# Development of Antilymphocyte Sera by Cross Immunization of Chimpanzees and Baboons[1,2] (37420)

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Tissue matching of recipient–donor pairs has substantially increased the graft survival of living related donors. These results indicate that lack of equivalent success between unrelated donors is due to the inability of the present battery of typing antisera to identify appropriate antigens. Typing antisera obtained from multiply transfused patients or multiparous women require extensive absorptions. Intradermal injections of humans with purified lymphocyte preparations have produced good typing antisera. Human isoimmunization incurs the risk of infection and sensitizing the volunteer, and high titers are not obtained without extensive immunization. Heteroimmunization may allow the production of high titered antisera in large volumes providing serum of a single specificity for matching many potential donor–recipient pairs. In an effort to provide an alternative source of potent antisera, chimpanzees were immunized with human leukocytes because their phylogenetic relationship to man would more likely allow production of iso-specific antisera than other species. After selective absorption with human leukocytes and erythrocytes, sera showed iso-antigen specificity, however, leukoagglutination titers ranged only to 1:32 (1).

The present study was designed to investigate methods of immunizing subhuman primates to determine a method of producing high-titered tissue typing antisera. Chimpanzees and baboons were cross immunized with peripheral blood lymphocyte preparations and the antibody responses were followed by hemagglutination, leukoagglutination and lymphocytotoxicity tests. We attempted to (a) determine the antibody response with long term stimulation at various lymphocyte doses, (b) compare the levels of response of three different antibody activities, (c) evaluate the use of adjuvants, and (d) describe efficient methods for immunization of primates.

*Materials and Methods. Serological techniques.* A modification of the lymphocyte microcytotoxic technique (LC) of Terasaki (2, 3) was used throughout the study. Fresh normal rabbit serum served as a source of complement after being absorbed with thrice washed baboon and chimpanzee erythrocytes. The endpoint for the LC test was the final serum dilution resulting in greater than 20% cell death as determined by dye exclusion. The defibrinated leukocyte agglutination technique (LA) of Zmijewski *et al.* (4) was employed. In the hemagglutination (HA) test, 0.1 ml of a 2% washed erythrocyte suspension was added to an equal volume of serial twofold saline dilutions of sera. Following incubation at room temperature for 30 min, the tubes were centrifuged at 600*g* for 3 min and the test read. The endpoint was determined as the last tube in which small clumps of cells were observed macroscopically.

In most cases, tests were performed on a single serum specimen with cells derived from two members of the opposite species and the

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results averaged. Titers against cells of these two sources rarely varied more than one dilution, so that one could not differentiate cell donors by any of the serological techniques.

*Antigen preparation.* Lymphocytes were obtained from the peripheral blood by a modification of the method of Perper *et al.* (5) and were used both in the LC test and for immunization of the animals. Contaminating erythrocytes were removed by hypotonic lysis. The number of cells in the antigen preparation was determined by counting with an hemocytometer. The pellet obtained from 40–50 ml of blood was diluted in Hank's balanced salt solution (HBSS) (6) to 2.0 ml then frozen at —20° until thawed for use in immunization, usually one week later.

A baboon spleen inoculum was aseptically prepared by homogenization of a previously frozen organ in a Sorvall Omnimixer apparatus (Ivan Sorvall, Norwalk, Conn.), straining through coarse meshed gauze and mixing with 10,000 IU of potassium penicillin G and 10 mg of streptomycin sulfate. The suspension was then diluted to 28 ml in HBSS and divided, yielding one 14-ml volume for each chimpanzee.

*Animals, immunization and bleeding schedules.* The animals were sedated with phencyclidine HCl for immunizations 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 inoculated at biweekly intervals with lymphocyte preparations derived from a member of the other species and frozen at —20° 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 (FCA) adjuvant (Difco, Detroit, Michigan). When inoculations were made intraperitoneally (ip), 1000 IU of the potassium penicillin G and 1.0 mg of streptomycin sulfate were mixed with the cells prior to injection. Intervals of immunization are indicated by a "V" over the weeks after the beginning of the studies as given in Figs. 1–6. Dosages are presented in Table I.

*a. Baboons immunized with chimpanzee lymphocytes without adjuvant.* Baboon Mike (*Papio cynocephalus*), a mature adult male, 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 a second 14-week period, was given ip. Baboon Monica (*Papio cynocephalus*), a mature adult female, received lymphocytes derived from chimpanzee Isabelle, using the same method and schedule of injections as described for baboon Mike.

