DT was used at 0.8 mg per ml in the control instead of 0.1 mg per ml because all the detectable DT remained associated with RLA as it was concentrated. The plates were incubated at room temperature. By 20 hours lines were visible in control plates containing DT and all 4 sera at all dilutions. No lines were visible in test plates which contained RLA. By 44 hours there were faint lines of precipitation of RLA with MARLA and AS 3 at all dilutions. No lines appeared at any time between RLA and control sera (NRTS and normal mouse ascites), whereas very strong lines were evident between DT and normal mouse ascites, weaker ones with NRTS. Lines did appear between RLA and MARLA and RLA and AS 3. Figure 5 shows the plates at 94 hours. Lines
Figure 5
Immunodiffusion of RLA

Center well contains 8 X RLA (14 mg protein per ml)
Well 1, undiluted MARLA
Well 2, 1:4 dilution of MARLA
Well 3, 1:8 dilution of MARLA
Well 4, undiluted Antiserum 3
Well 5, 1:4 dilution of Antiserum 3
Well 6, 1:8 dilution of Antiserum 3
Figure 6
Immunodiffusion of DT

Center well contains 0.8 mg DT per ml
Well 1, undiluted MARLA
Well 2, 1:4 dilution of MARLA
Well 3, 1:8 dilution of MARLA
Well 4, undiluted Antiserum 3
Well 5, 1:4 dilution of Antiserum 3
Well 6, dilution of Antiserum 3
Figure 6
of precipitation with MARLA were weak and those with AS 3 stronger and diffuse. DT alone reacted with undiluted AS 3 forming a double line (Fig. 6). A single line of identity formed with DT and dilutions of AS 3 and all concentrations of MARLA.

E. Immunoelectrophoresis (IEP)

The XM300 membrane retentates and ultrafiltrates of Extraction Exp. 2 were tested in IEP using MARLA. No lines of precipitation were seen with any of the ultrafiltrates. Two lines were formed with the 1 and 2 mg DT retentates and 5 or more with the 5 mg DT retentate. When 1, 2 and 5 mg per ml DT in 0.25 M sucrose were treated with MARLA, lines of precipitation similar but not identical to those obtained with XM300 retentates of RLA were seen.

F. Stability (Effect of various environmental conditions on activity and appearance of RLA)

1. Freezing, thawing and storage. RLA lost no ILC activity on 10 freeze - thaw cycles over a 2 week period. No activity was lost when it was stored at -20°C for 6 months. One sample was tested for ILC activity after 13.5 months of storage at -20°C with infrequent thawing over that period. The LCID$_{50}$ had not changed but the maximum inhibition of cytotoxicity obtainable was only 60 percent rather than 100 percent.
After being held at 4°C for 18 days, RLA lost half of its activity (LCID$_{50}$ of 16 rather than 32). However, no activity was lost when it was stored for one week at 4°C.

2. pH. Aliquots (0.5 ml) of RLA at neutral pH were brought to pH 5.0, 4.0, 3.0 or 2.0 with 0.1 N HCl or to pH 9.0, 10.0 or 11.0 with 0.1 N NaOH. (One sample was allowed to remain at pH 7.0). After 60 minutes at room temperature, the pH of all samples was returned to 7.0 with 0.1 NaOH or 0.1 N HCl. The samples were then tested for ILC activity (Fig. 7). Half the activity was lost at pH 2.0, pH 3.0, and pH 11.0. RLA samples lost no activity at pH 4.0 through pH 10.0.

It was interesting that the antigen preparation precipitated at pH 4 through 5 and redissolved at lower and higher pH. This property was used later in an attempt to separate DT activity from antigen activity.

3. Heating. Samples of RLA were tested simultaneously for ILC activity after being treated to 25°C, 37°C, 56°C, or 70°C for 30 minutes. When compared with an RLA sample which was newly thawed, no activity was lost after heating to 70°C. When RLA was held at 56°C for 30 minutes, the LCID$_{50}$ (8) was nearly the same as that of unheated RLA but only 60 percent rather than 100 percent inhibition of cytotoxicity could be obtained. (Undiluted, 1:2 and
Figure 7

Aliquots of RLA were brought to and held at the pH indicated for 1 hour before being returned to neutrality and tested for ILC activity. Two separate experiments are shown (O, △).
1:4 dilutions of RLA all inhibited 60 percent of the lympho-cytotoxic activity of MARLA, similar to the partial loss of ILC activity after storage at -20°C for a year.

4. Dialysis. A one (1) ml sample of RLA was dialyzed against 2 liters of 0.15 M NaCl for 24 hours at 4°C with stirring and one change of the dialysis bath. In the same manner, one (1) ml samples of RLA were dialyzed against tap water and against 0.25 M sucrose, the initial supporting medium. After dialyzing for 24 hours the RLA samples appeared cloudy to varying degrees, from a visible precipitate in the sucrose dialyzed sample to only slight cloudiness in the water dialyzed sample. After centrifugation at 10,000 g (max.) for 15 minutes, the supernatants were clear and varied from colorless (sucrose dialyzed sample) to yellow (saline and water dialyzed samples). The pellets resuspended easily to form suspensions.

Since RLA was known to precipitate at moderately acid pH, it seemed possible that precipitation was caused by a drop in pH during dialysis. Protein content, pH and ILC activity of the supernatant and pellet fractions were determined before and after dialysis (Table 2). Dialysis in sucrose induced the largest amount of precipitate and the lowest pH, 5.9. When RLA was brought to this pH with 0.1 N HCl slight cloudiness did develop and no precipitation occurred when RLA was dialyzed against 0.25 M sucrose
Table 2. pH, Protein Content and Antigen Activity of Dialyzed RLA Fractions.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Dialysis in</th>
<th>Tap Water</th>
<th>0.15 M NaCl</th>
<th>0.25 M Sucrose</th>
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<tr>
<td>pH</td>
<td>7.4</td>
<td>6.9</td>
<td>6.3</td>
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<tr>
<td>mg Protein per ml</td>
<td>2.00</td>
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</tr>
<tr>
<td>LCID\textsubscript{50}</td>
<td>8</td>
<td>&lt;1</td>
<td>4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} RLA samples were dialyzed in 4000 volumes of water, saline, or sucrose, centrifuged for 15 minutes at 10,000 g (max.), and the pH, protein content and antigen activity determined in supernatant and resuspended pellet fractions.
buffered with 0.02 M barbital, pH 7.3. The RLA sample with the next lowest pH (6.3), that dialyzed in saline, also had the next most precipitate, while RLA dialyzed in water had no change in pH (7.4) and only a slight amount of precipitate. All the ILC activity and nearly all the protein of the water dialyzed sample remained in the supernatant fraction after centrifugation at 10,000 g. Less of the activity (75 percent) and protein (65 percent) of the saline dialyzed sample remained in the supernatant and only 50% of the activity and 28% of the protein was recovered in the supernatant of the sucrose dialyzed sample. It should be noted that RLA lost no ILC activity due to dialysis only.

G. **Attempt to Remove DT from RLA**

Some of the above experiments led us to believe that DT may be bound to RLA, i.e., DT activity was found with antigen activity after ultracentrifugation, ultrafiltration, and Sepharose 6B chromatography.

Two methods were tried in an attempt to remove DT from RLA: precipitation by lowering the pH to 5.0 and precipitation by addition of phosphate. Preliminary experiments demonstrated that soluble DT is at least partially insoluble in 0.025 M, 0.05 M and 0.50 M potassium phosphate buffers in 0.25 M sucrose, at neutral pH, and that a fine white precipitate occurs when 0.05 M or 0.50 M potassium
is added to a solution of DT in 0.25 M sucrose.

Method 1: Removal of DT by precipitation with 0.50 M potassium phosphate. To 1.0 ml aliquots of RLA was added 0.10, 0.15, 0.10 or 0.30 ml of 0.50 M potassium phosphate in 0.25 M sucrose. The tubes were incubated at 37°C for 30 minutes and refrigerated overnight. They were centrifuged at 1000 g (max.), the pellet brought to original volume with 0.25 M sucrose, then pellets and supernatants tested for ILC and DT activity. Supernatants were passed through millipore filters to remove residual precipitate before testing. None of the pellets had any ILC activity nor any detectable DT activity (by rat lymphocyte death). The supernatants of RLA samples to which 0.10 and 0.15 ml phosphate had been added had lost 25 percent of the original activity. The supernatants to which 0.20 and 0.30 ml phosphate had been added lost 75 percent and 50 percent of their original activity, respectively. Although part of the antigen activity was apparently destroyed upon treatment of RLA with phosphate, the remaining activity, as well as low levels of DT activity, were found in the supernatants.

Method 2: Removal of DT by lowering pH. Kreger et al. (1971) and Kantor et al. (1972) found no precipitation of DT between pH 4 and 6.7 and this was confirmed by us. Therefore, DT should not precipitate at pH 4 through pH
5, as RLA does, unless it is bound to the antigen.

Six 1.0 ml aliquots of RLA in 2 separate experiments were gradually brought to pH 5 with 0.1 N HCl. The level of cloudiness was noted, the samples centrifuged for 10 minutes at 1000 g (max.), pellets brought to supernatant volume and both fractions neutralized with 0.1 N NaOH. They were stored at -20°C until tested for protein content, DT and ILC activity. As the pH was gradually lowered the following changes occurred: from pH 6.50 to 6.30 there was no change in appearance of RLA; at pH 5.80 to 5.70 a slight turbidity occurred; at pH 5.50 to 5.45 the cloudiness was moderate; and between pH 5.0 and 4.90 there was marked cloudiness which settled slowly on standing. After centrifugation the pellets were difficult to resuspend, with many clumps remaining. On being brought to neutral pH and standing a few minutes, all the clumps dissolved and the solution was once again clear with a yellow tint. Protein content and ILC activity were determined on individual samples and averaged. The major part (70 percent) of the total protein was found in the pellet which also contained 88 percent of the ILC activity.

It was difficult to determine unequivocally whether DT remained in the supernatant or pellet. The level of rat lymphocyte death induced by DT present in RLA was only slightly higher than that in controls (10 percent vs. 5 percent). However, the slightly higher cell death
in the pellets is in agreement with results obtained by precipitation with phosphate, i.e., DT activity was found in the fraction which contained antigen activity.

H. **Other Extracting Media**

Other isotonic media as supporting fluids to be used with DT for antigen extraction were evaluated for their effect on yield of rat cell membrane antigen.

1. **0.14 M NaCl + 0.01 M Tris-HCl.** Tris-HCl buffer in saline was used at both pH 7.5 and pH 8.6 with 2 levels of DT each; 0.2 mg and 0.32 mg (99 HD\textsubscript{50} per mg) per 50 x 10\textsuperscript{6} leukocytes. The extraction procedure was the same as that using 0.25 M sucrose. Results were identical at both pH and with both levels of DT. The LCID\textsubscript{50} of the 10,000 g supernatant was 18 but that of the 200,000 g (ave.) supernatants was only 3, a 6-fold loss of activity after ultracentrifugation.

2. **0.15 M NaCl.** The extraction procedure was the same as that using 0.25 M sucrose. To 40 x 10\textsuperscript{6} leukocytes per ml 0.10 mg DT (230 HD\textsubscript{50} per mg) was added. The 100,000 g supernatant had an LCID\textsubscript{50} of 8, but after ultracentrifugation only 1/8 of this activity remained in the supernatant. The LCID\textsubscript{50} of the pellet was 1.5, but it could not be completely dissolved in saline and part of the antigenic activity, therefore, was probably not available for neutralization of MARLA. The 200,000 g supernatant had only
25 percent of the protein content of RLA, corresponding roughly to antigen activity remaining.

The above media containing DT were compared with lithium diiodosalicylate (LIS) for ability to extract rat membrane antigens. LIS (0.1 M) in 0.25 M sucrose released no detectable rat lymphocyte antigen.

I. Attempts to Disperse RLA into Smaller Components

1. Addition of LIS to RLA. RIA was concentrated on an XM300 membrane and treated with 0.25 M LIS. The mixture was stirred at room temperature for 30 minutes, then mixed with a 3-fold excess volume of 0.25 M sucrose. After centrifugation to remove undissolved LIS the treated RLA sample was passed through an XM300 membrane at room temperature and the ultrafiltrate concentrated on a PM10 Diaflow membrane to bring it back to original volume. (Because LIS is cytotoxic washing of the ultrafiltrate 4 times was necessary before it could be tested for ILC activity.) After treatment of RLA with LIS, no additional activity was recovered in the XM300 membrane ultrafiltrate.

2. Addition of potassium phosphate to RLA. An equal volume of 1.0 M or 0.1 M potassium phosphate buffer, pH 6.9, in 0.25 M sucrose was added to a sample of RLA, mixed and incubated at room temperature for 60 minutes or 37 °C for 30 minutes. The samples were passed through an XM300 membrane at room temperature, and the ultrafiltrates concentrated
4-fold on XM100A membranes. The XM100A ultrafiltrates were concentrated 4-fold on PM10 membranes, and retentates and ultrafiltrates of all 3 filters were tested for ILC activity. (Concentration 4-fold was necessary because the inhibitory effect of 0.5 M phosphate on the ILC assay is removed by a 1:4 dilution, i.e., 0.125 M phosphate is not inhibitory.) Addition of 0.5 M or 0.05 M phosphate to RLA did not increase the activity recovered in the XM300 or XM100A ultrafiltrates.

3. **Addition of LiCl to RLA.** An equal volume of 2 M or 6 M LiCl was added to a sample of RLA, mixed, and incubated at 37°C for 30 minutes. The samples were then passed through XM300, XM100A and PM10 membranes at room temperature, brought back to original volumes and tested for ILC activity. No additional activity was recovered in the XM300 and XM100A ultrafiltrates over that obtained with untreated RLA.

