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The Ohio State University, Ph.D., 1972
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STUDIES ON BABOON XENOGENEIC HISTOCOMPATIBILITY 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

Anthony Schwartz, D.V.M.

*****

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1972

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In the race for the graduate degree, the one who has been most responsible for my crossing the finish line is my wife Claudia. Without her support and understanding it would have been impossible to continue at certain points. She was always on the sidelines, watching, cheering, and feeding the runner whenever necessary. The smaller fans, my sons Tommy and Eric, also watched, not often understanding why their father was still involved in such a sports event while other fathers worked for a living.

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INTRODUCTION

The inadequate supply of suitable kidneys and other organs for human transplantation has led investigators to consider organ transplantation between species (xenografting). Sub-human primates are the obvious candidates as sources of organs, due to their close phylogenetic relationship to man. Some investigators have performed xenografts of chimpanzee (Reemtsma et al., 1964) and baboon-derived kidneys (Starzl, 1964) into humans. Although there was one instance in which a chimpanzee kidney functioned for nine months in a patient (Reemtsma, 1968), most of the grafts were rejected within two months.

Antibody appears to play a prominent role in hyperacute and chronic allograft rejection in man (Hume, 1971), and is probably involved to a great extent in xenograft rejection (Reemtsma, 1971). Studying a subhuman primate xenograft model may provide data enabling prediction of the immunological response elicited by the grafting of humans with primate organs.

Two methods of immunosuppression likely to be investigated in xenografting are the use of antilymphocyte serum (ALS) and antigen-induced specific immunological tolerance. ALS acts equally well whether there is a strong or weak histocompatibility barrier between donor and recipient (Lance and Medawar, 1971). Thus, xenografts of skin from rats on mice or hamsters have survived several months
when chronic treatment of recipients with ALS was given, as have grafts from guinea pigs, rabbits and humans, on mice (Billingham and Silvers, 1971).

Since immunological tolerance is specific for the antigens involved, it would be an ideal mode of preventing graft rejection, as none of the immunologic reactivity of the host for tumors or infectious antigens would be affected. Utilizing this means of specific immunosuppression would require a supply of suitable antigens. For many years, attempts have been made to separate transplantation antigens from the cell membrane for the purpose of chemical identification (Mann and Fahey, 1971a). Although many agents have been employed as immunosuppressive adjuncts to the induction of tolerance, ALS pre-treatment appears to be the most appealing due to its high degree of effectiveness and low risk (Lance, 1971).

Antigens relevant to the immunosuppressive activity of ALS, or important to transplantation immunity in general, appear to be found on both internal and external membrane surfaces (Manson et al., 1968). Haughton (1966) tested the ability of whole lymphoid cells and homogenates of these cells to absorb cytotoxic H-2 antibodies from serum, and found that 80 per cent of antibodies were absorbed by whole cells. He contended that this may be an underestimation and that all H-2 antigens were probably on the plasma
membrane. Other investigators have isolated histocompatibility antigens from microsomal fractions of cell homogenates (Herberman and Stetson, 1965, Manson et al., 1963). While it was generally believed that microsomal fractions are vesicles derived only from the endoplasmic reticulum (pelleted by 100,000 x g for 60 minutes), the shear forces produced during homogenization have been found to be able to produce vesicles from the plasma membrane, which would also sediment in this fraction (Wallach, 1967). Manson et al. (1968), "toughened" the plasma membrane of lymphoid cells with a sulphydryl reacting compound (fluorescein mercuric acetate), allowing production of plasma membranes which could be isolated as cell ghosts. H-2 antigens were found in quantity at intracellular sites in the mouse lymphoid cell line studied (L-5178Y) using this technique.

Rapaport et al. (1965), reported that 4 fractions of normal human leukocyte homogenates, termed nuclear (755 x g pellet), mitochondrial-granular (20,000 x g pellet), microsome-rich (105,000 x g pellet), and fibrillar (105,000 x g supernatant), could sensitize human volunteers to reject skin grafts in an accelerated fashion. Centrifugation of the fibrillar fraction at 198,000 x g resulted in sedimentation of all of the activity. Electron micrographs of the sediment showed that it was made up of fine fibrils, and small fragments of membrane. The common active component of all of the fractions was concluded to be membranous material.
Xenoantigens have been found in a variety of centrifugal fractions of lymphocyte homogenates (Levey and Medawar, 1966a), though Lance et al. (1968), felt that the cytoplasmic membranes of lymphocytes carried the strongest antigens for the production of ALS. Moynihan and Grogan (1969) found that the most effective ALS for immunosuppression was derived by immunization with a subcellular fraction obtained by centrifugation at 8,000 x g for 15 minutes (following removal of the nuclear pellet). Hirose and Kurosawa (1971) used rabbit anti-mouse thymocyte serum in assays of inhibition of lymphocytotoxicity and inhibition of immunosuppressive ability, and found both the 8,000 x g pellet and the microsome-rich fraction of mouse thymocytes were active in inhibiting immunosuppression, though the nuclear and fibrillar fractions (of Rapaport et al., 1965) were not. While all fractions tested except the nuclear pellet had significant ability to inhibit cytotoxicity, most activity was in the microsome-rich fraction.

Various means of solubilization of allograft antigens have been utilized. Detergents have been used for this purpose, with findings that the antigens obtained are very difficult to separate from the solubilizing agent. If detergent is removed, though usually still active, the antigens are no longer soluble (Mann and Fahey, 1971a). The non-ionic detergents have been little studied. Triton X-100 extracts of H-2 antigens were digested with snake venom phospho-
lipase A by Kandutsch and Stimpfling (1963). This produced a material that was polydisperse on sedimentation, probably a collection of polymeric subunits or small membrane fragments. A portion of the antigenic activity was estimated to be slightly smaller than 200,000 molecular weight. Hilgert et al. (1969), found that complete removal of Triton X-100 from an antigen preparation by acetone-ether extraction resulted in loss of 2/3 of the H-2 activity. The latter authors stated that the antigenic activity could be separated from Triton X-114 by saturating the aqueous phase with ammonium sulfate, however the antigen was no longer soluble. Kandutsch and Stimpfling (1965) described the H-2 antigens obtained by Triton X-100 solubilization as high in lipid content, in addition to protein and carbohydrate constituents. Lipid extracted from their preparations contained none of the antigenic activity.

Hilgert et al. (1969), used cholate, which was found to be an efficient solubilizer of histocompatibility antigens and easier to remove than Triton X-100. Most of the solubilized material was retained near the excluded volume of Sephadex G-200. Metzgar et al. (1968), using desoxycholate, obtained a 30 per cent yield of antigens from human and chimpanzee cells.

Manson and Palm (1968) solubilized H-2 antigens from mouse microsomal lipoprotein preparations with sodium dodecyl sulfate.
(SDS). Mann and Fahey (1971a) found that whereas 0.02 mg of SDS per mg of membrane protein successfully solubilized HL-A antigens, ratios higher than that destroyed antigenic activity. Even after ion exchange chromatography to remove the strongly charged detergent, some sulfur-35 labelled SDS remained with the alloantigen. Davies et al. (1971), and Hammerling et al. (1971), employed a combination of SDS and starch stearate to solubilize H-2 and HL-A antigens. Only 3 to 5 per cent of the total membrane antigenic activity was solubilized, mainly in the form of components larger than 200,000 molecular weight. Small amounts of antigen were detected with molecular weights estimated at 60,000 and 120,000.

The organic solvent n-butanol has been used to extract histocompatibility antigens. Antigens so derived were 28 per cent lipid with molecular weights approximating 6 million (Manson and Palm, 1968).

The subject of sonication for the production of transplantation antigens has been extensively reviewed by Kahan and Reisfeld (1971). These were the first truly water-soluble materials having transplantation antigenic activity. Active antigens were solubilized with sonic energy (less than 16,000 cycles per second) but not with ultrasonic energy (greater than 16,000 cycles per second). Kahan and Reisfeld (1971) employed 9,000 to 10,000 cycles per second (at 15.5 W/cm²) for most of their work. When 90 per cent
of the cells were disrupted the nuclear membrane of the cells remained intact. In general, sonically derived antigens have been determined to be comprised of protein, with no detectable carbohydrate or lipid. In the case of solubilized guinea pig antigens a molecular weight of 15,000 was discovered (Kahan and Reisfeld, 1967, 1969), while HL-A antigens had a molecular weight of 34,600 (Kahan et al., 1968).

Autolytic liberation of histocompatibility antigens was first utilized by Nathenson and Davies (1966). The antigens derived were polydisperse, with molecular weights of over 200,000 and were not amenable to purification by polyacrylamide gel electrophoresis (Davies, 1967). Yields were limited to 1 to 2 per cent of total cellular antigen (Shimada and Nathenson, 1969).

Edidin (1967) solubilized H-2 antigens by employing trypsin in the presence of ethylene diamine tetraacetate (EDTA). He found that the material was included in Sephadex G-10 making this the smallest molecular component which has been reported to show alloantigenic activity. The chemical nature of the antigenic material was not determined.

Papain has been used to solubilize antigens, usually from crude cell membranes prepared from lymphoid cell sources (Mann et al., 1968, Nathenson and Davies, 1966, Shimada and Nathenson,
Based on cytotoxic inhibition data, between 50 per cent and 80 per cent of H-2 and HL-A antigens detected on whole cells was obtained in the crude membrane. Shimada and Nathenson (1969) described maximal release of H-2 antigen from their preparation after 60 to 90 minutes of incubation with crystalline papain at 37°C, with progressive destruction of the antigen thereafter. Mann et al. (1969), found crude papain to be more efficient than crystalline for HL-A antigens isolated from cultured lymphoid cells. After 1 hour of incubation at 37°C from 20 per cent to 30 per cent of the activity present in the crude membrane preparation was solubilized. It has been possible to separate two classes of both H-2 and HL-A antigens on the basis of molecular size. One (Class I) had molecular weight of 57,000 and the other (Class II) of about 35,000. These classes were seen to carry different antigenic determinants (Cullen and Nathenson, 1971). The two classes of antigenic specificities of the HL-A (Nathenson, et al., 1970) and H-2 systems were separated by Sephadex chromatography (Cullen and Nathenson, 1971), ion exchange chromatography (Davies, 1969), and polyacrylamide gel electrophoresis (Mann and Nathenson, 1969, Einstein et al., 1971). Mann and Nathenson (1969) combined H-2 and HL-A antigens and found that only slight differences in electrophoretic mobilities in polyacrylamide gels could be detected.
between antigens derived from members of the two species.

The chemical nature of papain-solubilized and purified HL-A antigens has been found to be primarily protein with about 6 per cent to 8 per cent carbohydrate (Mann et al., 1969). Similar findings were obtained for purified H-2 antigens (Maramatsu and Nathenson, 1970, Shimada et al., 1970, Shimada and Nathenson, 1969). Though most researchers feel that differences in alloantigenic specificity reside in the polypeptide and not carbohydrate moieties of the antigens (Mann and Fahey, 1971a), Sanderson has been a proponent of the importance of the latter (Sanderson et al., 1971). He found that periodate could destroy HL-A2 activity. In addition, pronase digestion produced a small component (included in Sephadex G-50) which could inhibit lymphocytotoxicity of immune serum and eluted with 90 per cent of the carbohydrate of the original antigen while about 90 per cent of the amino acids were either free or part of small inactive peptides. Final proof as to whether antigenic activity resides on the residual polypeptide fragment or the carbohydrate moiety is lacking.

Salts have been employed in the extraction of alloantigens. Mann and Fahey (1971b) have used TIS (Tris salt of 2-hydroxy-3,5-di-iodobenzoic acid) to release up to 12 per cent of the total HL-A antigenic activity detected on the lymphocyte surface.
Polyacrylamide gel electrophoresis of the solubilized material revealed two bands. These glycoproteins were found to have molecular weights of 60,000 and 90,000 (Mann and Fahey, 1971a, b). The major disadvantage of this salt was the difficulty to remove it from the solubilized proteins. Whereas activity was released with 0.1 M salt, destruction of all antigenic activity occurred with 0.2 M TIS.

Hypertonic salt solutions such as 3 M KCl have been thought to act by decreasing the orderly arrangement of water molecules in the medium surrounding the cell membrane, making it possible for the hydrophobic components of the membrane-embedded proteins to become detached from their lipid surroundings and become dispersed in the aqueous environment (Reisfeld and Kahan, 1972). This theory has been disputed by Mann (1972). Using the enzyme inhibitors iodoacetamide and diisopropylfluorophosphate, he was able to inhibit 3 M KCl induced release of HL-A antigens from whole cells or membrane preparations. Thus, it is possible that hypertonic salt may cause the release of membrane-bound proteolytic enzymes which then induce antigen release. Like sonication 3 M KCl treatment causes depolymerization of membrane components, though in contrast to sonication, there is extensive destruction of the internal architecture of the cell leading to release of DNA. Less lipoprotein appears to be released by 3 M KCl than by sonic energy (Reisfeld and Kahan,
11

1971). According to Reisfeld and Kahan (1971), up to 60 per cent of the HL-A activity in the crude lymphoid cell extract solubilized by KCl could be recovered as a highly purified monodisperse component with a relative migration (Rm) of 0.78 to 0.80 in 7.5 per cent acrylamide gels run at pH 9.6 at 0°C. The molecular weight of the isolated antigen has been estimated at 32,000 (Reisfeld and Kahan, 1971). Though the majority of work has been performed with lymphoid cell lines, Etheredge et al. (1972), solubilized HL-A antigens from human peripheral lymphocytes, using 3 M KCl. Work with cell membranes has been less successful (Mann and Fahey, 1971b).

This study was initiated firstly to follow the humoral immune responses of baboons to cross-immunization with peripheral lymphocytes, and secondly, to produce ALS of high titer for use as a reagent to aid in the study of xenogeneic histocompatibility antigens.

By utilizing a high titered serum at dilutions near the limit of its activity in serological tests, only antibody specificities in highest concentration would be operative. It is probably that these antibodies would be specific for the most important antigens in the system. The serological technique most widely utilized for following the level and distribution of antigen activity in fractions during purification is inhibition of complement-dependent lymphocytotoxicity (ILC). In order to kill lymphocytes, antibodies must
react with cell surface antigens. Likewise, if an antigen inhibits this reaction it must be on the cell surface and may be involved in transplantation reactions.

The chimpanzee, being one of the sub-human primates most closely related to man (Goodman, 1970), would lend itself well to the role of future xenograft donor to humans. Since chimpanzees are members of an endangered species, however, unless great strides are made in raising them in large numbers in a laboratory environment, they could not serve as a supply of organs. The next best choice for use as a source of organs for human xenografting is the baboon because of its availability and size. Therefore, we have attempted to solubilize baboon xenogeneic histocompatibility antigens for study of their potential use in baboon to chimpanzee, and eventually, baboon to man xenografts.
MATERIALS AND METHODS

Animals, Immunizations, Collection of Serum
and Serological Tests

1. Animals.
   a. Baboon Mike (Papio cynocephalus) was a mature adult male.
   b. Baboon Monica (Papio cynocephalus) was a mature adult female.
   c. Baboon Kate (Papio papio) was a mature adult female.
   d. Baboon Antoinette (Papio papio) was a mature adult female.
   e. Baboon Desdamona (Papio papio) was a mature adult female.
   f. Chimpanzee Rocky (Pan satyrus) was a juvenile male estimated at 7.5 years of age.
   g. Chimpanzee Isabelle (Pan satyrus) was a juvenile female estimated at 7.5 years of age.

2. Preparations of cells for use in immunizations and in serological tests.
   a. Erythrocytes for use in absorption of serum (heated and unheated), and for use as target cells for the hemagglutination and complement fixation tests, were derived from the blood of sheep, chimpanzees, or baboons. Fresh blood was diluted in
Alsevers solution to 50 per cent, and stored at 4°C. Cells were discarded if not used within three weeks except sheep erythrocytes (collected aseptically), which were used for up to seven weeks. In all cases erythrocytes were washed with 0.15 M NaCl and centrifuged at 615 x g for 10 minutes, a total of three times prior to use. When primate red cells were to be employed for absorption of rabbit complement or heated serum, which were used in the lymphocytotoxic antibody assay, they were washed as above. Special care was taken, however, not to remove the layer of leukocytes sedimenting on the surface of the erythrocyte pellet, as the removal of anti-leukocyte antibodies was desired to be as efficient as possible.

b. Viable peripheral lymphocytes were used throughout our studies as target cells in serological tests and as a source of antigen by salt extraction. A modification of the isopycnic centrifugation technique of Perper et al. (1968), was employed to separate lymphocytes from whole blood. Blood of the chimpanzees and baboons was taken by cephalic or femoral venipuncture, defibrinated by swirling with glass beads in an Erlenmeyer flask at 25°C, and the resulting fibrin clot was removed. A 10 ml volume of defibrinated blood was then mixed with 30 ml of Seligmann's Balanced Salt solution (SBSS), pH 6.7, modified from Noble et al. (1968), to contain 500 mg of disodium EDTA per liter.
The mixture was transferred to a siliconized 50 ml conical centrifuge tube and 10 ml of 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 383 x g (max.) for 25 minutes in a swinging bucket (GLC-1 Table Top Centrifuge, Ivan Sorvall, HL-4 rotor).

