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IMMUNE RESPONSES TO SKIN XENOGRAFTS AND EXTRATION OF XENOTRANSPLANTATION ANTIGENS

DISSertation

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

By

David L. Hines, B. Sc.

* * * * * *

The Ohio State University 1975

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INTRODUCTION

In man renal allografting is now considered an accepted method of treatment for terminal renal disease. Additionally, organs other than kidneys have been successfully transplanted and have further increased interest in transplantation as a method of treatment and means by which a severely afflicted individual may return to a productive and active life. The supply of suitable organs for human transplantation is limited and as a result consideration has been given to the use of organs from other species (xenografting).

The origin of the idea of cross-species transplantation is buried in history and many of man's earliest myths are filled with creatures embodying the parts of different animals. In the first three decades of this century, before the importance of immunological mechanisms in transplantation were recognized, several renal xenografts to man were attempted with little success, but it was established that an organ from a non-human species could be anastomosed to man and function (Neuhof, 1923). The modern era of xenografting began in the early 1960's after it was shown that immunosuppressive agents were successful in extending the survival of allografts. Xenografts of chimpanzee (Reemtsma
et al., 1964) and baboon (Starzl, 1964, and Hitchcock et al., 1964) kidneys into humans were tried. Most of these grafts were rejected within two months but in one instance a chimpanzee kidney functioned for nine months. These early clinical trials indicated the major barrier to normal function and survival of primate kidneys in humans is immunological in nature (Reemtsma, 1968).

Future success in clinical xenografting seems to depend on gains in knowledge about the mechanism of xenograft rejection, improvement in methods of immunosuppression and tissue matching, and the ability to produce specific immunological unresponsiveness to xenotransplantation antigens by the induction of tolerance or enhancement. These areas can best be approached by the study of a well defined animal xenograft model. A rodent model offers the advantages of low expense, the availability of genetically well defined animals, and a pre-existing wealth of information regarding allografting and allotransplantation antigens. For these reasons we believe it is advantageous to conduct basic studies in xenografting first in a rodent model and then attempt to apply the results to a subhuman primate model. The major disadvantage to the immediate use of a subhuman primate model is the value, in terms of cost and scarcity, of these animals.
The fate of any tissue transplant is determined by the genetic relation between the donor and the recipient. The tempo and intensity of primary allograft rejection depend upon the degree of genetic (i.e. antigenic) distinction between the donor and the recipient. Some antigens function as strong barriers to allotransplantation while others act as weak ones. Xenografts are generally rejected faster and with more vigor than are allografts and techniques which abet the survival of allografts are often inadequate for xenografts (Lance, 1970). Nevertheless, xenografts of skin from guinea pigs, rats, rabbits, and humans have survived several months on mice when the recipients were immunosuppressed by chronic treatment with antilymphocyte serum (ALS) (Lance and Medawar, 1968b).

Baldamus et al. (1973) described the rejection of rat skin grafts on untreated mice. The xenografts behaved in most respects as allografts of mouse skin but were rejected sooner with a median rejection time of 7.8 days. Histologic examination of the skin xenografts revealed evidence of rejection within 5 days of transplantation. There was an early infiltration of leukocytes, followed by edema, interstitial hemorrhage, and finally necrosis of donor cells. Cellular infiltration was more pronounced than that commonly seen in allograft rejection but in both cases it consisted primarily of mononuclear cells with polymorphonuclear leukocytes appearing only after necrosis had developed.
Second grafts of rat skin, placed one week after rejection of first grafts, survived for only 4-6 days and were apparently rejected without ever having been vascularized. Lance (1970) has noted that morphologically the rejection of a first-set rat skin graft by mice closely resembles the rejection of a second-set skin allograft.

Although it is apparent that the presence of antigenically foreign cells in a recipient generates both humoral and cell-mediated immune responses the relative role of these responses in graft rejection is not completely understood. Allograft rejection is usually attributed to cell-mediated immune responses but preformed antibody appears to play a prominent role in hyperacute rejection of organ allografts (Hume, 1969). Antibody is probably involved to a greater extent in xenograft rejection than in allograft rejection but cell mediated immune responses are still very important (Lance, 1970). Attempts to prolong xenograft survival must take into account the greater participation of antibody in xenograft rejection but cannot ignore the role of cell-mediated processes.

Since immunological tolerance and enhancement are specific for the antigens involved they would be ideal modes of preventing graft rejection as none of the immunologic reactivity of the host for infectious agents or tumors would be affected. Although many agents have been employed as immunosuppressive adjuncts to the induction of tolerance,
ALS pretreatment appears to be the most appealing due to its high degree of effectiveness and low risk (Lance, 1971). Lance et al. (1969) have shown that adult mice can be made tolerant to rat skin grafts by short intensive courses of ALS given to neonatally thymectomized animals combined with the injection of viable rat lymphoid cells. The tolerant animals were shown to be cellular chimeras and the xenografts survived for long periods (100-200 days). The induction of tolerance to xenografts using cell-free antigen preparations has been more difficult to demonstrate. Owen (1971) presented evidence that daily administered low doses (1 ug/kg) of cell-free antigens, derived from liver by sonication, would allow prolongation of skin, kidney, and liver xenografts in certain donor-recipient combinations. Daily injections of low doses of antigen for 5 weeks before grafting extended the survival of chimpanzee skin on rabbits from 6-9 days to 12-21 days. Similar antigen pretreatment prevented the hyperacute rejection of rat kidneys in rabbits and pig kidneys in dogs. Cerilli and Hattan (1972) showed prolonged survival of rat skin grafts on adult mice treated with a combination of immunosuppressive agents including ALS, procarbazine and acriflavine hydrochloride. The addition of large doses of cell-free antigen (obtained from repeatedly frozen and thawed rat lymphocytes), given before and after grafting, resulted in further prolonged survival (median
survival time 169 days) of rat skin xenografts on mice than when the immunosuppressive agents were used alone (median survival time 72 days).

Jeekel et al. (1974) demonstrated immunological enhancement of mouse skin grafts on rats treated with rabbit anti-mouse serum. The prolonged survival of the skin xenografts was attributed to the presence of enhancing antibodies in the passively transferred rabbit anti-mouse serum. However, the same antiserum which caused prolonged survival of mouse skin grafts on rats caused the accelerated rejection of mouse heart xenografts.

Antigens important to transplantation immunity appear to be on both internal and external membrane surfaces (Manson et al. 1968). Xenoantigens have been found in a variety of centrifugal fractions of lymphocyte homogenates (Levey and Medawar, 1966), though Lance et al. (1968a) felt that the cytoplasmic membranes of lymphocytes carried the strongest antigens for the production of ALS. Kano et al. (1972) have demonstrated that human species-specific antigens were widely dispersed on human lymphocytes while alloantigens have a more limited distribution. DeWitt et al. (1971) have reported similar findings in the rat and noted that there was about four times more species antigen than alloantigens on the lymphocyte surface.

Various means of solubilization of allograft antigens have been utilized (reviewed by Reisfeld and Kahan,
1971). These include detergents, organic solvents (n-butanol), sonication, autolysis, enzymes (papain and trypsin), and salts (hypertonic potassium chloride and the Tris salt of 2-hydroxy-3, 5 di-iodobenzoic acid). Hypertonic potassium chloride has been used to extract xenoantigens by two pairs of investigators.

Stroehmann and DeWitt (1972a) reported the extraction of a rat species antigen from lymphoid cells using hypertonic potassium chloride (3 M KCl). The xenoantigen was of a molecular size that eluted at the front of the inner bed volume of a Sephadex G-200 column. It was identified by its ability to block the cytotoxic activity of rabbit anti-rat serum.

Schwartz and Lang (1974) extracted baboon xenogeneic histocompatibility antigens from lymphocytes using 3 M KCl. 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 x g. The addition of Triton X-100, a non-ionic detergent, to the KCl extract allowed isolation of xenoantigen active components by polyacrylamide-gel electrophoresis. Papain was ineffective in solubilizing baboon xenoantigens in this system (Schwartz, 1972).

This study was initiated as part of a long-term project intended to investigate the feasibility of producing specific immunological unresponsiveness to facilitate
xenograft survival. Its goals were first to quantitate some of the normal immune responses to skin xenografts and second to extract cell-free xenotransplantation antigens which could be used in investigations of immunological tolerance or enhancement. The model system used was the grafting of rat skin on mice. This model was chosen because of the large amount of information available about allotransplantation in these species which can serve as a basis for comparison with xenografting.

Utilizing techniques involving intact cells as antigens, humoral immune responses were studied by hemagglutination and complement-dependent lymphocytotoxicity. Cellular immune responses were assayed by cytotoxicity of graft recipient lymphocytes for donor cells. Lymphoid cells were chosen as a source for extraction of xenoantigens because they were used as indicators of extracted antigen activity in the inhibition of complement-dependent lymphocytotoxicity assay, also they are known to be a rich source of alloantigens (Kahan and Reisfeld, 1971), and are easily obtained.
MATERIALS AND METHODS

**Animals**

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

**Skin Grafting Procedures**

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

a. **Preparation of donor skin.** Donor animals were sacrificed by ether anesthesia and/or cardiac exsanguination, shaved with clippers, and skin surfaces disinfected with 90 per cent ethanol. Pinch grafts, 1 cm. in diameter, were removed from the abdomens of rats or the lateral thoracic wall of mice, scraped cautiously with a scalpel to remove adherent fat, and placed on filter paper saturated with sterile saline. Grafts were kept at 4°C until used.
b. **Grafting technique.** Recipient mice for xenografting were anesthetized with ether, fur removed with clippers, and skin surfaces disinfected with 90 per cent ethanol. The graft bed was prepared by cutting with scissors a full thickness pinch graft, approximately 1 cm in diameter, from the dorsal thoracic wall. The removed recipient skin was discarded or replaced as an autograft. 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 plastic coated with neomycin sulfate (Myciquent, Upjohn) which was held in place by a compression dressing produced by wrapping the thorax with air vent tape (Dermicel, Johnson & Johnson) and the tape was then covered with a plaster cast. Allografts were done similarly. Where the recipients were rats, the graft was additionally held in place with four sutures of 0000 silk and the plaster cast coated with picric acid to discourage gnawing. Where animals received second grafts care was taken not to place the second graft on the site of the first.

c. **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, destruction of the epidermis including hemorrhage
and loss of pliability, and firmness of attachment to the
graft bed. A graft was considered rejected when greater
than 50 per cent of the donor epithelium no longer appeared
viable.

**Collection of Mouse Serum**

For following antibody production after xenografting
with rat skin, groups of four to ten mice were sacrificed
by cardiac exsanguination under ether anesthesia. Blood
from all animals within a group was pooled and allowed to
clot for two hours at room temperature. Erythrocytes were
removed by centrifugation, the serum heated at 56°C for 30
minutes, and then frozen at -20°C. Following immunizations
small volumes (0.3-0.5 ml) of mouse serum were obtained by
retroorbital sinus puncture using capillary tubes. After
the blood clotted at 25°C the capillary tubes were placed
at 4°C overnight to allow for clot retraction. The capil­
lar y tubes were then broken and all serum obtained from a
single group of 5 animals was pooled. After erythrocytes
were removed by centrifugation the serum was heated at 56°C
for 30 minutes to inactivate complement, and stored frozen
at -20°C. All mouse serums were slightly pink indicating
a moderate hemolysis of erythrocytes.

**Preparation of Cells for Use in Serological Tests**

a. **Viable 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). From 2 to 5 ml of cardiac blood was defibrinated by swirling for five minutes with 10-15 3 mm glass beads in a 25 ml Erlenmeyer flask. The defibrinated blood was then mixed with three volumes of Seligmann's balanced Salt Solution (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 16 x 125 mm plastic tube (Falcon Plastics, Los Angeles). Two to five ml of cold Ficoll-Hypaque solution (6.35 per cent Ficoll, Pharmacia, and 10 per cent diatrizoate sodium, Winthrop) was layered under the diluted blood. Centrifugation was then performed at 360 x 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 x g (max.) for 15 minutes. The supernate fluid was discarded and the pellet of cells loosened by flicking the tube. Contaminating erythrocytes were destroyed by hypotonic lysis. To each cell button was added 3 ml of distilled water followed by brief agitation with a vortex mixer. After 15 seconds (longer periods caused clumping of leukocytes) 1 ml of four times concentrated SBSS and
0.5 ml of heat inactivated (56°C for 30 minutes) normal rat serum (NRS) were added to the cell suspension. The NRS was necessary to maintain good viability during the subsequent centrifugation at 265 x g (max.) for 10 minutes. The pink supernate fluid was discarded and the cell button loosened by flicking the tube. The cells were then ready for dilution with appropriate media.