4. **Succinylation of RLA.** Two different amounts of succinic anhydride were added to 3 ml, two-fold concentrated RIA samples (pH 6.9, 4.0 mg per ml protein). The preparations were brought to pH 8.5 with 0.1 N NaOH in 0.25 M sucrose. To preparation 1, 2 mg solid succinic anhydride was added every 10 minutes for 30 minutes with constant stirring. The pH was kept between 8 and 9 with 0.1 N NaOH. A total of 8 mg succinic anhydride was added giving a 2/3 ratio,
mg succinic anhydride to mg antigen protein. To preparation 2, 4 mg solid succinic anhydride was added every 10 minutes for 50 minutes with constant stirring, the pH being held between 8 and 9. A total of 24 mg succinic anhydride was added, a 2-fold mg excess. One hour after the last aliquot of succinic anhydride was added, the preparations were diluted to 10 ml with 0.25 M sucrose and dialyzed against 0.02 M barbital, pH 7.3, in 0.25 M sucrose, overnight at 4°C to remove excess succinic anhydride. The preparations were then concentrated to original volume on a PM10 membrane at room temperature, after which they were passed through an XM300 membrane. Non-dialyzed, dialyzed, and ultrafiltrate samples were tested for ILC activity.

Both preparations became clearer after succinylation, but became slightly cloudy again after concentration. (RLA is crystal clear immediately after ultracentrifugation, but tends to cloud slightly on storage at 4°C or 25°C. Addition of heparin prevents this and also clears a preparation which has become cloudy.) Non-dialyzed and dialyzed samples of preparation 1 lost 67 percent of the activity present in an untreated sample while non-dialyzed and dialyzed samples of preparation 2 lost 75 percent of the original activity. No ILC activity was recovered in the XM300 membrane ultrafiltrates of either preparation.

We concluded that the addition of phosphate, LIS, LiCl, or succinic anhydride to RLA does not lead to
the development of small M active components (<300,000 M).
II. Immunogenicity and Xenotransplantation Antigen Activity of RLA

The studies discussed above indicated that RLA had met some of the criteria of a good antigen extract for transplantation studies. RLA was simple to prepare and contained a high yield of rat cell membrane xenoantigens as detected by the IIC assay. In addition, RLA maintained its activity on storage and during moderate changes of environmental conditions. We now turned our attention to whether RLA was immunogenic in mice and contained rat xenotransplantation antigens as evidence by its ability to modify the survival of rat skin grafts. These experiments were also designed to correlate the kinetics of the cellular and humoral immune responses of C3H/HeJ mice to WF/Mai RLA with the fate of rat skin grafted onto these mice. The contribution of antibody to the process of graft rejection or enhancement could be deduced from its in vitro effect on inhibition or potentiation of cell-mediated cytotoxicity (CMC) and by its HA and LC titers.

A. Humoral Immune Response

Groups of 10 C3H/HeJ mice in three separate experiments were injected with 0.2 ml RLA emulsified with 0.2 ml complete Freund's adjuvant (CFA). The dose was divided equally between I.P. and subcutaneous (S.C.) sites. Fifteen days later the animals received 0.4 ml RLA emulsified in CFA
with 0.6 ml being injected I.P. and 0.2 ml S.C. The third injection was given 50 days after the first. In this instance mice received 0.4 ml of two-fold concentrated RLA emulsified with 0.4 ml CFA; 0.6 ml was injected I.P. and 0.2 ml S.C. One control group of 5 mice received 0.25 M sucrose and CFA in the same dosage and at the same times. One additional experimental group of 10 mice received twice the concentration of RLA as the other three experimental groups at each injection. The antibody titers of this group were similar to those of the other three experimental groups so they were included in the calculation of mean titers (40 mice total). At various intervals all the mice were bled from the tail and the serum pooled. When sufficient ascites was available, the ascites was pooled instead of serum. Both serum and ascites were tested in HA and LC assays. Figure 8 shows the mean antibody titers of 4 groups of 10 mice ± S.E. Arrows along abscissa indicate day of injections. HA antibodies for rat erythrocytes were barely detectable in sera collected just before the second injection and reached a titer of 48 five days later. Similarly, a LC titer of 1.5 was obtained at 15 days and 5 days after the second injection the titer increased to 12.8. The maximum HA titer after the second RLA injection was 340 occurring 15 days following the second immunization. The maximum LC titer (37.4) occurred at the same time.
Forty mice were immunized with 0.2 ml RLA (0.4 mg protein) in an equal volume of CFA. Arrows represent booster injections in which mice received twice the amount of RLA as the previous injection in CFA. Serum or ascites was collected and pooled at the times indicated and tested for HA (dashed line) and LC (solid line) activity. The vertical bars represent S.E.
Both HA and LC titers dropped gradually. At day 50 the mice received their third and final injection. The HA and LC titers rose dramatically, then gradually leveled off around day 14 after the third injection. Maximum titers (HAT, 4096; and LCT, 512) were reached about day 70 (20 days after the third injection). The HA and LC activity remained high with a gradual decline over the next 50 days. By 120 days, the LC titer had fallen to 32 and the mean HA titer was 256. No HA or LC activity was found in sera or ascites from the control group of mice at any time.

B. Cellular Immune Response

The cellular immune response of mice to RLA was measured by three in vitro assays: lymphoblastic transformation (LBT), cell-mediated cytotoxicity (CMC) and antibody-dependent cell cytotoxicity (ADCC). In addition, the inhibiting or potentiating effect of serum or ascites on CMC was determined by assaying immune cell cytotoxicity on target cells in the presence of serum (ascites) collected at the same time in the immunization schedule as the spleen cells. We called this assay ADICC for antibody-dependent immune cell cytotoxicity.

Ninety C3H/HeJ mice were immunized according to the dosage and schedule described above for the study of the humoral immune response except that the third injection
was given 58 days instead of 50 days after the first injection and one-half the volume of CFA was used for the second and third injections. Six control mice received 0.25 M sucrose in place of RLA, incorporated in CFA. All assays including grafting were performed at 5 arbitrarily chosen intervals during the course of the experiment, as shown below. The day of the first immunization was considered day 0.

<table>
<thead>
<tr>
<th>Day of Immunization Interval</th>
<th>Day of Serum Collection</th>
<th>Day of LBT</th>
<th>Day of CMC</th>
<th>Day of ADCC</th>
<th>Day of ADICC</th>
<th>Day of Grafting</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>70</td>
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<td>87</td>
<td>88</td>
<td>89</td>
<td>89</td>
<td>90</td>
</tr>
</tbody>
</table>

Sera or ascites were collected from 20 mice at the specified times, pooled, tested for HA and LC activity, and used in the ADICC and ADCC assays. At each testing period two mice from the experimental group and one from the control group were sacrificed and spleen cells from each mouse were tested for LBT activity with several dilutions of RIA and with phytohemagglutinin-M (PHA-M). In addition,
two or three mice from the experimental group were sacrificed and spleen cells from each mouse were tested for CMC and ADICC activity. Splenocytes from two normal mice were pooled and used for CMC control and ADCC. Ten or more mice from the experimental group were grafted at each interval with WF/Mai rat skin.

The complete data from the LBT assays can be seen in Figures 9 through 13. The mitogen PHA-M was used to demonstrate that cells were viable and capable of responding in the LBT assay. PHA-M at 0.5 percent concentration was stimulatory in every case except in Interval II where failure to induce DNA synthesis was related to bacterial contamination of the mitogen. The viability of the cell preparation in this case was indicated by the proliferation induced by antigen treatment. Control animals were generally stimulated to a higher degree by PHA-M than were experimental mice, and PHA-M at 0.5 percent stimulated mouse cells to a much greater extent than PHA-M at 1.0 percent, which was often inhibitory.

Although there was some stimulation of control cells at certain concentrations of RLA, immune cells responded with greater uptake of $^3$H-thymidine at every interval, forming a bell-shaped curve with decreasing concentrations of RLA. The curves at Intervals I, III, and IV were very similar to one another with maximum stimulation occurring
Spleen cells from 3 mice were tested 12 days after the initial RLA injection (Interval I). In each group of 3 bars the left bar represents the control animal which received 0.25 M sucrose emulsified in CFA. The middle bar represents one experimental mouse and the right bar represents the second experimental mouse.
Spleen cells from 3 mice were tested 12 days after the second RLA injection (Interval II). In each group of 3 bars the left bar represents the control animal which received 0.25 M sucrose emulsified in CFA. The middle bar represents one experimental mouse and the right bar represents the second experimental mouse.
Spleen cells from 3 mice were tested 10 days after the third RLA injection (Interval III). In each group of 3 bars the left bar represents the control animal which received 0.25 M sucrose emulsified in CFA. The middle bar represents one experimental mouse and the right bar represents the second experimental mouse.
Spleen cells from 3 mice were tested 20 days after the third RIA injection (Interval IV). In each group of 3 bars the left bar represents the control animal which received 0.25 M sucrose emulsified in CFA. The middle bar represents one experimental mouse and the right represents the second experimental mouse.
Figure 13

Spleen cells from 3 mice were tested 30 days after the third RLA injection (Interval V). In each group of 3 bars the left bar represents the control animal which received 0.25 M sucrose emulsified in CFA. The middle bar represents one experimental mouse and the right bar represents the second experimental mouse.
at 10 µg RIA protein. At Interval II, maximum uptake was obtained at 40 and 20 µg RIA protein with individual animals differing in the concentration of RIA which induced maximum uptake. At the last interval the curve was shifted slightly to the right and maximum uptake was induced by 5 µg RIA protein.

The mean LBT response of the control mouse spleen cells to all concentrations of RIA in each test was compared at 2 standard deviations with the response of spleen cells from experimental animals. In the chart below "X" represents the cases where both experimental animals had significantly higher LBT responses than the mean of the control. "A" represents the cases where only one test animal had a significantly higher response and NS means the difference was not statistically significant.

<table>
<thead>
<tr>
<th></th>
<th>1:5</th>
<th>1:10</th>
<th>1:20</th>
<th>1:40</th>
<th>1:80</th>
<th>1:160</th>
<th>1:320</th>
</tr>
</thead>
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<tr>
<td>Interval I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>I</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>II</td>
<td>X</td>
<td>X</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>A</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

The experimental animals responded with significantly higher \(^3\)H-thymidine incorporation to nearly all concentrations
of RLA except in Interval II. At 1:40 through 1:160 dilutions in this interval the experimental animals actually had significantly lower responses than the control.

The LBT data is summarized in Table 3. The average stimulation index (S.I.) of experimental mice increased at each interval. Spleen cells from mice immunized with RLA were stimulated by RLA 2 to 4 times more than spleen cells from control mice immunized with the extracting medium only. The level of response increased with time following the third antigen injection. The dose of antigen necessary to elicit maximum response was greatest at Interval II. Individual animals differed in the level to which their lymphocytes responded to RLA and to PHA-M. It is interesting that control mice responded with greater uptake of $^{3}$H-thymidine than experimental mice when stimulated with PHA-M. Perhaps when animals are hyperimmunized not as many lymphocytes are responsive to stimulation with a mitogen.

There was no cell-mediated cytotoxicity (CMC) detected with immune lymphocytes at any time during the period of analysis. The specific $^{51}$Cr release (S.C.R.) ranged from 2.20 percent to -3.06 percent with a mean of -0.07 percent. S.C.R. of less than 5 percent was not considered significant. Since no significant CMC occurred it was not possible to measure serum inhibition of CMC. When the inhibition assays
Table 3. LBT of Spleen Cells from Mice Immunized with RLA plus CFA (Experimental) or 0.25 M Sucrose plus CFA (Control).

<table>
<thead>
<tr>
<th>Interval</th>
<th>Day Post-injection</th>
<th>RLA Stimulating Dose (µg Protein)</th>
<th>Response to RLA</th>
<th>Response to PHA-M (0.50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Experimental</td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CPM (Avg.) S.I.</td>
<td>CPM (Avg.) S.I.</td>
</tr>
<tr>
<td>I</td>
<td>12</td>
<td>10(^a)</td>
<td>32415 (^b) (2.7) (^c)</td>
<td>9558 (1.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27471 (1.7)</td>
<td>14793 (0.9)</td>
</tr>
<tr>
<td>II</td>
<td>27</td>
<td>20</td>
<td>15068 (2.1)</td>
<td>9906 (1.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15770 (2.9)</td>
<td>8491 (1.1)</td>
</tr>
<tr>
<td>III</td>
<td>68</td>
<td>10</td>
<td>22001 (2.3)</td>
<td>10337 (1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>19068 (3.0)</td>
<td>15338 (1.6)</td>
</tr>
<tr>
<td>IV</td>
<td>78</td>
<td>10</td>
<td>30619 (4.3)</td>
<td>11554 (2.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>32329 (3.6)</td>
<td>17564 (2.5)</td>
</tr>
<tr>
<td>V</td>
<td>88</td>
<td>5</td>
<td>62488 (4.5)</td>
<td>18075 (2.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>63957 (6.9)</td>
<td>40403 (2.9)</td>
</tr>
</tbody>
</table>

a The antigen dose is that which elicited maximum \(^3\)H-thymidine incorporation.

b CPM is the average of 3 separate determinations. Each value represents the results of one animal.

c Stimulation index (S.I.) is calculated as the CPM obtained with antigen or PHA-M divided by CPM obtained with medium only.
were run, however, instead of suppression or inhibition there was marked potentiation of CMC at Intervals II through V (Table 4).

When the serum pools were tested with the corresponding immune spleen cells collected at the same interval, no ADICC was observed at Interval I. The ADCC assay showed a minimal, base-line level of S.C.R. at Intervals I and II. ADICC was marked at Interval II, 13 days after the second injection, whereas significant levels of ADCC were not observed until Interval III, 11 days after the third injection. At Intervals II through V ADICC was greater than ADCC, and in all cases but one the difference was statistically significant (Table 4, Columns 3 and 5).