The cloudy layer just below the interface between the diluted serum and Ficoll-Hypaque was aspirated and centrifuged at 615 x g (max.) for 15 minutes. The supernatant fluid was collected and discarded, and the pellet of cells was loosened by vortex. Contaminating erythrocytes were removed by hypotonic lysis as follows: To each loosened button was added 3 ml of distilled water, followed by brief agitation with a vortex mixer. After 30 second (longer periods appeared to cause clumping of cells) of 1 ml of 4 times concentrated SBSS (at 25°C) was added to the mixture. Following vortexing, this mixture was centrifuged at 274 x g (max.) for 10 minutes. The pink supernatant fluid was discarded and the cells loosened, following which a suitable amount of baboon and chimpanzee erythrocyte absorbed normal rabbit serum (NRS) diluted 1/20 in Hank's Balanced Salt solution (HBSS, Hanks and Wallace, 1949) (NRS-HBSS) was added.

Total cell counts were obtained by use of either an hemocyto-
meter or a Model L Coulter Counter. Differential cell counts based on observation of at least 100 cells were performed on cover glass preparations stained with Wright's stain. From a total of 68 white cell counts performed on whole blood of chimpanzees over a period of 16 months, a mean of 32.5 per cent lymphocytes were found. Out of 54 lymphocyte preparations derived as above from the same chimpanzees, the mean differential cell count included 93 per cent lymphocytes (range of 65 per cent to 100 per cent), with 28 counts over 94 per cent and 49 counts over 84 per cent lymphocytes. Similarly, peripheral baboon blood (72 counts) had an average of 47.3 per cent lymphocytes, while the lymphocyte preparations contained a mean of 89 per cent lymphocytes (range of 57 per cent to 100 per cent) with 38 of 57 counts over 84 per cent lymphocytes.

When cell preparations were tested for protein content and/or used for immunization purposes, suspension of the cell buttons was performed in HBSS without NRS, following which the portion to be used for serological tests was further diluted immediately in NRS-HBSS. Following the hemolysis step, the cells to be extracted by 3 M KCl were diluted in 5 ml of 0.15 M NaCl and re-centrifuged at 274 x g (max.) for 10 minutes. The supernatant was then aspirated and the cells loosened, after which they were pipetted into the extracting medium.

3. Methods of immunization (routes and frequency). The animals
were sedated with phencyclidine HCl (Sernylan, Parke Davis), 1 mg per kg intramuscularly (IM), for injections of lymphocyte suspensions and for obtaining blood samples of more than 30 ml (chimpanzees were always sedated regardless of the blood volume required). Two animals of a given species were treated at bi-weekly intervals with lymphocyte preparations derived from a member of the other species (and frozen at -20°C) on the previous week. The suspension was thawed and inoculated alone or mixed with an equal volume of either Freund's incomplete (FIA) or complete adjuvant (FCA, Difco). When inoculations were made intraperitoneally (IP), 1,000 international units (I.U.) of potassium penicillin G and 1.0 mg of streptomycin sulfate were mixed with the cells prior to injection. Times of immunization are indicated by a "V" over the weeks after the beginning of the studies as given in Figs. 4 through 9. Dosages given are presented in Table 1.

a. Baboons immunized with chimpanzee lymphocytes without adjuvant. Baboon Mike received lymphocytes derived from chimpanzee Rocky. For the first 14 weeks, intradermal (ID) inoculations were made in 0.2 ml quantities into 10 sites over the back. The last series of inoculations made during the second 14 week period, was given IP. Baboon Monica received lymphocytes derived from chimpanzee Isabelle, using the same method and schedule of injections as described for baboon Mike.
### TABLE 1

**IMMUNIZATION OF CHIMPANZEEs AND BABOONS**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Weight (kg)b</th>
<th>Mean Bi-weekly Dosage of Lymphocytes x 10^6</th>
<th>Total Number of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chimpanzee Isabelle 27-40</td>
<td>27-40</td>
<td>34.0</td>
<td>45.3</td>
</tr>
<tr>
<td>Chimpanzee Rocky 27-40</td>
<td>27-40</td>
<td>19.2</td>
<td>37.4</td>
</tr>
<tr>
<td>Baboon Mike</td>
<td>23</td>
<td>23.3</td>
<td>.</td>
</tr>
<tr>
<td>Baboon Monica</td>
<td>16</td>
<td>21.5</td>
<td>.</td>
</tr>
<tr>
<td>Baboon Kate</td>
<td>16</td>
<td>.</td>
<td>22.7</td>
</tr>
<tr>
<td>Baboon Antoinette</td>
<td>14</td>
<td>.</td>
<td>20.4</td>
</tr>
</tbody>
</table>

^a Not including dosages given the chimpanzees at 40 weeks and thereafter.

^b Weight ranges indicate growth during the study.

^c Freund's incomplete adjuvant.

^d Without adjuvant.

^e With adjuvant.
b. Baboons immunized with chimpanzee lymphocytes with FIA.

Baboon Kate received all inocula (2.0 ml) admixed with an equal amount of FIA. For the first 14 weeks lymphocytes were derived only from chimpanzee Isabelle, whereas lymphocytes from Rocky and Isabelle were mixed for inoculation thereafter. In all cases the inoculum was divided in 0.2 ml amounts among approximately 10 ID sites over the back and 2 injections of 1 ml each given IM. Baboon Antoinette was immunized identically to Kate, however chimpanzee Rocky's lymphocytes alone were employed for inocula used over the first 14 weeks of injections. Serum samples continued to be studied through 9 weeks after the last immunization of baboon Antoinette to determine the persistence of antibody titers.

c. Immunization of chimpanzees Rocky and Isabelle.

Identical doses, routes and methods of immunization were used for each chimpanzee throughout the study, however different sources of baboon lymphocytes were used for the initial immunizations. Through the first 20 weeks of the study the two animals were inoculated without adjuvant. The ID route was employed through 14 weeks and the IP route from the 16th through the 20th week. During this period, and through the 30th week as well, Mike was the sole source of lymphocytes for Rocky, as was Monica for Isabelle. Beginning on the 22nd week FIA was mixed with the preparations, and the inoculum divided between IM and ID routes. From the 32nd through the 38th
weeks, pooled lymphocyte preparations derived from baboons Kate and Antoinette were employed.

On the 40th, 44th and 48th weeks pooled lymphocytes derived from several baboons in the Ohio State University Animal Colony were mixed with FIA. On those dates each chimpanzee received an inoculum containing $176 \times 10^6$, $420 \times 10^6$, and finally $1,240 \times 10^6$ cells (8.5, 21.7 and 39.54 mg protein, respectively). No immunizations were given from the 50th through the 58th weeks. On the 59th week a similarly derived pooled lymphocyte preparation was mixed with FCA and $690 \times 10^6$ cells (26.2 mg of protein) inoculated ID into each chimpanzee. The responses were followed by assay of biweekly bleedings. No further inoculations were given through the 66th week.

Aliquots of an aseptically prepared baboon spleen homogenate were injected into both animals on the 67th week. The previously frozen spleen weighed 10.82 grams after removing the capsule and surrounding fat. After the addition of 10 ml of HBSS it was homogenized in a 50 ml Sorvall Omnimixer bucket kept on ice. Homogenization for 1 minute at a power stat setting of 6 was repeated 3 times, with 1 minute intervals allowed in between for the purpose of cooling. HBSS, containing 10,000 I.U. potassium penicillin G and 10 mg of streptomycin sulfate, was used to bring the final dilution of the homogenate to 28 ml, after which it was strained through sterile gauze. Each chimpanzee received 14 ml of homo-
genate containing 700 mg of protein (17.5 mg/kg), divided between the IP (10 ml) and subcutaneous (4 ml) routes. The chimpanzees were bled weekly through the 71st (Isabelle) or 72nd (Rocky) weeks when the experiments were terminated.

4. Collection of serum. Chimpanzees and baboons were bled for serum as indicated by the points on the graphs in Figs. 4 through 9. The blood clotted at room temperature, and the clot was allowed to retract overnight at 4°C. Erythrocytes were removed by centrifugation, the serum was heated at 56°C for 30 minutes, and then frozen at -20°C. The final sera were obtained by adding whole blood to ACD (acid-citrate-dextrose) solution B anticoagulant (Travenol) at a ratio of 48 ml of blood to 12 ml of ACD. The blood was centrifuged at 15,900 x g for 20 minutes at 4°C, and the resulting plasma treated with 10 per cent CaCl₂ in 0.15M NaCl at a ratio of 2.5 ml of the latter solution added to each 48 ml of blood processed. During incubation at 37°C a fibrin clot formed. This was removed, and the serum so produced heated at 56°C for 30 minutes and frozen at -20°C.

5. Serological techniques. For following the results of immunizations of the chimpanzees and baboons, hemagglutination,
leukoagglutination and lymphocytotoxicity tests were performed to titrate antibody levels in the animals' sera. In most cases tests were performed on a single serum specimen with cells derived from two members of the opposite species and the results averaged. Duplicates generally agreed to within a two-fold dilution of serum, so that one could not differentiate cell donors by any of the serological techniques.

To follow the level and site of localization of antigenic activity in spleen and lymphocyte fractions, inhibition of lymphocytotoxicity, complement fixation and immunodiffusion tests were utilized.

a. Leukoagglutination (LA). The defibrinated leukocyte agglutination technique employed for evaluation of chimpanzee and baboon sera was used as described by Zmijewski et al. (1968).a

b. Hemagglutination (HA). For the direct HA test serum was heated at 56°C for 30 minutes and then diluted in a serial two-fold manner in 0.1 ml quantities. When the test was to be run simultaneously against red cells derived from two or more different animals, master dilutions were prepared, and 0.1 ml aliquots of the dilutions

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a Performed in the laboratory of Dr. R.L. St. Pierre.
transferred to the required number of 12 x 75 mm test tubes. Washed erythrocytes were diluted to 2 per cent in 0.15 M NaCl just prior to testing. To each tube containing 0.1 ml of a serum dilution was added 0.1 ml of the erythrocyte suspension derived from a member of the opposite species. The mixtures were shaken until homogeneous, and then incubated at ambient temperature for 30 minutes at which time they were centrifuged at 615 x g for 3 minutes. The tubes were individually flicked to observe for agglutination. Readings were performed as follows:

Positive: 4+ Solid button which resuspended as such.

3+ Some fragmentation but no pinkness in the supernatant.

2+ Large clumps with diffuse reddish coloring of the supernatant.

1+ Small clumps with diffuse reddish coloring of the supernatant.

Negative: + Very few small particles.
- No agglutination.

The last tube in the titration giving at least a 1+ was the endpoint and the reciprocal of that dilution was the titer recorded. Controls consisted of known positive and known negative sera.

c. Lymphocytotoxicity (LC). The LC activity of both baboon-anti-chimpanzee, and chimpanzee-anti-baboon sera, was
tested by a modification of the micro-method of Terasaki (Terasaki and McClelland, 1964; Mittal et al., 1968). The serum to be used was heated at 56°C for 30 minutes and serially diluted in 0.15 M NaCl. Using microliter syringes (Hamilton), 1 ul of each dilution was added in duplicate to wells of micro-test trays (Falcon Plastics), under drops of heavy mineral oil to prevent evaporation. One ul of a suspension of lymphocytes containing 4,000 cells was then added to each well, and the mixtures incubated at room temperature for 1 hour, after which 4 ul of undiluted chimpanzee-and/or baboon-erythrocyte-absorbed rabbit complement was added and the incubation repeated. At this time 5 ul of 5 per cent aqueous eosin Y dye was added, followed after several minutes by 4 ul of 36 per cent neutral formalin. A coverslip was placed on the tray and the percentage viability was then read, as measured by dye exclusion, on an inverted phase microscope. The reciprocal of the highest dilution of serum causing at least 20 per cent cell death was considered the endpoint (titer). Controls included undiluted pooled normal and/or pre-immune chimpanzee (NCS) or baboon (NBS) serum, an undiluted serum known to cause more than 95 per cent death of the tested cells, a complement control in which 0.15 M NaCl was substituted for serum and a cell control in which the cells were suspended in 0.15 M NaCl alone.

d. Inhibition of lymphocytotoxicity (ILC). To test the
ILC activity of baboon antigen preparations or whole lymphocytes, only the final serum (71 weeks) derived from chimpanzee Isabelle was employed. For the duration of the text this serum will be known as "immune chimpanzee serum." Baboon Desdamona was the sole source of target lymphocytes for these tests, which were performed exactly as the LC tests with the following modifications: When whole cells were tested for their absorptive capacity, a volume containing a known number of lymphocytes was added to an equal volume of suitably diluted serum and incubated with periodic swirling at room temperature for 30 minutes. The suspension was then centrifuged at 615 x g (max.) for 10 minutes, the pellet discarded, and 2 ul of supernatant was added to the micro-test tray. One ul of target lymphocytes was then added and the test continued from this point as was the LC test.

In contrast to this, when tissue antigens were tested for their capacity to inhibit cytotoxicity, 1 ul of suitably diluted serum was added to the well, following which 1 ul of serially diluted antigen was added. The mixtures were incubated at 25°C for 30 minutes and lymphocytes were added and the test completed. In both tests inhibitory activity was determined by comparing the test results to a titration of immune chimpanzee serum similarly incubated but with 0.15 M NaCl substituted for antigen.
The dilutions of immune chimpanzee serum used in the ILC test were selected to cause 70 to 80 per cent cell death (usually 1/512 and defined for our purposes as 1 cytotoxic unit), and the highest dilution causing 90 per cent cell death (usually 1/256 and called 2 cytotoxic units). Occasionally one half the latter dilution was also tested (4 cytotoxic units). The dilution of antigen causing 50 per cent inhibition of cytotoxicity (LCID₅₀) was estimated by the Reif modification of the van Krogh equation (Reif, 1966), as described by Kahan and Reisfeld (1971). In a similar fashion the number of intact cells required to absorb 50 per cent of the cytotoxic activity of a dilution was estimated (AD₅₀). The total number of LCID₅₀'s/ml was obtained by multiplying the reciprocal of the dilution at the LCID₅₀ by 1,000, since the 1 ul of antigen is used in estimation of the latter figure. See Appendix I for methods of estimation of the AD₅₀, LCID₅₀ and the "absorptive yield."

e. Quantitative micro-complement fixation (CF). The technique of Levine (1967) was employed to test the CF activity of soluble antigens. For the tests 1/25 to 1/200 dilutions of the 71 week serum of chimpanzee Isabelle (immune chimpanzee serum) were always employed. The serum was prepared by heating at 56°C for 30 minutes, followed by absorbing twice, at 4°C for 10 minutes each time, with an equal amount of packed three-times washed sheep
erythrocytes. If a particular batch of serum proved anti-complementary it was processed as described in Appendix II. Sheep blood was stored at 4°C for at least one week prior to use in the test. Lyophilized guinea pig serum was the source of complement (Microbiological Associates) at the highest arithmetic dilution (1/125-1/225) giving 90 per cent hemolysis. Rabbit anti-sheep hemolysin (Sylvana Co.) was diluted 1/1000 for use. The degree of hemolysis was determined spectrophotometrically at 413 nm after removal of residual erythrocytes by centrifugation, and recorded as per cent CF. This was estimated by subtracting the absorbancy of the test mixture, from the mean of the absorbancies of the 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 CF (C'50) was estimated by a logarithmic plot using the van Krogh equation as described for the ILC test (See Appendix I). In this case the y-axis was the log of the dilution of antigen, and the x-axis the log [\(|\%CF/(100-\%CF)|\)], also noted as \(\log \left(\frac{100-Y}{Y}\right)\). Y is a measure of the residual complement in the test mixture. The number of C'50's per ml was given by the reciprocal of the antigen dilution at the C'50 level.

f. Immunodiffusion. Double diffusion was carried out in 0.5 per cent agarose gel (electrophoresis grade, General Biochemicals), most often in a solution of 0.15 M NaCl containing 0.02 per
cent sodium azide. Wells were cut either 6 mm in diameter, and 3 mm apart or 7 mm in diameter and 3 mm apart. Following overnight refrigeration, the wells were aspirated dry and antigen and serum preparations added. The plates were sealed with parafilm if necessary, and incubated at 4°C for at least 2 weeks with periodic examination. Triton X-100 in a concentration of 0.1 percent in suitable buffer was added to the gel, for studies of 3 M KCl Extracts 4 and 6.

Studies on Baboon Spleen Antigens

Baboon Kate was sedated with phencyclidine HCl as described above, and after 30 minutes was anesthetized by intravenous injection of pentothal sodium to effect. She was exsanguinated by means of a common carotid artery canula. The spleen was then aseptically excised, placed on ice and perfused with lactate ringers solution (Travenol) at 4°C until the venous effluent was clear. Each 1 liter bottle of the latter solution contained 0.01 percent procaine HCl (Novacain, Winthrop) and 1,000 USP units of sodium heparin (Liquemin Sodium "10", Organon).