Total cell counts were obtained by use of a hemocytometer. 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 and were based on observation of at least 100 cells. Typical differential cell counts were 90 per cent lymphocytes, 5-7 per cent monocytes, and the rest neutrophils. Viability measured by ability to exclude 2.5 per cent eosin Y dye was usually greater than 95 per cent.

b. Erythrocytes for hemagglutination and complement fixation tests were collected from blood of rats and sheep respectively. Rat erythrocytes were obtained from the cell pellet following Ficoll-Hypaque separation of defibrinated blood for preparation of lymphocytes. After removal of the lymphocyte cell layer from the Ficoll-Hypaque gradient, the remaining serum, Ficoll-Hypaque and the leukocyte buffy coat were aspirated and discarded leaving the erythrocyte pellet. The erythrocytes were washed three times with 10 ml
of 0.15 M NaCl, centrifuging at 650 x g for 10 minutes. The washed cells were then used immediately. Sheep erythrocytes were derived from whole blood collected aseptically and diluted to 50 per cent with Alsever's solution. Sheep erythrocytes were used only after storage at 4°C for a period of one to eight weeks.

**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 SBSS. A cell suspension was made by pressing the whole organs through a 60 gauge 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 x 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 Freund's complete adjuvant (FCA, Difco). Fifty C3H/HeJ mice were injected intraperitoneally
with 0.4 ml of the cell-FCA suspension (5 x 10^7 cells per animal). All animals received an identical injection two weeks later, 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 injection.

Ascites fluid was collected weekly, from 3-5 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 x g for 10 minutes at room temperature. The supernate was placed at 4°C for 18 hours and the lipid pellicle 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 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.
Rabbit anti-mouse lymphocyte serum was prepared by primary 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 lymphocytotoxicity titer of 1024 for mouse spleen lymphocytes and a direct hemagglutination titer of 1024 for mouse erythrocytes.

**Serological Techniques**

To titrate antibody levels following skin grafting or immunization, direct hemagglutination and lymphocytotoxicity tests were performed. Inhibition of lymphocytotoxicity, complement fixation, immunodiffusion, and immunoelectrophoresis were used to test for antigen activity in lymphocyte fractions.

a. **Direct hemagglutination (HA).** Serial two-fold dilutions of heat inactivated serum were made in 0.1 ml quantities with 0.15 M NaCl. Freshly prepared, washed, packed rat erythrocytes were diluted to a 2 per cent 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 12 x 75 mm glass test tube containing 0.1 ml of a serum dilution. The mixtures were shaken and incubated at room
temperature for 30 minutes. Following centrifugation at 600 x g for 3 minutes, the tubes were individually flicked 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 supernate.
- 2 + Large clumps with a diffuse redness of the supernate.
- 1 + Small clumps with a diffuse redness of the supernate.

**Negative**
- + Very few small particles.
- - No agglutination seen.

The last tube in the titration giving at least a 1+ was the endpoint and serum titer was recorded as the reciprocal of that dilution. Controls consisted of known positive and negative sera and 0.15 M NaCl alone. All sera were tested at least twice and if the endpoint varied more than one tube dilution four separate titrations were performed. Final titers were expressed as the average of all tests.

b. **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). Serial two-fold dilutions of heat
inactivated serum were made in 0.1 ml quantities with 0.15 M NaCl. All dilutions were tested in duplicate and to conserve serum 1 ul aliquots of the same serum dilutions used in DH assays were often employed. Using microliter syringes with a repeating dispenser (Hamilton Company, Whittier, California), reagents were added to wells of microtest I trays (Falcon Plastics), under a drop of heavy mineral oil to prevent evaporation. To 1 ul of diluted serum in each well was added 1 ul of a suspension of lymphocytes in Hank's Balanced Salt Solution (HBSS, Hanks and Wallace, 1949) containing 4,000 cells and the mixtures incubated at room temperature for 1 hour. Then 4 ul of guinea pig complement (Microbiological Associates, Bethesda, Maryland) diluted one to eight with HBSS was added to each well. After further incubation for 1 hour at room temperature 5 ul of 5 per cent eosin Y dye in 0.15 M NaCl was added followed in 3-5 minutes with 4 ul of 36 per cent 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 either immediately or after settling overnight at 4°C. The reciprocal of the highest serum dilution causing 50 per cent cell death was considered the endpoint (titer). Controls included using known positive and negative sera, the serum being tested without the addition of complement, a complement control in which 0.15 M
NaCl was substituted for serum, and a cell control in which the cells were suspended in HBSS with 25 per cent normal rat serum. For estimation of antibody levels following skin grafting, all sera were tested three times in duplicate and the 50 per cent cell death endpoint (and 95 per cent confidence limits) estimated by probit analysis of the pooled data.

c. Inhibition of lymphocytotoxicity (ILC). MARLA was used to detect the presence of rat lymphocyte surface antigens by inhibition of 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 ul of suitably diluted antibody (MARLA) was added to the well followed by 1 ul 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 as in the LC assay. Controls were the same as for the LC test with the inclusion of 0.15 M NaCl and the appropriate extracting medium substituted for antigen. When whole cells were tested for their absorptive capacity a cell suspension was prepared identically to that used for antigen extractions except in HBSS and the cells were washed once in 10 ml of HBSS. A 1.0 ml aliquot of cell suspension containing a known number of cells was placed in a 10 x 75 mm glass test tube and centrifuged at 600 x g for 10 minutes. The supernate was carefully removed and
discarded. The cell pellets were resuspended in 1.0 ml of MARLA suitably diluted with HBSS and incubated one hour at room temperature with mixing every 10 minutes. The mixture was then centrifuged at 600 x g for 10 minutes and 1 ul aliquots of the supernate tested in quadriplicate for residual LC activity. The cell pellet was then resuspended in the remaining supernate and the number of cells recounted using a Coulter counter.

The dilution of MARLA used in the ILC assay was selected to cause 90 per cent cell death (usually 1/64 dilution). The amount (micrograms) of antigen required to cause a 50 per cent inhibition of lymphocytotoxicity (LCID_{50}) was estimated by the method described by Gotze and Reisfeld (1974). The activity of each antigen dilution was calculated as a percentage of inhibition as:

\[
\text{per cent inhibition} = 100 - \frac{\text{per cent cells killed in presence of inhibitor}}{\text{per cent cells killed without antiserum}} \times \frac{\text{per cent cells killed in absence of inhibitor}}{\text{per cent cells killed without antiserum}}
\]

The number of intact cells necessary to absorb 50 per cent of the cytotoxic activity of a dilution of MARLA (AD_{50}) was estimated in a similar manner.

d. Quantitative micro-complement fixation (CF). The technique of Levine (1957), modified so that all volumes of
reagents used was one-half that originally described, was used to test the CF activity of antigen preparations. A 1/200 dilution of MARLA was normally used and at this dilution no anti-complementarity was observed. However, upon repeated freezing and thawing some aliquots of MARLA developed anticomplementary activity. Anticomplementarity was removed by millipore filtration (0.45 µ filter, Millipore Corp., Bedford, Mass.) and the addition of a final concentration of 10 per cent freshly reconstituted lyophilized guinea pig serum (Microbiological Associates). After incubation at 37°C for 30 minutes remaining complement activity was destroyed by incubation at 56°C for 30 minutes, and MARLA was then stored at -20°C. MARLA treated in this manner and tested in serial dilution from 1/500 to 1/3200 using a hypertonic sucrose extract antigen preparation containing 5.2 CF₅₀'s per ml, had a 50 per cent endpoint greater than 1/800. Lyophilized guinea pig serum was the source of complement and was used at the highest dilution (1/150 - 1/225) giving 90 per cent hemolysis. Rabbit anti-sheep hemolysin (Sylvana Co.) was used at a 1/1000 dilution. The degree of hemolysis was determined spectrophotometrically at 413 nm after centrifugal removal of residual erythrocytes. The per cent CF activity of antigen dilutions was estimated by subtracting the absorbency of the test mixtures from the mean of the absorbancies of serum, complement, and antigen controls and dividing the latter figure by the
mean of the absorbancies of the controls. The highest dilution of antigen causing 50 per cent complement fixation (CF$_{50}$) was estimated by arithmetic interpolation. The number of CF$_{50}$'s per ml was given by the reciprocal of the antigen dilution at the CF$_{50}$ level.

e. **Immunodiffusion (ID).** Double immunodiffusion was carried out in 0.5 per cent agarose gel (electrophoresis grade, General Biochemicals) in a solution of 0.15 M NaCl containing 0.02 per cent sodium azide. Fifteen ml of agar was poured into a 100 by 15 mm plastic petri dish (Falcon Plastics) and wells were cut 6 mm in diameter, 3 mm apart. Following overnight refrigeration the wells were aspirated dry and reagents added. The plates were incubated at 4°C for two weeks with periodic examination.

f. **Immunoelectrophoresis (IEP).** Immunoelectrophoresis was performed as described by Scheiddegger (1955) using Gelman Immunoelectrophoresis equipment. Using 1 per cent 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.
Cell Mediated Cytotoxicity (CMC)

For in vitro assessment of cell-mediated immune responses following immunization or skin grafting, cytotoxicity of lymphoid cells on target cells having the appropriate membrane antigens was used. The technique was a modification of the \textsuperscript{51}Cr release method described by Berke et al. (1972).

a. Cell preparation. Animals were sacrificed by cardiac exsanguination under ether anesthesia. Cell suspensions were prepared in cold phosphate buffered saline (Dulbecco, 1954) containing 25 per cent fetal calf serum (Grand Island Biological Co.) heated at 56°C for 30 minutes, 100 units per ml penicillin, and 100 ug per ml streptomycin (Grand Island Biological Co.), hereafter referred to as PBS-FCS. Effector spleen cells were prepared by teasing the organs with forceps in 10 ml PBS-FCS, followed by agitation of the cell suspension with a Pasteur pipette, filtration through a single layer of cotton gauze, and separation by the Ficoll-Hypaque method described above for separation of rat blood lymphocytes. The erythrocyte lysis step which caused extensive clumping of the cells was omitted. Effector lymph node cells were prepared from axillary lymph nodes by teasing the nodes with needles, agitation of the cell suspension with a Pasteur pipette, and filtration through a single layer of cotton gauze. Lymph node cell
suspensions were centrifuged at 600 x g for 10 minutes, the supernate discarded, and the cells resuspended in PBS-FCS. All effector cells were kept refrigerated until used.

Target cells were prepared from rat cardiac blood by the Ficoll-Hypaque technique described previously or from spleens of normal mice as described for preparation of effector spleen cells. Target cells at a concentration of $1 \times 10^7$ per ml were labeled with 100 uCi of sodium chromate ($^{51}$Cr) solution (Amersham/Searle Corp., Arlington Heights, Illinois) by incubation at 37°C for 30 minute with occasional shaking. Labeled target cells were washed four times with 10 ml cold PBS-FCS by centrifugation at 300 x g for 10 minutes and allowing the cells to stand at 4°C for 30 minutes prior to the third washing (Wigzell, 1965).

Total and viable cell counts were done for both target and effector cells using a hemocytometer and 2.5 per cent eosin Y dye. Target cells were used only if viability was greater than 85 per cent and at a concentration of $2.5 \times 10^5$ viable cells per ml. Effector cells were adjusted to $2.5 \times 10^7$ viable cells per ml. The viability of effector spleen cells was always greater than 85 per cent and that of lymph node cells averaged greater than 60 per cent with a range of 54 to 78 per cent.

b. CMC test procedure. $2.5 \times 10^6$ viable effector cells and $2.5 \times 10^4$ viable target cells, an effector to target cell ratio of 100:1, were placed in a total volume
of 1.0 ml of PBS-FCS in 35 mm tissue culture plates (Falcon Plastics). The plates were incubated on a Rocker Platform (Bellco Glass, Inc., Vineland, N.Y.) at 5 cycles per minute at 37°C for four hours.

At the end of the incubation period 1 ml cold PBS-FCS was added to each plate and the contents transferred by Pasteur pipette to 12 x 75 mm glass tubes. The tubes were centrifuged at room temperature at 415 x g for 7 minutes and 1.0 ml of the supernate transferred to 10 x 75 mm glass tubes for counting. Radioactivity of the supernates was assayed in a well-type gamma counter (Nuclear-Chicago) counting for 10 minutes or 1000 total counts. For determination of 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 three times before centrifugation.