Two randomly chosen dilutions of antiserum were used in the first 3 intervals for measuring the degree of potentiation by serum. After observing that a clear endpoint had not been reached, higher dilutions of antiserum were used for testing at the remaining 2 intervals. Higher dilutions of serum (1/50 to 1/200) demonstrated a substantial increase in the difference between ADICC and ADCC at Interval IV as compared to early periods. With both immune and normal cells serum potentiation appeared to peak at Interval III or IV, 11 to 21 days following the third injection. A significant decrease in activity was observed 31 days following the third injection (Interval V).
Table 4. Antibody-Dependent Cell Cytotoxicity with Normal Mouse Spleen Cells (ADCC) and with Immune Mouse Spleen Cells (ADICC) after Immunization with RLA^a

<table>
<thead>
<tr>
<th>Interval</th>
<th>Antibody</th>
<th>Titer</th>
<th>Percent Specific $^{51}$Cr Release</th>
<th>ADCC</th>
<th>ADICC</th>
<th>Percent $^{51}$Cr Release Due to Immune Cells Only (ADICC Release - ADCC Release)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>I</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>9.1</td>
<td>5.4</td>
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<tr>
<td></td>
<td></td>
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<td>-6.0</td>
<td>-1.7</td>
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<td>-1.2</td>
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<td>5.9^b</td>
<td>8.4^b</td>
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<td>81.4</td>
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<td>64.1</td>
<td>71.3</td>
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<td>(70.4)</td>
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<tr>
<td>IV</td>
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<td>53.3^b</td>
<td>54.7^b</td>
<td>38.6^b</td>
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<td>82.1</td>
<td>75.0</td>
<td>64.1</td>
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<td>(61.6)</td>
<td>(75.9)</td>
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<td>(75.9)</td>
<td>(68.9)</td>
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<tr>
<td>V</td>
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<td>31.5^c</td>
<td>33.0^d</td>
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<td>--</td>
<td>(47.7)</td>
<td>(43.1)</td>
<td>(40.4)</td>
</tr>
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</table>

^a Each value represents the specific $^{51}$Cr release calculated from triplicate tests except for Interval I, where duplicates were used in some cases. Values in parentheses are averages. The effector to target cell ratio was 200:1 in all assays at each interval except Interval I, where 125:1 for ADCC and 100:1 for ADICC were used. Dilutions are final dilutions of antiserum or normal serum. The serum for each interval was pooled from 20 mice.

^b Statistical analysis: ADCC and ADICC were compared at the same antiserum dilution. P < .001, ADCC compared to average ADICC.

^c P < .01, ADCC compared to average ADICC.

^d P < .05, ADCC compared to average ADICC.

^e P < .02, ADCC compared to average ADICC.
The sudden rise in ADCC at Interval III corresponded to a marked increase in LC activity (30 fold). It also corresponded to the first time that target cells, incubated with heat inactivated antiserum only, released significantly greater $^{51}$Cr than did target cells incubated with normal serum. This occurred with antisera from Intervals IV and V as well but not with antisera from Intervals I and II (Table 5, Column 2). (When antisera from Intervals III, IV and V were incubated with target cells in the LC assay in the absence of complement and the cells subsequently examined for Eosin Y dye exclusion, lysis of target cells was not evident.) When the S.C.R. for ADCC and ADICC was calculated after substraction of the counts by antiserum alone, using the equation below, data was obtained as shown in Table 5 (Columns 3 and 4).

\[
\% \text{ S.C.R. = } \frac{(\text{CPM } \bar{c} \text{ AS + EC}) - (\text{CPM } \bar{c} \text{ AS - CPM } \bar{c} \text{ NS}) - (\text{CPM } \bar{c} \text{ NS + EC})}{\text{Max. CPM - CPM } \bar{c} \text{ NS + EC}} \times 100
\]

\[
\text{CPM } \bar{c} \text{ AS + EC} = \text{CPM with a dilution of antiserum plus effector cells and target cells}
\]

\[
\text{CPM } \bar{c} \text{ AS} = \text{CPM with same dilution of antiserum and target cells}
\]

\[
\text{CPM } \bar{c} \text{ NS} = \text{CPM with same dilution of normal serum and target cells}
\]
Table 5. ADCC and ADICC Calculated after Subtracting Counts Released Due to Antiserum Alone.

<table>
<thead>
<tr>
<th>Interval</th>
<th>Percent Specific (^{51})Cr Release Due to AS Only(^a)</th>
<th>(\text{ADCC})</th>
<th>(\text{ADICC})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1:10)</td>
<td>(1:40)</td>
<td>(1:50)</td>
</tr>
<tr>
<td>I</td>
<td>0 0 0 -- -- --</td>
<td>9.1 5.4 -- -- --</td>
<td>-6.9 -1.2 -- -- --</td>
</tr>
<tr>
<td>II</td>
<td>0 0 0 -- -- --</td>
<td>5.9 8.4 -- -- --</td>
<td>29.2 30.0 -- -- --</td>
</tr>
<tr>
<td>III</td>
<td>33 33 -- 20 11</td>
<td>16.4 16.9 -- -- --</td>
<td>32.0 34.1 -- -- --</td>
</tr>
<tr>
<td>IV</td>
<td>-- -- 22 24 16</td>
<td>-- -- 27.0 18.1 16.2</td>
<td>-- -- 56.0 42.5 55.5</td>
</tr>
<tr>
<td>V</td>
<td>-- -- 21 25 15</td>
<td>-- -- 15.5 12.0 16.9</td>
<td>-- -- 27.6 20.1 26.8</td>
</tr>
</tbody>
</table>

\(\text{a} \quad \text{CPM with AS} - \text{CPM with NS} \times 100; \text{AS, antiserum; NS, normal serum.}\)

\(\text{b} \quad \text{Each value represents the specific} \(^{51}\)\text{Cr release from target cells calculated as described in text.} \)

For other details see legend, Table 4.
\[ \text{CFM} \oplus \text{NS} + \text{EC} = \text{CFM with same dilution of normal serum plus effector cells and target cells} \]

In Intervals I and II there were no changes from data in Table 4 because antisera from these intervals by themselves did not cause \(^{51}\text{Cr} \) release from target cells. This result was not surprising for antiserum from Interval I because it had no HA or LC activity. However, since antiserum from Interval II had moderate HA and LC titers (Table 4), lack of cytotoxic activity without complement may indicate a qualitative as well as a quantitative difference between this antiserum and the antisera of later intervals. The greatest difference between immune and normal cells was found in Interval II with a mean 6-fold increase of ADICC over ADCC. In Interval III there was 1.6 to 2.3 times more S.C.R. with immune cells than with normal cells. In Interval IV there was 2.1 to 3.0 times more S.C.R. with immune cells and in Interval V this ratio dropped to 2.0 to 1.7 times more S.C.R. with immune cells than with normal cells.
C. Correlation of In Vitro Immunological Activity with Skin Graft Survival Time

HA and LC titers, LBT stimulation indices (S.I.) and S.C.R. from labeled rat lymphocyte target cells in CMC, ADICC and ADCC assays of RIA immunized and control mice are summarized in Table 6 along with graft rejection data.

There was an abrupt increase in both HA and LC titers at Interval II and another abrupt increase in LC titer, about 30-fold, at Interval III while HA titer increased only three-fold. After Interval III there was little change in HA and LC titers.

The LBT S.I. increased at each successive interval with the biggest increase, 1.5-fold each, coming between Intervals III and IV and Intervals IV and V.

No immunized mouse had any CMC activity in any of the intervals tested; the values listed deviate from zero within the normal experimental error of the assay. S.C.R. less than 5 percent was not considered significant.

ADCC and ADICC S.C.R. was calculated after subtraction of counts released with target cells incubated with antiserum alone, according to the equation in the text. Only the data obtained with the highest antiserum dilution used at each interval are shown. The pattern which emerged during the course of the experiment is quite clear. There
Table 6. *In Vitro* Immunologic Activity and Skin Graft Rejection after Immunization with RLA.

<table>
<thead>
<tr>
<th>Interval</th>
<th>Day Post-1° Inj.</th>
<th>Day Post-2° Inj.</th>
<th>Day Post-3° Inj.</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LBT&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent Specific 51&lt;sup&gt;c&lt;/sup&gt;Cr Release</th>
<th>Graft Rej.&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
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<tr>
<td>I</td>
<td>11-14</td>
<td></td>
<td></td>
<td>0</td>
<td>&lt;1</td>
<td>-2.7</td>
<td>7.3±0.6 (1/9)</td>
</tr>
<tr>
<td>II</td>
<td>26-29</td>
<td>11-14</td>
<td></td>
<td>2048</td>
<td>32</td>
<td>1.2</td>
<td>9.1±0.7 (2/10)</td>
</tr>
<tr>
<td>III</td>
<td>67-70</td>
<td>52-55</td>
<td>9-12</td>
<td>6144</td>
<td>850</td>
<td>0.85</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>77-80</td>
<td>62-65</td>
<td>19-22</td>
<td>6144</td>
<td>770</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>V</td>
<td>87-90</td>
<td>72-75</td>
<td>29-32</td>
<td>8192</td>
<td>1536</td>
<td>-0.35</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> HA and LC titers are the mean of 2 determinations.

<sup>b</sup> LBT Stimulation Index (S.I.) is the average of the maximum response for 2 animals.

<sup>c</sup> The ADCC and ADICC values are those adjusted for CPM released by antiserum alone (see text). CMC and ADICC values are the average of 2 mice except in Interval III, where they are the average of 3 mice.

<sup>d</sup> S.C.R. for antiserum only was calculated according to the equation shown in Table 5. The number in parentheses refers to the antiserum dilution used for ADCC, ADICC, and AS only and was the highest dilution tested at each interval.

<sup>e</sup> WF/Mal rat skin graft rejections by C3H/HeJ mice are given as mean survival time in days (MST) ± standard error of the mean (S.E.) and/or numbers of white grafts (W.G.) out of total mice grafted.
was only a small increase in ADCC at Interval II, but an enormous increase in ADICC. At Interval III there appeared to be an increase in ADCC (about 2-fold) whereas little change occurred in ADICC. This may be because ADICC S.C.R. (before subtracting counts due to antiserum alone) was approaching maximum releasable $^{51}$Cr and thus the sensitivity of the test was decreased. At Interval IV the antiserum dilution was increased with a concomitant rise in ADICC while ADCC values remained similar to those at Interval III. There was a decrease in ADICC at Interval V but no decrease in ADCC.

At Interval I the MST of rat skin on 8 mice (there was one white graft and one technical failure) was not significantly longer than that of controls (16 un-immunized mice). There was one white graft in this group of mice whereas no white grafts were observed in over 100 untreated grafted mice. (White grafts are skin grafts which have never become vascularized and are typical of secondary hyperacute xenograft rejection (Baldamus, et al., 1973).

During Interval II, the MST of rat skin grafts on 8 immunized mice was significantly greater than the MST of immunized mice in Interval I ($P < .001$) and unimmunized mice ($P < .001$). There were two white grafts in this group indicating that the immune response to RLA of some
mice resulted in hyperacute rejection whereas the immune response of other mice resulted in enhanced survival. Since pooled sera were used for *in vitro* assays, individual animals may have differed substantially from the group in regard to their humoral and/or cell-mediated immune response. In half the grafts more edema than normal was present during rejection.

In Interval III the grafts of the 10 immunized mice were rejected hyperacutely as white grafts. Unexpectedly by day 6, 7 out of 10 grafted mice died, while no deaths occurred with immunized mice not grafted.

In Interval IV all the immunized mice rejected their grafts as white grafts. Once more grafted mice died; 9 out of 14 were dead by day 6.

In Interval V, all 10 immunized mice had white grafts but no mice had died by day 9 when the experiment was terminated.

In a pilot experiment 5 mice, immunized for the humoral immune response experiment (Fig. 9), were grafted 40 days after they received their third RLA injection, 8 days later than the mice grafted in Interval V. At that time their HA titer was 512 and LC titer was 32. The MST was not significantly different from controls.
III. Use of RLA to Prolong Skin Xenografts

Since RLA was a stable, soluble xenoantigen preparation with potent xenotransplantation activity, it appeared to be suitable for xenograft prolongation experiments. Three different procedures were used in an attempt to induce rat graft prolongation in mice.

A. Combination treatment of anti-lymphocyte serum and RLA.

B. Immunization of mice with RLA to produce active enhancement.

C. Administration of anti-RLA to produce passive enhancement.

A. Xenograft Prolongation with RLA in Combination with Anti-Lymphocyte Serum (RLA-BPV-ALS Experiments)

Experiment 1. Sixteen days before receiving primary rat skin grafts, groups of C3H/HeJ mice received either an intraperitoneal (I.P.) injection of 1.0 ml of concentrated RLA or an intravenous (I.V.) injection of 0.5 ml of concentrated RLA containing 10 I.U. of heparin per ml to prevent in vivo aggregation of RLA with activation of the clotting system. Six days before grafting the mice received 0.3 ml of Bordetella pertussis vaccine (BPV) I.V. (Eli Lilly and Company). Mice were injected I.P. with 0.5 ml of rabbit anti-mouse lymphocyte serum (ALS) on days -2, 0, 2, 4, and 6 relative to grafting day 0.
Control mice received either ALS plus BPV or nothing. The basic design of Experiment 1 and Experiment 2 are shown in Table 7 and discussed below.

We used BPV to induce a lymphocytosis to augment the effectiveness of ALS (Festenstein et al., 1969, and Pinto et al., 1974). ALS acts principally by destroying lymphocytes in peripheral blood, thus a greater number of lymphocytes should be eliminated with the use of BPV. Due to prior RLA treatment a greater proportion of the circulating lymphocytes should be specific for RLA antigens.