1. Homogenization and initial differential centrifugation of baboon spleen homogenate. The spleen was stripped of its surrounding fat and capsule and aseptically cut into approximately 1 gram pieces. For each gram of tissue, 1.0 ml of Medium A (0.25 M sucrose, 0.025 M KCl, 0.005 M Mg Cl₂, and 0.05 N Tris-HCl, pH 7.6) was added.
The tissue was then homogenized with a Sorvall Omnimixer at a power stat setting of 6 for two one minute cycles (separated by one minute intervals) with the canister immersed in an ice bath. After homogenization, Medium A was added to a final tissue concentration of 20 per cent (w/v). The mixture was then homogenized an additional one minute. The homogenate was centrifuged at 15,900 x g (max.) for 30 minutes using a Sorvall RC-2B centrifuge with an SS-34 rotor (see Fig. 1). The pellet (Fraction I) was collected and frozen at -70°C. The supernate was then centrifuged at 140,000 x g (max.) for 75 minutes, at 4°C, in an IEC B-60 ultracentrifuge using an A-211 rotor. The resulting supernatant (Fraction II) was decanted and frozen at -70°C. The pellet was quantitatively transferred to a glass tissue grinder in which it was resuspended to 24 ml in Medium B (0.0005 M KH$_2$PO$_4$, 0.0005 M K$_2$HPO$_4$, 0.0005 M MgCl$_2$). The latter suspension was again subjected to ultracentrifugation as above, and the supernatant fluid (Fraction IV), decanted and frozen at -70°C. The pellet (Fraction III), was resuspended in an all-glass grinder with Medium B, and frozen at -70°C.

2. Preparation of crude spleen membranes (CrSM).
   a. Sephadex G-200 chromatography of Fraction II. Sephadex G-200 column A (vide infra) excluded pool concentrates derived
Fig. 1. — Preparation of baboon spleen fractions.
from 6 chromatographic separations of 3.14-fold concentrated Fraction II, were combined, dialyzed against 200 volumes of 0.14 M NaCl at 4°C for 10 hours and frozen at -20°C.

b. Sephadex G-200 chromatography of Fraction IV.

Fraction IV was thawed and concentrated 5-fold to 4 ml by nitrogen positive pressure ultrafiltration (Eastman H-35 membrane) and was then run through G-200 column A (vide infra). Fractions of the excluded peak (56.5 ml) were pooled, concentrated to 5 ml, then dialyzed against 0.14 M NaCl as above and frozen.

c. Extraction of Fraction I (15,900 x g pellet).

Serial progressively hypotonic extractions of this fraction was carried out on ice by a modification of the technique of Männ et al. (1960). The pellet (13 ml) was thawed and added to 45 ml of 0.14 M NaCl. This suspension was subjected to 10 plunges of an all-glass grinder following which it was centrifuged at 15,900 x g (max.) for 30 minutes at 4°C and the opalescent supernatant fluid collected and stored at 4°C. This procedure was repeated two times on the resulting pellets. Two further extractions of Fraction I were then performed with 0.07 M NaCl, and, finally, three extractions were performed with 0.01 M NaCl. A total of 300 ml of extract derived above was pooled, brought to a concentration of 0.14 M NaCl, and concentrated to 26 ml.
The concentrated extract was passed through Sephadex G-200 column A in 5 ml volumes. Fractions of the excluded peaks of all column runs of this extract were pooled, concentrated to 24 ml, dialyzed against 0.14 M NaCl and then frozen at -20°C.

d. Fraction III was extracted in a manner similar to that of Fraction I, after which 48 ml of extract was concentrated to 9 ml. This was passed through Sephadex G-200 column B as above, and the fractions of the excluded peaks pooled and concentrated to 8.3 ml. Following dialysis against 0.14 M NaCl it was frozen at -20°C.

e. The Sephadex G-200 excluded pooled concentrates of Fraction I-IV were combined and further concentrated to 15 ml by positive pressure ultrafiltration, then divided in 1 ml aliquots (now known as CrSM), and frozen at -20°C.

3. Papain digestion of baboon crude spleen membranes (CrSM).

A modification of the technique of crude papain digestion described by Mann et al. (1968, 1969), was employed. For this purpose solutions 1 and 2 were prepared as follows: Solution 1 consisted of crude papain (Sigma Papain Type II, 2.4 units/mg., Sigma Chemical Co.), 749 units per 100 ml, 0.02 M Tris (Sigma 7-9, Sigma Chemical Co.), 0.14 M NaCl, and 0.029 M L-cysteine (Mann Labs.), adjusted to pH 8.6. Solution 2 was 0.5 M 2-Iodoacetamide (J.T. Baker) adjusted to pH 7.6. Both solutions were prepared fresh prior to use and held in an ice bath; solution 2 was kept in the dark.
Several 1 ml aliquots of CrSM were thawed and placed in a 37°C water bath. To each was added 1 ml of Solution 1. At 15, 30, 60 and 120 minutes, 0.2 ml of Solution 2 was added to a reaction mixture which was then mixed and held on ice. For the zero (0) time digest, Solutions 1 and 2 were added to 1 ml of CrSM simultaneously. Controls consisted of the following mixtures:

a. **Saline control.** 1 ml of 0.14 M NaCl was added to 1 ml of Solution 1, to which Solution 2 was added without incubation.

b. **Undigested control.** An aliquot of CrSM was thawed and kept at 0°C.

c. **Cysteine controls.** 1 ml of Solution 1 without papain was added to 1 ml of CrSM and at 0 and at 30 minutes of incubation at 37°C, distilled water instead of Solution 2 was added. After mixing, the control solutions were kept at 0°C.

d. **Cysteine plus iodoacetamide controls.** Incubation times of 0 and 30 minutes were used as in c., with the addition of solution 2 instead of distilled water at those times.

e. **Temperature controls.** An aliquot of CrSM was incubated with an equal volume of 0.14 M NaCl for 0 or 30 minutes at 37°C after which water instead of solution 2 was added.

Immediately following digestion the 0, 15, 30, 60 and 120 minute test digests were centrifuged using an IEC B-60 ultra-
Centrifuge in a SB-405 rotor at 140,000 x g (max.) for 60 minutes at 4°C. The supernatants were collected and stored on ice, and the pellets resuspended to 1 ml (one half the original volume) using a 7 ml capacity all-glass tissue grinder kept on ice for homogenization. For tests of CF activity the undigested control (b., above) was centrifuged similarly.

**Extraction of Baboon Lymphocytes by 3 M KCl**

Peripheral lymphocytes from 200 ml of blood derived from baboon Desdamona were extracted by the method of Reisfeld et al. (1971). To each 10⁹ lymphocytes prepared as described above was added 20 ml of 3 M KCl containing Sorensen's phosphate (Diem, 1962) buffered saline, pH 7.4. The mixture was shaken overnight (16 to 18 hours) at 4°C using a mechanical reciprocal shaker at 60 cycles per minute. This resulted in a gel-like opalescent material which was centrifuged at 163,000 x g (avg.) for 1 hour at 4°C. The resultant supernatant fluid was dialysed against 3 changes of 200 volumes of 0.15 M NaCl for 18 to 24 hours. The pellet was resuspended in 0.15 M NaCl by use of a glass tissue grinder and kept on ice until tested.

The dialysed 163,000 x g supernatant was then centrifuged at 1,500 x g (avg.) for 20 minutes at 4°C. This sediment was resuspended as was the 163,000 x g pellet. The supernatant of 1,500 x g centrifugation was termed the "KCl extract." Triton X-100
detergent (Rohm and Haas) was added to the KC1 extracts to 0.1 per cent in studies of KC1 Extracts 4, 5 and 6.

**Sephadex G-200 Column Chromatography**

Sephadex G-200 (Pharmacia Fine Chemicals) was separated into several fractions by sieving, and the 40 to 63 micron fraction was used for all the work to be described. The gel was prepared and the columns poured and run by the methods described by Fischer (1970). Following swelling in the eluant buffer in a boiling water bath for 5 hours, the gel was cooled at 4°C and poured into columns.

Column A was 2.6 x 100 cm, and was run by upward flow at 11.4 ml per hour with the aid of 2 flow adaptors and a peristaltic pump. It was equilibrated and eluted with a buffer containing 0.2 M Tris, 0.5 M glycine, and 0.5 per cent mannitol, pH 8.0 (Kahan and Reisfeld, 1967). The column was partially characterized as follows: The total volume (Vt) of the column including the volume of the column occupied by gel (91.5 x 2.6 cm) and the connecting tubing was 490 ml. A void volume (Vo) of 200 ml was obtained from the elution volume (Ve) at the inflection point of the 19S peak of a 2.5 ml sample of normal human serum applied to the column (Fig. 2). The volume of the gel was calculated by the formula $V_x = V_t - V_o (= 290$ ml). Average partition coefficients (Kav) of the separated components were estimated by the formula
Fig. 2. Partial characterization of Sephadex G-200 column A. Normal human serum (2.5 ml) was applied, and the effluent was collected in 3 ml fractions. Peaks of absorbance ($A_{280}$) in order of elution are 19S, 7S and 4.5S (Fischer, 1970).
SEPHADEX G-200
COLUMN A
HUMAN SERUM
\[ Kav = \frac{Ve - Vo}{Vx} \]
The Kav of the 19S, 7S and 4.5S peaks of the normal serum run were 0.00, 0.17 and 0.36, respectively, as read by the absorbance at 280 nm (Fig. 2).

Column B was a 1.5 x 83 cm gel column prepared similarly to Column A but run without flow adaptors, and with downward, instead of upward flow, at 5.8 ml per hour. The Vt of column B was 149.7 ml and the Vx, 91 ml. The equilibrating and eluting buffer for studies of CrSM supernatants after digestion with papain was Tris, 0.01 M; NaCl, 0.14 M; pH 8.6 (Mann et al., 1969). For later studies, 0.1 per cent Triton X-100 in continuous system electrophoresis buffer, pH 7.8 (see the Polyacrylamide Gel Electrophoresis of 3 M KCl Extracts section) was exchanged for the former eluant.

Column B was partially characterized with separate runs of 0.5 ml of normal human serum and 1.0 ml of a crude papain solution containing 7.5 mg of the enzyme (using the Tris, NaCl, pH 8.6 buffer). Fig. 3 shows the Ve (Vo) of the 19S peak (Kav of 0.00) to be 58.7 ml with the Ve and Kav 70.7 ml and 0.13 for the 7S peak, and 85 ml and 0.29 for the 4.5S peak. Crude papain had a single peak of absorption at 280 nm with the Ve and Kav being 119 ml and 0.66, respectively.

**Polyacrylamide Gel Electrophoresis of 3 M KCl Extracts**

1. **Discontinuous buffer system.** In most instances a
Partial characterization of Sephadex G-200 column B. Normal human serum (0.5 ml) and crude papain (7.5 mg in 1.0 ml) were applied separately and eluted in 2 ml fractions. Peaks of absorbance ($A_{280}$) in order of elution are 19S, 7S and 4.5S of serum (Fischer, 1970) and papain.
SEPHADEX G-200
COLUMN B

HUMAN SERUM

PAPAIN

A$_{280 \text{ nm}}$

ml ELUTED
modification of the discontinuous alkaline polyacrylamide gel electrophoresis method of Davis (1964) was employed.

Only when running 3 M KCl Extract 1 was a sample gel used (2.5 per cent acrylamide, pH 6.7). In addition, this was the only time 5 x 40 mm, 7.0 per cent separation gels (pH 8.9) were employed. For 3 M KCl Extracts 2 and 3, the sample was incorporated in 10 per cent sucrose (total volume 0.1 to 0.2 ml) and layered onto the surface of the stacking gel (2.5 per cent, pH 6.7) prior to the run; thereafter (KCl Extracts 4, 5 and 6) the stacking gel was discarded as well, and the sample was layered directly onto the running gel. For separations of KCl Extracts 2 through 6 the gels employed were "semi-preparative," 6 x 100 mm in size. When using this system in electrophoresis of KCl Extracts 4, 5 and 6, 0.1 per cent Triton X-100 was present in the sample, gels and all buffers.

Electrophoresis was performed in a Buchler analytical gel electrophoresis apparatus with a Beckman Duostat power supply. For the first 3 KCl extracts this was performed at a constant current of 3 ma per gel with tap water running through the cooling jacket. At a constant current of 1 ma per gel for 30 minutes, followed by 2 ma per gel, thereafter, electrophoresis of the last 3 extracts was performed in a 4°C walk-in refrigerator with the apparatus lower reservoir immersed in an ice bath, and the cooling
jacket filled with ice water. Following electrophoresis the temperature of the lower reservoir was 2°C and the upper reservoir was 7°C.

Cutting of the 5 x 40 mm gels was performed by hand at 2 mm intervals and individual fractions were pulverized with a glass rod in 12 x 75 mm tubes, using the CF diluent of Levine (1967) for elution. After at least 2 hours at 4°C with periodic shaking, the fractions were tested for immunological activity. In one case the 2 mm fractions were placed directly into the wells of immunodiffusion plates for testing. For all but KCl Extract 1, 6 x 100 mm gels were automatically cut and pulverized in 2 mm fractions by a Gilson Gel Fractionator (Gilson Medical Electronics), eluting the fractions with 0.3 ml of CF diluent.

Protein containing components were stained by soaking the gels in 1 per cent amido schwarz stain in 7 per cent acetic acid for at least 4 hours. Removal of excess stain was accomplished by serial changes of 7 per cent acetic acid, and the gels were then stored in tubes for photography.

For determining the relative migration (Rm) of active components, one drop of 1 per cent bromphenol blue was mixed with the sample on the gels to be stained as above. The Rm of active components was estimated by dividing the midpoints of their positions on the gel by the midpoint of migration of the bromphenol
blue tracking dye. Fixed gels swelled in length by 10 per cent. Each stained and fixed gel was measured individually before and after staining.

2. Continuous buffer system, pH 7.8. For initial experiments with a pooled extract, and with KCl Extract 4, a continuous buffer system was utilized which was developed by Doctor George Milo of the Department of Veterinary Pathobiology. The gels prepared had an acrylamide concentration of 7.85 per cent. The Milo buffer system consisted of NaH$_2$PO$_4$ (0.03 M), Na$_2$HPO$_4$ (0.045 M), Tris (0.028 M), Na$_2$EDTA (0.001 M), pH 7.8. Gels were polymerized, following which pre-electrophoresis for 1 hour at 5 ma per gel was performed, using Milo's buffer with 0.1 per cent Triton X-100. At Dr. Milo's suggestion, gels were pre-electrophoresed as above, and then allowed to soak in the buffer, containing detergent, for at least 1 week, which was felt to allow the detergent to saturate the gel. Within 10 minutes prior to electrophoresis Triton X-100 was added to KCl Extract 4, previously concentrated by dialysis against 50 per cent sucrose in electrophoresis buffer. Though Milo had suggested electrophoresing at room temperature, the ice bath technique described for the Davis system in the previous section was utilized. Separations were carried out at 2 ma per gel until the bromphenol blue tracking dye had entered the gel at which time the constant
current was increased to 4 ma per gel. Electrophoresis of the Sephadex G-200 included pool of Extract 4 was performed with gels treated the same way but 0.1 per cent Triton X-100 was incorporated into the gel during polymerization.

To test whether the level of Triton X-100 in the running gels could reach 0.1 per cent merely by pre-electrophoresis and then soaking of the gels in buffer containing the detergent, assessment of the concentration of detergent was carried out by evaluating the ultraviolet light absorbance of the material eluted from gel fractions. In aqueous solutions, Triton X-100 has an absorbance peak at 275.5 nm (Kelly and Greenwald, 1958). Serial gel fractions were prepared by the Gilson Gel Fractionator as described in the previous section, with elution of the 2 mm gel fractions performed with 0.3 ml of CF buffer. Gels polymerized without detergent but soaked in it (in gel tubes) for at least two weeks, were tested for absorption, as were gels prepared with 0.1 per cent detergent in the monomer solution prior to polymerization. The first fraction of the gel prepared without detergent was estimated to contain approximately 0.03 per cent, and subsequent fractions about 0.15 per cent, using a nomogram supplied by Rohm and Haas. In contrast, similarly prepared fractions derived from the gels polymerized with 0.1 per cent Triton X-100 gave the predicted absorbance. With an average molecular weight
of 628 it is probable that some detergent would enter the gels, as attested to by the absorbance observed above. Since polymerization of the detergent occurs (personal communication, Rohm and Haas) into molecules of up to 5,000 moles (with molecular weights in the range of 31,000), it is doubtful that merely soaking the gels could allow the large molecular weight constituents to enter very far, at least in the time allotted.

Concentration of Antigen Preparations

Concentration was accomplished by one of two methods. Preparations were often dialyzed against 100 volumes of 50 per cent sucrose containing the buffer with which it was desired that the solution be equilibrated. This was performed with magnetic stirring at 4°C, after which excess sucrose was removed by dialysis against 100 volumes of buffer solution with 10 per cent sucrose. At times, positive pressure (nitrogen) ultrafiltration was performed at 4°C with magnetic stirring, using a Diaflo chamber (Amicon) at 50 to 70 lbs. per in.² applied to an Eastman H 35 membrane.