Effector cell suspensions from the spleen or pooled axillary lymph nodes from each animal were assayed for CMC activity in triplicate or quadriplicate. Results are expressed as the percentage of maximal $^{51}$Cr release, given as a per cent CMC, according to the equation (Cerottini and Brunner, 1971):

$$\text{per cent CMC} = 100 \times \frac{\text{counts per minute released in the presence of immune cells} - \text{counts per minute released in the presence of normal cells}}{\text{maximum counts per minute released by freeze-thawed cells} - \text{counts per minute released in the presence of normal cells}}$$
Radioactivity released from target cells in the presence of normal cells was 1-2 per cent less than that released from target cells incubated alone. Maximal release represented 80-90 per cent of total radioactivity incorporated into the target cells. Data was considered only when release of radioactivity in the presence of normal cells was less than 33 per cent.

**Sephadex G-200 Column Chromatography**

Sephadex G-200 (Pharmacia Fine Chemicals) was separated into several fractions by sieving and the 88 to 120 micron fraction was used. The gel was prepared and the columns poured and run by the methods described by Fischer (1970). Following swelling in the eluant solution on a boiling water bath for 5 hours, the gel was cooled to 4°C and poured into columns.

Column A was 2.6 x 90 cm, and was run at 4°C by downward flow at 15.6 ml per hour with a peristaltic pump. It was equilibrated and eluted with 0.75 M sucrose. Eluant was collected in 3.0 ml fractions.

Column B was 2.6 x 89 cm, and run identical to column A. It was equilibrated and eluted with a buffer containing 0.75 M sucrose; 0.3 M potassium phosphate, dibasic; and 0.60 M sodium phosphate, monobasic, final pH of 7.0 (referred to as sucrose-phosphate buffer or 0.5 M phosphate). The column was partially characterized with separate runs.
of 5.0 ml and 4.0 ml of normal rat serum.

Concentration of Antigen Preparations

Concentration was accomplished by positive pressure (nitrogen) ultrafiltration at 4°C or 25°C with magnetic stirring, using a Diaflo Chamber (Amicon) at 60 lbs. per square inch applied to an Eastman H 35 membrane.

Estimation of Protein Concentration

The protein concentration of samples was estimated by one of two methods. When possible a modification of the method of Lowry as described by Chase and Williams (1968) was used. Bovine plasma albumin (Armour) was used as a standard. Samples were tested in duplicate with dilutions in 0.15 M NaCl and 0.1 N NaOH. With samples containing sucrose-phosphate this method could not be used because precipitation occurred with the addition of the Folin-Ciocalteu reagent. In these cases protein concentration was estimated by comparison of the optical density of the sample at 280 nm to a standard curve derived from the optical density at 280 nm of bovine plasma albumin in sucrose-phosphate.

Extraction of Rat Xenoantigens

The spleen, thymus and mesenteric lymph nodes were removed aseptically from rats, sacrificed by cardiac
exsanguination under ether anesthesia, and placed directly in the extracting solution (usually 0.75 M sucrose). Cell suspensions were prepared immediately 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 equal 10 ml of cell suspension per animal sacrificed. The cell suspension was incubated at 25°C for 2 hours in a 50 ml conical glass centrifuge tube with occasional mixing by inversion. Following incubation the suspension was centrifuged at 1300 x g (max.) for 20 minutes to remove whole cells (see Fig. 1). The pellet was either discarded or resuspended in an equal volume of extracting solution and reextracted by incubation overnight at 4°C. The supernate was centrifuged at 10,000 x g (max.) for 15 minutes at 4°C using a Sorvall RC-2B centrifuge with an SS-34 rotor. The resulting pellet was resuspended in an equal volume of extracting solution. The supernate of the 10,000 x g centrifugation was then centrifuged at 200,000 x g (ave.) for 3 hours at 4°C in an IEC B-60 ultracentrifuge using an SB-405 rotor. The supernate was removed by aspiration. The pellet was resuspended in a volume of extracting fluid equal to that of the supernate.
Rat spleen, thymus and mesenteric lymph nodes

Preparation of cell suspensions in extracting medium

Incubation 25°C
2 hours

1300 x g (max.)
20 minutes

Pellet  
Supernate

10,000 x g (max.)
15 minutes

Pellet  
Supernate

200,000 x g (ave.)
3 hours

Pellet  
Supernate (HSE)

Fig. 1 Procedure for preparation of rat xenoantigens
EXPERIMENTAL RESULTS

Skin Xenograft Rejection in Untreated Mice

Primary grafts of WF/Mai rat skin on C3H/Hej mice were firmly attached to the graft bed and well vascularized by day 3. The earliest sign of rejection was erythema. Erythematous portions of the graft became frankly hemorrhagic with a concurrent loss of pliability during the next 24 to 48 hours and when 50 per cent of the surface was hemorrhagic the graft was considered rejected. Even after the entire surface of the graft appeared to have lost viability many of the grafts remained firmly attached to the graft bed for up to one week. When grafts like these were peeled from the recipient there was extensive bleeding.

The mean graft survival time ± standard error of the mean (MST ± S.E.) for 152 primary grafts was 5.6 ± 0.1 days (Table 1).

Secondary grafts of rat skin 2, 3 or 4 weeks after primary graft rejection survived for only 3 days and showed two types of rejection patterns (Table 2). Most secondary grafts appeared to have undergone a hyperacute ("white graft") rejection. Grafts rejected in this manner appeared never to have been vascularized for they remained pale (the donor epidermis was white) and when peeled from the recipient...
### TABLE 1

REJECTION OF PRIMARY RAT SKIN GRAFTS ON UNTREATED MICE

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (55) (^{(a)})</td>
<td>1</td>
<td>5</td>
<td>21</td>
<td>13</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>B (38)</td>
<td>3</td>
<td>1</td>
<td>11</td>
<td>13</td>
<td>6</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>C (45)</td>
<td>3</td>
<td>12</td>
<td>15</td>
<td>9</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D (14)</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Totals (152)</strong></td>
<td>7</td>
<td>19</td>
<td>49</td>
<td>41</td>
<td>25</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Figures in parenthesis indicate number of animals in group. Each group was grafted as an entirely separate experiment and at different times.
### TABLE 2

REJECTION OF SECONDARY RAT SKIN GRAFTS ON UNTREATED MICE

<table>
<thead>
<tr>
<th>Time of Secondary Grafting after Primary Graft Rejection</th>
<th>Number of mice</th>
<th>Number of &quot;White Grafts&quot;(a)</th>
<th>Number of Vascularized Grafts(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 days</td>
<td>14</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>21 days</td>
<td>14</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>28 days</td>
<td>28</td>
<td>24</td>
<td>4</td>
</tr>
</tbody>
</table>

(a) All grafts were rejected on day 3 after secondary grafting.
there was little or no bleeding even if the graft originally was firmly attached. The less frequent type of secondary graft rejection appeared as an accelerated form of primary graft rejection in that the grafts seemed to have been vascularized but were extensively hemorrhagic on day 3. When grafts in this condition were peeled from the recipient there was extensive bleeding.

**Antibody Responses of Untreated Mice Following Primary Grafting of Rat Skin**

Groups of 5 to 10 mice were sacrificed at various times from day 0 to day 34 after primary grafting and serum from each group pooled. Antibody titers of pooled sera are shown in Figure 2. Mouse sera obtained prior to grafting had no detectable pre-existing HA or LC antibodies. HA antibodies were first detectable on day 6, rapidly increased to a titer greater than 256 by day 10, decreased to 64 by day 13, and increased again to greater than 512 by day 27. LC antibodies were first detectable on day 7 and while lower than HA titers, showed a similar pattern of activity. Serum from autografted mice had no detectable HA or LC anti-rat antibodies.

Immunoelectrophoresis of rat serum developed with mouse serum obtained on the 12th day after primary grafting gave no lines of precipitation.
Fig. 2. Antibody responses of untreated mice following primary grafts of rat skin. (——) direct hemagglutination, (-----) lymphocytotoxicity. Brackets indicate 95 per cent confidence intervals.
Antibody Responses Following Secondary Grafting of Rat Skin On Mice Previously Rejecting Primary Grafts

Groups of 4 to 10 mice were sacrificed from 1 to 15 days after receiving second rat skin grafts either 2, 3 or 4 weeks after rejection of primary grafts. As shown in Figure 3 titers of LC antibodies increased following secondary grafting to levels greater than 4 times those following primary grafting. HA antibody responses were similar. When mice received secondary grafts at 4 weeks after primary graft rejection a significant drop in LC and HA titers occurred immediately after grafting.

Cellular Immune Responses Following Primary Grafting of Rat Skin on Untreated Mice

Spleen and axillary lymph node cells from mice which had received a primary rat skin graft 5 to 10 days earlier were examined in vitro for direct cytotoxicity for $^{51}$Cr labeled rat lymphocytes. This time period was chosen to correlate with the time of in vivo graft rejection and maximum HA and LC serum antibody responses. Cells from each mouse were assayed individually and data from 3 to 5 mice were pooled for each day tested. As indicated in Figure 4 the per cent of cell mediated cytotoxicity (CMC) was never very high when examined at an effector to target cell ratio of 100:1 with 4 hours incubation. Spleen cells from day 6 after grafting gave the highest average CMC, 7.0 per cent, while lymph node cells were never above 4.2 per cent.
Fig. 3. Lymphocytotoxic antibody responses of mice receiving secondary rat skin grafts at (---) 2, (---) 3, or (-----) 4 weeks after primary graft rejection. Brackets indicate 95 per cent confidence intervals.
Fig. 4. Cell-mediated cytotoxicity of mouse cells for rat lymphocytes following primary rat skin grafts. 

(—■—) mouse spleen cells, (— —) mouse lymph node cells. Mean per cent maximal $^{51}$Cr release ± standard error. Effector to target cell ratio of 100:1 with a four hour incubation at 37°C. Numbers in parenthesis indicate number of mice tested for each day.
The limit of using a maximum of 4 hours incubation was imposed by the high spontaneous release of radiolabel from the target cells; about 15 per cent at 4 hours with an increase of about 10 per cent per hour thereafter. The release of $^{51}$Cr from labeled cells has been shown to correlate with loss of cell viability as measured by dye exclusion (Sullivan et al., 1972). An effector to target cell ratio of 100:1 was the maximum which could be used and allow cells from each mouse to be assayed in quadruplicate.

Controls for the assay system consisted of testing effector cells from mice and rats that had received skin grafts or had been immunized with lymphocytes. Spleen cells, from two Lewis rats which had received primary skin allografts from histoincompatible WF/Mai rats seven days before, were tested, using labeled WF/Mai rat lymphocyte target cells, giving an average of 14.2 per cent CMC. The target cells were the same as for the rat to mouse xenograft experiments. These results (14.2 per cent CMC) compare favorably to the 12 per cent CMC obtained seven days after primary skin allografts using two other rat strains as reported by Peter and Feldman (1972). Spleen and axillary lymph node cells from five C3H mice (H2$^{k}$), which had received skin allografts from A/J mice (H2$^{a}$) seven days before, were tested as effector cells using $^{51}$Cr labeled A/J spleen
cells as targets. The spleen cells gave an average per cent CMC ± S.E. of 26.9 ± 1.9 per cent while the lymph node effector cells gave 16.1 ± 2.5 per cent CMC. These figures are comparable to the 35 per cent for spleen cells and 25 per cent for axillary lymph node cells reported by Canty and Wunderlich (1971) using a similar mouse allograft system and testing seven days after grafting. Spleen and lymph node cells from two C3H mice which were autografted 8 days before testing using WF/Mai rat lymphocyte target cells gave 2.3 and 3.0 per cent CMC respectively. Finally, it was shown that mouse spleen cells were capable of killing rat lymphocyte target cells. Spleen cells from two mice, immunized by four injections of 2 x 10^7 rat lymphocytes in Freund's complete adjuvant, gave 32.3 and 12.3 per cent CMC when tested using WF/Mai rat target cells.

It was concluded that the cellular immune response of mice, as measured by the CMC assay testing spleen and axillary lymph node cells, was lower following primary skin xenografts than following primary skin allografts.