In a preliminary experiment to determine the most effective dose, route and day of BPV administration, the vaccine was injected into 6 mice I.P., 6 mice subcutaneously (S.C.) in the neck region and 9 mice I.V. Results are shown in Table 8. The I.P. route produced variable cell counts; higher leukocyte counts were apparently induced with 0.25 ml than with 0.30 ml BPV. Very little if any leukocytosis was induced by S.C. injection of the vaccine. The highest counts occurred after I.V. injection of BPV and peaked at day 4 after injection. The last 5 mean values are those obtained in RLA-BPV-ALS xenograft prolongation Experiment 1 (Table 7) when 0.30 ml of BPV was injected I.V. Leukocytes were counted on day 4 after BPV injection, which was 2 days before grafting. The WBC count was 3 to 4-fold higher than normal at that time.
Table 7. Regimen of Treatment for Xenograft Prolongation with RLA, *Bordetella pertussis* Vaccine (BPV) and Anti-Lymphocyte Serum

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Mice</th>
<th>Treatment</th>
<th>Dose</th>
<th>Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13</td>
<td>RLA (I.P.)</td>
<td>7 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>RLA (I.P.)</td>
<td>14 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>C</td>
<td>14</td>
<td>RLA (I.V.)</td>
<td>7 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>D</td>
<td>11</td>
<td>RLA (I.V.)</td>
<td>14 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>E</td>
<td>16</td>
<td>None</td>
<td></td>
<td>-16</td>
</tr>
<tr>
<td>A,B,C,D,E</td>
<td></td>
<td>BPV (I.V.)</td>
<td>0.3 ml</td>
<td>-6</td>
</tr>
<tr>
<td>A,B,C,D,E,E</td>
<td></td>
<td>ALS (I.P.)</td>
<td>0.5 ml</td>
<td>-2,0,2,4,6</td>
</tr>
<tr>
<td>A,B,C,D,E,E</td>
<td>Skin Graft</td>
<td>--</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>16</td>
<td>RLA (I.V.)</td>
<td>8 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>B</td>
<td>19</td>
<td>None</td>
<td></td>
<td>-16</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>BPV (I.V.)</td>
<td>0.3 ml</td>
<td>-2</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>ALS (I.P.)</td>
<td>0.5 ml</td>
<td>2,4,6</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>Skin Graft</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>13</td>
<td>RLA (I.V.)</td>
<td>8 mg Protein</td>
<td>-16</td>
</tr>
<tr>
<td>B</td>
<td>17</td>
<td>DT + Heparin</td>
<td>0.7 mg DT (I.V.) 5 I.U. Heparin</td>
<td>-16</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>BPV (I.V.)</td>
<td>0.2 ml</td>
<td>-6</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>ALS (I.P.)</td>
<td>0.5 ml</td>
<td>-2,0,2,4,6</td>
</tr>
<tr>
<td>A,B</td>
<td></td>
<td>Skin Graft</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 8. Leukocyte Counts* After Administration of *Bordetella pertussis*
Vaccine.

<table>
<thead>
<tr>
<th>Route</th>
<th>Dose</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.P.</td>
<td>0.25 ml</td>
<td>22,200±2421b</td>
<td>25,000±3897</td>
<td>19,300±603</td>
<td></td>
</tr>
<tr>
<td>I.P.</td>
<td>0.30 ml</td>
<td>11,800±1058</td>
<td>12,200±1557</td>
<td>9,600±2553</td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td>0.25 ml</td>
<td>11,000±650</td>
<td>11,500±1501</td>
<td>9,600±379</td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td>0.30 ml</td>
<td>11,200±3763</td>
<td>10,600±1833</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.V.</td>
<td>0.20 ml</td>
<td>26,000±7451</td>
<td>20,000±5571</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23,020±3615c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25,980±4797d</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I.V.</td>
<td>0.30 ml</td>
<td>21,600±2303</td>
<td>26,900±3308</td>
<td>21,700±1762</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28,000±7394e</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>26,100±5292f</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>33,100±6315g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>37,100±4158h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28,200±6951i</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mice were bled from the tail and leukocytes counted with a Coulter Counter. Normal WBC, 8760±1660.

b Mean ± standard deviation.

c Mean WBC of 5 mice in Group A, RLA-BPV-ALS Exp. 3.

d Mean WBC of 5 mice in Group B, RLA-BPV-ALS Exp. 3.

e Mean WBC of all mice (15) in Group A, RLA-BPV-ALS Exp. 1.

f Mean WBC of all mice (14) in Group B, RLA-BPV-ALS Exp. 1.

g Mean WBC of 5 mice in Group C, RLA-BPV-ALS Exp. 1.

h Mean WBC of 5 mice in Group D, RLA-BPV-ALS Exp. 1.

i Mean WBC of 5 mice in Group E, RLA-BPV-ALS Exp. 1.
(The normal leukocyte count for C3H/HeJ mice was 8760 ± 1660 per mm$^3$ (mean ± S.D.) for tail vein blood.) Mean WBC for animals in RIA-BPV-ALS Experiment 3 are also shown in Table 8. They received 0.2 ml BPV instead of 0.3 ml to reduce loss of mice.

LC and HA antibody titers for rat cells two days prior to grafting were determined for all groups in RIA-BPV-ALS Experiment 1. We tested sera on this day rather than on the day of grafting because ALS was injected on day -2 and it contains cross-reacting antibody to rat antigens (LC titer of 512 and HA titer of 32). LC titers were negative in all groups, and only Group C (0.5 ml of 8 x RLA I.V.) produced detectable HA antibody (HAT of 1).

Two different concentrations of RLA were injected by 2 different routes. By this approach differences in MST could be related to amount and/or route of antigen administration. Graft survival is shown in Table 9. Three (A, B, and D) of the 4 groups which received RLA had significantly longer MST ($P < .001$, $P < .01$, $P < .01$, respectively) when compared to Group E, which received BPV plus ALS but no RLA. The MST of Group C was not very different from those of Groups A, B and D, but the range of graft survival for individual animals was greater.

Based on Student's one-tailed t test, the MST of Group
Table 9. Rat Skin Graft Survival on Mice Treated with Rat Lymphocyte Antigen, *Bordetella pertussis* Vaccine and Anti-Lymphocyte Serum, Experiment 1.a

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>RLA Concentration and Route of Injection</th>
<th>Mg of Protein and Route of BPV and ALS</th>
<th>MST ± SE</th>
<th>Pd</th>
<th>Day of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4 x I.P.</td>
<td>7</td>
<td>Yes</td>
<td>25.0±1.4</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>B</td>
<td>8 x I.P.</td>
<td>14</td>
<td>Yes</td>
<td>23.2±2.0</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>C</td>
<td>8 x I.V.</td>
<td>7</td>
<td>Yes</td>
<td>19.3±2.0</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>D</td>
<td>16 x I.V.</td>
<td>14</td>
<td>Yes</td>
<td>20.3±1.1</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>0</td>
<td>Yes</td>
<td>14.3±1.7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>0</td>
<td>No</td>
<td>5.8±0.6</td>
<td>---</td>
</tr>
</tbody>
</table>

a See Table 7 for details of treatment.

b *Bordetella pertussis* Vaccine and Rabbit Anti-Mouse Lymphocyte Serum.

c Mean graft survival time ± standard error.

d Level of significance for two-tailed Student's t test, Groups A, B, C and D compared with Group E, Group E compared with Group F.

e Final number of mice in group and reason it was less than original number.

Group A: 4(9 mice died by day 11)  
Group B: 6(5 mice died by day .4)  
Group C: 8(6 technical failures)  
Group D: 8(1 mouse died before grafting, 2 technical failures)  
Group E: 14(2 mice died by day 5)  
Group F: 16(2 technical failures)
C was significantly longer (P < .05) than Group E. One mouse in control Group E retained his rat skin graft 2 to 4 times longer than any other mouse in that group. Pinto et al. (1974) found similar results in a minority of their control mice after injection of BPV plus ALS in an allograft model. Without averaging this mouse with Group E, the MST ± S.E. was 12.9 ± 1.1 and the P values for Groups A, B and C were <.001, while that of Group C was <.02. The condition of well accepted skin grafts with fully grown hair is shown for one mouse in Group B and one in Group C on day 28 (Figures 14 and 15).

A number of mice died unexpectedly in Groups A, B, D and E, although no deaths occurred in Groups C and F. More of the mice receiving RLA I.P. died (14/24) than did mice receiving RLA by the I.V. route (1/25). The fact that there were 2/16 deaths in Group E, which received no antigen, meant that BPV or ALS or the combination was responsible for some deaths. No deaths were encountered in previous experiments with numerous C3H/HeJ mice injected with ALS alone or in combination with rat antigen extracted with hypertonic sucrose (Hines and Lang, submitted for publication). In addition, when 13 mice were injected I.V. with BPV only, no deaths occurred. Therefore, the combination of BPV and ALS resulted in death for approximately one-tenth of the animals (3/41). This deleterious effect
PHOTOGRAPH OF MOUSE TREATED WITH RIA, BORDETELLA PERTUSSIS AND ANTI-LYMPHOCYTE SERUM, THEN GRAFTED WITH RAT SKIN

EXPERIMENT 1.

Figure 14

Mouse from Group B on day 28 (14 mg Protein Antigen I.P.). All mice (13) but one in control Group E (no antigen) had rejected their grafts by day 17.
PHOTOGRAPH OF MOUSE TREATED WITH RLA, BORDETELLA PERTUSSIS AND ANTI-LYMPHOCYTE SERUM, THEN GRAFTED WITH RAT SKIN

EXPERIMENT 1.

Figure 15
Mouse from Group C on day 28 (7 mg Protein Antigen I.V.).
was potentiated by injection of RLA by the I.P. route, increasing mortality to 50 percent (14/24).

There was no significant difference in MST based on dose of antigen. However, when individual graft survival times of mice injected with RLA I.P. (Groups A and B) were combined and compared with the MST of mice injected with RLA I.V. (Groups C and D), there was significant prolongation of grafts on mice receiving RLA I.P. (P of 0.02).

The MST of control Group E (which received ALS plus BPV) was significantly longer (P < .001) than the MST of Group F (the untreated controls). This was expected and consistent with the results of others in the field of xenotransplantation.

**Experiment 2.** The regimen used in Experiment 1 (Table 7) was modified to follow more closely that of Pinto et al. (1974), which had been so successful in prolonging mouse allografts. In this experiment RLA was injected I.V. only, in order to minimize the loss of mice previously observed with I.P. injection. Group A received 0.7 ml of a 6-fold concentrated RLA preparation containing 8 mg protein and Group B received no antigen. Heparin was added to 14.0 I.U. per ml RLA before concentration. BPV and ALS were given as shown in Table 7, Experiment 2.
Out of 16 Group A mice grafted with rat skin, there were no deaths and 1 technical failure. Out of 19 grafted mice in control Group B, which received BPV plus ALS but no RIA, 7 died during the course of the experiment with 2 of the 7 dying on the day of grafting. Including control mice from Experiment 1, 9 out of 35 animals receiving only BPV plus ALS died.

Some of these grafts did not show the usual appearance observed during the rejection process. Most of the grafts at days 3 through 6 were not healthy pink in color but mainly white with pink in a portion of the grafts only. They were not, however, "white grafts" and had healed in around the borders of the graft bed. When rejection was occurring these grafts became more and more edematous, then some hardened and finally crusted over. Unfortunately, it was not easy to grade these grafts and determine the day of 50% rejection. In both control and experimental groups some of the mice rejected their grafts in the usual, easy to score fashion, but a much smaller proportion did so in the control group (3 out of the 12 surviving animals). In the group which received RIA, 10 out of 15 rejected their grafts in the normal manner. Table 10 shows the MST of 50% graft rejection for mice which rejected their grafts in the normal manner, and MST of edematous grafts which were based on 100% graft rejection. (With the edematous
Table 10. Rat Skin Graft Survival on Mice Treated with Rat Lymphocyte Antigen, *Bordetella pertussis* Vaccine and Anti-Lymphocyte Serum, Experiment 2.

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>RLA Concentration</th>
<th>Mg of Protein Antigen</th>
<th>50% Rejection ( \text{MST ± SE} )</th>
<th>Day of Rejection</th>
<th>100% Rejection ( \text{MST ± SE} )</th>
<th>Day of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6 x</td>
<td>8</td>
<td>12.4 ± 1.2</td>
<td>6, 10, 10, 10, 10, 14, 15, 16, 16, 17</td>
<td>19.8 ± 0.5</td>
<td>19, 19, 19, 21, 21e</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
<td>0</td>
<td>11.0 ± 2.5</td>
<td>6, 13, 14</td>
<td>14.2 ± 0.9</td>
<td>10, 11, 12, 15, 15, 15, 16, 16, 18e</td>
</tr>
</tbody>
</table>

See Table 7 for details of treatment

The mice which rejected their grafts in the usual manner were scored at day of 50% graft rejection.

Mean graft survival time in days ± standard error.

Mice whose grafts could not be scored at day of 50% rejection were scored at day of 100% rejection. See text for details.

Fifteen final animals in Group A (1 technical failure).

Twelve final animals in Group B (7 mice died).

The level of significance according to Student’s t test for day of 100% graft rejection, Group A compared with Group B. The difference in MST of 50% rejection between Groups A and B was not significant (NS).
grafts it was impossible to determine 50% rejection. Criteria for 100% graft rejection were a thick, grossly wet, whitish graft, which, when pulled back, resulted in very little bleeding, or alternatively, a hard, partially separated graft which also resulted in very little or no bleeding when pulled away from the graft bed.) The MST (50% rejection of Group A, which received antigen, was not significantly different from that of Group B, the control animals. There was, however, a significant difference in MST based on 100% graft rejection between experimental and control groups (P < .001). Graft prolongation in Experiment 1 was significantly longer than in Experiment 2 for experimental mice (P < .001). This may be due to the use of 2 fewer ALS injections for the mice in Experiment 2.

**Experiment 2.** Results of the third RLA-BPV-ALS experiment are shown in Table 11. The experimental group (A) in this experiment was identical to Group C, Experiment 1. The control group received DT plus heparin in place of RLA. Although the MST of the experimental group was longer than the MST of the control group by 2 days, the difference was not statistically significant. Unexpectedly, 4 experimental mice died on day 18 when their grafts were 100 percent viable, thus having negative influence on the statistical difference. The results of this experiment
Table 11. Rat Skin Graft Survival on Mice Treated with Rat Lymphocyte Antigen, *Bordetella pertussis* Vaccine and Anti-Lymphocyte Serum, Experiment 3.a

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>Mg of Antigen Protein</th>
<th>MST ± SE^b^</th>
<th>P^c^</th>
<th>Day of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>8</td>
<td>18.9 ± 1.9</td>
<td>N.S.</td>
<td>8, 13, 15, 19, 20, 22, 24, 25^d^</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>16.8 ± 1.5</td>
<td></td>
<td>7, 7, 7, 13, 13, 14, 16, 19, 19, 20, 21, 21, 23, 23, 23</td>
</tr>
</tbody>
</table>

^a^ See Table 7 for details of treatment.

^b^ Mean graft survival time ± standard error.