Electron Microscopy of the Pellets of 3 M KCl Extractions

Electron microscopy was carried out in the laboratory of Dr. Abramo Ottolenghi of the Department of Medical Microbiology. The technique of Bowman et al. (1971), was followed. Pellets
were fixed in 2 per cent glutaraldehyde in 0.25M sucrose and
0.05 M HEPES buffer (Calbiochem), pH 7.4 (EM buffer), overnight
at 4°C. The supernatant fluid was removed and the pellet re-
suspended in 1 per cent OsO₄ in EM buffer for 1 hour in an ice
bath. This fluid was then decanted and the sediment washed
twice in EM buffer. The resulting sediment was then dehydrated
in 50, 70, and 95 per cent ethanol successively for 15 minutes
each, followed by 2 dehydrations with 100 per cent ethanol (10
minutes each) and then 2 treatments with propylene oxide (10
minutes each). The next treatment was with a 1:1 mixture of pro-
pylene oxide and Spurr's Complete Embedding Medium (Polysciences)
for 1 hour, followed by another bath in 100 per cent Spurr's
medium. Samples were then placed in BEEM capsules containing
Spurr's medium and incubated in an oven at 70°C overnight. The
samples were cut by a Porter-Blum MT-2 Ultramicrotome (Ivan
Sorvall). Sections were stained in 2 per cent aqueous uranyl acetate
and Reynold's lead citrate, and then examined with an Hitachi
HU-12 electron microscope.

Chemical Analyses of Antigen Preparations

1. Protein. The protein concentration in samples was
estimated by a modification of the method of Lowry et al. (1951),
as described by Chase and Williams (1968). Sample readings were
compared with a standard curve established with dilutions of
bovine plasma albumin (BPA). Dry BPA (Armour) was dissolved in 0.02 N HCl and its concentration determined spectrophotometrically by the method of Kaziro et al. (1961), with the formula \( A = 0.640 \text{ liter g}^{-1} \text{ cm}^{-1} \) at 278 nm. The preparation was thus estimated to contain 16 per cent water, as reflected in the standard curve. Duplicate dilutions in 0.15 M NaCl of the sample to be tested were made using microliter syringes (Hamilton).

2. **Hexose.** Acrylamide gel fraction pools were tested for the presence of hexose by a modification of the anthrone method of Umbreit et al. (1959). Preliminary dialysis of the pools was performed with 2 changes of 200 volumes of 0.15 M NaCl over a 24 hour period at 4°C. The samples tested were brought to 0.75 ml with 0.15 M NaCl following which 1.5 ml of 0.2 per cent anthrone (Eastman) in 95 per cent \( \text{H}_2\text{SO}_4 \) was added to the mixtures. These were vigorously mixed by vortex, and placed in a 95 to 98°C water bath for 3 minutes. After cooling the mixtures were read for absorbance at 620 nm, using 0.15 M saline as a blank sample. The concentration of hexose was estimated by comparison with a standard curve prepared by the same technique using various concentrations of glucose dissolved in 0.15 M NaCl. Duplicates of each dilution of antigen were assayed.
3. Desoxyribonucleic Acid (DNA). DNA determinations were performed on acrylamide gel pools by Bonting and Jones' (1958) micro-modification of the Ceriotti (1954) indole method. To each duplicate 1 ml sample containing 1 N NaOH was added an equal quantity of a freshly prepared solution containing 0.02 per cent indole and 5 N HCl. Mixing was performed by vortex, following which the tubes were placed in a boiling water bath for 10 minutes. The samples were extracted twice by shaking for 4 minutes with 10 ml of chloroform, after which the water layer was centrifuged to remove residual quantities of the extracting medium. The samples were then read for absorbance at 490 nm, and DNA levels were estimated by comparison with a standard curve produced by performing the above test with arithmetic dilutions of Desoxy Ribonucleic Acid Di Sodium (Highly Polymerized, Nutritional Biochemicals).
EXPERIMENTAL RESULTS

The following outline of the contents of this section of the text is supplied for the convenience of the reader:

Cross Immunization of Baboons and Chimpanzees with Peripheral Lymphocyte Preparations

1. Baboons immunized with chimpanzee lymphocytes without adjuvant.
2. Baboons immunized with chimpanzee lymphocytes mixed with Freund's incomplete adjuvant (FIA).
3. Chimpanzees immunized with baboon lymphocytes and spleen.

Studies on Baboon Spleen Antigens

1. Immunological activity of fractions of baboon spleen obtained by differential centrifugation.
2. Chromatographic separation of "soluble" spleen antigens.
3. Effect of absorption of immune chimpanzee serum on the precipitating activity of baboon spleen Fraction II concentrate.
4. Papain digestion of baboon crude spleen "membranes" (CrSM).

Extraction of Baboon Lymphocytes with 3 M KCl

1. Lymphocytes extracted.
2. Gross and electron microscopic appearance of fractions formed during KCl extraction.
3. Chemical studies of the three fractions of KCl extraction.
4. **Immunological activity of 3 M KCl extracts.**

5. **The effect of freezing, heating and concentration, on the immunological activity of KCl extracts.**

6. **Studies on the solubility of 3 M KCl extracts.**

7. **The effect of 6 M urea on the solubility of the antigens.**

8. **Treatment of 3 M KCl extracts with Triton X-100.**
Cross Immunization of Baboons and Chimpanzees with Peripheral Lymphocyte Preparations

1. Baboons immunized with chimpanzee lymphocytes without adjuvant. As shown in Fig. 4 Baboon Mike did not develop measurable levels of LC, or LA antibodies. HA antibodies were detected beginning on the 10th week, reached a maximum level of 16 on the 14th week and were negative by the 24th week. Mike was the only baboon of the four immunized which had no pre-existing HA antibody against chimpanzee erythrocytes.

   Baboon Monica (Fig. 5) had a pre-inoculation HA antibody titer of 16 which fluctuated at first following the initiation of the immunization program but by 16 weeks became stable at 64-128. LC antibody was first detected at 12 weeks reaching low but persistent levels of 4 to 8 by the 16th week. LA titers were not observed until the 24th week with a maximum level of 4 detected on the last (28th) week of the study.

2. Baboons immunized with chimpanzee lymphocytes mixed with Freund's incomplete adjuvant (FIA). The responses of the two baboons, Kate and Antoinette (Figs. 6, 7), were very similar to one another. Both had pre-existing HA antibody titers which rapidly increased to peak levels of 4096 and 1536 by 5 1/2 weeks. With continued biweekly immunizations, however, the HA levels gradually declined. LC responses were first observed 10 days after the first
Fig. 4. Antibody responses of baboon Mike to immunization with chimpanzee lymphocytes without adjuvant. Titers are recorded as the reciprocal of the last positive dilutions of sera. HA is hemagglutination, LC is lymphocytotoxicity and LA is leukoagglutination. "V" designates the time of each inoculation. All inoculations to the left of "ID" were given intradermally and all to the right of "IP" were given intraperitoneally.
Fig. 5. Antibody responses of baboon Monica to immunization with chimpanzee lymphocytes without adjuvant. See legend, Fig. 4, for further details.
Fig. 6. Antibody responses of baboon Kate to immunization with chimpanzee lymphocytes in Freund's incomplete adjuvant. All doses were given by both intradermal (ID) and intramuscular (IM) routes. Titers are recorded as the reciprocal of the last positive dilutions of sera. HA is hemagglutination, LC is lymphocytotoxicity and LA is leukoagglutination. "V" designates the time of each inoculation.
Fig. 7. Antibody responses of baboon Antoinette to immunization with lymphocytes in Freund's incomplete adjuvant. See legend, Fig. 6 for further details.
Immunization and maximal LC titers of 1024 to 2048 were reached by 7 1/2 to 9 1/2 weeks. Antoinette's immunizations were stopped 9 weeks prior to the end of the study (Fig. 7). The results in Fig. 7 show that HA and LC titers persisted throughout this period. LA antibodies first appeared at 10 days (Antoinette) and 24 days (Kate), reached maximum levels by 6 to 8 weeks, and remained very stable at titers of 8 to 16 thereafter.

3. Chimpanzees immunized with baboon lymphocytes and spleen. Neither animal had detectable pre-immunization levels of antibody by any of the three tests employed. The antibody responses of Rocky during the first 22 weeks of immunization (without adjuvant) are shown in Fig. 8. Rocky developed low levels of HA antibodies by the 4th week, peaking at 32 by the 10th week and dropping to 4 by the 20th week. LC antibodies were first detected by 8 weeks, attaining a maximal titer of 4 by 12 weeks. No LA antibodies could be detected in Rocky's sera during this period. Chimpanzee Isabelle's responses were similar to those of Rocky's for this period (Fig. 9), though an LA titer of 3 was detected when testing the serum of Isabelle at 22 weeks.

Two weeks after adding FIA to the inocula markedly increased levels of LC and HA antibody activities were obtained in the sera of both animals (Figs. 8, 9), and after 4 weeks (the 26th week of the study), LA antibody activity appeared in Rocky's serum.
Fig. 8. Antibody responses of chimpanzee Rocky to immunization with baboon lymphocytes and then a spleen inoculum. HA is hemagglutination, LC is lymphocytotoxicity, and LA is leukoagglutination. Titers are recorded as the reciprocals of the last positive dilutions of sera. ID denotes use of the intradermal route: IP, intraperitoneal; IM, intramuscular; SQ, subcutaneous. INC. ADJ. indicates incorporation of Freund's incomplete adjuvant in the inoculum. COMP. ADJ., incorporation of Freund's complete adjuvant. Dosages shown by arrows represent the total protein of lymphocyte preparations inoculated at those times. "V" designates the time of each inoculation.
Fig. 9. Antibody responses of chimpanzee Isabelle to immunization with baboon lymphocytes and then a spleen inoculum. See legend, Fig. 8, for further details.
Increased antigen dosages given at 40, 44 and 48 weeks represented approximately 4, 10 and 30 times the previous mean biweekly dosage of cells. Though neither chimpanzee responded to the 4-fold increase, Isabelle showed a transient 4-fold rise in LC titer to 1024 two weeks after both the 10-fold and 30-fold increase in antigen dose. Rocky's LC titer was elevated (8-fold) only at 2 weeks after the 30-fold increased dose given on week 48. Once immunizations were stopped LC and HA titers fell in both animals' sera. It appeared that Isabelle's HA titers began falling even before inoculations were discontinued.

At 59 weeks, FCA was admixed with the lymphocyte suspensions (14-fold increased dosage) and following inoculation HA and LC titers were seen to rise transiently in the sera of both animals. Isabelle's HA titer, which had fallen from its previous high of 256 to 2, increased to 16 two weeks after this injection, and then dropped to 4 by 8 weeks later. Her LC titer, which had dropped to 128, increased to 1024 by 4 weeks after she received the inoculum, falling, once again to 128 by 3 weeks later (Fig. 9). Rocky's responses were similar (Fig. 8).

Finally, inoculation of each of the chimpanzees with baboon spleen homogenate (see MATERIALS AND METHODS) resulted in an 8-fold increase (to 1024) in Isabelle's LC titer by 2 weeks, which reached 8192 by 4 weeks, (64-fold increase). Her HA level increased as well, but not as greatly (from 8 to 128 by 2 weeks
after the injection. Rocky's LC and HA titers rose to 512 (128-fold and 32-fold, respectively) within 1 week but decreased sharply thereafter, dropping to 32 and 16 respectively.

Studies on Baboon Spleen Antigens

1. Immunological activity of fractions of baboon spleen obtained by differential centrifugation. As presented in MATERIALS AND METHODS (Fig. 1) the baboon spleen was homogenized and separated by differential centrifugation into four fractions: I through IV. Over 90 per cent of the ILC activity (tested with immune chimpanzee serum) was present in Fraction I, which contained almost 54 per cent of the total protein (Table 2). Fraction II had 6.2 per cent of the activity and 37.3 per cent of the protein, while Fractions III and IV, which contained 8.8 per cent of the protein had 2.2 per cent of ILC activity. Wistar rat spleen fractions prepared identically to baboon Fractions I and II failed to exhibit any ILC activity in this system.

Serial 2-fold dilutions of each of the fractions were made from 1/1 (undiluted) through 1/128, and placed in immunodiffusion plates in 6 mm wells cut 3 mm apart. The dilutions were tested against both pooled normal chimpanzee serum (NCS) and immune chimpanzee serum. Similar plates were prepared in which the immune chimpanzee serum was tested against Wistar rat spleen Fractions I and II, pooled normal baboon serum (as antigen), and the final
### TABLE 2

**INHIBITION OF LYMPHOCYTOTOXICITY BY BABOON SPLEEN FRACTIONS**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg) in Fraction</th>
<th>Per Cent of Total Protein</th>
<th>(ug/ul) Protein at LCID&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total LCID&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt; x 10&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Per Cent LCID&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1,130</td>
<td>53.9</td>
<td>0.095</td>
<td>11,900</td>
<td>91.6</td>
</tr>
<tr>
<td>II</td>
<td>783</td>
<td>37.3</td>
<td>0.977</td>
<td>802</td>
<td>6.2</td>
</tr>
<tr>
<td>III</td>
<td>152</td>
<td>7.3</td>
<td>0.692</td>
<td>220</td>
<td>1.7</td>
</tr>
<tr>
<td>IV</td>
<td>32</td>
<td>1.5</td>
<td>0.501</td>
<td>64</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2,097</strong></td>
<td><strong>100.0</strong></td>
<td><strong>...</strong></td>
<td><strong>12,966</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup> Using 2 cytotoxic units of immune chimpanzee serum.
serum of baboon Kate (as antigen). Precipitation activity was noted only in the plates in which immune chimpanzee serum was tested against baboon Fractions I and II, in both cases showing a single line adjacent to the 1/1, 1/2 and 1/4 antigen dilution wells. A single line of identity was seen when the two fractions were tested together against immune chimpanzee serum. All precipitating activity of the Fraction I pellet was removed by washing with 0.15 M NaCl. Fraction II was concentrated from 94 to 30 ml (3.14-fold) by positive pressure ultrafiltration, with no loss of total ILC activity. The immunodiffusion pattern of Fraction II had changed with concentration: At this time at least three components were observed when the latter was tested against immune serum in the same system employed for testing the unconcentrated Fraction II (Fig. 10). No activity was noted in a control plate in which the same antigen was tested with NCS. Information as to the chromatographic separability of the immunologically active components of Fraction II was desired.

2. Chromatographic separation of "soluble" spleen antigens. The results to be described below are typical of those obtained in duplicate studies. Since concentrated Fraction II was cloudy, it was centrifuged at 48,900 x g (max.) at 4°C for 20 minutes. The resulting pellet was resuspended in 0.15 M NaCl and centrifuged again at 48,900 x g. The supernate was discarded and the washed
Fig. 10. Immunodiffusion activity of 3.14-fold concentrated spleen Fraction II. The central row of 3 wells contains final serum of chimpanzee Isabelle. Wells around the outside in a clockwise fashion from the upper left contain serial 2-fold dilutions of 3.14-fold concentrated baboon spleen Fraction II from 1/1 through 1/128. Photographed after fourteen days incubation at 4°C.
pellet resuspended to the pre-centrifugation volume of
Fraction II in 0.15 M NaCl. The original supernatant fluid
of Fraction II (3.1 ml) was run through G-200 column A (described in MATERIALS AND METHODS) and eluted in 3 ml volumes.
Fractions were studied for their absorbancy at 280, 260 and
406 nm (Fig. 11). Three peaks were obtained. The first peak eluted with the void volume (i.e. it was equal to or greater than 800,000 to 1 million molecular weight). The second peak had a Ve of 331 ml and a Kav of 0.46. Absorbancy at 280 nm was somewhat greater than at 260 nm (Fig. 11). The major absorbancy at 406 nm coincided with the second peak indicating that much of the protein in the peak was probably hemoglobin. The third peak appeared completely included in the gel (5,000 molecular weight or less), absorbing primarily at 260 nm, with a Ve of 480 ml, and Kav of 0.97. As shown in Fig. 12, the fractions were combined into eleven pools which were concentrated by nitrogen positive pressure ultrafiltration at 4°C (MATERIALS AND METHODS) to approximately 1 ml each. Table 3 summarizes the analysis of these pools for protein and ILC activity. For comparison, uncentrifuged concentrated Fraction II, the 48,900 x g supernatant of Fraction II, and the washed pellet of Fraction II were tested. The percentages of ILC activity found in the various pools of the column are given in Table 3 and
Fig. 11  Sephadex G-200 column A - Chromatographic separation of 3.1 ml of baboon spleen Fraction II.
TABLE 3
SEPHADEX G-200 COLUMN POOLS OF BABOON SPLEEN FRACTION II

<table>
<thead>
<tr>
<th>Pool</th>
<th>Total ug Protein</th>
<th>ug/ul Protein at LCID$_{50}^b$</th>
<th>Total LCID$_{50}^b$</th>
<th>Per Cent LCID$_{50}^b$ In Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11,300</td>
<td>0.741</td>
<td>15,300</td>
<td>32.3</td>
</tr>
<tr>
<td>2</td>
<td>3,610</td>
<td>0.199</td>
<td>18,100</td>
<td>38.3</td>
</tr>
<tr>
<td>3</td>
<td>3,140</td>
<td>0.479</td>
<td>6,570</td>
<td>13.9</td>
</tr>
<tr>
<td>4</td>
<td>4,320</td>
<td>.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5,520</td>
<td>6.31</td>
<td>873</td>
<td>1.8</td>
</tr>
<tr>
<td>6</td>
<td>6,800</td>
<td>3.98</td>
<td>1,710</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>4,080</td>
<td>5.76</td>
<td>707</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>2,520</td>
<td>5.13</td>
<td>492</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>2,530</td>
<td>2.04</td>
<td>1,240</td>
<td>2.6</td>
</tr>
<tr>
<td>10</td>
<td>825</td>
<td>0.708</td>
<td>1,160</td>
<td>2.5</td>
</tr>
<tr>
<td>11</td>
<td>322</td>
<td>0.182</td>
<td>1,170</td>
<td>2.5</td>
</tr>
<tr>
<td>Total</td>
<td>44,967</td>
<td>.</td>
<td>47,322</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Centrifugation of Fraction II (48,900 x g max.)

| Whole Fraction II | 70,750 | 0.562 | 126,000 |
| Whole Supernate   | 54,800 | 1.32  | 41,100  |
| Whole Pellet      | 2,680  | 0.049 | 55,000  |

$^a$ Derived data from 3.1 ml sample of a 3.14-fold concentrate of Fraction II.