Characterization of Mouse Anti-rat Lymphocyte Ascites Used for Detection of Rat Xenoantigens

1. Reactivity with rat cell-surface antigens. Mouse anti-rat lymphocyte ascites (MARLA) had HA and LC antibody titers of 4096 and 256 respectively. Normal C3H/Hej mouse
serum was negative in both tests. Absorption with washed, pooled, viable rat spleen, thymus, and mesenteric lymph node cells indicated $2.3 \times 10^6$ cells (the AD$_{50}$ of MARLA) were necessary to reduce by 50 per cent the LC titer of 1.0 ml of a dilution of MARLA causing 90 per cent cell death.

2. **Demonstration of antibody in MARLA to rat xenotransplantation antigens.** Six mice received daily 0.3 ml injections of MARLA intraperitoneally beginning the day before grafting and continuing for seven days after grafting. Three of these animals received primary skin grafts exactly as described above, the other three received primary grafts that were incubated with the epidermal surface up for 2 hours at 4°C on filter paper saturated with undiluted MARLA. All six grafts were rejected in the hyperacute "white graft" manner previously described for rejection of secondary grafts. In a second experiment twelve mice received daily intraperitoneal injections of 0.1 ml of MARLA beginning the day of grafting and continuing for five days. All twelve animals received primary rat skin grafts with four animals receiving grafts that were incubated in vitro with MARLA. All twelve grafts were rejected as primary grafts in untreated animals with a mean survival time of 5.9 days. The hyperacute rejection seen in the first experiment demonstrated the presence of antibodies to rat xenotransplantation antigens in MARLA.
3. **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 (Figure 5) it was important to determine if MARLA contained antibodies to rat serum. No CF or ILC activity was obtained using MARLA and normal rat serum as antigen. Also no lines of precipitation were seen in ID or IEP 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 xenoantigens.

**Extraction of Rat Xenoantigens with 0.75 M Sucrose**

1. **Extraction procedures and appearance of fractions formed.** Nine separate 0.75 M sucrose extractions of rat cells were performed. Six extractions (6, 7, 9, 10, 11, and 12) were performed as described in Materials and Methods (Figure 1). In extraction experiment 4, the cell suspension was prepared by forcing the organs through a 60 gauge wire screen instead of teasing with forceps and needles. In extraction experiments 3, 4 and 5 the 10,000 x g (max.) centrifugation step was omitted. The total cells extracted, volume of extracting solution, and the protein content of the fractions are listed in Table 3.
<table>
<thead>
<tr>
<th>Extraction Experiment</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracted (x 10⁹)</td>
<td>0.52</td>
<td>0.43</td>
<td>2.0</td>
<td>2.1</td>
<td>0.86</td>
<td>1.2</td>
<td>1.6</td>
<td>0.56</td>
<td>0.70</td>
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<tr>
<td><strong>Extracting Volume (ml)</strong></td>
<td>14</td>
<td>18</td>
<td>15</td>
<td>21</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td><strong>1300 x g (max) supernate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>4.00</td>
<td>5.85</td>
<td>N.T. (a)</td>
<td>N.T.</td>
<td>2.90</td>
<td>N.T.</td>
<td>2.75</td>
<td>N.T.</td>
<td>1.75</td>
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<tr>
<td><strong>10,000 x g (max) supernate</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>N.D. (b)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3.00</td>
<td>2.44</td>
<td>N.T.</td>
<td>2.44</td>
<td>1.73</td>
<td>1.80</td>
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<tr>
<td><strong>10,000 x g (max) pellet</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Protein (mg/ml)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.T.</td>
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<td>N.T.</td>
<td>0.14</td>
<td>0.10</td>
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<tr>
<td><strong>200,000 x g (ave) supernate</strong></td>
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<tr>
<td>Protein (mg/ml)</td>
<td>2.88</td>
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<td>2.73</td>
<td>2.28</td>
<td>1.58</td>
<td>1.97</td>
<td>1.95</td>
<td>1.32</td>
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<tr>
<td><strong>200,000 x g (ave) pellet</strong></td>
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</tr>
<tr>
<td>Protein (mg/ml)</td>
<td>0.68</td>
<td>1.44</td>
<td>N.T.</td>
<td>N.T.</td>
<td>0.48</td>
<td>N.T.</td>
<td>0.42</td>
<td>0.28</td>
<td>0.28</td>
</tr>
</tbody>
</table>

(a) N.T., not tested.
(b) N.D., step was not done.
(c) Pellet was resuspended in volume of extracting solution equal to volume of the supernate.
Erythrocytes represented about 15 per cent of the total cells and were not included in estimating the total cells extracted. The 200,000 x g supernate contained an average of 45.44 (range 20.13 to 131.86) mg protein per $10^9$ cells extracted.

After incubation in 0.75 M sucrose for 2 hours at 25°C the cells appeared intact with a viability of about 90 per cent by eosin dye exclusion. Wright's stained smears of the cells showed crenated erythrocytes with other cells appearing to have a normal nucleus, a reduced cytoplasm volume, and convolution of the cell membrane. The 1300 x g (max) pellet was bright red, highly viscous, and could not be completely resuspended even with vigorous shaking on a vortex mixer. Microscopic examination (100 X) of the resuspended 1300 x g pellet showed intact cells which looked similar to the cell suspension before centrifugation, but many of the cells were clumped. Wright's stained smears of the resuspended 1300 x g pellet showed intact cells both free and in small clumps. The clumps appeared to be held together by a fibrous, dark blue stained material. The 1300 x g supernate was cloudy and pink. (In extraction 4 there was a large pellicle of material on the top of the tube following the 1300 x g centrifugation. This was removed and discarded.) The 10,000 x g (max.) and 200,000 x g (ave) pellets were grey and bright red respectively. Both pellets easily resuspended in 0.75 M sucrose with the
suspensions appearing white and translucent. These pellets appeared to remain as homogenous suspensions upon storage at 4°C or upon freezing and thawing. The 10,000 x g (max.) supernate was pink and less cloudy than the 1300 x g supernate, while the 200,000 x g (ave) supernate was pink and clear. Material in the 200,000 x g supernate (designated Hypertonic Sucrose Extract, HSE) was given a working definition of "soluble," and this fraction was chosen for further study.

In extraction experiments 3, 4 and 6 the 1300 x g pellet was reextracted using an equal volume of 0.75 M sucrose as in the initial extraction. The re-extraction was carried out by incubation at 4°C for 18 hours followed by 1300 x g (max) and 200,000 x g (ave) differential centrifugations. The 200,000 x g supernate was clear and colorless, the pellet was similar but much smaller than in the first extraction. The re-extraction 200,000 x g supernates of experiments 3, 4 and 6 contained 0.18, 0.13 and 0.20 mg protein per ml respectively.

2. Xenoantigen activity of 0.75 M sucrose extract fractions.

a) Inhibition of lymphocytotoxicity (ILC). Fractions of seven separate 0.75 M sucrose extraction experiments were tested for ILC antigen activity using MARLA (Table 4). A positive ILC test indicates the presence of cell-surface
## TABLE 4

### INHIBITION OF LYMPHOCYTOTOXICITY ANTIGEN ACTIVITY OF 0.75 M SUCROSE EXTRACT FRACTIONS

<table>
<thead>
<tr>
<th>Extraction Experiment</th>
<th>Fraction</th>
<th>3</th>
<th>4</th>
<th>6</th>
<th>7</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>means</th>
</tr>
</thead>
<tbody>
<tr>
<td>1300 x g (max) supernate</td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>2224</td>
<td>2509</td>
<td>N.T. (a)</td>
<td>3584</td>
<td>N.T.</td>
<td>5092</td>
<td>N.T.</td>
<td>3352</td>
</tr>
<tr>
<td></td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>240</td>
<td>614</td>
<td>N.T.</td>
<td>242</td>
<td>N.T.</td>
<td>171</td>
<td>N.T.</td>
<td>317</td>
</tr>
<tr>
<td>10,000 x g (max) supernate</td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>N.D. (b)</td>
<td>N.D.</td>
<td>2000</td>
<td>1499</td>
<td>N.T.</td>
<td>3077</td>
<td>2174</td>
<td>2188</td>
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<tr>
<td></td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>N.D.</td>
<td>N.D.</td>
<td>60</td>
<td>85</td>
<td>N.T.</td>
<td>91</td>
<td>80.4</td>
<td>79.1</td>
</tr>
<tr>
<td>10,000 x g (max) pellet</td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.T.</td>
<td>13.9</td>
<td>N.T.</td>
<td>12.9</td>
<td>N.T.</td>
<td>12.9</td>
</tr>
<tr>
<td></td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.T.</td>
<td>21.0</td>
<td>N.T.</td>
<td>21.0</td>
<td>N.T.</td>
<td>22.6</td>
</tr>
<tr>
<td>200,000 x g (ave) supernate</td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>438</td>
<td>1200</td>
<td>568</td>
<td>1724</td>
<td>2381</td>
<td>3571</td>
<td>1212</td>
<td>1588</td>
</tr>
<tr>
<td></td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>34.0</td>
<td>161.0</td>
<td>13.0</td>
<td>63.0</td>
<td>78.0</td>
<td>85.0</td>
<td>34.3</td>
<td>66.9</td>
</tr>
<tr>
<td>200,000 x g (ave) pellet</td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/mg protein</td>
<td>4.3</td>
<td>4.2</td>
<td>N.T.</td>
<td>5.8</td>
<td>N.T.</td>
<td>13.8</td>
<td>N.T.</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells</td>
<td>8.2</td>
<td>251.0</td>
<td>N.T.</td>
<td>63.9</td>
<td>N.T.</td>
<td>71.0</td>
<td>64.3</td>
<td>91.7</td>
</tr>
</tbody>
</table>

(a) N.T., not tested.

(b) N.D., fractionation step not done.
antigen components. Results have been expressed both as activity per mg. of protein and as total activity of the fraction per $10^9$ cells extracted. Hypertonic sucrose alone, normal rat serum, or similarly extracted fractions of C3H/Hej mice spleens did not inhibit the LC activity of mouse anti-rat lymphocyte ascites.

A mean total of 66,900 LCID$_{50}$'s per $10^9$ cells extracted (range from 13,000 to 161,000) was obtained in the HSE ($200,000 \times g$ supernates). The "absorptive yield" of an antigen preparation is a measure of the inhibitory activity of an extract compared with the absorptive capacity of the whole cells from which the extract was obtained. Absorptive yield can be estimated by using the ratio of the 50 per cent absorptive dose (AD$_{50}$) to the LCID$_{50}$ when the latter is expressed as cell equivalents. The AD$_{50}$ for MARLA was 23,160 cells (obtained as described in Materials and Methods). The average number of cells extracted to obtain one LCID$_{50}$ in the HSE's was 14,939 ($1 \times 10^9/66.9 \times 10^3$). Thus the average absorptive yield was calculated as 23,160/14,939, or 155 per cent for HSE.

Total ILC activity per $10^9$ cells was distributed approximately evenly between the $200,000 \times g$ supernate and pellet. The supernate contained a mean of 51.5 per cent (range 34.8 to 80.5) while the pellet contained a mean of 48.5 per cent, based on the five extraction experiments where both fractions were tested.
The 200,000 x g supernate from the re-extraction of the 1300 x g pellet in extraction experiment 3 contained 5560 LCID<sub>50</sub>'s per mg protein equal to a yield of 26.9 x 10<sup>3</sup> LCID<sub>50</sub>'s per 10<sup>9</sup> cells extracted. The same fraction from experiment 6 contained no ILC activity.

b. Complement fixation (CF). Results of testing fractions of nine 0.75 M sucrose extractions for CF activity are shown in Table 5. The CF test, in contrast to the ILC test, is not specific for the presence of cell-surface antigen components. Anticomplimentary activity was not noted in any fraction tested. As a specificity control fractions of a 0.75 M sucrose extract of C3H/Hej mice spleens were negative. The CF test appeared to be more sensitive than the ILC test in that antigen was detectable at a much higher dilution in the former, however, in all but one fraction (extraction experiment 3, 200,000 x g pellet) the total ILC activity per 10<sup>9</sup> cells extracted was greater than the total CF activity.

The number of CF<sub>50</sub>'s obtained in the HSE per 10<sup>9</sup> cells varied from 2000 to 13,100 with a mean of 4,600. The HSE contained a mean of 30.9 per cent (range 24.0 to 39.0 per cent) of the activity distributed between the 200,000 x g supernate and pellet for 7 extractions where both were tested.