^c^ P value based on Student's t test for significance.

^d^ Four mice out of 13 in Group A died on day 18.
are consistent with those of Experiment 1, Groups C (8 x RLA I.V.) and E (no RLA), i.e., the difference in MST between these groups also was not significant. There was no significant difference between Group C, Experiment 1, and Group A, Experiment 3; nor was there a significant difference between control groups in these experiments, showing that DT plus heparin does not lead to prolonged graft survival.

B. Xenograft Prolongation Using RLA Alone (Active Enhancement)

Experiment 1. Ten C3H/HeJ mice were injected I.V. 17 days before grafting with 0.5 ml of 8-fold concentrated RLA containing 5 I.U. heparin. A statistically significant difference in MST was observed (P < .001) between grafts on these test animals (12.0 ± 0.8) and grafts on the untreated control mice (5.8 ± 0.5) (Table 12). This study was repeated (Experiment 2 below) using the same mg RLA protein per mouse but differing slightly in other respects.

Experiment 2. Ten mice were injected I.V. 16 days before grafting with 0.7 ml of 6-fold concentrated C3H mouse lymphocyte antigen which was prepared exactly like RIA. This group controlled for specificity of the rat lymphocyte antigen, since the C3H antigen contained delta toxin, sucrose, protein, and heparin in the same concentrations as RLA.
## Table 12. Rat Skin Graft Survival Times on Mice Immunized with RLA. (Combined data from Experiments 1, 2 and 3).

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>Injected I.V.</th>
<th>MST ± SE ( ^a )</th>
<th>( p ^b )</th>
<th>Day of 50% Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>RLA(^c)</td>
<td>9.0 ± 0.5</td>
<td>&lt;.001</td>
<td>9, 10, 10, 11, 11, 12, 12, 13, 15, 17 (12.0 ± 0.8, Exp.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6, 6, 6, 7, 7, 7, 8 (6.8 ± 0.3, Exp. 2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6, 7, 7, 8, 8, 8, 9, 9, 9, 9 (7.8 ± 0.3, Exp. 3)</td>
</tr>
<tr>
<td>B</td>
<td>RLA(^d)</td>
<td>8.3 ± 0.7</td>
<td>&lt;.01</td>
<td>6, 6, 8, 9, 9, 9, 11 (Exp. 2)</td>
</tr>
<tr>
<td>C(_1)</td>
<td>Nothing (Exp. 1)</td>
<td>5.8 ± 0.5</td>
<td></td>
<td>4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 6, 8, 10, 10 (Exp. 1)</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_2)</td>
<td>C3H Antigen (Exp. 2)</td>
<td>6.3 ± 0.4</td>
<td></td>
<td>5, 5, 6, 6, 6, 7, 7, 8 (Exp. 2)</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_3)</td>
<td>DT + Heparin (Exp. 3)</td>
<td>7.2 ± 0.4</td>
<td></td>
<td>5, 6, 6, 7, 8, 8, 8, 8, 8, 9 (Exp. 3)</td>
</tr>
</tbody>
</table>

Avg. Control MST 6.3 ± 0.3

---

\( ^a \) Mean graft survival time in days ± standard error.

\( ^b \) Groups A and B compared with average MST of Group C.

\( ^c \) Eight mg RLA protein were injected into mice 16 or 17 days before grafting.

\( ^d \) Two injections of 8 mg RLA protein were given; one on day -16, the second on day -4.
The MST of Group A (6.8 ± 0.3), which received a single RLA injection, was not significantly different from that of Group C mice (6.3 ± 0.4, P < .20), which received C3H antigen (Table 12). When Group A was compared with untreated controls the MST was not significantly different either (.05 < P < .10). The MST of Group B (8.3 ± 0.7), which received 2 RLA injections was significantly different from the MST of Group C1, the untreated controls (P < .01), and Group C2, the control group which received C3H antigen (P = .02).

Some of the mice in Experiment 2 did not reject their grafts in the usual, easy-to-score fashion (one in Group A, 3 in Group B, and 2 in Group C2. Their grafts were too edematous to be of use.) This may be because the dose of 6X RLA and 6X C3H antigen could have contained from 9.8 I.U. heparin to 6 times that amount (58.8 I.U.), depending upon whether heparin was concentrated along with antigen.

Experiment 2. Twelve mice (Group A) were injected I.V. 16 days prior to grafting with 0.5 ml of 8-fold concentrated RLA containing 5 I.U. heparin. Another 12 mice (Group C3) received DT and heparin (5 I.U.) I.V. in place of RLA 16 days before grafting. The amount of DT given (.7 mg) was the amount used to prepare RIA. The slightly increased MST of Group A (7.8 ± 0.3 compared to 7.2 ±
0.4) was not statistically significant (Table 12). The experimental group which received 2 RLA injections also had a significantly longer MST $(P < .01)$ when compared to the combined control MST. When the combined Group A was compared with the MST of the control from Experiment 3, which had the longest control MST, the difference was still significant $(P < .02)$.

C. Xenograft Prolongation Using Anti-RLA Alone (Passive Enhancement)

**Experiment 1.** Ascites fluid from C3H mice injected with RLA in CFA was collected, heated to $56^\circ$C for 30 minutes, and HA and LC titers determined. C3H ascites fluid from mice injected with CFA only was also collected, heat inactivated, and used as control ascites. Some low titered ascites was combined with the control ascites to provide sufficient fluid volume for this experiment. The resulting pool had no LC titer and a mean HA titer of 1.5. Five mice (Group A) were injected I.V. with 0.6 ml low titered anti-RLA ascites (Antiserum 1:HAT, 190; LCT, 6) on the day of grafting. Five mice in Group B$_1$ were injected I.V. on day 0 with 0.6 ml anti-RLA ascites of moderate titer (Antiserum 2:HAT, 4096; LCT, 150). Five control mice (Group C) were injected I.V. on day 0 with 0.6 ml of control ascites.
The MST of Group A (8.8 ± 0.3) which received low titered anti-RLA was longer than that of Group C (7.5 ± 0.5), which received control ascites, but the difference was not significant (0.05 < P < 0.10). The MST of Group Bı (10.0 ± 0.0), which received moderately titered anti-RLA was significantly longer than the MST of Group C (P < .01). This experiment was very important because it demonstrated enhancement in a skin xenograft model.

As occurred in the above experiments, a minority of the mice in this experiment rejected their grafts in the edematous manner. One animal from each group had soggy, whitish grafts which never appeared healthy. The occurrence appeared unrelated to antibody titer. Of the 5 additional animals in each group that received anti-RLA ascites I.P. all but one in each group had edematous grafts. The reason for this is unknown but may be related to the small amount of heparin present in the ascites fluid.

**Experiment 2.** In a second experiment anti-RLA ascites was injected I.V. in 0.3 ml aliquots on day 0 and day 3 instead of 0.6 ml on day 0. The control ascites and low-titered Antiserum 1 were the same as used in Experiment 1 and were given to Groups C and A respectively. Antiserum 2 was replaced by a higher titered serum (Antiserum 3; HAT, 3070; LCT, 512) and was given to Group Bı. The ascites
fluid was collected from different immunization experiments and pooled according to Ig titer alone, irrespective of the time following immunization.

The MST of both Group A \((9.4 \pm 0.6)\) and \(B_2\) \((9.8 \pm 2.2)\) were longer than Group C \((8.0 \pm 0.5)\), but the difference was not significant. The most important information from this experiment was the wide variation in graft survival times of individual animals in Group \(B_2\), which received the high titered Antiserum 3 (Table 13). A couple mice rejected their grafts like untreated mice on days 5 and 7. Another 2 mice had greatly prolonged graft survival of 13 and 14 days, while the remaining five mice all rejected their grafts hyperacutely as white grafts. These results are similar to results obtained in the Immunization Experiment (Interval II, Table 6). At that time both hyperacute rejection and prolonged graft survival were seen.

Even though in Experiment 1 one injection of anti-RLA was given and in Experiment 2 two were given, the total volume injected was identical (0.6 ml). In addition, there appeared to be no real differences in graft survival time between the experiments. We, therefore, combined the data from both experiments for groups in which the same anti-RLA ascites pool had been used. The combined data are shown in Table 13. When Group A, which received the low titered antiserum, was compared with Group C, which
Table 13. Rat Skin Graft Survival Times on Mice Injected with Anti-RLA Ascites Fluid, Experiments 1 and 2.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>No. of Mice</th>
<th>Antiserum No.</th>
<th>HA</th>
<th>LC</th>
<th>MST ± SE</th>
<th>F</th>
<th>Day of 50% Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12</td>
<td>1</td>
<td>190</td>
<td>6</td>
<td>9.2 ± 0.4</td>
<td>&lt;.02</td>
<td>7, 7, 8, 9, 9, 9, 9, 10, 10, 11, 12</td>
</tr>
<tr>
<td>B\textsubscript{1}</td>
<td>4</td>
<td>2</td>
<td>4096</td>
<td>150</td>
<td>10.0 ± 0.0</td>
<td>&lt;.001</td>
<td>10, 10, 10, 10</td>
</tr>
<tr>
<td>B\textsubscript{2}</td>
<td>9</td>
<td>3</td>
<td>3070</td>
<td>512</td>
<td>7.8 ± 0.3</td>
<td></td>
<td>5, 7, 13, 14 (plus 5 white grafts)</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>0</td>
<td>1.5</td>
<td>0</td>
<td>7.8 ± 0.3</td>
<td></td>
<td>6, 7, 7, 8, 8, 8, 8, 9, 9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mice were injected with a total of 0.6 ml anti-RLA ascites or control ascites, either in one or two injections and grafted with rat skin. The data are pooled from Experiments 1 and 2 for Groups A and C.

\textsuperscript{b} HA and LC titers are the average of 2 determinations on each ascites pool.

\textsuperscript{c} Mean graft survival time ± standard error.

\textsuperscript{d} Groups A and B\textsubscript{1} compared with Group C.
received the control ascites, there was significant graft prolongation ($P < .02$). The use of a moderately titered antiserum resulted in even longer graft prolongation (10 days compared with 7.8 days, $P < .001$). But when a high-titered antiserum was injected into the mice, at least half of them rejected their grafts hyperacutely as white grafts.
DISCUSSION

The major objective of this study was to prepare cell-free rat xenotransplantation antigens (RLA) suitable for attempts at prolonging rat skin xenografts on mice. Treatment of rat lymphoid cells dispersed in 0.25 M sucrose with very small amounts of *Staphylococcus aureus* delta toxin (DT) led to extraction of cell membrane antigens as shown by inhibition of cytotoxic xenoantiserum and elicitation of LC antibody. These antigens were judged to be soluble by the criterion that they were not sedimentable after ultracentrifugation for 3 hours at 200,000 g (ave.). When injected into mice, RLA led to hyperacute rejection or prolongation of rat skin xenografts depending on the procedure employed. RLA, therefore, contained xenotransplantation antigens.

Initial experiments determined that for antigen extraction simple incubation of *S. aureus* DT with rat lymphocytes in isotonic sucrose was superior to the use of LIS (which resulted in no detectable antigen release) and to hypertonic sucrose. Using the latter medium, Hines and Lang (submitted for publication) were able to extract 70,000 LCID$_{50}$ per $10^9$ rat lymphoid cells, an absorptive yield of 155%. This was much more antigen that Stroehmann and Dewitt (1972a) were able to extract from rat spleen and thymus cells using 3 M KCl. They obtained 2000 LCID$_{50}$ per $10^9$ cells which was about equal to the recovery of a rat alloantigen. Schwartz and Lang (1974) using 3 M KCl extracted baboon xenogeneic histocompatibility
antigens from blood lymphocytes with a yield of 87,000 LCID₅₀ per 10⁹ cells, an absorptive yield of 57%. Using DT and 0.25 M sucrose we were able to solubilize 500,000 LCID₅₀ per 10⁹ lymphoid cells, an absorptive yield of 500%. Possible reasons for the greater ILC activity in RLA than in an equivalent number of whole lymphocytes include:

1) inaccessibility of some cellular antigens to MARLA during absorption because of cell settling during overnight incubation at 4°C, 2) some cellular fragmentation during antigen preparation precluding the counting of those cells when determining antigen yield, and 3) loss of antigenic activity during washing of cells (cells are not washed during RLA preparation). Hines and Lang (submitted for publication) and Stroehmann and Dewitt (1972a) noted that washing of cells prior to extraction resulted in lower recovery of antigen. Loss of antigen was also observed during preparation of cell membranes (Davies, 1973). These effects are most likely due to the release of some surface antigens by autolytic enzymes present in and around cell membranes (Davies, 1973). In any case, solubilization of lymphocyte antigen with DT resulted in high yields of activity. No additional antigen was released when cell pellets were retreated with DT, suggesting complete elution and solubilization of xenogenic activity by the initial treatment.

In contrast to the high ILC activity recovered from
lymphocytes after treatment with DT, we obtained no activity when a preparation of purified rat RBC was similarly treated. This is consistent with several observations: 1) human RBC do not contain HL-A antigens and there is evidence that Ia antigens are absent on murine red cells; 2) Kano et al. (1972 and 1973) observed that some mouse species antigens are associated with H-2 antigens and some human species antigens have the same distribution as HL-A antigens; 3) the experiments of Staines and Davies (1973) suggest that H2 and mouse species antigens reside on the same molecule; and 4) Staines (1974) found that it is the H-2 molecules themselves that carry the xenoantigens which are dominant in xenoimmunization. From our observations it seems probable that dominant species antigens and alloantigens (or Ia antigens) exist together in the rat and that they are absent or poorly represented on rat RBC.

RLA is a heterogenous preparation containing many cellular and serum proteins. This was demonstrated by the finding of multiple protein bands after PAGE of RLA. Technical problems such as acrylamide gel cytotoxicity prevented us from detecting the presence or absence of antigenic activity in gel cuts. Since it would be desirable to know the number of antigenically distinct xenoantigens in the preparation, and the ILC assay cannot provide this information, we turned to ID and IEP.