$^b$ Using 2 cytotoxic units of serum.
illustrated in the bar graph in Fig. 12.

Centrifugation at 48,900 x g of concentrated Fraction II resulted in sedimentation of more ILC activity than the total detected in the pools of the column. While 18 per cent of the ILC activity of Fraction II could not be accounted for, 43.7 per cent was found in the 48,900 x g pellet. Of the remaining 38.3 per cent located in the pools of the G-200 column, 31.9 per cent was detected in the fractions of the excluded peak. Thus, only 6.4 per cent of the total ILC activity of Fraction II was smaller than the exclusion limit of Sephadex G-200 and this was spread throughout the collected fractions. Apparently the ILC activity in this supposedly soluble (140,000 x g max.) fraction of the baboon spleen resided, in reality, on large fragments which probably reaggregated on concentration.

The only strong precipitating antigen activity which could be detected in any of the 11 pools of the Sephadex G-200 column was discovered in pools 7, 8 and 9. A portion of Fig. 12 graphically represents the highest titered components of the pools. Each of pools 7, 8 and 9 were seen to contain two strong precipitating components. A single strong line of identity was observed when the three pools were tested against immune chimpanzee serum in wells of the same plate. The two strong lines seen in the plates run previously, apparently did not separate under the
Fig. 12. Sephadex G-200 column A – Chromatographic separation of baboon spleen Fraction II: Absorbance at 280 nm, and immunological activity of the fractions. The upper portion of the figure shows the points of division of the pools listed in Table 3. The three horizontal lines above the scan of the fractions for absorbancy (280 nm) indicate the relative positions of the serum peaks presented in Fig. 2. The location of precipitating and ILC antigen activities in the pools is found in the central portion of the figure and in the bargraph. The titer of precipitating (immunodiffusion) activity indicates the highest dilution of the pools at which one of the components was detectable.
conditions of the test. A diffuse precipitate was seen adjacent to the serum well which may have represented the fainter third component seen to be present in whole concentrated Fraction II (Fig. 10), but not obvious in tests performed with any of the G-200 pools (see above).

3. **Effect of absorption of immune chimpanzee serum on the precipitating activity of baboon spleen Fraction II concentrate.**

To answer the question as to whether the precipitating antigens in Fraction II were found on the surface of baboon cells, an effort was made to determine the level of precipitating antibody in the standard immune chimpanzee serum used in our study, and to attempt to inhibit that reaction by absorption of the antiserum with either baboon lymphocytes or erythrocytes.

a. **Titration of immune chimpanzee serum.** A 1/8 dilution of whole 3.14-fold concentrated Fraction II was placed in the center well of an immunodiffusion plate, surrounded by 1/1 to 1/32 dilutions of immune serum. The 2 dense lines noted in previous studies with Fraction II were usually fused, remaining visible through a 1/8 dilution of serum. A third weaker component was present as well.

b. **Absorption of immune serum with peripheral blood lymphocytes.** Baboon Desdamona was bled and peripheral blood lymphocytes were prepared. Each serial 2-fold dilution of the cell suspension was then centrifuged at 615 x g for 10 minutes, the super-
nates discarded, and 0.4 ml of heat-inactivated, undiluted immune chimpanzee serum was added to each pellet. Mixture by reciprocal shaker (60 cycles per minute) for 1 hour at 25°C was followed by centrifugation at 615 x g for 10 minutes, and the supernatant serum was collected from each tube. These absorbed sera were serially diluted in a 2-fold manner from 1/1 through 1/4096. Unabsorbed serum was similarly titrated. By eosin Y dye exclusion it was noted that the lymphocytes were 99 per cent viable following their use in absorbing serum.

The absorbed sera were then tested by both LC and by immunodiffusion against whole spleen Fraction II as in section a. Absorption with 2.54 x 10^8 cells resulted in decreasing the titer of the undiluted serum in the LC test from 2048 to 32 (98.5 per cent). In immunodiffusion studies there was no significant difference noted in the titer or pattern of the lines of any of the unabsorbed or absorbed serum-containing plates.

c. Absorption of immune chimpanzee serum with baboon erythrocytes. Fresh thrice washed erythrocytes were prepared from the blood of baboon Desdamona, taking care at each wash to aspirate the upper layer of cells which appeared contaminated with leukocytes. To one ml of packed erythrocytes (approximately 10^{10} cells) was added 1 ml of heat inactivated immune chimpanzee serum. The suspension was shaken gently at 25°C for thirty minutes and
then centrifuged to remove the erythrocytes. This absorption was repeated, and the hemoglobin tinged supernatant aspirated and tested by HA and immunodiffusion tests. Before absorption the serum contained antibodies with a titer of 128 in the HA test, while following absorption no activity was noted. Absorption did not, however, appear to eliminate the presence of the major precipitating antibody components of the serum, though the level of intensity of the lines was slightly decreased.

d. Conclusion. Though not proven beyond a doubt, it was concluded that the antigens detected in precipitation experiments were not of cell surface origin.

4. Papain digestion of baboon crude spleen "membranes" (CrSM).

a. Effect of papain digestion on ILC activity. From table 4 it is apparent that crude papain had an inhibitory effect on the ILC activity of the preparation (see MATERIALS AND METHODS). By 15 minutes more than 98 per cent of ILC activity had been lost and it was completely undetectable by 30 minutes. About one third was lost at zero time when compared to the ILC activity detected in the undigested preparation. There was no evidence of solubilization of ILC activity. In control experiments it was found that treatment of the CrSM without papain but with cysteine alone, or with both cysteine and iodoacetamide, both at zero time and after 30 minutes of incubation at 37°C, failed to have any detrimental
TABLE 4

EFFECT OF PAPAIN DIGESTION OF BABOON SPLEEN MEMBRANES ON PROTEIN CONTENT AND INHIBITION OF LYMPHOCYTOTOXICITY

<table>
<thead>
<tr>
<th>Preparation Tested</th>
<th>Protein-mg/ml</th>
<th>LCID50/ml&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>Pellet&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Undigested</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>0 minutes</td>
<td>11.0</td>
<td>3.38</td>
</tr>
<tr>
<td>15 minutes</td>
<td>10.5</td>
<td>3.04</td>
</tr>
<tr>
<td>30 minutes</td>
<td>11.0</td>
<td>2.80</td>
</tr>
<tr>
<td>60 minutes</td>
<td>10.2</td>
<td>2.43</td>
</tr>
<tr>
<td>120 minutes</td>
<td>10.1</td>
<td>2.28</td>
</tr>
<tr>
<td>Saline control&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.38</td>
<td>. .</td>
</tr>
</tbody>
</table>

<sup>a</sup> Using 2 cytotoxic units of immune serum.

<sup>b</sup> Centrifugation at 140,000 x g for 60 minutes.

<sup>c</sup> All values in digest derived from protein determination on test sample less protein content of saline control (due to papain content).

<sup>d</sup> Not done.

<sup>e</sup> Contained all reactants (including papain) except antigen for which saline was substituted.
effect on ILC activity.

b. Effect of papain digestion on precipitating antigen activity. Strong precipitation lines were not observed in tests with CrSM. There was some indication of weak precipitating activity producing a broad diffuse line. Papain digestion had no significant effect on this activity which was present in both the 140,000 x g supernatant and pellet of CrSM.

c. Effect of papain digestion on CF activity. The ultracentrifugal supernatants of the undigested and 0 to 120 minutes papain digests of baboon spleen membranes were diluted from 1/40 through 1/640 and tested by CF assay. Antigens were similarly titrated for simultaneous use as antigen controls. The undigested CrSM supernatant was anticomplementary at the 1/40 (92 per cent) and 1/80 (61 per cent) dilutions. This activity decreased rapidly after the addition of papain. Thus, by 15 minutes only the 1/40 dilution showed any significant anticomplementary activity (22 per cent). All of the figures for CF activity to be discussed were calculated taking the degree of anticomplementarity of the antigen controls into consideration. Fig. 13 shows the results of papain digestion. As with ILC activity, it was apparent that much of the CF activity was destroyed at zero time. After 15 minutes, when essentially no ILC activity could be detected either in the supernatant or the pellet,
Fig. 13. Papain digestion of baboon crude spleen membranes (CrSM): The effect on CF activity of 140,000 x g (max.) supernates. Large circles in the graphs of undigested and of the 0 minute digest of CrSM indicate that anticomplementarity was so great at those dilutions that true CF activity analysis was not possible. Arrows indicate the postulated increase in CF activity, if anticomplementarity were nonexistent.
30-0 UNDIGESTED
0-0 0 MIN.
0-0 15 MIN.
0-0 30 MIN.
0-0 60 MIN.
0-0 120 MIN.

% CF

BABOON SPLEEN CRUDE
MEMBRANE PREPARATION
PAPAIN DIGESTION

ANTIGEN DILUTION
CF activity of the supernatant appeared to become stable, maintaining approximately 30 per cent CF at the 1/40 dilution of antigen through 120 minutes.

d. **Effect of papain on protein concentration.** The overall protein concentration of the CrSM appeared to decrease slightly and gradually over the period of digestion (Table 4).

e. **Chromatographic separation of the supernatants of CrSM before and after papain digestion.** The following three Sephadex G-200 chromatographic separations were made: The first was of the undigested supernatant; the second was of the zero time digest supernatant; and the third, a pool of the 15 through 120 minute digest supernatants. In succession, 0.5 ml of each the preparations was run through column B. Effluents were collected in 2 ml aliquots.

A single peak of absorbance at 280 nm was observed with the void volume of the undigested CrSM supernatant, while two peaks of absorbance at 280 nm were detected when each of the digests was tested. The second peak was found where papain had eluted previously, with a Kav of 0.66 (See Fig. 3.). Alternate fractions of each of the three columns were tested for CF activity, with an antigen control run simultaneously on each fraction tested.

All of the CF activity of the undigested preparation eluted with the void volume with a maximum CF level of 50 per cent in unconcentrated fractions. The zero time digest showed a decrease in maximum
activity to 18 per cent CF in the excluded peak. No activity of any significance remained in any of the fractions of the column run with the 15 to 120 minute digest pool. No CF activity was detected in any of the included fractions of any of the three chromatographic separations.

Extraction of Baboon Lymphocytes with 3 M KCl

1. Lymphocytes extracted. Six separate 3 M KCl extractions of baboon peripheral blood lymphocyte preparations, ranging from 5.78 x 10^8 to 9.79 x 10^8 cells, were performed. Total and differential counts of the cells extracted may be found in Table 5. Lymphocytes comprised 87 per cent or more of the cell populations, except for extraction experiment 4, where only 73 per cent lymphocytes were found.

2. Electron microscopic and gross appearance of fractions formed during KCl extraction. All 163,000 x g (avg.) pellets formed during extraction were white and firm. Electron microscopy of this pellet of extraction experiment 3 (Fig. 14) demonstrated some membranous constituents on a background containing much fibrillar material, possibly composed of DNA. Many partially disrupted and some apparently intact mitochondria and Golgi material were observed in the pellet.

In 4 of the 6 KCl extraction experiments, the 1,500 x g (avg.) sediments, derived after saline dialysis of the 163,000 x g super-
TABLE 5

EXTRACTION OF BABOON LYMPHOCYTE PREPARATIONS WITH 3 M KCl
CELL COUNTS AND PROTEIN DETERMINATIONS

<table>
<thead>
<tr>
<th>Extraction Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells Extracted</strong></td>
</tr>
<tr>
<td><strong>Total x 10^8</strong></td>
</tr>
<tr>
<td>5.78</td>
</tr>
<tr>
<td>99</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td><strong>Per cent mononuclear</strong></td>
</tr>
<tr>
<td><strong>Per cent polymorphs</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein Content in Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fraction</strong></td>
</tr>
<tr>
<td>163,000 g (avg.) pellet</td>
</tr>
<tr>
<td>1,500 g (avg.) sediment</td>
</tr>
<tr>
<td>KCl extract</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

*Not done*
Fig. 14. Electron micrograph of a 163,000 x g pellet formed during 3 M KCl extraction of baboon peripheral lymphocytes (magnified 30,000 times).
natants, had the gel-like consistency described by Reisfeld et al. (1971). Of the other 2 experiments, in extraction 1 a powdery precipitate rather than a gel formed during dialysis, leading to the formation of a discrete pellet after 1,500 x g centrifugation. Dialysis failed to result in any sediment in the case of extraction experiment 5. Electron microscopic examination of a 1,500 x g sediment (extraction experiment 4) showed only the presence of fibrillar material (Fig. 15), probably DNA.

The 1,500 x g supernate (to be termed the KCl extract) was clear and colorless. It was gelatinous in consistency in all cases except for Extract 1 which was less viscous.

3. Chemical studies of the three fractions of KCl extraction. Reisfeld et al. (1971), stated that the 1,500 x g sediment consisted mainly of DNA, leaving the supernatant free of DNA by both chemical and radioactive labeling experiments. When Extract 6 was electrophoresed DNA was found to be present in a pool of the first four fractions of the polyacrylamide gel (Table 10). During the production of KCl Extract 1, dialysis and subsequent removal of the 1,500 x g pellet resulted in a concomitant increase in the $A_{280}/A_{260}$ ratio from 0.474 to 1.455, indicating a significant loss of nucleic acids. In the case of KCl Extracts 2 and 3, however, the absorbance ratios were 0.68 and 0.76, respectively, probably indicating nucleic acid contamination.
Fig. 15. Electron micrograph of a 3 M KCl 1,500 x g (avg.) sediment of baboon peripheral lymphocytes after re-centrifugation at 163,000 x g to pellet (magnified 30,000 times).
As listed in Table 5, the KCl extracts contained from 20 per cent to 47 per cent (mean of 37.5) of the total protein in the three fractions.

4. Immunological activity of 3 M KCl extracts

a. Inhibition of lymphocytotoxicity (ILC). Table 6 summarizes the results of testing 6 KCl extracts for ILC activity. Using 1 cytotoxic unit of immune chimpanzee serum a mean total of 169,000 LCID$_{50}$'s was obtained in 5 KCl extracts, corresponding to an average absorptive yield (see Appendix I) of 51 per cent. Using 2 cytotoxic units of serum a mean total of 83,000 LCID$_{50}$'s was obtained in 5 KCl extracts corresponding to an average absorptive yield of 54 per cent. In relation to the average total activity of the 3 fractions of 5 extraction experiments, the KCl extracts contained a mean of 46 per cent of the activity when measured with 1 cytotoxic unit and 48 per cent when measured with 2 cytotoxic units. The data from extraction experiment 4 are not used in the above information.

b. Complement fixation (CF) by 3 M KCl extracts. CF tests with KCl extracts appeared to be more sensitive than ILC tests in that antigen at much higher dilution was detectable by the former. Table 7 lists the dilutions at which the C'50 was obtained, and the total number of C'50's in each of the last 4 KCl extracts. The C'50 dilutions of the fresh (not previously concentrated or frozen)
### TABLE 6

**INHIBITION OF LYMPHOCYTOTOXICITY BY FRESH 3 M KCl EXTRACTS OF BABOON LYMPHOCYTE PREPARATIONS**

<table>
<thead>
<tr>
<th>Activity in Extract</th>
<th>KCl Extract Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>$\text{LCID}_{50} \times 10^3$</td>
<td>77.2 ND</td>
</tr>
<tr>
<td>Per cent of total $\text{LCID}_{50}$</td>
<td>35.7 ND</td>
</tr>
<tr>
<td>$\text{LCID}_{50} \times 10^3/10^9$ cells extracted</td>
<td>135.5 ND</td>
</tr>
<tr>
<td>Absorptive yield (per cent)</td>
<td>30.4 ND</td>
</tr>
</tbody>
</table>

---

*ND* Not done

*As compared with sum of LCID$_{50}$'s in all 3 fractions.*

*Derived from formula AD$_{50}$/LCID$_{50}$ in cell equivalents (see Appendix I).*
### TABLE 7

**COMPLEMENT FIXATION BY FRESH 3 M KCl EXTRACTS OF BABOON LYMPHOCYTES**

<table>
<thead>
<tr>
<th>Extract Number</th>
<th>1 and C'50</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution of KCl extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/538</td>
<td>1/123</td>
<td>1/234</td>
<td>1/209</td>
<td></td>
</tr>
<tr>
<td>Total units in KCl extract</td>
<td>ND</td>
<td>7,530</td>
<td>2,500</td>
<td>3,140</td>
<td>3,340</td>
<td></td>
</tr>
<tr>
<td>Total units/10&lt;sup&gt;9&lt;/sup&gt; cells</td>
<td>ND</td>
<td>7,700</td>
<td>2,770</td>
<td>5,020</td>
<td>4,650</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> There is one C'50/ml at this dilution.