The 200,000 x g supernates from the re-extraction of the 1300 x g pellet of experiments 3, 4 and 6 contained
TABLE 5

COMPLEMENT FIXATION ANTIGEN ACTIVITY OF 0.75 M SUCROSE EXTRACT FRACTIONS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extraction Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>1300 x g (max) supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>160</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>17.2</td>
</tr>
<tr>
<td>10,000 x g (max) supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>N.D.(b)</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>N.D.</td>
</tr>
<tr>
<td>10,000 x g (max) pellet</td>
<td>N.D.</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>N.D.</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>88</td>
</tr>
<tr>
<td>200,000 x g (ave) supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>6.8</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s x 10&lt;sup&gt;3&lt;/sup&gt;/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>909</td>
</tr>
<tr>
<td>200,000 x g (ave) pellet</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>16.1</td>
</tr>
</tbody>
</table>

(a) Not tested.
(b) Fractionation step not done.
an average of 419 CF$_{50}$'s per mg protein and $1.6 \times 10^3$ CF$_{50}$'s per $10^9$ cells extracted. Thus re-extraction of the original cells led to the recovery of additional (but less) soluble CF activity.

c. Immunelectrophoresis (IEP) and immunodiffusion (ID). HSE from extractions 3, 4, 9, 10, and 11 was tested in IEP using MARLA and no lines of precipitation were seen. However, three lines were seen when the HSE of extractions 10 and 11 were tested using rabbit anti-rat serum (Figure 5) indicating rat serum components were present. HSE from extraction 4 gave no lines in IEP using normal mouse serum.

The 1300 x g supernate, 200,000 x g supernate, and 200,000 x g pellet of extraction 3 were diluted and tested with undiluted MARLA in ID. The only activity detected after two weeks of incubation at 4°C was a single faint line close to the antigen wells containing undiluted and 1/2 diluted 1300 x g supernate. Therefore, while HSE contained ILC and CF antigen activity no precipitating antigens were detectable using MARLA.

Studies on Hypertonic Sucrose Extract

1. Effect of refrigeration, freezing, and heating on immunological activity of HSE.

   a) Storage at 4°C. Hypertonic Sucrose Extract 3 and Extract 5 kept at 4°C for 12 and 14 days respectively and then retested showed a 38 and 39 per cent decrease in CF
Fig. 5. Immunoelectrophoresis of Hypertonic Sucrose Extract 10, top well, and normal rat serum, bottom well. Developed with rabbit anti-rat serum. Anode is on the left.
activity from that obtained immediately after preparation. HSE 3 and 4 refrigerated for 12 and 14 days respectively exhibited no change in ILC activity when retested. HSE 4 had a 52 per cent increase in CF activity after 14 days at 4°C and HSE 3 showed an increase of 7 per cent in CF activity over the original result when retested again after 19 days. All hypertonic sucrose extracts after storage at 4°C for 24 hours were still clear but had developed a few flocculent particles. It was considered that the variations in CF activity might have been due to aggregation of some of the antigens detected in the CF test and a subsequent uneven distribution of the aggregates among the aliquots tested. A sample of HSE 5, which had been stored at 4°C for 14 days, was centrifuged at 200,000 x g (ave) for 3 hours at 4°C. The resulting supernate had 31 per cent less CF activity than the uncentrifuged sample.

It was concluded that storage at 4°C had no affect on ILC titers but resulted in an overall decrease in CF activity. Additionally, there was some evidence of aggregation of the antigens detected in the CF test.

b) Freezing at -20°C. Freezing had similar effects on the immunological activity of HSE as storage at 4°C. After being frozen for 3 days HSE 3 had no change in ILC titer. Flocculent particles similar to those seen upon storage at 4°C were present upon thawing. A sample of HSE 6, which had been frozen for 4 days, was centrifuged at
200,000 xg (ave) for 3 hours at 4°C. An uncentrifuged aliquot of this preparation showed a 38 per cent decrease in CF titer from that of the original fresh preparation and the centrifugated aliquot had a further 20 per cent reduction in CF titer, however both preparations had the same ILC titer as the original fresh preparation.

HSE 10 was examined for the effect of repeated freezing and thawing. After original CF and ILC testing of the fresh preparation HSE 10 was frozen and thawed 3 times in 9 days. Testing after the third thawing showed a 40 per cent decrease in CF titer and a 72 per cent decrease of ILC titer from that of the original. After the second thawing the ILC titer was the same as the original. Centrifugation at 200,000 x g for 3 hours, of a sample after the third thawing, resulted in no further reduction in ILC titer but a 11 per cent further decrease in CF titer as compared to the uncentrifuged portion. It was concluded that freezing at -20°C resulted in a loss of CF activity and aggregation of some CF antigens, whereas two freeze-thaw cycles did not adversely effect ILC titers.

c) Heating. Samples of HSE 3 were tested simultaneously after being heated to 37°C or 60°C for one hour. Samples had been stored at 4°C for 12 days before heating and titers were compared to an aliquot of unheated sample. There was no loss of CF activity at 37°C and a 39 per cent loss at 60°C. There was no change in ILC titer at either
2. Sephadex G-200 column chromatography of HSE

a) Chromatography eluting with 0.75 M sucrose. A 5.0 ml sample of HSE 4 containing 15.75 mg protein, 1560 CF<sub>50</sub>'s and 25,860 LCID<sub>50</sub>'s was run through G-200 Column A eluting with 0.75 M sucrose. The 3 ml fractions were studied for their absorbancy at 280 and 413 nm (Figure 6). Two peaks were obtained. The first peak eluted with the void volume (previously determined using normal rat serum) thus containing components equal to or greater than 800,000 to 1 million molecular weight. The second peak eluted after the column volume, thus was probably of 5,000 molecular weight or less. As shown in Figure 6 fractions were combined into seven pools which were then concentrated by positive pressure ultrafiltration at 4°C to approximately 5 ml each. Following concentration of pool II (the first peak) a brown viscous material was noted on the ultrafiltration membrane. This precipitate was washed from the membrane with 5 ml of 0.75 M sucrose and saved. All seven concentrated pools and the precipitate from the concentration of pool II were tested for CF and ILC activity. Antigen activity was found only in pool II and the precipitate from pool II, therefore all activity eluted in the first peak.

Pool II contained 0.95 mg protein, 1005 CF<sub>50</sub>'s and 8,350 LCID<sub>50</sub>'s representing 12.4 per cent, 64.4 per cent, and 32.3 per cent respectively of the total protein and
Fig. 6. Sephadex G-200 Column A chromatographic separation of 5.0 ml Hypertonic Sucrose Extract 4.

(______) absorbency at 280 nm., (- - -) absorbency at 413 nm. The line at the top indicates the portions of eluent pooled and concentrated to the original sample volume for CF and ILC testing.
Fig. 6.
antigen activity applied to the column. The pool II precipitate contained 4.0 per cent of the protein, 14.1 per cent of CF activity, and none of the ILC activity of the original sample. Thus, the first peak represented 16.1 per cent of the total protein; 78.5 per cent of the CF$_{50}$'s and 32.3 per cent of the LCID$_{50}$'s of the original sample.

Precipitation of antigen activity in pool II during concentration indicated possible aggregation of some components and the precipitate could only be partially resuspended. Pool II and the precipitate of pool II were centrifuged at 200,000 x g (ave) for 3 hours. Testing of the resulting supernates indicated only 15.4 per cent of the CF activity but all of the ILC activity of pool II remained soluble, while 12.3 per cent of the CF activity of the precipitate remained in the supernate.

All antigen activity eluted in a single peak equal to the void volume of the column and thus was of large molecular weight. Additionally there appeared to be different solubilities of the antigens detected in the CF and ILC tests. Ultracentrifugation of the antigen active pool resulted in pelleting of 84.6 per cent of the CF activity but no reduction of ILC activity. Also chromatography resulted in some purification of antigen components since the LCID$_{50}$ dose in micrograms of protein of pool II was 0.375 compared to 0.609 for the original sample and CF$_{50}$'s per milligram protein increased 3.2 fold in pool II as compared to the
original sample.

b) Chromatography eluting with 0.75 M sucrose and 0.5 M phosphate (sucrose-phosphate). Four samples of HSE derived from three separate extraction experiments, were run through G-200 column B eluting with sucrose-phosphate, pH 7.0. The samples were applied to the column after dilution of HSE with an equal volume of 0.75 M sucrose and 1.0 M phosphate, pH 7.0, giving a final concentration of 0.5 M phosphate and 0.75 M sucrose. Collected fractions were studied for absorbancy at 260, 280 and 413 nm. Three of the samples were fresh preparations and the fourth had been stored at -20°C in sucrose-phosphate for 2 days. The elution patterns for all samples were very similar. Figure 7 shows the results using a fresh 12 ml sample of HSE 6. The pattern of absorbancy at 280 nm is very similar to that using hypertonic sucrose alone (Figure 6). The first peak eluted with the void volume and the second peak was completely included in the gel. However, the pattern of absorbancy at 413 nm, presumably hemoglobin, was different with sucrose-phosphate than with sucrose. Two peaks were seen at 413 nm in 0.75 M sucrose, roughly approximating those seen at 280 nm, but in sucrose-phosphate only a single, broad, completely included peak was observed. Figure 8 shows the elution pattern of a 10 ml sample of HSE 7 which had been frozen at -20°C for 2 days before chromatography.
Fig. 7. Sephadex G-200 Column B chromatographic separation of fresh Hypertonic Sucrose Extract 6 eluting with 0.75M sucrose and 0.5M phosphate, pH 7.0. Sample volume of 12.0 ml.

(---) absorbancy at 280 nm, (----) absorbancy at 413 nm.

The line at the top indicates the portions of effluent pooled and concentrated for CF and ILC testing.
Fig. 8. Sephadex G-200 Column B chromatographic separation of Hypertonic Sucrose Extract 7 after storage at -20°C for two days. Eluted with 0.75M sucrose and 0.5M phosphate, pH 7.0. Sample volume 10.0 ml. (-----) absorbency at 260 nm, (---) absorbency at 280 nm, (-----) absorbency at 413 nm.
Figure 8 also depicts the pattern of absorbancy at 260 nm, which was very similar to the pattern at 280 nm.

Column fractions were combined into three pools as indicated in Figure 7. The pools were concentrated by ultrafiltration to approximately the original sample volume. During concentration no precipitate collected on the ultrafiltration membrane as happened using only 0.75 M sucrose. All the concentrated pools were slightly cloudy and pool II was pink. The concentrated pools were tested in ILC and CF assays (Table 6). Antigen activity was found only in pool I. A mean of 33.3 per cent of the CF activity applied to the column was recovered, however 78.5 per cent was recovered using sucrose alone. The reverse was found with ILC activity, for the percentage of original sample activity recovered was at least two-fold greater in sucrose-phosphate than in 0.75 M sucrose. It was concluded that although treatment of HSE with phosphate enhanced the solubility of the antigen components it did not greatly affect their size.

3. Effects of dialysis on antigen activity of HSE. Samples of HSE 6 and concentrated pool I of HSE 6 chromatographed on Column B using sucrose-phosphate were dialyzed against 1400 and 600 volumes respectively of 0.15 M NaCl for 72 hours at 4°C. As the sucrose concentration decreased during dialysis a fine white precipitate appeared in the dialysis bag containing HSE 6. No precipitate was noted
### TABLE 6

**RECOVERY OF ANTIGEN ACTIVITY FROM SEPHADEX G-200 COLUMN B**

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Total CF&lt;sub&gt;50&lt;/sub&gt;'s Applied</th>
<th>Total LCID&lt;sub&gt;50&lt;/sub&gt;'s Applied</th>
<th>Total CF&lt;sub&gt;50&lt;/sub&gt;'s Recovered</th>
<th>Percent CF Activity Recovered</th>
<th>Total LCID&lt;sub&gt;50&lt;/sub&gt;'s Recovered</th>
<th>Percent of LCID Activity Recovered</th>
</tr>
</thead>
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<tr>
<td>5</td>
<td>2000</td>
<td>N.T. (a)</td>
<td>528</td>
<td>26.4</td>
<td>N.T.</td>
<td>N.T.</td>
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<tr>
<td>6</td>
<td>1168</td>
<td>17,400</td>
<td>485</td>
<td>41.5</td>
<td>15,515</td>
<td>89.1</td>
</tr>
<tr>
<td>7a</td>
<td>659</td>
<td>15,000</td>
<td>422 (b)</td>
<td>32.0</td>
<td>19,162</td>
<td>63.9</td>
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<tr>
<td>7b</td>
<td>659</td>
<td>15,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Not tested.