When RLA was incubated with either MARLA or anti-RLA
ascites (AS 3), one or several lines of precipitation were observed in both ID and IEP. Because DT forms nonspecific lines of precipitation with serum lipoproteins (Kreger et al., 1971; Kantor et al., 1972), it is difficult to identify lines due to antigen-antibody precipitation. We believe, however, that the lines observed in ID plates containing RLA and either MARLA or anti-RLA represented true antigen-antibody reactions for the following reasons: No lines appeared when RLA was incubated with normal mouse ascites or normal rat serum, whereas lines of precipitation were formed between DT and all 4 sera. A weak line appeared between RLA and MARLA and much stronger ones formed between RLA and AS 3, while lines of equal density were obtained between DT and MARLA and DT and AS 3 (Fig. 6). Furthermore, the spur seen in Fig. 5 points to MARLA, the antiserum which should possess fewer antibody specificities to RIA. (MARLA does not contain antibody to rat serum whereas AS 3 probably does.) Obviously, an unequivocal demonstration of RLA antigen activity in ID would necessitate testing the gamma globulin fraction of MARIA or AS 3 with RLA.

Evidence that there are at least two major antigens in RIA was provided by experiments conducted to determine stability of RIA. One (or more) antigen was labile when RIA was heated for 30 minutes at 56°C while the other(s) was stable. The heated sample had an LCID<sub>50</sub> which was identical to that of an unheated sample but only 60% inhibition
of cytotoxic antibody could be obtained. Forty per cent of the antigenic activity had been destroyed. Similar separation of activity into labile and stable fractions was observed when RLA was stored at \(-20^\circ C\) for 13 months. Whether or not the same antigens were involved is unknown although the same per cent of total activity was destroyed in each case. It is interesting that Davies (1973) has found a similar variability in temperature lability for allospecificities. This suggests similarity between allo-antigens and xenoantigens.

The means by which DT releases membrane antigens can only be surmised. It is known that it is a non-enzymatic lysin since the absolute number or per cent of total erythrocytes lysed, when incubated with a fixed amount of DT, decreases with increasing cell concentration. It is also known to be surface-active and capable of disrupting leukocytes (Kreger et al., 1971). In our system we believe it acts by damaging the cell membrane after which it remains bound to small membrane fragments. Microscopic examination of DT-treated lymphocytes revealed whole leukocytes with a tendency to aggregate, and crenated or lysed erythrocytes. This suggested membrane damage which was confirmed by the inability of DT-treated lymphocytes to exclude 0.3% Trypan Blue Dye. In contrast to these results were those of Hines and Lang (submitted for publication) who found 90% viability
of lymphocytes by dye exclusion after extracting with hypertonic sucrose. Disruption of nuclear membranes would probably have resulted in a highly viscous preparation due to the release of DNA. RIA extracts were never viscous; extracts prepared with LIS, on the other hand, were highly viscous.

We believe that DT remains bound to membrane fragments because it could not be separated from antigenic activity by a variety of methods: Sepharose 6 B chromatography, ultrafiltration, ultracentrifugation, acid precipitation, phosphate precipitation, and precipitation by dialysis. However, DT is prone to aggregation and under non-dissociating conditions will appear to be of large M (Kantor et al., 1972) and, therefore, difficult to separate from other large M components. The acid precipitation data show that DT is bound to antigenic fragments. DT alone does not precipitate at pH 4 to 6, whereas DT activity (although minimal) was found in the pellet along with antigen activity after acid treatment and centrifugation.

Our inability to separate antigenic activity from the extracting agent, DT, presents no or little health hazard for in vivo use for two reasons. The amount of DT required to extract membrane antigens is considerably under the minimal lethal dose (MLD<sub>50</sub>) for mice. We injected RLA which originally contained 0.4 mg DT and the MLD<sub>50</sub>
(I.V.) for mice is 2.5 mg (Kreger, 1971). Secondly, the activity of DT in solution decreases with time when stored at -20°C (our observations) while the activity of RLA remains stable for at least 6 months. So simple storage of the antigen for 3-4 months should minimize any potential problems with DT. In addition, the probable binding of DT to antigenic components in RLA perhaps interferes with its further action on cell membranes. The absence of precipitation lines in ID with normal mouse ascites suggests that DT is indeed bound to RLA and may not be available to precipitate serum lipoprotein.

RLA contained xenoantigens which were soluble after ultracentrifugation at 200,000 g for 3 hours in isotonic sucrose. The activity of most transplantation antigen extracts sediments after centrifugation for 1 hour at 163,000 g or lower speeds. Thus, barring a large lipid content of RLA, it appeared to be smaller in size than most transplantation antigen extracts. However, the RLA antigen active components were large by several criteria. Nearly all activity was excluded from Sepharose 6B and by ultrafiltration studies only 13% of the total activity was of M less than 300,000. Similar large M for transplantation antigens have been found by other workers except when enzymatic methods of extracting alloantigens were employed. DiPadua et al. (1973) extracted murine alloantigens with the detergent...
NP40 which were excluded from Sephadex G-200. Stroehmann and DeWitt (1972a) reported a rat xenoantigen, extracted with 3 M KCl, eluted at the front of the inner bed volume of a Sephadex G-200 column. Schwartz and Lang (1974) found that a baboon xenoantigen extracted with 3 M KCl sedimented during centrifugation at 163,000 g when it was brought to isotonicity. Hines and Lang (submitted for publication) extracted rat xenoantigens (with hypertonic sucrose) of a M which were excluded from a Sephadex G-200 column, even under dissociating conditions. All the antigenic activity was retained by an ultrafilter which excludes molecules of M greater than 300,000 (our unpublished results). Brent et al. (1973), Owen (1971) and Cerilli and Hattan (1972, 1974) extracted alloantigens and xenoantigens by a variety of methods and all were sedimented by ultracentrifugation.

On the other hand, small M histocompatibility antigens (34,000-44,000) can be extracted with papain or by autolysis, but, as previously discussed, most of these preparations have poor biological activity. Perhaps there is a M limit under which the optimum antigenic configuration for presentation to immunocompetent cells is no longer attainable. That the purified molecules are antigenic is certain but loss of contiguous membrane structures appears to weaken their immunogenicity and, perhaps, is undesirable from the standpoint of induction of unresponsiveness to graft
antigens. Nevertheless, investigating the possibility that small soluble antigens in RIA may have aggregated or adsorbed onto larger molecules, we attempted to disperse RIA into smaller M components capable of passing through an XM 300 Diaflow filter (excludes molecules of $M > 300,000$). We knew from our own observations that RIA aggregated slowly on standing at $25^\circ C$ or $4^\circ C$ and more quickly during concentration. Heparin (14 I.U./ml) prevented this aggregation but did not disperse any aggregates which may have originally been present, i.e. it did not allow more activity to pass through an XM 300 filter. Addition of LILS, potassium phosphate, LiCl, or succinic anhydride in several concentrations to either RIA or the XM 300 membrane retentate of RIA did not disperse the antigen into components capable of passing through the filter. In addition, these agents either interfered with the ILC assay by their cytotoxicity or anti-complementariness, or, in the case of succinic anhydride, by decreasing antigenicity of RIA.

Support for the accumulating evidence that the antigen was of high $M$ came from studies where physiological saline or Tris-buffered saline was used instead of isotonic sucrose as the lymphocyte suspending medium. All the active components released by DT treatment of cells in these media were found in the supernatant after centrifugation at 10,000 g but most of them ($83-87\%$) sedimented upon ultracentrifugation at 200,000 g for 3 hours.
Perhaps 0.25 M sucrose contributes to the solubility of RLA by exerting a buoyancy effect on macromolecules, or by virtue of its highly hydrated nature. The density of a solution determines the buoyancy effect on suspended molecules and viscosity is an important factor in sedimentation rate. The density (d_{20}^4) of isotonic sucrose is approximately 1.03 and its viscosity is 1.8 centipoise while the density and viscosity of 0.15 M saline are < 1.005 and close to 1.0, respectively. Hines and Lang (submitted for publication) have suggested that the high density and viscosity of hypertonic sucrose are responsible for the solubility of their rat xenoantigen (HSE) during ultracentrifugation. It appears that the same phenomena may be responsible for the solubility of RLA. Hines and Lang also showed (by dialysis of antigen against water and saline) that hypertonic sucrose was necessary to maintain the solubility and, indeed, the antigenic activity of their rat xenoantigen extract. Similarly, Schwartz and Lang (1974) found that their 3 M KCl extracted xenoantigen was no longer soluble when removed from its hypertonic extracting medium. In contrast, we found RLA to be completely stable and nonsedimentable (at 10,000 g) after dialysis against water. It would have been interesting to see if the antigen-active moiety in water would have remained soluble after ultracentrifugation. The data with DT extraction in saline suggest it would have sedimented.
The precipitation of RLA active components during dialysis against saline and 0.25 M sucrose was most likely due to a drop in environmental pH. This aggregation was probably caused by removal of dialyzable ions and small molecular weight components which are necessary to maintain the antigen at neutral pH. The alkalinity of tap water may have prevented precipitation of the water dialyzed sample of RLA.

A second objective of this study was to quantitate and compare various in vitro parameters of the immune response of mice to RLA. Immunization with RLA in CFA induced high titers of HA and LC antibodies. Average maximum HA and LC titers for 4 experiments were 4096 and 512, respectively. In one experiment a LCT of 2048 was attained. These were even higher titers than the maximum obtained after secondary rat skin xenograft rejection (Hines et al., 1976), and indicate that RLA is a potent xenoimmunogen. These high antibody titers contrast with the absence of CMC following immunization with RLA but similar results were reported by Hines and Lang (submitted for publication) and Rao et al. (1974). Both groups of investigators found no CMC after inoculation of mice with soluble or semi-soluble antigen, but a high level after immunization with whole cells. Rao et al. worked with an allograft model without adjuvant and Hines and Lang used CFA and a xenograft model.
It, therefore, appears that cell-free antigen elicits an immune response which is deviated away from T-cell mediated cytotoxicity. This may have important ramifications when attempting to induce unresponsiveness.

Despite the absence of cytotoxic T cells, spleen cells from immunized mice were stimulated by RLA in a LBT assay. The slight to 2-fold stimulation of control cells was possibly due to the known mitogenic effect of DT for lymphocytes (Evans and Lack, 1969) or to a normal blastogenic response of mouse lymphocytes to rat species antigens. The stimulation of immune cells by RLA was always significantly greater (1.7 to 3.5 times) by 2 standard deviations than the response of control cells.

There are several possible explanations for the absence of cytotoxic T cells and the presence of responding cells in the LBT assay, which is generally regarded as an assay for cell-mediated immunity. The responding cells in the LBT assay may be the same subpopulation of T cells which respond by $^{3}$H-thymidine uptake in the mixed leukocyte culture test. This subpopulation of T cells has been shown to be different from the T cells which mediate CMC and the antigenic determinants which stimulate cytotoxic T cells and the MLC-responding T cells are different (LD and SD determinants) (Bach, 1973). A second possibility is that the LBT-responding cells represent an abortive attempt
to mount a cell-mediated response. Such a phenomenon is not unknown in immunology. For instance, in some diseases only the blastogenic response remains intact. The patient's cellular immune capacity is deficient in that it lacks the ability to respond in the MIF or skin test assays. A third possibility is that the LBT responding cells are T helper cells. This is supported by recent observations of Reinisch et al. (1976) which showed that CFA has a differential effect on T cell functions. Helper T cell activity was enhanced, while at the same time, cytolytic T cell function was depressed. This depression appeared to be mediated by CFA-induced splenic suppressor T cells. Finally, the possibility that B cells as well as T cells respond with increased thymidine uptake in the LBT assay has not been ruled out.

Several groups of investigators (Peter and Feldman, 1972; Berke and Sullivan, 1973; Sonis et al., 1975; Rao et al., 1974) have shown that antisera to target cells inhibit in vitro CMC in an allogeneic model. In contrast to these results we found that antiserum to RLA induced rather than inhibited cytotoxicity to rat target cells by immune mouse spleen cells. The test was performed similarly to that for antibody-dependent cell cytotoxicity (ADCC) with the effector cells being immune rather than normal spleen cells. ADCC has been extensively investigated since
the report by Moller in 1965 (Moller and Svehag, 1972).

Antibody-treated target cells undergo cytolysis without complement upon addition of nonimmune effector cells in both xenogeneic and allogeneic systems. We found ADCC with normal spleen cells using antisera from mice immunized with RLA, but much greater cytolysis when immune spleen cells were used as effector cells (ADICC). In Interval II (13 days after the second RLA injection) there was 3.6 to 9.7 times more S.C.R. in ADICC than ADCC. In Intervals III and IV (11 and 21 days after the third RLA injection, respectively) there were 2 to 3 times more S.C.R. with immune cells.

We believe that the differences between ADICC and ADCC obtained in the last 3 intervals (III, IV and V) could be even greater for the following reasons. 1) The heated antisera from Intervals III, IV and V caused release of $^{51}$Cr without addition of complement or effector cells. This decreased the total number of target cells available for cytolysis by effector cells, making the assay less sensitive. (S.C.R. was 80 per cent in several cases, approaching maximum $^{51}$Cr release.) 2) Normal IgG has been shown by Scornik et al. (1974a) to inhibit ADCC by competing for Fc receptors on effector cells. Moller and Svehag (1972) have deduced that as few as 2 IgG antibody molecules per effector lymphocyte could induce cytotoxicity. Therefore,
at low dilutions of antiserum, the large amount of "non-specific" immunoglobulin may block all or most Fc receptors. At high dilutions of antiserum Fc receptors would not be blocked, and because of the high degree of sensitivity of ADCC target cell killing would be seen. Thus, there would be more opportunity for the greater number or more effective immune cells to cause cytolysis of target cells. 3) At higher antiserum dilutions there would likely be no or very little $^{51}$Cr release without effector cells present.

We found a decrease in $^{51}$Cr release with increasing antiserum dilution.

There are 2 possible reasons for the killing of target cells by antiserum alone without effector cells or complement. The most obvious one is that a small amount of active complement remained in the heat inactivated FCS (used at 1:4) causing some target cell lysis with high titered antiserum. The second possibility is that some of the target cells (rat peripheral blood lymphocytes) are acting as effector cells. The target lymphocyte suspension undoubtedly contains K lymphocytes, with Fc receptors, which can kill other lymphocytes to which the Fab ends of IgG are bound.