<sup>b</sup> Tests not done on preparation prior to freezing and/or concentration.
extracts ranged from 1/123 to 1/538 with total yields up to 7,530 C'50's. The number of C'50's obtained in the fresh KCl extract from \(10^9\) cells varied from 2,770 to 7,700 with mean of 4,540. KCl Extract 4 had the lowest total CF yield of all of the extracts, yet was previously seen to have the highest yield of ILC activity (see Table 6). Anticomplementary activity was not noted in any of the KCl extracts, even after they were concentrated. NCS showed a minimal level of CF reactivity with the KCl extracts, e.g., in one test, with immune chimpanzee serum the C'50 was 1/574, while with NCS at the highest concentration of antigen tested (1/40), only 9.5 per cent CF occurred.

c. Immunodiffusion. The three fractions of extraction experiment 1 were diluted and the various dilutions tested both against NCS and immune chimpanzee serum. The only activity detected after 3 weeks of incubation at 4°C, was in the form of 2 diffuse, weak lines when immune serum was reacted with the KCl extract. One line was visible through the 1/16 dilution of antigen and was closer to the serum well than the second line which was visible through a dilution of 1/8.

5. The effect of freezing, heating and concentration, on the immunological activity of KCl extracts.

a. Effect of freezing at -20°C. Two KCl extracts re-tested by CF test after storage at -20°C for 2 to 4 weeks, had
lost 70 per cent (KCl Extract 2), and 73 per cent (KCl Extract 3) of initial activity. The third KCl extract tested (KCl Extract 4), however, had 41 per cent higher activity. ILC activity was re-tested in only 2 cases after 4 weeks storage. Extract 4 had lost 74 per cent of its initial activity, while KCl Extract 5 lost none. Since the original estimates of ILC activity in KCl Extract 4 may have been erroneously high (see Table 6), the loss of ILC activity by this extract on freezing may be inaccurate. Therefore, the only conclusion drawn from the above data is that freezing at -20°C adversely affected CF titers of fresh KCl extracts.

b. Heating. Aliquots of KCl Extract 5 were tested simultaneously after being kept at 0°C for 30 minutes, or heated to 56°C for 30 minutes. There was no loss of ILC activity at 56°C as compared with the ILC activity of the aliquot kept at 0°C (CF activity was not tested).

c. Concentration by dialysis against 50 per cent sucrose. KCl Extracts 3 and 4 were concentrated 5- and 7.3-fold, respectively, by dialysis against 50 per cent sucrose (see MATERIALS AND METHODS). The concentrates exhibited no loss of total CF activity when compared with the unconcentrated extracts.

6. Studies on the solubility of 3M KCl extracts. Several attempts were made to fractionate and to test for soluble
antigenic material in the KCl extracts.

a. Polyacrylamide gel electrophoresis. KCl extract 1, kept at 4°C after concentrating 4-fold by positive pressure ultrafiltration, was subjected to polyacrylamide gel electrophoresis by the method of Davis (1964). The sample gel contained 164 ug of extract protein. Following electrophoresis, CF activity was detected only in the sample gel (43 per cent) and the first 2 mm fraction of the running gel (34 per cent). The only definite ILC activity was detected in the sample gel. A single precipitin line was detected in tests with the sample gel fractions. A weak single line was found, in addition, in tests of running gel fractions 2 and 3.

Using 6 x 100 mm gels without sample gels, 0.15 ml of KCl Extract 2, concentrated 5.44-fold, and containing 417 C'50's per ml, was electrophoresed and the gel fractions tested for CF activity. The only activity of eluted gel fractions was seen in the stacking gel (sample gels were no longer used after KCl Extract 1), where 97 per cent CF was detected. A pellet of amido schwarz positive material was observed on top of the stacking gel.

Unconcentrated extract 3 was electrophoresed just after preparation. Though at least 10 stained components resulted from electrophoresis of 0.2 ml of extract (538 C'50's ml) only the stacking
gel exhibited definite evidence of CF activity (the C'50 of the stacking gel was obtained when it was diluted to 1/52).

In no case, therefore, was CF activity detected in the included fractions of the running gel. When a sample gel was employed all of the activity was restricted to it, and the first fraction of the running gel. In three different attempts without the use of a sample gel the only activity detected was in the stacking gel. ILC activity, in one test, was found restricted to the sample gel. Therefore, the only definite evidence that any antigenic material had passed into the running gel (as detected by the serum employed) was the finding, with KCl Extract 1, of an extremely weak line of precipitation in the second and third fractions of a 5 x 40 mm gel. Stronger precipitating activity was associated with the sample gel of that electrophoresis experiment.

b. Sephadex G-200 chromatography. A 0.5 ml sample of previously frozen KCl Extract 1, containing 1.15 mg of protein, was run through Sephadex G-200 column B (MATERIALS AND METHODS). The only definite peak of CF activity involved 4 consecutive fractions eluting with the void volume, which had CF levels of 83 to 91 per cent.

c. Ultracentrifugation. Aliquots of fresh KCl Extracts 4 and 5 were centrifuged at 163,000 x g for 60 minutes. The
pellets were resuspended in 0.15 M NaCl to the original volume. There was no ILC or CF activity detectable in the resulting supernatant of KCl Extract 4 (Table 8). The pellet of KCl Extract 5 contained 98 per cent of the CF activity, and 95 per cent of the ILC activity (Table 8).

The results of these attempts led to the conclusion that the important components, which had previously been resistant to sedimentation at 163,000 x g in the presence of 3 M KCl, were no longer soluble once KCl was removed.

7. The effect of 6 M urea on the solubility of the antigens. After storage for 1 month at -20°C KCl Extract 3 was thawed, and dialyzed for 16 hours against the Davis (1964) reservoir buffer, pH 8.3, containing 6 M urea, at 4°C. The extract was then concentrated 5-fold by dialysis against the same urea solution, containing, in addition, 40 per cent sucrose. Stacking and running gels were prepared by substituting 6 M urea for water. Two gels, each run with 0.2 ml of the sample, were cut by the Gilson Gel Fractionator. CF tests of the fractions demonstrated that the stacking gels had identical CF titers of 1/210 (while the concentrated sample had a titer of 1/960). No CF activity was detected in any of the fractions of the running gel though two duplicate gels had as many as 20 lines when stained with amido schwarz reagent.
<table>
<thead>
<tr>
<th></th>
<th>Extract 4</th>
<th>Extract 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Triton X-100</td>
<td>No Triton X-100</td>
</tr>
<tr>
<td></td>
<td>LCID$_{50}$</td>
<td>LCID$_{50}$</td>
</tr>
<tr>
<td>Fraction</td>
<td>2 Units</td>
<td>1 Unit</td>
</tr>
<tr>
<td>Pellet</td>
<td>1,178</td>
<td>4,780</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ After centrifugation at 163,000 x g (avg.) for 60 minutes.

$^b$ Number of lymphocytotoxicity 50 per cent inhibitory doses in 1 ml employing indicated number of cytotoxic units of serum.

$^c$ Number of 50 per cent complement fixing doses in 1 ml.

$^d$ Pellet resuspended to volume of supernatant with a glass tissue grinder at 0°C.

$^e$ No detectable CF at a 1/5 dilution.
8. Treatment of 3 M KCl extracts with Triton X-100.

a. Effect of addition of 0.1 per cent Triton X-100 on immunological reactivity. KCl Extracts 5 and 6 were dialyzed against 50 per cent sucrose solutions containing Davis reservoir buffer, pH 8.3, and 0.1 per cent Triton X-100, for concentration (6.1- and 6.9-fold, respectively). Sufficient detergent was added to the extract prior to this procedure to yield a final concentration of 0.1 per cent. When these concentrates were tested simultaneously with aliquots of fresh extract, total CF activity of KCl Extract 5 was seen to have dropped by 26 per cent, and KCl Extract 6, by 60 per cent. In addition, total ILC levels were estimated to have fallen by 88 per cent and 65 per cent respectively. As discussed previously, similar concentration of KCl Extracts 3 and 4 in the absence of Triton X-100 resulted in no loss of CF activity. Detergent was added in the same concentration to fresh, unconcentrated KCl Extract 4 prior to freezing for 1 month at -20°C. No effect on ILC activity was demonstrated, however the CF level was 62 per cent lower than the same extract treated by freezing without detergent.

b. Effect of addition of Triton X-100 on the solubility of KCl extracts. Triton X-100 was added to an aliquot of KCl Extract 5 to 0.1 per cent, after which it was kept at 0°C for 8 hours. The mixture was then centrifuged at 163,000 x g for 1 hour. The pellet was resuspended with the aid of an all glass
tissue grinder in 0.15 M NaCl. The resuspended pellet and supernatant were tested for immunological activity. There was a mean loss of about 70 per cent of the ILC and 54 per cent of the CF activity from the sum of the supernatant and pellet fractions as compared with simultaneous tests performed without detergent (see Table 8). The supernate contained 27 per cent of the ILC activity and 11 per cent of the CF activity. This was an absolute increase of 175 per cent in ILC activity in the supernatant, and a 317 per cent increase in supernatant CF activity (Table 8).

c. Effect of Triton X-100 on the CF and ILC tests.

When Triton X-100 was in samples tested for CF activity, complete hemolysis of the indicator sheep red cell suspension occurred with 0.1 per cent detergent, either undiluted, or diluted 1/5, while 1/10 and 1/20 dilutions were somewhat (25 per cent and 12 per cent, respectively) anticomplementary. A test of the susceptibility of lymphocytes in the ILC test to lysis by the detergent indicated (by dye exclusion) that 100 per cent of cells were killed by an antigen solution containing undiluted detergent at 0.1 per cent concentration, while a 1/2 dilution had no adverse effect. Each 2 mm fraction of acrylamide gel produced by the Gilson Gel Fractionator was calculated to be 0.06 ml in volume. The machine added 0.3 ml of diluent, effecting a 1/6 dilution of detergent and gel. The latter dilution did not affect the ILC assay. For the CF tests, the eluted material was used diluted 1/2, yielding
a final gel (and detergent) dilution of 1/12. The slight anti-complementary activity of the resulting fractions was very constant, and did not interfere with assessment of CF.

d. Polyacrylamide gel electrophoresis and Sephadex chromatography.

1) Continuous buffer system, pH 7.8. First electrophoresis experiments with Triton X-100 followed the technique of Milo per se (see MATERIALS AND METHODS). For the purpose of attempting to achieve a final concentration of 0.1 per cent Triton X-100 in polyacrylamide gels, the gels had been pre-electrophoresed and then soaked for 2 weeks in buffer containing 0.1 per cent Triton X-100. The detergent was added to the samples, as well, to 0.1 per cent, 10 minutes prior to electrophoresis. Previously concentrated, pooled and frozen aliquots of KCl Extracts 2 and 3 were run by this method (0.1 ml per gel). CF activity in the resulting duplicate gels (running gel only) was limited to the first and second 2 mm fractions. In immunodiffusion experiments, hand-cut fractions of a third gel were tested using 0.5 per cent agarose containing detergent and pH 7.8 buffer. Precipitation was observable from the first through the fourteenth 2 mm fractions (Rm of 0.40). There were three distinct components: A single broad line was present, starting from the first fraction and continuing through the
seventh fraction. At this point the line split into two components, one of which was detectable through gel fraction ten, and the other through fraction eleven. The third component was first seen as a diffuse precipitate near the serum well at fraction 7, gradually becoming more discrete, and finally disappearing by fraction 14.

In another experiment three aliquots of 0.2 ml each of pooled KCl Extracts 2 and 3 were incubated with 0.1, 0.5 or 1.0 per cent Triton X-100 and run on gels prepared identically to those described in the last paragraph. Immunological activity of the gel fractions was not investigated; merely the effect of different concentrations of detergent in the sample on the appearance of stained gels was studied. The 0.1 per cent and 0.5 per cent detergent runs were identical in appearance, while the pattern (but not the number of components) in the 1.0 per cent detergent run was distorted (Fig. 16). Trailing of stained material from the top (cathode end) of the gels was observed. Fig. 16 also shows a comparison of NBS run on gels by the discontinuous method of Davis (with neither sample nor stacking gel) with detergent in the sample and gel, with the same serum run on the Milo gel as described above. It is obvious that a much better separation of components of serum was obtained when the Davis method was employed.
Fig. 16. Polyacrylamide gel electrophoresis using the continuous buffer system, pH 7.8: Pooled KCl Extracts 2 and 3, previously incubated with 0.1, 0.5 and 1.0 per cent Triton X-100 (first 3 gels from left to right). The second gel from the right is a simultaneously performed electrophoresis of 5 ul of normal baboon serum by the same system. The gel on the far right is the same as the latter but performed using the discontinuous buffer system, pH 9.4 (modified from Davis, 1964). Amido schwarz stain. The cathodal end is at the top.
Following 7.35-fold sucrose concentration of fresh KCl Extract 4, with equilibration with pH 7.8 electrophoresis buffer, the preparation was divided into 2 aliquots, to each of which was added detergent to 0.1 per cent. One aliquot was electrophoresed in polyacrylamide gels and the other chromatographed on Sephadex G-200. Eleven gels were electrophoresed after adding 0.1 ml of the sample (containing 292 ug of protein, 800 LCID$_{50}$'s and 560 LCID$_{50}$'s as detected by 1 and 2 cytotoxic units, respectively, and 240 C'50's) to each. Two gels were stained with amido schwarz, 1 was cut by hand and the fractions tested for immunodiffusion activity, and the remaining 8 gels were sectioned on the gel fractionator. In the latter case all of the same numbered fractions from each gel were pooled. CF activity was restricted to the first 3 fractions of the gels (100 per cent, 87 per cent, and 65 per cent CF, in that order), while no other fractions displayed any activity above 8 per cent CF. Testing for ILC revealed definite activity to be limited to the first fraction (50 per cent and 22 per cent as detected by 1 and 2 cytotoxic units, respectively). Immunodiffusion results were very similar to those obtained for the pooled KCl Extracts 2 and 3 (see above). The pattern of staining of the gels indicated a poor separation (Fig. 17).

Sephadex G-200 chromatography (column B) was performed with
Fig. 17. Polyacrylamide gel electrophoresis of fresh 3 M KCl Extract 4 containing 0.1 per cent Triton X-100. The tube on the far left contains a gel electrophoresed using the continuous buffer system, pH 7.8. Detergent was not in the gel during polymerization (see text) but had been added to the 0.1 ml sample prior to electrophoresis. Center tube: Electrophoresis of the active G-200 included peak of KCl Extract 4, with detergent in the gel at the time of polymerization; continuous buffer system, pH 7.8. Tube on right: Same as center tube but run with discontinuous buffer system modified from Davis (1964), with Triton X-100 in the sample, gel and buffer. Amido schwarz stain.
concentrated KCl Extract 4. The sample contained a total of 2.79 mg of protein, 7,350 LCID<sub>50</sub> and 5,150 LCID<sub>50</sub> (as detected by 1 and 2 cytotoxic units, respectively) and 2,221 C<sub>50</sub>'s.

Due to the content of Triton X-100 in the effluent, aliquots were diluted 1/2 for testing for ILC activity, and 1/10 for CF. Two peaks of activity were detected in aliquots of the effluent. The first peak appeared with the void volume (Fig. 18), showing 52 per cent CF, and 50 per cent, and 13 per cent ILC as measured by 1 and 2 cytotoxic units of serum, respectively. In the second peak, highest levels of ILC activity detected were 63 per cent and 19 per cent when measured by 1 and 2 cytotoxic units of serum, respectively. Three fractions in the peak exhibited low (7 per cent to 9 per cent) CF activity. The latter peak appeared at a Ve of 103 ml (Kav of 0.49), falling between the sites of elution of papain and human albumin (approximately 20,000 and 70,000 molecular weight, respectively, see Fig. 3). Protein determinations were performed on the fractions of the column (Fig. 18). The pattern was virtually identical to one previously observed (but not shown here), after chromatography of KCl Extract 1 on the same column (B) without the presence of detergent (see section 4. b., above).

The fractions of the second active peak from the G-200 column were pooled and concentrated by dialysis against 50 per cent
Fig. 18. Sephadex G-200 column B run with 0.92 ml of fresh 3 M KCl Extract 4. The column had been equilibrated with the continuous system electrophoresis buffer, pH 7.8, containing 0.1 per cent Triton X-100. CF data, as well as protein concentrations of the 3 ml fractions, are given. ILC activity was measured by both 1 and 2 cytotoxic units of immune chimpanzee serum.
SEPHADEX G-200
COLUMN B

3M KCl EXTRACT
0.1% TRITON X-100

% ACTIVITY

ml ELUTED

PROTEIN 4g/ml
sucrose to contain 260 µg of protein per ml (7.2-fold). A total of 4 gels were run (0.25 ml per gel), which had been polymerized with detergent in the monomer solution. No definite activity could be detected in any of the fractions by either the CF test or the ILC test. The stained gel showed what appeared to be a single major protein peak (Fig. 17) with some components migrating in front of it. Aliquots of the same pooled G-200 fractions were also electrophoresed by the method of Davis (1964) with detergent incorporated into the gels (only running gels were used). There was much better separation of the stained components, virtually covering the whole range of migration in the gels (Fig. 17).