(b) Concentrated pools from sample numbers 7a and 7b were combined before testing.
in the pool I sample. After resuspension of the precipitate the dialyzed HSE 6 contained only 15 per cent and 44 per cent of CF and ILC activity respectively compared to undialyzed HSE 6. Dialyzed pool I had lost all detectable CF and ILC activity. Dialyzed HSE 6 was centrifuged at 200,000 x g (ave) for 3 hours at 4°C. The resulting clear supernate had no detectable CF or ILC activity. The white pellet would not resuspend in 0.75 M sucrose and was not tested.

A sample of HSE 10 was dialyzed against 1000 volumes of distilled water for 2 days at 4°C. Precipitation occurred during dialysis as described above. Dialyzed HSE 10 had 52 per cent less CF activity than undialyzed material and the supernate from centrifugation of the dialyzed HSE at 200,000 x g had no detectable CF activity.

It was concluded that dialysis of antigen active extracts against isotonic saline or distilled water resulted in loss of antigen activity and the remaining antigen active components were no longer soluble after centrifugation at 200,000 x g.

**Studies on Extraction of Rat Xenoantigens Using Other Extracting Medias**

Several investigations were made on the ability to extract "soluble" xenoantigen activity by the previously described procedure (Figure 1) but using extracting medias
other than 0.75 M sucrose. The results are summarized in Tables 7 and 8 and discussed below.

1. Hanks' Balanced Salt Solution (HBSS). The fractions resulting from the differential centrifugation were similar in physical appearance to those previously described for 0.75 M sucrose with the exception that the 200,000 x g pellet was firmer, fibrous, and would not resuspend homogenously. The 1300 x g supernate contained considerable CF and ILC antigen activity, but comparing HBSS with 0.75 M sucrose major differences were found in the relative distribution of this activity in the other fractions. The most important difference was the relative distribution of activity between the 200,000 x g supernate and pellet. With 0.75 M sucrose the 200,000 x g supernate contained an average of 31.2 per cent CF and 51.5 per cent ILC activity of the totals while with HBSS the 200,000 x g supernate contained only 3.4 per cent of the total CF activity and had no detectable ILC activity. Large differences were also noted between the distribution of activity between the 10,000 x g centrifugation step fractions.

2. 0.25 M sucrose. Use of 0.25 M sucrose (isotonic sucrose is 0.27 M) as the extracting media in two experiments produced results very similar to the use of HBSS with regard to the total antigen activity and distribution in fractions. The 200,000 x g supernates from both experiments contained little CF activity, 6.4 and 4.3 per cent of the
### TABLE 7

EXTRACTION OF RAT LYMPHOCYTES: CELL COUNTS, EXTRACTING VOLUME, AND PROTEIN CONTENT OF FRACTIONS

<table>
<thead>
<tr>
<th>Extracting Medium</th>
<th>Hank's Balanced Solution</th>
<th>0.25 M Sucrose</th>
<th>0.25 M Sucrose</th>
<th>0.77 M Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cells Extracted (x $10^9$)</td>
<td>0.77</td>
<td>0.24</td>
<td>1.30</td>
<td>0.68</td>
</tr>
<tr>
<td>Extracting Volume (ml)</td>
<td>10</td>
<td>10</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>Protein (mg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,300 x g supernate</td>
<td>2.23</td>
<td>2.25</td>
<td>1.10</td>
<td>N.T. (a)</td>
</tr>
<tr>
<td>10,000 x g supernate</td>
<td>2.06</td>
<td>2.20</td>
<td>1.33</td>
<td>2.08</td>
</tr>
<tr>
<td>10,000 x g pellet (b)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.14</td>
</tr>
<tr>
<td>200,000 x g supernate</td>
<td>1.38</td>
<td>1.35</td>
<td>1.00</td>
<td>1.38</td>
</tr>
<tr>
<td>200,000 x g pellet (b)</td>
<td>0.35</td>
<td>0.50</td>
<td>0.14</td>
<td>0.33</td>
</tr>
</tbody>
</table>

(a) Not tested.

(b) Pellet was resuspended in volume of extracting medium equal to volume of the supernate.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Extracting Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hank's Balanced Solution</td>
</tr>
<tr>
<td>1300 x g Supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>575</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>16,600</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>5,393</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>88,800</td>
</tr>
<tr>
<td>10,000 x g Supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>239</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>6,400</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>931</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>19,200</td>
</tr>
<tr>
<td>200,000 x g Supernate</td>
<td></td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>7</td>
</tr>
<tr>
<td>CF&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>117</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/mg protein</td>
<td>0</td>
</tr>
<tr>
<td>LCID&lt;sub&gt;50&lt;/sub&gt;'s/10&lt;sup&gt;9&lt;/sup&gt; cells extracted</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Not tested.
total activity of the 200,000 x g fractions, and the one supernate tested had no detectable ILC activity.

Two experiments were done to see if CF activity of the 0.25 M sucrose 10,000 x g supernate would remain in the supernate during a 200,000 x g centrifugation if the sucrose concentration was increased to 0.75 M before centrifugation. Samples of the 0.25 M sucrose 10,000 x g supernate were diluted with an equal volume of 1.25 M sucrose to give a final concentration of 0.75 M sucrose and then centrifuged at 200,000 x g (ave) for 3 hours at 4°C. The resulting pellets were resuspended in a volume of 0.75 M sucrose equal to the supernate and both supernate and pellet tested in the CF assay. The control consisted of the 10,000 x g supernates diluted with an equal volume of 0.25 M sucrose before ultracentrifugation. The first experiment was done using 10,000 x g supernate which had been stored at 4°C for 6 days. It indicated the sucrose concentration had little effect on the distribution of CF activity. At final sucrose concentrations of 0.75 M and 0.25 M the 200,000 x g supernate contained 19 and 14 per cent respectively of the total CF activity. However, when this experiment was repeated using freshly extracted 0.25 M sucrose 10,000 x g supernate different results were obtained. Using fresh extract the 200,000 x g supernate at a sucrose concentration of 0.75 M contained 48 per cent of the total CF activity while the supernate at a sucrose
concentration of 0.25 M contained only 4.4 per cent of the total CF activity. Thus, using fresh extract it seemed that increasing the sucrose concentration to 0.75 M before ultracentrifugation resulted in pelleting less of the CF antigen activity extracted with 0.25 M sucrose than if the sucrose concentration remained at 0.25 M.

3. **0.77 Mannitol.** The osmotic pressure of 0.77 M mannitol is very close to that of 0.75 M sucrose and thus its use as an extracting medium would presumably expose the cells to a hypertonic environment similar to that created by the hypertonic sucrose. In contrast to the use of HBSS or 0.25 M sucrose, the use of 0.77 M mannitol resulted in the recovery of considerable ILC activity in the 200,000 x g supernate. The mannitol 200,000 x g supernate contained 31.9 per cent of total ILC activity of the sum of 200,000 x g supernate and pellet, compared to an average of 51.5 per cent for five extractions using 0.75 M sucrose. In terms of LCID$_{50}$'s per $10^9$ cells extracted the mannitol 200,000 x g supernate contained 33 per cent of the average obtained for seven extractions with 0.75 M sucrose. The CF activity of the mannitol 200,000 x g supernate was 16.7 per cent of the total of the 200,000 x g supernate and pellet, and was 18.9 per cent of the average activity per $10^9$ cells extracted for ten extractions using 0.75 M sucrose.

4. **Conclusions.** The use of HBSS and 0.25 M sucrose resulted in little CF and no ILC activity in the 200,000 x
g supernates and thus were ineffective in extracting xen-antigens that were "soluble" (not pelleted by centrifugation at 200,000 x g for 3 hours). Mannitol was effective in extracting soluble rat xenoantigens but less so than 0.75 M sucrose. Increasing the sucrose concentration to 0.75 M before ultracentrifugation of a fresh 10,000 x g supernate extracted with 0.25 M sucrose, resulted in more CF activity in the supernate than if the sucrose concentration was maintained at 0.25 M. This may have been due to a buoyant effect on the CF antigens resulting from a greater density of the 0.75 M sucrose, or alternatively, it may have been due to the ability of the higher sucrose concentration to prevent aggregation of CF active components.

**Immunogenicity and Xenotransplantation**

**Antigen Activity of HSE**

The studies discussed above indicated HSE and the material which eluted as the first peak from Sephadex G-200 chromatography of HSE contain rat xenoantigens detectable by CF and ILC assays. We now turned our attention to whether this material was immunogenic in mice and contained rat xenotransplantation antigens as evidenced by its ability to modify rejection of rat skin grafts on immunized mice.

Twenty five C3H/Hej mice were injected weekly for three weeks with 0.2 ml of antigen active material (material eluting as the first peak from Sephadex G-200 Column B)
emulsified with an equal volume of Freund's complete adjuvant (FCA, Difco). The first two injections were given both subcutaneously (SC) and intraperitoneally (IP) as equally divided doses, the third was given only IP. Serum samples were collected weekly by retroorbital sinus puncture beginning six days after the first injection. Direct hemagglutinating (HA) antibodies for rat erythrocytes were first detected 6 days after the third injection and reached a titer of 64 one week later. Tests for lymphocytotoxic antibodies (LC) were negative. As controls, serum from animals receiving sucrose-phosphate emulsified with FCA had no detectable HA or LC antibody activity while serum from animals receiving per week $2 \times 10^7$ rat lymphoid cells emulsified with FCA had a HA titer of 4096 and a LC titer of 1024 after three injections. Since the total dosage the test animals received during these initial three injections was low (1.24 mg protein, 22 CF$_{50}$'s, and an estimated 2250 LCID$_{50}$'s) they were boosted with two identical injections of freshly prepared HSE. The first booster injection of 0.2 ml of HSE emulsified with FCA contained 40 CF$_{50}$'s and 931 LCID$_{50}$'s, and was given in doses divided equally between SC and IP sites two weeks after the third initial injection. The second booster injection was given one week later by IP route only. Six days after the first booster injection test animals had a HA titer of 1024 and a LC titer of 8. Six days after the second booster HA
and LC titers were 4096 and 32 respectively. The control animals receiving booster injections of sucrose-phosphate with FCA failed to develop detectable antibody, while the control animals receiving a single booster injection of $2 \times 10^7$ rat cells with FCA 3 weeks after their third initial injection showed no change in serum HA and LC titers.

Six days after the final booster injection mice from all groups were tested for cell-mediated cytotoxicity (CMC) of spleen cells using $^{51}$Cr labeled rat blood lymphocytes as target cells. Spleen cells from three animals having received HSE gave percentages of cell-mediated cytotoxicity of only 3.0, 1.2, and 0.5, while spleen cells from two animals which received whole rat cells gave CMC percentages of 32.3 and 12.3. Spleen cells pooled from two animals which received only sucrose-phosphate gave 5.3 per cent CMC.

The remaining mice from all three groups, along with normal mice, received primary rat skin grafts one week after the final booster injection. All grafts were evaluated daily beginning on day 3. For calculation of mean survival times, grafts which underwent hyperacute ("white graft") rejection were considered rejected on day 3 (Table 9). All grafts on 6 mice immunized with rat lymphocytes and 11 of the 17 grafts (64.3 per cent) on mice immunized with HSE were hyperacutely rejected. The remaining 6 grafts on mice immunized with HSE were rejected in a manner and time sequence similar to primary rat xenografts on normal
<table>
<thead>
<tr>
<th>Group</th>
<th>Immunizing Preparation</th>
<th>No. of Animals</th>
<th>No. of &quot;White Grafts&quot;</th>
<th>M.S.T.±S.E. (b)</th>
<th>p (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>14</td>
<td>0</td>
<td>6.2±0.3</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>Hypertonic Sucrose Extract with FCA</td>
<td>17</td>
<td>11</td>
<td>4.1±0.4</td>
<td>0.001</td>
</tr>
<tr>
<td>C</td>
<td>Rat Lymphocytes with FCA</td>
<td>6</td>
<td>6</td>
<td>3.0±0.0</td>
<td>0.001</td>
</tr>
<tr>
<td>D</td>
<td>Sucrose-Phosphate Buffer with FCA</td>
<td>8</td>
<td>0</td>
<td>6.0±0.5</td>
<td>N.S. (d)</td>
</tr>
</tbody>
</table>

(a) See text for details of immunization procedure.

(b) Mean survival time (days) ± standard error.

(c) Probability compared with group A using Student's t test.

(d) Not significant.
mice and mice immunized only with sucrose-phosphate. Twelve days after grafting, all mice were sacrificed and pooled serum from each group assayed. Animals previously immunized with HSE had a LC titer of 256, increased from a titer of 32 at the time of grafting. Unimmunized animals and animals immunized only with sucrose-phosphate had LC titers of 64 and 32 respectively.