Several investigators used very high dilutions of antiserum to study ADCC. Schirrmacher et al. (1975) diluted rabbit anti-chicken RBC serum 1:25,000 or 1:100,000; Scornik et al. (1974) diluted mouse anti-chicken RBC serum to 1:1000
or 1:10,000; Moller and Svehag (1972) used rabbit anti-
sheep serum at dilutions of 1:500 and 1:1000; and Harding
and MacLennan (1972) found that a serum dilution in excess
of $10^5$ induced ADCC. Notably, these authors all worked
with a xenogeneic system. A way of obtaining the same
end result without using high antiserum dilutions is to
wash the target cells after pre-incubation with antiserum
and before addition of effector cells. Gordon et al.
(1973) working in a human allogeneic system and Simpson
et al. (1973) working with a mouse tumor xenograft model
employed this technique. It is important that the latter
investigators found no ADCC if target cells were not washed
before effector cells were added. They used a 1:40 antiserum
dilution.

Other groups of workers utilized low dilutions of
antiserum similar to those we used in ADCC studies. Sonis
et al. (1975) diluted mouse alloantiserum and rat xenoantiserum
from 1:10 to 1:80, and Rao et al. (1974) diluted mouse
alloantiserum to 1:50 or 1:100. Thus, the parameters in
the systems used to study ADCC vary widely. It seems probable
that in our system the differences detected between immune
and normal splenocytes in mediating antibody-dependent
cytotoxicity are minimum ones.

Potentiation of CMC rather than inhibition by recipient
anti-donor serum has been found by others. Sonis et al.
(1975) utilized a 200:1 effector to target cell ratio and low antiserum dilutions of serum raised to whole cells. In the mouse allogeneic system, they found slight inhibition of CMC with antiserum and in the rat xenogeneic model at a 1:80 antiserum dilution, they reported potentiation of CMC from 36 per cent to 68 per cent lysis. This compares with 33.5 per cent lysis for ADCC using normal rat spleen cells and antibody-treated mouse target cells. The percent lysis obtained in potentiation of CMC is similar to ours in ADICC. They suggested an additive effect of CMC and ADCC in their xenogeneic model but our results clearly show that immune cells effect much higher antibody-dependent cytotoxicity than normal cells without any participation of CMC. This could be due to a larger number of effector cells capable of participating in antibody-dependent cytotoxicity.

A similar potentiation rather than inhibition of CMC was found with activated effector cells in human renal transplant recipients (Gordon et al., 1973). Patients' leukocytes activated *in vitro* by culturing with PHA, pokeweed, PPD or Mitomycin-treated lymphoblasts, caused 4 to 8 fold more cytotoxicity against donor-similar target cells in the presence of autologous serum than without serum. It is notable that these workers found no ADCC without activation of leukocytes. Almost identical results were reported
by Connolly et al. (1975). They stimulated human blood leukocytes in vitro with mitogens or allogeneic cells and found augmented effector cell activity in the ADCC assay. Thus, these results with human leukocytes are fully consistent with ours and show that stimulated effector cells are much better mediators of ADCC than normal cells.

It would be interesting to know, in our system, if this effect is specific for RLA-immunized cells or if non-specifically-stimulated mouse splenocytes are equally effective in augmented antibody-dependent cytotoxicity against rat target cells.

Results directly contradictory to ours and the studies above were reported by Schirrmacher et al. (1974). They found that in the presence of optimal concentrations of antibody spleen cells from immune and normal mice were equally effective in producing cytotoxicity. Their system differed from other workers in that antigen-coated chicken erythrocytes served as target cells and the S.C.R. after 18 hours incubation was determined for splenocytes obtained from mice following a single injection of a soluble protein antigen such as BSA in CFA. Near the time they assayed immune cells from mice (3 or 4 weeks post-immunization), we found no ADICC and low levels of ADCC. Had they hyper-immunized their animals perhaps they would have found results
similar to ours.

The spleen, as well as peripheral blood, contains several cell types which could be the mediator(s) of ADCC, e.g. T cells, B cells and macrophages. Moller and Svehag (1972) ruled out T cells by demonstrating that Mitomycin C suppressed CMC but had no effect on the mediators of ADCC, and heparin inhibited ADCC but not PHA-induced CMC or cytotoxicity due to sensitized lymphocytes. In addition, they showed that the Fc fragment of antibody was essential for ADCC and all B cells and macrophages but only a small proportion of T cells have Fc receptors. These authors felt that the mediators of ADCC were B cells rather than macrophages because they had used iron powder to remove phagocytic cells from human leukocytes. In addition, on microscopic examination of the killing cells there was no evidence for granulocytes or macrophages. Very recent evidence that a subpopulation of B lymphocytes mediates ADCC comes from studies by Chess et al. (1976) and Nelson et al. (1976). Both groups found it was the immunoglobulin-negative subset of B cells (sheep RBC rosette negative) which mediated human allogeneic ADCC, while xenogeneic ADCC could also be mediated by PMN and macrophages.

Scornik and Cosenza (1974) thoroughly investigated the effector cells of ADCC in peritoneal exudate and spleens of mice. They found that while both populations of cells
were quite capable of ADCC the effector cells in each population were different. In peritoneal exudate the effector cell was the macrophage as shown by its resistance to aminophylline and EDTA, and increased activity after culture for several days. In the spleen, although macrophages may be partially responsible for ADCC induced by adherent splenic cells, the cytotoxicity induced by unseparated spleen cells was completely inhibited by EDTA. Upon several days culture of unseparated spleen cells, no cytolytic capacity remained. They concluded that even though cytotoxic cells functionally similar to macrophages are present in the spleen, they play little or no role in target cell destruction when unseparated spleen cells are used as effector cells. Since their model system was also a mouse xenogeneic one (CRBC conjugated with TNP hapten as target cells) it is quite likely that the effector cells in our model are also B lymphocytes. Attempts to separate macrophages from these B lymphocytes would be difficult since Scornik and Cosenza (1974) found that adherence to glass or plastic is not a reliable method for separating effector cell types responsible for ADCC. Furthermore, we found no noticeable spleen cell adherence after 4½ hours incubation in plastic tissue culture dishes at 37°C.

On the other hand, spleens from RIA immunized mice were grossly enlarged and contained about twice as many
mononuclear cells as normal spleens. It is possible that there was a greater proportion of macrophages among these more numerous mononuclear cells.

By comparison of skin xenograft survival with in vitro immune responses of mice following immunization with RLA plus CFA, we made several observations. We found no correlation of graft rejection with the LBT response. There was a gradual increase in S.I. during the immunization schedule while the MST of skin grafts varied from no change, to increased, to decreased values. Using the MLC assay to predict human kidney graft survival, Cochrun et al. (1973) found a positive correlation; positive MLC, decreased chances of survival and vice-versa. Sasportes et al. (1973), however, found no correlation of human skin graft survival and a positive MLC. A negative MLC correlated with good survival. Similarly, no correlation was found between MLC and rejection or quiescent phases of human renal transplant recipients (Stiller et al., 1976). Our results with the LBT assay support the observations of the latter 2 groups of authors.

Since CMC was not detectable at any time during the course of the experiment, hyperacute rejection was most likely due to antibody. Two different types of cytotoxic antibody could be responsible -- complement-dependent or lymphocyte-dependent antibody. That these 2 types of anti-
body are, in fact, different was shown by Gordon et al. (1973). While our results seem to indicate that complement-dependent antibody is primarily responsible, lymphocyte-dependent antibody cannot be ruled out. The sensitivity of the test probably was not great enough to detect the full contribution of ADICC to graft rejection.

Our results demonstrating hyperacute rejection of skin xenografts by antibody (Intervals III, IV and V, Immunization Experiment) support those of Baldamus et al. (1973) and Hamilton and Gaugas (1972). These workers reported rapid destruction of skin xenografts on immunosuppressed mice by I.V. administration of anti-donor antibody. Contrary to the results of Jooste et al. (1973) and those of Gerlag et al. (1975), however, we did not find ingrowing grafts completely resistant to the destructive effects of antibody. White grafts never heal in or become vascularized and we have shown in the Immunization Experiment (Table 6) that this type of rejection is mediated without participation of CMC and, thus, by antibody. Our experiments in passive immunization support this contention further. Five out of 9 grafts were rejected hyperacutely as white grafts after injection of antiserum to RIA. Our results are in agreement with Hines (1975) who found that passive transfer of mouse anti-rat lymphocyte antibody led to white graft hyperacute rejection of rat skin.
Several other investigators have attempted to correlate ADCC with graft rejection. D'Apice et al. (1976) found no correlation between ADCC and human renal transplant rejection. Stiller et al. (1976) found a negative correlation (P=0.05) with a greater frequency of ADCC during quiescent phases of human kidney transplant rejection. Prevost et al. (1975) also found increased ADCC during progression of leukemia or lymphoma in monkeys. While it is difficult to make any firm conclusions, our results suggest a positive correlation between ADCC and hyperacute rejection. Perhaps ADCC is more important in xenograft than in allograft rejection. In the literature xenogenic ADCC appears to be a more sensitive phenomenon than allogeneic ADCC (based on the higher antiserum dilutions used in most xenogeneic systems). Since the contribution of CMC to primary xenograft rejection is less than to allograft rejection, other immunologic mechanisms assume greater importance. The predominance of mononuclear cells found at the graft site may well include antibody-secreting B-cells as well as ADCC effector cells. Harding and MacLennan (1972) found that in a mouse allogeneic system damage to mastocytoma cells was exclusively attributable to thymus-dependent lymphocytes, but in rats using the same target cell ADCC was operative. Simpson et al. (1973) attempted to assess the in vivo biological significance of the ADCC measured in vitro. B mice (adult thymectomized, irradiated
and bone marrow reconstituted) were grafted with hamster
tumor cells subcutaneously and then treated with lymphocyte-
dependent antibody (LDA) or normal serum or syngeneic spleen
cells. Under these conditions they found LDA ineffective
in controlling and eliminating tumor cells whereas spleen
cells caused total tumor suppression. The absence of LDA
activity in these in vivo experiments may be due to several
causes. The LDA used by these authors, while found to
have high titers when target cells were washed, had no activity
at any dilution when target cells were not washed before
addition of effector cells. This indicates that their
system was different from ours and that of other investigators.
In addition, 1) effector cells were from B mice and may
not be the same as normal ADCC effector cells, and 2) the
more numerous or efficient antibody-dependent cytotoxic
effector cells are found in hyperimmunized mice (our results).
Therefore, ADCC or ADICC as an important effector mechanism
in xenograft rejection should not be ruled out. In addition,
the exquisite sensitivity of this mechanism argues for
its in vivo action. It is important to remember, too, that
in our experiments mice rejected their grafts hyperacutely.
Perhaps ADICC, besides being possibly more important in
xenogeneic systems, is also more important in hyperacute
than in primary graft rejection.

Graft prolongation by immunization with RLA in
CFA was found in Interval II (29 days post-primary injection, 14 days post-secondary injection). The MST was 9.1 ± 0.7 compared with 5.8 ± 0.5 days for normal animals with P < .001. There were, however, 2 white grafts out of 10 grafts. Perhaps these 2 animals had higher cytotoxic and hemagglutinating antibody titers as well as higher ADICC activity than the other 8. It was impossible to test the animals individually for every immunologic parameter. Suggestive that there was more variation in immune activity in this interval than at other intervals was the significant 2 fold difference in ADICC between the 2 mice tested (30 per cent vs. 58 per cent S.C.R.) while at other intervals the differences were not significant. Perhaps the animal with the higher ADICC level would have rejected its graft hyperacutely while the animal with the lower ADICC would have had prolonged graft survival.

While enhancement has been often demonstrated in allograft models, it has been more difficult to obtain with xenografts. Owen (1971) has achieved it with antigen alone and Jeekel et al. (1974) and Corry and Kelley (1974) achieved it by passive immunization with anti-donor antiserum. The latter authors' results were very similar to ours in that at a certain dilution of cytotoxic antiserum 9 rat hearts were enhanced in mice and 2 were hyperacutely rejected. In our experiment, 8 skin grafts were prolonged and 2 were
hyperacutely rejected when pooled antiserum had a LCT of 1:32. The results of Corry and Kelley were obtained with a single antiserum. They suggested a diluting out of destructive components to allow its enhancing ability to be manifested. Our results could be explained similarly, i.e. a low LCT alone results in enhancement, or to a qualitative difference (greater enhancing ability) in antiserum produced early in immunization compared to a more destructive ability later. Perhaps a reduced affinity for antigen or a larger non-complement-fixing proportion of early antibody could cause afferent or efferent blocking of graft antigens or even central inhibition by antigen-antibody complexes.

The third objective of this project was induction of graft unresponsiveness with our antigen preparation. We achieved it 3 ways: injection of RIA in conjunction with ALS and *Bordetella pertussis* vaccine, injection of RLA alone (active enhancement) and injection of anti-RLA (passive enhancement). We detected no LC and no or slight HA antibody 2 days prior to grafting (the mice received RLA on day -16). This is in agreement with Owen (1971), who found no hemagglutinins against either allograft or xenograft donors after multiple antigen injections, and Kilshaw *et al.* (1974) who found no HA or LC antibodies after alloantigen injection. Two doses of RLA and 2 different routes of administration were used in our RLA-BPV-ALS Experiment
1. We found no difference between 7 and 14 mg protein RLA but a longer MST when the I.P. route was employed (MST of 25.0 (7 mg I.P.) and 23.2 (14 mg I.P.) vs. 19.3 (7 mg I.V.) and 20.3 (14 mg I.V.), \( P = .02 \)). These MST were compared with a control value of 14.3 days for mice receiving no antigen (\( P \leq .001 \) for I.P. route and \( < .01 \) for I.V. route). The advantage of the I.P. route of antigen injection was offset by the high mortality rate (about 50%). BPV contributed to this mortality as seen by a few mouse deaths in control groups (2/18 in Experiment 1 and 7/19 in Experiment 2). \( E. \) pertussis acts as an adjuvant in the formation of anaphylactic antibodies and it also increases the susceptibility of mice to passive anaphylaxis, but it is doubtful that this effect caused death in this case. The mice appeared to weaken gradually over several days or weeks. Munos and Bergman found that BPV increases susceptibility to anoxia or cold stress (from Pinto et al., 1974) and this appears to be the effect which contributed to the mouse deaths here.