2. Discontinuous buffer system of Davis (1964). KCl Extracts 5 and 6 were prepared as usual up to the time of concentration by dialysis against 50 per cent sucrose, at which point sufficient Triton X-100 was added to each extract to reach a final concentration of 0.1 per cent by the time dialysis and concentration was completed. Table 9 lists the number of gels of each extract, whose same numbered fractions obtained by the Gilson Gel Fractionator were pooled following electrophoresis. In addition, total protein, CF and ILC activity data are given that were obtained from tests of the extracts prior to electrophoresis. These data reflect the total amount of active material
<table>
<thead>
<tr>
<th>Number of Gels Pooled</th>
<th>Total Protein</th>
<th>Total LCID$_{50}$'s</th>
<th>Total C'50's</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract 5</td>
<td>2.52 mg</td>
<td>4,900</td>
<td>17,900</td>
</tr>
<tr>
<td>Extract 6</td>
<td>2.20 mg</td>
<td>5,240</td>
<td>25,200</td>
</tr>
</tbody>
</table>
placed on the gels. Electrophoresis of KCl Extract 5 was performed until the tracking dye front reached 79.5 mm while KCl Extract 6 was run to 95 mm. Two additional gels were electrophoresed for the purpose of staining.

Pooled, undiluted Gilson gel fractions (final concentration of gel was 1/6) were tested by ILC and 1/2 dilutions of these by CF test. All of the immunological results were calculated by comparison with similar tests performed with numbered 2 mm fraction pools from gels electrophoresed with sucrose and buffer serving as the sample. The results of immunological tests were similar for the two electrophoretically separated KCl extracts. There appeared to be 4 peaks of activity as shown for KCl Extract 6 in Fig. 19. The first peak at the top (cathode end) of the gel exhibited high levels of ILC and CF activity. Both ILC and CF activity were apparent in the second peak as well (Fig. 19). A third component, well separated from the second, only exhibited activity in the ILC test. A fourth small peak of ILC activity migrating faster than the latter could be detected as well.

Due to the method of running the ILC test there was a limited possibility that what had been interpreted as soluble surface-derived antigens binding specific antibodies, could, in reality, be complement fixation by soluble antigen-antibody
Fig. 19. Complement fixation and inhibition of lymphocytotoxicity (ILC) by polyacrylamide gel fractions of KCl Extract 6. ILC activity was measured by both 1 and 2 cytotoxic units of immune chimpanzee serum. Electrophoresis by a modification of the discontinuous system of Davis (1964) was performed with gels polymerized to contain 0.1 per cent Triton X-100. Detergent was also in the electrophoresed samples. Fractions of the 100 x 6 mm gels were cut at 2 mm intervals after the dye front had moved 95 mm.
POLYACRYLAMIDE GEL ELECTROPHORESIS

3M KCl EXTRACT 6

0.1% TRITON X-100

% ACTIVITY

2mm GEL FRACTION POOL
complexes (not necessarily of surface specificity). It is possible that complement fixation could occur, to the point of decreasing the ability of detecting surface-active antibodies still free in the reaction mixture. Therefore, a two-stage ILC test was devised and run as follows: To 0.05 ml of serial 2-fold dilutions of the 4 preparations to be tested (see below), was added 0.05 ml of immune chimpanzee serum containing 1 cytotoxic unit. After 30 minutes of incubation at 25°C, 0.05 ml of a baboon lymphocyte suspension containing 4,000 cells per ul was added. After mixing, incubation continued for 1 hour, at which time HBSS (20 volumes) was added to each mixture with thorough agitation. The contents were centrifuged lightly (274 x g) for 10 minutes to pellet the cells. The supernate was aspirated and the cells resuspended in 0.05 ml of NRS-HBSS. One ul of this suspension was added to duplicate microtest wells already containing 2 ul of HBSS, complement was added and the test completed as usual from this point (see MATERIALS AND METHODS).

The antigen preparations tested by the above protocol were whole KCl Extract 5, pooled peak 2 (fractions 6 through 9), pooled peak 3 (fractions 17 and 18) and pooled inactive fractions from between peaks 2 and 3 (fractions 11 through 15), of Extract 6 (see Fig. 19). The whole KCl extract showed a higher degree of
ILC in the two-stage test than it did when tested simultaneously in the standard plate test (the LCID^50 was obtained at a 1/22 dilution in the plate test and at a 1/37 dilution in the 2 stage test). Though the undiluted pooled fractions from between peaks 2 and 3 exhibited no ILC activity, peaks 2 and 3 inhibited cytotoxicity of 37 per cent and 47 per cent, respectively. Peak 4 was not tested. Therefore, it appeared that the fractions tested contained surface components.

The first peak of activity detected in KCl Extracts 5 and 6 electrophoresis runs had the highest levels of immunologic activity in gel fraction pool 2 (Fig. 19), having a mean Rm in the two runs of 0.035. Peak 2 had an Rm of 0.164 in KCl Extract 5 and an Rm of 0.150 in KCl Extract 6, with a mean Rm of 0.157. The Rm of peak 3 in KCl Extract 5 was more widely disparate from the Rm of the same peak in KCl Extract 6. Peak 3 of KCl Extract 5 had an Rm of 0.45 while that of KCl Extract 6 had an Rm of 0.37, yielding a mean Rm of 0.41. Peak 4 of KCl Extract 5 had an Rm of 0.63 and of KCl Extract 6, 0.64.

Gel fractions 4 through 10 of KCl Extract 5 (peak 2) were pooled, concentrated by dialysis against 50 per cent sucrose, and electrophoresed as was the original extract. Activity in the resulting pooled gel fractions was restricted to a single peak with an Rm of 0.14. Though no activity was detected at the
top of the gel, 25 per cent ILC (as detected by 1 cytotoxic unit), and 30 per cent CF activity were found in fractions of this peak.

The first peak of activity in KCl Extracts 5 and 6 was at or near the top of the gels and was related, at least in part, to the amido schwarz stained material remaining there following electrophoresis. The second peak of activity was located in a position centered between the two more densely stained lines near the top of the gel as shown in Fig. 20. Whereas the third peak of activity corresponded with the next lower (toward the anodal end) dense line having an $R_m$ of 0.37 in the gels of Extract 6 (Fig. 20), the third peak in gels of Extract 5 ($R_m$ of 0.46) did not coincide with the stained component. There was no stained line associated with peak 4.

Table 10 shows the results of protein (Lowry), hexose (anthrone), and DNA (indole) analyses of selected gel fraction pools of KCl Extracts 5 and 6. In all cases a blank gel pool was run simultaneously and used as the "zero" point for purposes of calculation. Peak 1 of KCl Extract 6 was highly contaminated with DNA, in addition to having a hexose level approximately 25 per cent of that of protein in the peak. DNA and hexose levels of peak 2 of KCl Extract 6 were both 5 per cent of the concentration of protein. Neither hexose nor DNA
Fig. 20. Polyacrylamide gel electrophoresis of 3 M KCl Extract 6. Electrophoresis was performed by a modification of the discontinuous system of Davis (1964) with gel and sample containing 0.1 per cent Triton X-100. The cathodal end is up. Amido schwarz stain. Arrows denote peaks of activity as enumerated in the text.
TABLE 10
CHEMICAL ANALYSIS OF ACTIVE POLYACRYLAMIDE GEL FRACTIONS

<table>
<thead>
<tr>
<th>Fraction Tested</th>
<th>Gel Fractions Pooled</th>
<th>ug/ml Protein</th>
<th>ug/ml Hexose</th>
<th>ug/ml DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1</td>
<td>1 - 4</td>
<td>42</td>
<td>11</td>
<td>21.6</td>
</tr>
<tr>
<td>Peak 2</td>
<td>6 - 9</td>
<td>40</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Peak 3</td>
<td>17 - 18</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peak 4</td>
<td>29 - 31</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Re-electrophoresis of Extract 5-Peak 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active Peak</td>
<td>4 - 7</td>
<td>11</td>
<td>Not done</td>
<td>0</td>
</tr>
</tbody>
</table>
-could be detected in peak 3 nor in the gel fractions pooled at the position of peak 4 of KCl Extract 6. The re-electrophoresed peak 2 fractions of KCl Extract 5 contained 11 ug per ml of protein with no detectable DNA.
DISCUSSION

This study allowed analysis of the antibody responses of pri-
mates following xenogenic stimulation without the interference
of transplanted tissue, immunosuppression, or a rejection episode.
The responses following intradermal injection of 2 baboons and 2
chimpanzees with low doses of lymphocyte preparations led to the
development of only low levels of hemagglutinating (HA) and lympho-
cytotoxic (LC) antibody even when stimulated biweekly for as long
as 20 weeks. No leukoagglutinating (LA) activity was observed.
The incorporation of adjuvant (FIA) into a similar dose of anti-
gen led to a significant rise in HA and LC antibody as well as a
moderate increase in LA titers. Further stimulation with larger
doses of antigen in adjuvant increased the response in chimpan-
zees only transiently.

The lower response obtained with antigen without adjuvant
seemed to be related to antigen dose when compared to studies
with other species. It has been shown that immunization with
heterologous lymphocytes in the range of $10^9$ cells per inoculum
without adjuvant produced high titered sera in rabbits (Levey
and Medawar, 1966b; Spreafico, 1970; Martin, 1969) and horses
(Betel et al., 1970, Iwahashi et al., 1970). These doses were
approximately 40 times the number of cells used in our study
(Table 1). Metzgar et al. (1965), however, made monthly intra-
venous injections of chimpanzees for as long as 8 months with mixed human leukocyte preparations containing $1 \times 10^9$ leukocytes, $3.5 \times 10^9$ platelets and $3 \times 10^9$ red blood cells and obtained LA titers of 4 to 32 which were similar to those of our animals. The results obtained by these workers are not directly comparable with our studies because their antisera were absorbed with erythrocytes prior to titration. Johanssen and Seiler (1972) described one chimpanzee immunized repeatedly with human peripheral blood lymphocytes without adjuvant first by the intravenous and then the subcutaneous route. Nine inoculations of $10^6$ to $10^8$ cells were given over a period of 30 weeks resulting in maximum LC titers of 2 to 8, which are very similar to the results described here. Although the difference between primates and rabbits in their response to heterologous stimulation could be attributed to the dose of antigen per kg body weight, this could not be the explanation for the difference in regard to heterostimulation of horses. Perhaps the lower response level in our system could have been due partly to the lower immunogenicity of cells for a closely related species.

The apparent lack of sufficient antigen and/or its low immunogenicity was overcome in our studies by mixing FIA with the injected preparations, resulting in very substantial LA titers in the case of the previously unimmunized baboons. That the chimpanzees did not respond as well might be explained by more rapid
clearance of antigen due to the presence of circulating antibodies resulting from previous injections.

Peripheral lymphocytes were used as a source of antigen because they are rich in transplantation antigens (Kahan and Reisfeld, 1971) and are relatively easy to isolate from the peripheral blood with 90 per cent or greater purity (Perper et al., 1968). By this method we were able to obtain a source of antigen from a single animal over a long period of time without injury, and therefore the animals were available for immunization as well.

It was necessary to sedate the animals with phencyclidine HCl prior to bleeding and immunization, and therefore we selected a biweekly injection schedule for fear that the animals might not do well on a long term study if they were sedated more frequently. It was convenient to bleed one pair of animals one week for antisera and at the same time to obtain lymphocytes that could be used the following week for immunization of members of the other species. By this schedule, however, it was necessary to store the lymphocytes frozen (-20°C) until used for immunization. Although the majority of investigators preparing ALS have employed living cells, it is not certain that frozen and thawed cells are less immunogenic.

The number of animals in our study was certainly too small to draw statistically valid conclusions regarding the sequence of appearance of different antibody activities, however these activities
were consistently observed in the order of HA, LC and LA. This is most easily seen in Rocky’s sera (Fig. 8), however similar results were obtained with the other animals immunized without adjuvant. In the case of baboon Mike, only an HA response was seen (Fig. 4). Although Monica had pre-existing HA antibodies, the sequence of appearance of the various antibody activities was the same (Fig. 5). Chimpanzee Isabelle’s responses were quite similar to those of Rocky’s though her LC and LA antibodies appeared simultaneously. Though this sequence was not clearly evident with the two baboons immunized with FIA incorporated into the inoculum, the LA antibodies were the last to appear in baboon Kate’s serum (Fig. 9). In contrast to our findings James et al. (1970), working with a horse antihuman lymphocyte serum model, found lymphoagglutinins and hemagglutinins appearing simultaneously whereas lymphocytotoxic antibodies were detected later.

It is very probable that some of the antigens detected in our heterosystem by the three serological tests were not identical. In most cases the HA antibody level was initially higher than that of the LC even if the two appeared simultaneously. It appears that as the immunization continued HA antibody titers tended to decrease gradually, while LC antibody levels became higher. For example, Isabelle’s HA titer began to
fall in the face of prolonged immunization (Fig. 9). This was not unexpected. Due to the method of preparation of the inoculum the amount of contaminating erythrocytic stroma was small, and therefore insufficient to allow persistence of the HA antibody level. Injection of an erythrocyte-rich spleen homogenate resulted in an increase in HA titer (Fig. 9). Our impression is in agreement with similar conclusions Metzgar and Seigler (1970) made for another heterospecific system (chimpanzees immunized with human cells). Careful absorption studies would, however, be required for confirmation.

Freund's complete adjuvant (FCA), when injected into the chimpanzees, resulted in abscess formation locally at all intradermal sites of inoculation over the back, as well as in posterior cervical, inguinal and axillary lymph nodes. Some discomfort on the part of the chimpanzees attended the formation of these abscesses, especially during the first three weeks following injection. Gross lymphadenopathy persisted through the termination of the experiments three months later. Chimpanzee Rocky's abscessed axillary and posterior cervical nodes opened and drained 10 weeks following inoculation of the FCA. Beginning 4 weeks after this injection Rocky also developed a persistent neutrophilic leukocytosis with total peripheral white cell counts elevating to between 20,000 and 57,000 per mm$^3$. Moor-Jankowski
(personal communication, 1971) has suggested that by dividing
the inoculum among several deep intramuscular sites, and de­
creasing the total FCA in half to 1.0 ml, such untoward
sequellae might not have occurred, and perhaps would have re­
sulted in higher levels of response.

Activity in the ILC test was detected in all 4 fractions
of differential centrifugation of baboon Kate's spleen.
Fraction I (15,900 x g pellet) was probably quite similar to
the combination of the nuclear and mitochondrial-granular
pellets of Rapaport et al. (1965), made up of nuclei, residual
whole cells, mitochondria, and large membrane fragments.
Fraction III (105,000 x g avg., pellet) was essentially identical
to the microsome-rich pellets of both Rapaport et al. (1965),
and Hirose and Kurosawa (1971), made up of ribosomes, endoplasmic
reticulum, and small plasma membrane fragments. Fraction II was
the 105,000 x g (avg.) supernatant of Fraction III, and Fraction
IV was the wash of the latter. The fact that nearly 92 per cent
of the ILC activity was present in Fraction I (Table 2) is not
surprising since more than half of the total protein was in that
fraction and it probably contained the highest concentration of
large membrane fragments. Fraction II contained 37 per cent of
the total protein of the extract but only about 6 per cent of the
ILC activity due possibly to the large amount of soluble protein of cytoplasmic and serum origin. Probably the most surprising aspect of the study of baboon Kate spleen fractions was the low amount of total reactivity in Fraction III, which in Hirose and Kurosawa's study (1971) was the most potent in the ILC test. The difference may be due to the different means of homogenization employed. They used a Teflon homogenizer while we employed a Sorvall Omnimixer. While we have noted that virtually all cells are disrupted by this technique, it is possible that this form of homogenization may produce larger membrane fragments which will sediment at lower speeds, thus increasing the levels of ILC activity of the low speed pellet while decreasing the expected ILC levels of the microsomal pellet.

We found that on further manipulation (3.14 fold concentration by ultrafiltration) Fraction II formed an insoluble precipitate which sedimented at 48,900 x g. This sediment contained 44 per cent of the ILC activity of the fraction, with another 38 per cent eluting with the void volume of Sephadex G-200. These results are consistent with the work of Rapaport et al. (1965), which showed that all of the activity of the "fibrillar" fraction (105,000 x g supernatant) sedimented at 198,000 x g. We conclude as did they that the active components were, in reality, small
membrane fragments and not truly soluble.

The precipitating antigens of Fraction II did not appear to be associated with ILC activity. The fact that absorption with whole lymphocytes failed to reduce the ability of serum to form strong precipitating lines with antigen extracts would indicate that these are not surface antigens and, therefore, not of potential importance in transplantation. It is possible, but unlikely, that they are surface antigens in such low concentrations that absorption with the numbers of cells employed was insufficient to affect a reduction of precipitating activity. In addition they could be surface antigens of no importance in the LC test (though possibly important in transplantation).