From the above experiments we concluded that HSE contained rat xenoantigens capable of eliciting the production of both HA and LC antibodies. Furthermore, since nearly two-thirds of these mice hyperacutely rejected primary rat skin grafts and the mean graft survival time was significantly reduced when compared to grafts on unimmunized animals, we concluded HSE contained rat xenotransplantation antigens.

**Attempted Xenograft Prolongation**

A single injection of donor strain tissue extracts (crude membrane preparations) prior to grafting and a short course of ALS at the time of grafting were found by Brent et al. (1973) and Cerilli et al. (1974) to prolong skin allograft survival in mice. The means by which this prolongation was obtained is not known but may involve a combination of tolerance and enhancement (Kilshaw et al. 1974). This protocol was modified slightly in an attempt to prolong survival of rat skin xenografts on mice using 0.75 M
sucrose extracted fractions of rat cells.

Sixteen days before receiving primary rat skin grafts, groups of C3H/Hej mice received intraperitoneal injections of 1.0 ml of either HSE (1.67 mg protein, 525 CF$_{50}$'s, and 7120 LCID$_{50}$'s) or 200,000 x g pellet resuspended in 0.75 M sucrose (0.44 mg protein, 1080 CF$_{50}$'s and 6670 LCID$_{50}$'s). The mice were also injected intraperitoneally with 0.5 ml of rabbit anti-mouse lymphocyte serum (RAMLS) on days -2, 0, +2, +4 and +6 in relation to grafting. Control mice received either antigen alone, RAMLS alone, or nothing. LC and HA antibody titers for rat cells on the day of grafting are indicated in Table 10. Both antigen fractions were immunogenic but antibody titers were lower in mice treated with RAMLS. Regretfully serum from animals receiving only RAMLS was not tested but RAMLS was known to have cross-reacting antibody to rat antigens (LC and HA titers of 64). Graft survival is shown in Table 11. Although both antigen preparations were immunogenic neither antigen alone altered graft survival compared to untreated animals. Treatment with RAMLS alone significantly extended graft survival (p < 0.015). The slight increases in mean graft survival time when treatment with either HSE or the 200,000 x g pellet was combined with RAMLS were not significant when compared to treatment with RAMLS alone.
### TABLE 10

**SERUM ANTIBODY LEVELS OF MICE INJECTED WITH RAT LYMPHOCYTE FRACTIONS AND ANTILYMPHOCYTE SERUM**<sup>(a)</sup>

<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>Antigen Treatment</th>
<th>ALS&lt;sup&gt;(b)&lt;/sup&gt;</th>
<th>Antirat Antibody Titers&lt;sup&gt;(c)&lt;/sup&gt;</th>
<th>Lymphocytotoxicity</th>
<th>Direct Hemagglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HSE</td>
<td>Yes</td>
<td>Negative</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>200,000 x g Pellet</td>
<td>Yes</td>
<td>Undiluted</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>C</td>
<td>HSE</td>
<td>No</td>
<td>4</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>D</td>
<td>200,000 x g Pellet</td>
<td>No</td>
<td>4</td>
<td></td>
<td>512</td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>Yes</td>
<td>N.T. (d)</td>
<td></td>
<td>N.T.</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>No</td>
<td>Negative</td>
<td></td>
<td>Negative</td>
</tr>
</tbody>
</table>

<sup>(a)</sup>Lymphocyte fractions given intraperitoneally 16 days before testing, antilymphocyte serum given 2 days before testing. See text for more details.

<sup>(b)</sup>Rabbit anti-mouse lymphocyte serum.

<sup>(c)</sup>Reciprocal of last serum dilution giving positive test.

<sup>(d)</sup>Not tested.
<table>
<thead>
<tr>
<th>Mouse Group</th>
<th>Antigen Treatment</th>
<th>ALS (b)</th>
<th>M.S.T. ± S.E. (c)</th>
<th>Day of Rejection</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>HSE</td>
<td>Yes</td>
<td>16.9 ± 3.1 (10) (d)</td>
<td>6,9,10,12,14,15,16,22,27,38</td>
</tr>
<tr>
<td>B</td>
<td>200,000 x g pellet</td>
<td>Yes</td>
<td>17.8 ± 3.3 ( 9)</td>
<td>6,8,9,13,15,22,25,27,35</td>
</tr>
<tr>
<td>C</td>
<td>HSE</td>
<td>No</td>
<td>7.1 ± 0.8 ( 8)</td>
<td>4,5,6,6,7,9,10,10</td>
</tr>
<tr>
<td>D</td>
<td>200,000 x g pellet</td>
<td>No</td>
<td>6.7 ± 0.7 ( 7)</td>
<td>5,5,6,6,7,8,10</td>
</tr>
<tr>
<td>E</td>
<td>None</td>
<td>Yes</td>
<td>14.1 ± 2.4 ( 8)</td>
<td>5,8,10,13,16,16,16,18,27</td>
</tr>
<tr>
<td>F</td>
<td>None</td>
<td>No</td>
<td>6.8 ± 0.6 ( 6)</td>
<td>5,6,6,7,8,9</td>
</tr>
</tbody>
</table>

(a) See text for details of treatment

(b) Rabbit anti-mouse lymphocyte serum

(c) Mean graft survival time ± standard error

(d) Number of mice in group
DISCUSSION

One of the objectives of this study was to examine the normal immune responses to skin xenografts to provide information which could later be compared to the immune responses of animals experimentally manipulated in attempts to prolong xenograft survival. Additionally, it was hoped that quantitative information regarding humoral and cellular immune responses might provide some indication of the relative role of each in skin xenograft rejection.

Grafts of rat skin on mice provided a consistent model for study. For primary grafts the ranges and mean graft survival times of different groups of animals were very similar (Table 1). Secondary grafts were always rejected faster than primary grafts but two qualitatively different types of secondary rejection were noted. Preformed anti-donor antibody probably played a major role in secondary hyperacute or "white graft" rejection and may be heavily involved in the accelerated form of secondary graft rejection where vascularization of the graft appeared to have occurred.

Cell mediated cytotoxicity of mouse spleen and lymph node cells was two to four times lower following skin xenografts than allografts. Berke et al. (1971) have also
reported levels of CMC activity of rat lymph node cells, following mouse skin xenografts, less than one-half those found following skin allografts. Other investigators have found evidence of lower cell-mediated immune responses to xenoantigens than to alloantigens. Wilson and Fox (1971) reported that rat lymphocytes were less reactive to human, hamster, guinea pig, and mouse cell surface antigens in one-way mixed lymphocyte cultures (MLC) than to major histocompatibility alloantigens. Widmer and Bach (1972) found there was a great variation in the response of xenogeneic MLC but in some combinations the xenogeneic response was as great as in allogeneic mixtures. Jones and Lafferty (1969) noted the intensity of the normal lymphocyte transfer (NLT) reaction in the skin of sheep was not as great in xenogeneic as in allogeneic combinations and declined as the phylogeneic separation of donor and recipient increased.

The lower levels of cellular immune responses reported with xenogeneic combinations in MLC and NLT reactions suggest that in the circulating lymphocyte pool there are fewer cells reactive to xenogeneic cellular antigens than to alloantigens. Additional evidence supporting this concept and extending it to include other cell populations is the finding that mouse lymph node and spleen lymphocytes and human blood lymphocytes non-specifically stimulated in
vitro with phytohemagglutinin are less cytotoxic to xenogeneic than to allogeneic target cells (Stejskal et al., 1973).

The finding of lower levels of cell mediated immunity following xenografting than allografting does not in itself imply that cellular immunity plays less of role in the rejection of xenografts than of allografts. The lymphocytes used as effector cells in the CMC assay were only from axillary lymph nodes and spleen and it is possible these cell populations may not have accurately reflected the total number of cytotoxic cells present in the graft recipients. Mononuclear cell infiltration of skin xenografts occurs quicker and is more pronounced than that seen with skin allografts (Baldamus et al., 1973), but the identity and reactivity of these cells is not known. It is possible that the total number of cytotoxic cells generated in the lymph nodes and spleen in response to skin xenografts is equal or greater than the number generated in response to allografts but either more of the cytotoxic cells migrate to the graft site or the time of residence of the cytotoxic cells within the lymphoid organs before migration is less with xenografts than with allografts. Either of these two possibilities would lead to the finding of few cytotoxic cells within these organs following xenografts than following allografts, but there is no evidence to support either concept. Although it is known that cells
differ in susceptibility to the cytotoxic action of sensitized lymphocytes (Brunner et al., 1970), the rat cells used as targets in the xenogeneic CMC assay were quite susceptible to the cytotoxic action of both allogeneic cells sensitized by skin allografts and of xenogeneic mouse cells sensitized in vivo by repeated injections of rat lymphocytes.

We have concluded that skin xenografts elicit lower levels of cell-mediated immunity as detected by the CMC assay than do skin allografts, and feel CMC activity was less following skin xenografts than allografts because fewer cytotoxic cells were present in response to the xen-antigens. In allograft immunity in the mouse cytotoxic cells detected in CMC assays have clearly been identified as specifically sensitized thymus-derived lymphocytes (Cerrottini et al., 1971), but the picture is less clear in xenograft systems. Beverley and Simpson (1972) showed that cytotoxic activity of spleen and lymph node cells from mice immunized with hamster tumor cells was abolished by treatment with anti-theta serum and complement. Similar evidence for the participation of thymus-derived cells in a xenogeneic cytolytic reaction was obtained by Lonai et al. (1971) in studies of rat lymphocytes sensitized in vitro on mouse fibroblasts. However, in several studies (reviewed by Cerrottini and Brunner, 1974) using combinations of target cells, antibody, and normal lymphoid cells from
different species an antibody-dependent cytotoxicity mediated by non-sensitized lymphoid cells has been observed. The lymphoid effector cell in this case is apparently not thymus-derived, is characterized by surface receptors for the Fc portion of IgG, and its identity is still unknown. The identity of the effector cells in our CMC assay was not established but it would be of interest to know if the CMC activity of spleen and lymph node cells following skin xenografting would be affected by treatment of the effector cell population with anti-theta serum plus complement.

Serum antibody was first detected on day 6 after primary xenografting, a time when many grafts were already undergoing extensive destruction. It is probable that antibodies were produced before this but were undetectable because of rapid complexing with graft antigens and became detectable only when they were present in excess. Hildemann (1967) has demonstrated antibody production by mouse spleen and lymph node cells as early as 2-3 days after skin allografting but serum antibody is not detectable before day 7 (Canty and Wunderlich, 1971). After both primary and secondary grafting HA antibody titers were higher than LC titers but changes in titer were parallel. We do not know if the two assays were detecting the same antibody populations but it is reasonable to assume that difference in titer were due primarily to different sensitivities of the assays. Both HA and LC titers remained elevated for up to
34 days after primary grafting and rapidly increased following secondary grafting. The increase for LC titers after secondary grafting was greater than four-fold but HA titers increased slightly less. The decline in titers seen from about day 8 thru 14 after primary grafting may have been due to decreasing levels of serum IgM.

Following primary xenografting LC titers were low (16 or less). This was noted also by Hamilton and Gaugas (1972) following primary grafting of hamster skin on adult mice. In contrast Lance et al. (1969) using a different assay technique reported LC titers greater than 640 in the serum of mice as early as seven days after receiving primary grafts of rat tail skin. However, Lance detected antirat LC titers in the range of 32-64 and antirat hemolysins of 64-128 in normal mouse serum, whereas we found no LC or HA activity to rat cells in normal mouse serum. These variances may have been due to the use of different rat and mouse strains.

The passive transfer of mouse anti-rat lymphocyte ascites (MARLA) to otherwise normal adult mice led to the hyperacute rejection of primary rat skin grafts. Thus anti-donor antibody is capable of causing accelerated rejection of skin xenografts as well as vascularized organ xenografts (Perper and Najarian, 1967). Reports by Bal-damus et al. (1973) and Hamilton and Gaugas (1972) have
indicated that flourishing rat and hamster skin xenografts on immunosuppressed mice can be rapidly destroyed by the intravenous administration of anti-donor antibody. The grafts showed signs of rejection within one hour after antibody administration and the reaction, requiring both complement and polymorphonuclear neutrophils (Winn et al., 1973), resembled an Arthus reaction. Grafts were completely rejected within 24-48 hours. Transfer of $10^5$ sensitized lymph node cells (from mice receiving a secondary skin graft 8 days previously) or $10^6$ normal cells to immunosuppressed mice with grafts expected to survive for at least four weeks also resulted in xenograft rejection but with an initial delay of 6 days (Hamilton and Gaugas, 1972).