In the second experiment with RIA, BPV and ALS only the I.V. route of antigen administration was used. MST were considerably shorter in both groups and the grafts appeared more edematous than in Experiment 1. When grafts were evaluated for 100% rejection, there was significant (\( P \leq .001 \)) prolongation in the group which received RIA.
(19.8 days vs. 14.2 days). In this experiment we decreased by 2 the number of ALS injections and, thus, also changed the day of BPV injection from d-6 to d-2 (ALS given on days 2, 4 and 6). RLA was also prepared slightly differently. It was concentrated 6-fold instead of 8-fold and heparin was added before concentration rather than afterwards. It seems unlikely, however, that this slight change in RLA contributed to the altered appearance of grafts since more control animals (9/12) had edematous-looking grafts than animals which received RLA (5/15). We concluded that the original regimen with 5 ALS injections was superior to the one used by Pinto et al. (1974) for inducing graft prolongation in our xenograft model. Apparently, the optimum immunosuppressive level needed to induce specific unresponsiveness with donor antigens is greater for xenografts than for allografts.

In the third RIA-BPV-ALS experiment the I.V. route of antigen injection was again used. This experiment was conducted exactly like the first one, i.e. 0.5 ml of 8 fold concentrated RLA containing 5 I.U. of heparin was injected I.V. 16 days before grafting. ALS was injected 5 times in 0.5 ml aliquots. A smaller amount of BPV (0.2 ml) was injected to minimize its toxicity, resulting in WBC which were only slightly lower than those obtained with 0.3 ml BPV. The slightly longer MST of the experimental
group was not significantly different from control animals. The deaths of 4 experimental mice on day 18 when their grafts were 100 per cent viable had a negative effect on the MST. When MST of experimental and control groups were compared to their counterpart groups of Experiment 1, there were no significant differences between them. We concluded that the I.V. route of antigen injection in our system was less efficacious than the I.P. route, inducing significant graft prolongation in only 1 group out of 3. The high mortality rate incurred with the I.P. route when combined with BPV administration precluded our using it in Experiments 2 and 3. We were able to demonstrate xenograft prolongation with a single donor antigen injection and a short course of ALS in one of 3 RLA I.V. groups and both RIA I.P. groups. Neither Brent et al. (1973) nor Cerilli and Hattan (1974) were able to do so in their models. Very recently, Hines and Lang (submitted for publication) have succeeded in inducing significant rat skin graft prolongation (P < .05) on mice with a single donor antigen injection and a short course of ALS. The greater significance of our results (in groups which received RLA I.P.) probably rests on the more potent antigen extract. Ours was 4 times more concentrated than theirs. Perhaps even longer graft survival could be achieved without the use of BPV which we found to be toxic to mice, contrary to the observations of Pinto et
If prolonged unresponsiveness to xenografts can be consistently achieved with a single antigen injection and a short course of ALS, it would be very advantageous and of great importance, indeed. The patient's minimal immune response would result in barely detectable antibody levels (presumably enhancing antibody) which could be maintained thereafter by antigens eluting from the graft. Monitoring of the recipient's immune response to donor antigen could detect a rise in antibody titer or cell-mediated immunity which would be a signal to administer more immunosuppression. This could tip the finely balanced immunologic scales in favor of enhancement rather than rejection.

In the first active enhancement experiment we obtained a 2 fold increase in MST of antigen-treated mice over untreated controls (12.0 vs. 5.8 days) with $P < .001$. (The control value was very similar to the MST of 5.6 ± 0.1 days reported by Hines et al. (1976) for 152 grafts in the same rat-mouse model.) We believe xenograft prolongation by a single antigen injection not to have been previously reported. Owen (1971) achieved kidney xenograft prolongation in rabbits with multiple antigen injections but neither Brent et al. (1973) using a single antigen injection nor Sumerska et al. (1974) using 2 or more injections were able to prolong
skin allografts in animals which differed at a major histocompatibility locus.

The results of our second experiment, however, were different. Animals which received a single RLA injection 16 days before grafting rejected their grafts in 6.8 days compared with 6.3 days for the C3H antigen control group. When 2 RLA injections were given the MST was increased to 8.3 days which was statistically longer than controls. A small proportion of these grafts, however, appeared more edematous than usual. The concentrated RLA was prepared in a slightly different manner which may account for both the edematous grafts and the shorter MST. The amount of heparin was increased from 10 I.U. per ml to 14 I.U. per ml and it was added before RLA was concentrated. Perhaps the additional heparin interfered with the normal rejection or enhancement process, which seems unlikely unless the heparin became concentrated along with RLA during positive pressure ultrafiltration. The fact that only one graft was rejected edematously in the group which received a single antigen injection and 3 were rejected edematously in the group which received 2 RLA injections lends support to this possibility. Besides interfering with the blood clotting process, heparin suppresses the cytotoxic effect of granulocytes and macrophages (Moller and Svehag, 1972). It is not impossible, therefore,
to visualize how it could interfere with the enhancing activity of antibody, whatever the mechanism. There is evidence that antigen-antibody complexes are the real mediators of enhancement and perhaps macrophages are an integral component of their action. They could possibly present the complexes to T or B lymphocytes, resulting in inactivation of these cells. In addition, if ADCC is an important mechanism in xenograft rejection, the normal rejection process would be impaired because heparin suppresses ADCC (Moller and Svehag, 1972).

Results of the third active enhancement experiment were similar to those of the second. Animals which received RLA had a slightly longer MST (7.8 days) than controls (7.2 days) but the difference was not significant. When data from the 3 experiments were pooled, the MST (9.0) of experimental animals was significantly longer ($P < .001$) than that of controls (6.3 days). The reason mice in Experiment 1 which received RLA had graft survival times longer than experimental mice in Experiments 2 and 3 is unknown. Our results are encouraging enough that more research in active enhancement of xenografts should be attempted. Since skin grafts are more difficult to maintain than organ grafts (Davies, 1973), it may be easier to induce enhancement of renal xenografts than skin xenografts. This has been shown to be true in one allograft model at
least. When rat renal allografts are permanently accepted through enhancement mechanisms, skin grafts from the same donor are rejected. (Reviewed by Fabre and Morris, 1975.)

Passive enhancement is more likely to be used clinically than active enhancement because of the dangers of inducing glomerulonephritis, anaphylactic shock, etc., with the latter. For this reason and because it seemed plausible that sensitization to graft antigens may be initiated mainly by donor passenger leukocytes, and enhancement, therefore, due to their elimination, we attempted passive enhancement of xenografts. We injected into mice a total of 0.6 ml antiserum to RLA in one or 2 injections and grafted the mice with rat skin. Antisera of 4 different titers were used to correlate enhancement with cytotoxic titer. The interpretation of the results was based solely on the antibody titers of the ascites and not on differences in class of antibody as would be considered if all the ascites in one pool had come from a single interval after immunization. Of course there may well be a qualitative difference in antibody in the pools but they were not selected specifically for that reason.

A MST of 9.2 ± 0.4 was obtained with the low titered antiserum (HAT = 190, LCT = 6) and an MST of 10.0 ± 0.0 with the medium titered antiserum (HST = 4096, LCT = 150). Both were significantly longer than the MST of the control group, with P < .02 and P < .001, respectively. The control group received
the lowest titered antiserum available which, although it had no LCT, still had a HAT of 1.5. This may explain its MST of 7.8 ± 0.3 compared with 5.8 days for untreated mice. A fourth group of animals received high titered antiserum (HAT = 3070, LCT = 512). Survival times varied from white graft rejection to 14 days, with 5 of 9 mice rejecting their grafts hyperacutely and 2 having prolonged graft survival. These results are similar to those obtained in the Immunization Experiment, Interval II (Table 6), i.e. two mice out of 10 rejected their grafts hyperacutely and the MST of the remainder was significantly longer than that of untreated controls. Pooled antiserum from this interval had a LCT of 32 and HAT of 2048. Considering both the dilution factor (0.6 ml of 512 LCT as compared with total titer of 32) and the greater proportion of hyperacutely rejected grafts in the passive enhancement experiment, the results in these experiments are consistent with each other and similar to those of Corry and Kelley (1974). These authors found enhancement of 2 and hyperacute rejection of 8 rat hearts when 0.25 ml of a 1:8 dilution of cytotoxic antiserum (1:256) was administered to mice. At a 1:16 dilution 4 hearts were enhanced while 6 were hyperacutely rejected. Thus, in their xenograft model a similar proportion of enhanced to hyperacutely rejected grafts as ours was obtained with a smaller amount of cytotoxic antiserum (0.25
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ml of essentially a 1:16 - 1:32 LC titered serum compared to 0.6 ml of a 1:512 LC titered serum in ours.) This suggests that rat hearts at least are more susceptible to cytotoxic antisera than is rat skin. The results of Jeekel et al. (1974) are consistent with this suggestion and enlarge the phenomenon to include mouse hearts and skin. They showed that with an amount of anti-donor xenoantiserum sufficient to induce passive enhancement of mouse skin on rats, hyperacute rejection of mouse hearts resulted.

The reason for the greater sensitivity of heart grafts than skin grafts to cytotoxic antisera may be that they possess a greater number of major histocompatibility or dominant species antigens. On the other hand, and more likely, the number of antigens per cell may be similar but the heart as an organ may be more sensitive to the death of a small proportion of its cells than skin would be. A highly vascular organ like heart would be more susceptible to ischemia than skin would be. A third possibility is that hearts possess fewer important xenotransplantation antigens than skin with cell death occurring when a similar proportion (for example 10%) of all critical antigenic sites in cells of both types of graft were attacked. If the latter explanation were true, it would be easier to reconcile the ostensibly contradictory experimental observation that skin grafts are more difficult to maintain than organ grafts. In this
case, it is easy to visualize that when sufficient blocking antibody is present to cover up the fewer antigenic sites on organs, some antigenic sites on skin would still be susceptible to afferent or efferent immunologic mechanisms resulting in rejection.

Since our results as well as those of Corry and Kelley (1974) have demonstrated that a low to moderately titered or a diluted high-titered cytotoxic antiserum can result in enhancement of xenografts, the balance between the cytotoxic and enhancing effect of graft-directed antiserum must be very fine, indeed. Several explanations are possible. There could be a larger proportion of non-complement-fixing antibodies in the serum, which can exert their effect only by diluting out the complement-fixing ones. Or the majority of IgG antibodies may be complement-fixing and dilution lessens the probability that 2 will attach in close enough proximity to fix complement. In these cases enhancement could be effected by covering up of a portion of graft antigens and preventing sensitization (afferent arm) or killing (efferent arm) of graft cells. A third possibility is related to the presence of passenger leukocytes in grafts. A certain number of cytotoxic antibodies would be beneficial in prolonging graft survival by eliminating these potentially sensitizing agents; but additional cytotoxic antibodies would cause destruction of the graft. By diluting the
antiserum just enough the optimum number of antibodies should be attained. There is evidence in the literature that loss of donor passenger leukocytes leads to prolonged rat kidney graft survival (Stuart, 1973). (Retransplantation of well established renal grafts to new hosts of the recipient strain led to rejection in slightly delayed fashion. This was attributed to loss of donor type leukocytes in the grafts.) But to explain the greater sensitivity of heart grafts to cytotoxic antiserum by this hypothesis, it is necessary to further hypothesize that hearts contain fewer passenger leukocytes than skin grafts.

While our results cannot distinguish which of the above hypotheses may be correct, they do demonstrate that enhancement of xenografts is possible. Obviously, there are many simultaneously occurring immunologic events in enhancement as well as in rejection, and it is very difficult to isolate and deduce the contribution of any one of them. It is gratifying to realize, however, that many similarities exist between allografts and xenografts, and that someday xenografts in humans may be a realized method of immunologic therapy.
SUMMARY

The goals of this research were 3-fold: 1) to obtain and characterize a stable, potent xenotransplantation antigen extract; 2) to correlate the immune response to the rat antigen extract as measured in vitro with xenograft survival; 3) to induce xenograft prolongation with the aid of the antigen extract.

Staphylococcus aureus delta toxin was used to extract rat lymphocyte membrane antigens (RLA). The antigen activity, as measured by inhibition of lymphocytotoxicity, was stable to moderate environmental changes of pH, heat, ionic strength, isotonicity, freeze-thawing and storage. RLA was heterogeneous as shown by polyacrylamide gel electrophoresis. At least two dominant antigens were demonstrated by the reaction of the antigen preparation with antiserum in immunodiffusion and by its differential lability on heating and storage. The antigen-active components, although soluble based upon ultracentrifugation in isotonic sucrose, were of large molecular weight as determined by Sepharose chromatography and ultrafiltration. Attempts to disperse the antigens into smaller molecular weight components by treatment with several agents, known for their ability to disperse aggregates, were unsuccessful. Delta toxin could not be separated from antigen activity and probably is complexed to antigen-
active membrane fragments.

The antigen preparation was highly immunogenic in mice. Hemagglutination and lymphocytotoxicity titers of 4096 and 512 respectively were attained in mice by 3 injections of RLA emulsified in complete Freund's adjuvant. The blastogenic response of spleen cells from mice immune to RLA increased with time following immunization. There were no cytotoxic cells demonstrable at any time, indicating the concomitant hyperacute white graft rejection was mediated by antibody. Whether this antibody was complement-dependent or lymphocyte-dependent is unknown. The antibody-dependent cell cytotoxicity of immune spleen cells was considerably higher than that of normal cells following the second RLA injection and continuing to the termination of the experiment.

Rat skin xenograft prolongation on mice was achieved with RLA in three ways: by injection of RLA in conjunction with anti-lymphocyte serum, by active enhancement with RLA alone and by passive enhancement with anti-RLA. Injections of high-lymphocytotoxic-titered anti-RLA could produce either enhanced graft survival or hyperacute rejection, attesting to the fine immunologic balance between xenograft enhancement and hyperacute rejection.
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