Papain digestion destroyed more than 98 per cent of the xenogeneic ILC activity of our crude spleen membrane preparation within 15 minutes at 37°C. Mann et al. (1969), used the same protocol with a crude membrane preparation produced by hypotonic lysis and repeated extraction of the cell pellets. He found human alloantigenic yields (by ILC) after 1 hour of incubation at 37°C to be in the order of 20 per cent to 30 per cent of the activity present in the membrane preparation. The great sensitivity of xenoantigen(s) to the effects of papain cannot be explained, though involvement of important proteins in their primary structure or in the structure of carriers of the antigens is inferred. It must be noted that the level of anticomplementarity of our crude membrane preparation fell at about the same rate as did reactivity in the ILC test. One might wonder,
then, whether the ILC activity detected, could have been due in part to the anticomplementarity of the antigen preparation. Though not mentioned in the text, we performed a two-stage ILC test, identical to that described for use with KCl extraction-derived antigens. That is, initial reaction of antigen and antibody in a tube was followed by addition of target cells. After incubation of the latter mixture, a 20-fold dilution by buffer was made, and low speed centrifugation (274 x g) for 10 minutes pelleted the cells. The supernatant was aspirated and discarded, the cells were added to a microtest tray, and the test completed in a routine manner. Therefore, but for a slight contamination by the 1/20 dilution of supernatant fluid, the cells were added without antigen, and sensitized by varying amounts of antibody. The level of ILC detected by the two-stage tube test was the same as in the standard plate test, performed simultaneously, inferring that neither complement fixation, nor anticomplementarity was the determining factor.

The possibility should be kept in mind that although ILC activity was apparently destroyed by papain, destruction of only one or a few of the antigens may have been sufficient to prevent the ones not destroyed from being detected by the ILC test. For example, if both antibodies A and B are in the serum at the dilution employed, and antigen A is destroyed but not antigen B, the residual antigen B, which has been
solubilized, can absorb out antibody B, but antibody A is still available to kill the target lymphocytes. This is, of course, the major disadvantage in working in an undefined system where no monospecific antisera are available.

CF activity fell and then became stable at 15 minutes at quite low levels though ILC activity had been destroyed. This can be explained by postulating that the antibodies involved could be detecting different antigens in part, (e.g. there was a weak precipitating system present which was unaffected by the digestion procedure). The initial drop in CF activity was probably related to the loss of the very antigens active in the ILC test.

It is quite possible that further experimentation with this technique, testing different reaction temperatures and concentrations of enzyme, might result in more positive results.

By several criteria discussed in the text 3 M KCl extraction performed by the method of Reisfeld et al. (1971), resulted in formation of a semisoluble material. The immunologically active components of the extracts could be sedimented at 163,000 x g (avg.) for 1 hour, would not pass into 7 per cent acrylamide gels and eluted with the void volume of Sephadex G-200. This is, of course, very different from the high yields of alloantigenic material with a molecular weight of 32,000 as obtained by Reisfeld. In our heteroantigen system the salt extraction of intact baboon
peripheral lymphocytes could have resulted in the formation of rather large fragments containing the important antigens, or, perhaps, the antigens may have been truly soluble, small molecules which reaggregated following removal of hypertonic salt. This would be similar to the findings of Smit et al. (1972), i.e., pyrophosphate extracted baboon spleen-derived alloantigens failed to remain soluble after removal of the solubilizer. A third possibility is that large molecules unrelated in structure to the antigens in question adsorbed the latter once hypertonic salt was removed. The DNA contaminating the preparations may, thus, have had a detrimental effect. Prior removal of DNA by filtering through glass or nylon wool or by the use of desoxyribonuclease might have been valuable in this regard. The findings that xen-antigens solubilized by 3 M KCl were limited to the excluded volume of Sephadex G-200 were similar to those of Mann and Fahey (1971b) with HL-A alloantigens solubilized from lymphoid cell membranes by 3 M KCl.

If non-covalent bonds held these antigens in an aggregated state, compounds acting to break these weak forces, such as urea or detergents, were thought of as possible remedies of the situation. Six molar urea had no effect on de-aggregating a previously frozen and thawed preparation. Perhaps if a fresh preparation were incubated with the agent during concentration, greater success
would have been attained. In addition, concentrations as high as 10 molar urea could be tried to enhance the de-aggregation (Reisfeld and Small, 1966).

Triton X-100 added in the concentration of 0.1 per cent to both the extract and to 7 per cent acrylamide gels (Davis, 1964) allowed demonstration of solubilized surface located xenoantigens. In two separate experiments Triton X-100 was added to 0.1 per cent to fresh KCl extracts. When electrophoresed on the gels described above, four active peaks were detectable. Though the molecular weights of the active components are not known, three of them were well included in the gels. The first component was primarily at the top of the gel having its peak activity in the second 2 mm fractions of the 79.5 and 95 mm gel runs. This is probably a large molecular weight, complex antigen. Preliminary chemical analysis of this peak showed it to be heavily contaminated with DNA and high in hexose (Table 10). Due to the fact that both ILC and CF activity could be detected in this peak it probably represented material similar to, though perhaps slightly smaller than that which had failed to enter Davis gels at all before the addition of Triton X-100.

Peak 2 had an Rm of about 0.16, and migrated in the same region as does IgG in the Davis gel system. It is interesting that Smit et al. (1972), using pyrophosphate to extract baboon spleens,
solubilized a glycoprotein with a molecular weight of about 150,000, and an Rm of 0.17, also using the Davis system, but apparently with SDS in the gels. Further investigation would be necessary to draw any conclusions as to the identity of the two antigenic components. We are currently attempting to obtain alloantisera which would be most helpful along these lines. Chemical analysis of peak 2 showed that carbohydrate (hexose) was present to a level of about 5 per cent of the quantity of protein in this peak, in addition to a similar concentration of DNA (Table 10). This peak was characterized by both CF and ILC activity, and could be re-electrophoresed to the same location.

The third peak had a mean Rm of 0.41. There was no hexose or DNA present at the 3 per cent level of sensitivity. More interesting is the fact that, as with the fourth peak (Rm 0.63), although ILC activity was evident, there was no CF activity detectable. This phenomenon could be explained by postulating that only one heteroantigenic determinant was present on the molecule (i.e. a hapten). To be active in the CF test the proximity of two IgG antibodies is required. Thus, at least two antigenic determinants would have to be on the same fragment.

With or without the addition of detergent to polyacrylamide gels and KCl extracts, precipitating activity was seen to enter the first few fractions at electrophoresis. It was not possible, for unknown reasons, to demonstrate CF activity at this location.
Future studies employing KCl extraction should be attempted using a large source of antigen, such as a freshly obtained spleen. We have been hampered by the small amounts of antigen we had to work with. Simultaneously running tests for the presence of alloantigens would be most instructive. Tanigaki et al. (1972), showed that heteroantisera to human HL-A antigens were capable of reacting with all specificities in the HL-A system that were tested. This inferred the presence of a common heteroantigen, even on the fragments derived from different HL-A subloci. It would not be surprising to discover that alloantigenic activity is associated with our solubilized components.

Lipid was not tested for in the above fractions due to the limited quantity of material with which we had to work. Though most lipoprotein was probably removed during 3 M KCl extraction, it is possible that there would be a significant amount present as was found by Kandutsch and Stimpfling (1965), working with Triton X-100 solubilized H-2 antigens.

We currently have no knowledge as to whether the antigenic specificities on the four soluble components are different or identical, but it is appealing to think that they might represent progressively smaller polymeric units of a membrane antigen complex.
SUMMARY

These initial investigations into an inter-subhuman primate xenograft model had the goals of studying the immune responses of chimpanzees and baboons to cross-immunization with peripheral lymphocyte preparations, and obtaining high titered antilymphocyte sera derived from these animals for use as reagents in the study of baboon xenogeneic histocompatibility antigens.

Two chimpanzees and 4 baboons were cross-immunized biweekly with peripheral blood lymphocytes prepared by isopycnic centrifugation. The inocula were given with or without Freund's incomplete (FIA) or complete (FCA) adjuvant, and humoral immune responses were followed by hemagglutination (HA), leukoagglutination (LA), and lymphocytotoxicity (LC). Chimpanzees and baboons responded poorly or not at all to immunization with each other's lymphocytes at dosages of less than about $10^6$ lymphocytes per kg, when adjuvant was not admixed. When FIA was added to preparations containing a similar amount of antigen, baboons developed LC titers of 4096 to 8192, and LC titers of chimpanzees (4 to 8), rose to 256. HA antibody levels increased similarly after incorporation of
adjuvant, while LA levels increased but remained low.

When the antigen-FIA dose was increased to $1 \times 10^7$ to $3 \times 10^7$ cells per kg of body weight ($42 \times 10^7$ and $124 \times 10^7$ total cells, respectively) peak titers of 512 to 1024 (LC) appeared in the chimpanzees' sera two weeks following the injection, and then fell to 128 to 256 two weeks later. Administration of approximately $1.7 \times 10^7$ cells per kg ($69 \times 10^7$ total cells) with FCA resulted in little change in LC response over that observed when FIA was used. Inoculation of baboon spleen homogenate intraperitoneally into chimpanzees, resulted in a substantial elevation of both LC and HA antibody titers.

A baboon spleen homogenate was fractionated by differential centrifugation, following which the $140,000 \times g$ (max.) supernate was subjected to Sephadex G-200 chromatography. Nearly all the antigen in the homogenate detectable by chimpanzee anti-baboon lymphocyte serum assayed by inhibition of lymphocytotoxicity (ILC) was related to membrane fragments. Almost 92 per cent of ILC activity in the homogenate sedimented at $15,900 \times g$, and over 93 per cent of the ILC activity in the "soluble" ($140,000 \times g$ supernatant) fraction, upon concentration, either became sedimentable at $48,900 \times g$, or eluted with the excluded volume of Sephadex G-200.

Papain digestion of a crude spleen membrane preparation failed to cause solubilization of antigens, but resulted in
their rapid destruction as detected by either complement fixation (CF) or ILC tests. While essentially all ILC activity was destroyed by 15 minutes of incubation of the preparation with crude papain at 37°C, CF levels decreased through 15 minutes of incubation, and then became stable through 120 minutes of observation.

Extraction of peripheral baboon lymphocytes by 3 M KCl resulted in a large yield of xenoantigenic material. These antigens sedimented at 163,000 x g and failed to enter 7 per cent polyacrylamide gels run in an alkaline discontinuous system. Adding Triton X-100 at 0.1 per cent to the KCl extracts resulted in partial loss of immunologic (CF and ILC) activity, but appeared to release at least three antigenic components which entered 7 per cent acrylamide gels with mean relative migrations (Rm) of 0.16, 0.41, and 0.63. The component with an Rm of 0.16 as well as active material restricted to the first few fractions of the gels could be detected by both ILC and CF tests using chimpanzee anti-baboon lymphocyte serum in the test. The other components (Rm 0.41 and 0.63), could only be detected with the ILC test.
APPENDIX I

CALCULATION OF INHIBITION OF LYMPHOCYTOTOXICITY (ILC)

Arithmetic plots were made of the per cent ILC obtained by testing serial 2-fold dilutions of antigens (in terms of ug protein per ml). These plots were also made to illustrate the numbers of whole cells causing inhibition (absorption) of a certain percentage of the cytotoxic activity from the serum. (Fig. 21). To calculate the 50 per cent endpoint of the ILC (LCID50) caused by an antigen preparation, logarithmic plots were made wherein the log of the protein content in ug per ml was plotted against the term \( \log \left( \frac{100-P}{P} \right) \), using the Reif modification of the van Krogh equation (Reif, 1966) as given below (Equation I).

\[
\log g = \log G + \frac{1}{M} \log \left( \frac{100-P}{P} \right) \quad \text{Equation I}
\]

In equation I, \( g \) is the ratio of the total quantity of cellular antigen to the total quantity of serum. In our tests the quantities of each was the same, leaving \( g \) as the protein concentration or the total number of cells in that volume. \( P \) is the per cent of the potency of the antiserum remaining free after absorption (100 minus the percentage inhibition). The figure 100 - \( P \) is the percentage inhibition of cytotoxicity.
Fig. 21. Inhibition of lymphocytotoxicity of immune chimpanzee serum by peripheral baboon lymphocytes. Arithmetic plot of pooled results derived from 3 different tests using 1, 2, and 4 cytotoxic units of serum and various numbers of lymphocytes to absorb.
G is equal to g when P is 50 per cent. Log G is the y intercept, and can be read directly from the plots (Fig. 22), giving the \( LCID_{50} \). Finally, \( 1/M \) is the slope of the plot where M is the avidity coefficient of the antigen-antibody complex.

**Inhibition of cytotoxicity by absorption with whole peripheral baboon lymphocytes.** The 50 per cent absorptive dose (AD\(_{50}\)) for whole peripheral lymphocytes was determined for 1, 2 and 4 units of immune chimpanzee serum (final serum, chimpanzee Isabelle). The arithmetic (Fig. 21), and the logarithmic (Fig. 22) plots, and the AD\(_{50}\)'s so derived, were obtained from the results of three separate experiments performed at different times using baboon Desdamona's lymphocytes for absorption and as target cells. As is the case for determining the LCID\(_{50}\), the AD\(_{50}\)'s were estimated by the y-intercept of the logarithmic plots (Fig. 22). The results show that 15,130, 6,610 and 2,290 cells per ul were the AD\(_{50}\)'s for 1 ul of dilutions of serum containing 4, 2 and 1 cytotoxic units, respectively. By plotting the log of the protein concentration in the lymphocyte preparations (in ug per ml) against the term \( \log \left( \frac{100-P}{P} \right) \), the LCID\(_{50}\) was observed to be 569, 282 and 81 ug per ml of lymphocyte protein for 4, 2 and 1 cytotoxic units of serum.

The "absorptive yield" of an antigen preparation is a measure of the inhibitory activity obtained in an extract.
Fig. 22. Logarithmic plot using the Reif modification of the van Krogh equation for estimation of the 50 per cent endpoint of inhibition of lymphocytotoxicity. Points were taken from the arithmetic curves in Fig. 21, where inhibition was greater than 20 per cent and less than 80 per cent. The AD50 can be estimated for whole lymphocytes derived from a baboon inhibiting 1, 2 and 4 cytotoxic units of immune chimpanzee serum.

1 unit; 2 units; 3 units.
as compared with the absorptive capacity of the number of whole cells from which the extract was obtained. It may be estimated using the ratio of the AD$_{50}$ to the LCID$_{50}$ when the latter is expressed as cell equivalents. Thus, if the number of cells extracted to obtain one LCID$_{50}$ is estimated to be 7,500 (cell equivalents), and if the AD$_{50}$ is 2,290 cells, then the absorptive yield would be calculated as 2,290/7,500, or 30.4 per cent.
APPENDIX II

TREATMENT OF CHIMPANZEE SERUM TO AVOID ANTICOMPONENTARITY

The final serum of chimpanzee Isabelle, which was the immune serum always utilized in the CF test, had been prepared by treating the blood with ACD solution, followed by centrifugal removal of cells and the addition of sufficient CaCl$_2$ to promote formation of a fibrin clot. With each subsequent thawing of the serum, some precipitation of fibrin occurred. Though this did not hamper its use in the ILC or LC tests, it appeared that the precipitate was somewhat anticomplementary, i.e., as much as 20 per cent or more at the 1/25 to 1/50 dilutions of serum used in early tests. To avoid this anticomplementarity several steps were taken. It was found that heating a second time at 56°C for 30 minutes coupled with centrifugation of the serum at 48,900 x g for 30 minutes at 4°C, caused a great deal of the fibrin to precipitate, leaving future thaws of the serum less likely to form such a precipitate. This did not appear to affect specific activity, but did significantly suppress anticomplementarity of the serum.

In order to avoid this problem it was also necessary to dilute the serum sufficiently. In one test (see Fig. 23), the immune serum, after absorption with sheep erythrocytes, re-heating, and centrifugation as above, was serially diluted from
Fig. 23. Complement fixation test. Titration of immune chimpanzee serum with and without antigen. The antigen was a 1/20 dilution of 2-fold concentrated baboon lymphocyte KCl Extract 3. The reciprocal of the serum dilution is given on the X-axis.
COMPLEMENT FIXATION:
TITRATION OF IMMUNE
CHIMP. SERUM WITH
AND WITHOUT
ANTIGEN

% CF

SERUM DILUTION

TEST

SERUM CONTROL

100
80
60
40
20
0

50 100 200 400 800 1600 3200
1/50 through 1/3,200 and tested alone (antibody control), or with a 1/20 dilution of a KCl extract containing 8 C'50's at that dilution (the test). At a 1/50 dilution of serum, the antibody control was 20 per cent anticomplementary, whereas by the 1/200 dilution no such activity could be detected. Titration of the serum with antigen demonstrated that the 50 per cent endpoint dilution of serum is somewhat greater than 1/800.
BIBLIOGRAPHY


Reif, A., "An Experimental Test of Two General Relationships to Describe the Adsorption of Antibodies by Cells and Tissues." Immunochemistry, 3 (1966), 267-78.