Since the primary barrier to the survival of skin allografts and xenografts is immunological in character it has been suggested by Lance (1969) and others that the more vigorous rejection of skin xenografts may be due to a greater participation of humoral antibody in the rejection process. This suggestion is supported by our findings of lower CMC following skin xenografts than allografts and that passive transfer of MARLA led to accelerated rejection of skin xenografts. It should be noted that most investigations of the effects of passive transfer of antibody on the outcome of skin allografts have shown either no effect or prolonged survival (Winn, 1970). A credible theoretical explanation for a greater role of humoral antibodies in
the rejection of skin xenografts than allografts has been offered by Baldamus et al. (1973). Whether or not the combination of humoral antibodies with donor antigens causes damage to skin grafts depends upon the ability of the immune complexes formed to activate a variety of mechanisms serving as effector pathways for immune and inflammatory responses. With inflammation initiated by immune reactions it is reasonable to expect the intensity of the response to be determined by the concentrations of primary reactants i.e., antigen and antibody. In graft rejection the extent of antigen-antibody interaction is limited by the concentration of relevant antigens on the surface of the cells of the graft. The concentration of relevant antigens present in skin allografts and accessible to combine with humoral antibody may not be sufficient to incite an effective inflammatory response for tissue destruction. Skin xenografts, on the other hand, possess a greater number and concentration of macromolecular substances foreign to their hosts (Kano et al., 1972; Sachs, et al., 1971; and DeWitt et al., 1971) and, accordingly, there is a greater opportunity for the accumulation of antigen-antibody complexes at the cell surfaces. This in turn may lead to more effective mobilization of effector substances and to more vigorous attack on the transplanted tissues.

The second objective of this study was the preparation of cell-free rat xenotransplantation antigens suitable
for use in investigations on producing immunological tolerance or enhancement to prolong the survival of skin xenografts. Hypertonic sucrose treatment of rat lymphoid cells led to the extraction of xenoantigens that were capable of causing accelerated rejection of rat skin xenografts on immunized mice. Thus, HSE contained material capable of fulfilling the criteria for xenotransplantation antigens in that it modified the rejection time of primary xenografts. Additionally, these antigens were subcellular components that resisted pelleting during ultracentrifugation at 200,000 x g for 3 hours. Solubility of the antigen is a property generally regarded as advantageous in the production of immunological tolerance to tissue antigens (Medawar, 1963).

HSE contained antigens detectable in both ILC and CF assays using MARLA but no antigens detectable by precipitation in ID or IEP. For an antigen to inhibit lymphocytotoxic antibody it must also be present on the cell surface and therefore may be important in transplantation. On the other hand, detection of antigen by the CF test implies nothing of its cellular location. Additionally, neither test gives an indication of the number of reacting antigens. While it is reasonable that both assays detected some of the same antigens there were several instances where manipulations of HSE resulted in different recoveries of the two antigen activities. ILC activity was stable during storage.
at -20°C and 4°C and resisted heating to 36° and 60°C for one hour. In contrast, declines in CF activity and/or aggregation of CF detected antigens were noted after storage at -20°C and 4°C and heating to 60°C. The stability of the ILC activity of HSE was particularly pleasing since it allowed storage without loss of activity.

Immunization of mice with HSE led to the production of LC and HA anti-rat antibodies. Spleen cells from mice immunized with HSE had no CMC activity against rat target lymphocytes but spleen cells from mice immunized with whole rat lymphocytes were very active (32.3 per cent CMC). Eleven of seventeen primary rat skin grafts on HSE immunized mice and six of six on mice immunized with whole rat cells were rejected as "white grafts." Thus HSE contained rat xenoantigens that were serologically detectable and antigens with demonstrable biologic xenotransplantation activity. We do not know if the serologically detected and xenotransplantation antigens are the same, but it is tempting to speculate that at least some of them are.

The extraction of rat xenoantigens with 0.75 M sucrose is a simple and reproducible procedure giving good yields of antigen. HSE from pooled rat spleen, thymus, and lymph node cells contained an average of 66,900 LCID\textsubscript{50}'s and 7,600 CF\textsubscript{50}'s per 10\textsuperscript{9} cells extracted, an absorptive yield of 155 per cent. Additional but smaller amounts of antigen were recovered by re-extraction of the
1300 x g pellet. Stroehmann and DeWitt (1972a, 1972b) using 3 M KCl to extract rat spleen and thymus cells recovered a rat species-specific antigen, identified by lymphocytotoxic inhibition of rabbit antirat lymphocyte serum, in an amount about equal to the recovery of a rat alloantigen at 2000 LCID_{50}'s per 10^9 cells. Schwartz and Lang (1974) using 3 M KCl extracted baboon xenogeneic histocompatibility antigens from blood lymphocytes with yields of 87,000 LCID_{50}'s per 10^9 cells, an absorptive yield of 57 per cent, and 4,540 CF_{50}'s per 10^9 cells (Schwartz, 1972).

Since the cells were not washed before extraction HSE contained serum components, small amounts of hemoglobin, and had a large protein content. Washing the cells prior to extraction resulted in a lower recovery of antigen (noted also by Stroehman and DeWitt, 1972a) and suggests the antigens may have been loosely bound to the cell surface. No CF or ILC activity was detectable in rat serum, however, and also MARLA was not reactive with rat serum components. Because of the large amount of protein in HSE its specific activity, in terms of antigen active units per mg protein, was lower than some of the other fractions.

A hypertonic environment contributed to the extraction of xenoantigens that resisted pelleting at 200,000 x g in the extracting medium. Isotonic HBSS and 0.25 M sucrose
were ineffective in extracting soluble antigens. Hypertonic mannitol was effective but less so than sucrose. It is possible that hypertonic conditions led to more cell fragmentation during preparation of the cell suspensions. However, ninety per cent of the cells maintained their integrity, i.e. still excluded vital dyes and appeared intact, following extraction with 0.75 M sucrose.

Even though HSE contained xenoantigens which were not pelleted during ultracentrifugation at 200,000 x g for 3 hours, the antigen active components still appeared to be large since all activity was excluded from Sephadex G-200 (similar to Stroehmann and DeWitt, 1972b). Hypertonic sucrose treatment of lymphoid cells may have resulted in the formation of membrane fragments or possibly small soluble antigens which may have aggregated or adsorbed to larger molecules. Investigating the latter two possibilities HSE was treated with 0.5 M phosphate under conditions sufficient to break the bounds of the haptoglobin-hemoglobin complexes contaminating the preparation (compare absorbency at 413 nm in Figures 6 and 7). Under these conditions all antigen activity was still excluded from G-200 but there was a two-fold increase in recovery of ILC activity from the column and there was no aggregation of the antigen components during concentration of the column eluant. Perhaps greater success would have been obtained with use of stronger de-aggregating agents such as urea,
detergents, or more chaotropic ions. Two findings indirectly suggest HSE did not contain antigen activity simply because of a buoyant effect of 0.75 M sucrose (d[^20] of 1.0965) on very large antigen components during centrifugation. Reultracentrifugation of HSE even after freezing did not result in pelleting of ILC activity, but less CF activity was still soluble. Extraction with 0.77 M mannitol, considerably less dense (d[^20] of 1.0476) than 0.75 M sucrose, was effective but 0.25 M sucrose with a density (d[^20] of 1.0311) close to that of mannitol was not. Ultracentrifugation of fresh 0.75 M sucrose extracts for longer periods, up to eighteen hours, might be informative in this regard.

Regardless of the size of the xenoantigen components of HSE the sucrose contributed to their solubility. After dialysis against distilled water or isotonic NaCl precipitation occurred and no soluble immunologic activity was detectable. This is similar to the finding of Schwartz (1972) that 3 M KCl extracted baboon xenohistocompatibility antigens were no longer soluble after removal of the solubilizer.

We believe HSE is a good material for use in studies on inducing unresponsiveness to skin xenografts. It contains serologically detectable xenoantigens and biologically active xenotransplantation antigens in at least semisoluble
form. Good yields of antigens are obtained from relatively small amounts of material and the extract can be stored with little loss of activity. The relative impurity of the antigens in HSE should not be viewed as detrimental. Xenografts undoubtedly contain more substances capable of eliciting immune responses in the recipient than do allografts and at present there is no evidence that any one of these is more important than others in determining the fate of xenografts. Unless there is a particular substance that is dominant in determining the survival of xenografts, working with materials that contain a large number of xenantigens may increase the possibility of prolonging graft survival. It is interesting in this regard that the cytotoxic antibody response in xenoimmunization with insoluble mouse cell membranes has been shown to be directed against antigenic determinants associated with soluble major allohistocompatibility (H-2) antigens (Staines, 1974). For prolonging xenograft survival it would be advantageous if there is a particular immunodominant substance.

The failure to obtain significantly longer graft survival in our single attempt at graft prolongation using hypertonic sucrose extracted antigens was not discouraging. The protocol used was a slight modification of one used successfully with allografts but it is already known that most procedures sufficient for prolonging allograft survival are less efficient with xenografts (Lance et al.,
1969). Indeed, we were encouraged by the slight increases in mean graft survival time that was seen when treatment with antigen fractions was combined with ALS and believe further experiments using larger amounts of antigen are warranted.
SUMMARY

These investigations of a xenograft model had the goals of determining the immune responses of normal mice to grafts of rat skin, and the extraction of cell-free rat xenotransplantation antigens suitable for use in studies on production of immunological unresponsiveness to prolong xenograft survival.

Primary grafts of rat skin on mice were initially accepted but by macroscopic examination were undergoing extensive destruction by 5-6 days after grafting. The mean graft survival time for greater than 150 primary grafts was 5.6 days. Lymphocytotoxic (LC) and direct hemagglutinating (HA) antibodies were first detectable 7 and 6 days respectively after grafting. LC and HA levels were roughly parallel but while HA titers reached a peak of 512, LC titers were never above 24. Both were still elevated as late as 34 days after grafting. Cellular immunity, as measured by the cell mediated cytotoxicity (CMC) assay using mouse spleen and lymph node cells reacting with $^{51}$Cr-labeled rat lymphocytes, was low after primary xenografting. Peak CMC levels of 7.0 per cent were reached 6 days after xenografting and were less than 1/2 to 1/4 the CMC responses seen after rat-to-rat or mouse-to-mouse skin allografts.
Secondary grafts of rat skin on mice were rejected within 3 days and showed two types of rejection. The most common was a form of hyperacute "white graft" rejection in which the grafts were never vascularized, the second appeared macroscopically at least to be an accelerated form of rejection similar to what was seen after primary grafting. Both LC and HA antibody titers increased rapidly after secondary grafting with LC titers reaching levels greater than four times those following primary grafting.

The ability of humoral antibody alone to cause accelerated primary skin xenograft rejection was demonstrated by the passive transfer of mouse anti-rat lymphocyte ascites, to mice receiving primary rat skin grafts, resulting in hyperacute "white graft" rejection.

Extraction of cells from rat lymphoid organs with hypertonic (0.75 M) sucrose resulted in large yields of xenoantigenic material detectable by serologic procedures and with demonstrable xenotransplantation antigen activity in vivo. These antigens resisted pelleting during ultracentrifugation at 200,000 x g for 3 hours but were nevertheless large since they were excluded from Sephadex G-200 during column chromatography. Extracted cell surface xenoantigens detected by inhibition of lymphocytotoxic antibody (ILC) were stable during storage at 4°C and -20°C but antigens detectable by complement fixation (CF)
underwent some aggregation and loss of activity. During removal of the sucrose by dialysis all xenoantigen activity precipitated and was no longer soluble. Hank's Balanced Salt Solution and 0.25 M sucrose were ineffective in extracting soluble xenoantigenic material but mannitol at a hypertonicity equal to that of 0.75 M sucrose was effective but less so than sucrose.

Soluble rat xenoantigens extracted with 0.75 M sucrose were immunogenic in mice leading to the production of LC and HA antibodies but negligible levels of detectable cell-mediated cytotoxicity. Hyperacute "white graft" rejection of primary rat skin grafts on the immunized mice indicated the presence of rat xenotransplantation antigens in the hypertonic sucrose extract (HSE). In an attempt to prolong xenograft survival, treatment of mice with anti-lymphocyte serum plus hypertonic sucrose extracted rat xenoantigens gave a slight but not significant increase in mean graft survival of primary rat skin grafts.
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