*b. Baboons immunized with chimpanzee lymphocytes with FIA.* Baboon Kate (*Papio papio*), a mature adult female, received all inocula (2.0 ml) admixed with FIA (2.0 ml).

TABLE I. Immunization of Chimpanzees and Baboons.[a]

| Animals immunized | Wt (kg)[b] | Mean biweekly dosage of lymphocytes × $10^6$ | | Total no. of injections |
|---|---|---|---|---|
| | | Without adjuvant | With adjuvant[c] | |
| Chimpanzee Isabelle | 27–40 | 34.0 | 45.3 | 10[d] then 9[e] |
| Chimpanzee Rocky | 27–40 | 19.2 | 37.4 | 11[d] then 9[e] |
| Baboon Mike | 23 | 23.3 | — | 14 |
| Baboon Monica | 16 | 21.5 | — | 14 |
| Baboon Kate | 16 | — | 22.7 | 16 |
| Baboon Antoinette | 14 | — | 20.4 | 17 |

[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.

For the first 14 weeks, lymphocytes were derived 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 (*Papio papio*), a mature adult female, was immunized in the above manner, however, only lymphocytes from chimpanzee Rocky were employed for inocula 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.* Chimpanzees Rocky and Isabelle (*Pan satyrus*), a male and a female, respectively, were both juveniles (estimated at 7.5 yr). 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 for the first 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 were lymphocytes from Monica for Isabelle. Beginning on the 22nd week FIA was mixed with the preparations, and the inoculum split 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 week 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 $17.6 \times 10^7$, $42 \times 10^7$, and finally $124 \times 10^7$ cells (8.5 mg, 21.75 mg, and then 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 $69 \times 10^7$ cells (26.2 mg of protein) inoculated id into each chimpanzee. The responses were followed by assay of biweekly bleedings. No further inoculations were made through the 66th week.

A baboon spleen homogenate, prepared as described above, was inoculated into both animals on the 67th week. Each animal received homogenate containing 700 mg of protein, (17.5 mg/kg) divided between ip (10 ml) and subcutaneous (4 ml) routes. The chimpanzees were bled weekly through the 71st (Isabelle) or 72nd (Rocky) week when the experiments were terminated.

*Results. Baboons immunized with chimpanzee lymphocytes without adjuvant.* As shown in Fig. 1, 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 preexisting HA antibody against chimpanzee erythrocytes.

Baboon Monica (Fig. 2) had a preinoculation HA antibody titer of 16 which fluctuated at first following the initiation of the immunization program but by 16 weeks, remained stable at 64–128. LC antibody was first detected at 12 weeks reaching low but persistent levels of 4–8 by the 16 weeks. LA titers were not observed until the 24th

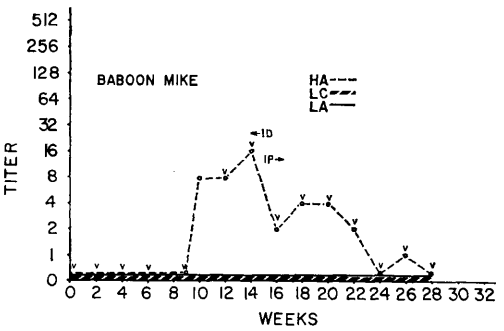


FIG. 1. Antibody responses of the baboon Mike to immunization with chimpanzee lymphocytes without adjuvant. Titers are recorded as the reciprocal of the highest dilution of serum giving positive reaction. HA is hemagglutination, LC is lymphocytotoxicity and LA is leukoagglutination. "V" designates the time of each inoculation. All inoculations to the left of the "ID" were given intradermally and all to the right of "IP" were given intraperitoneally.

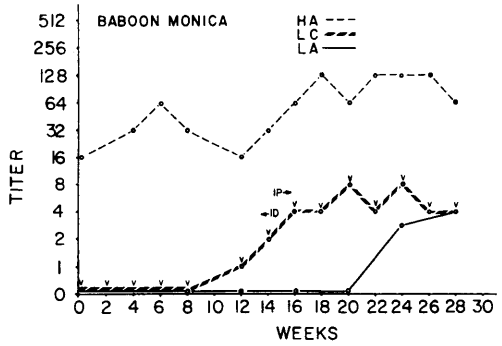


FIG. 2. Antibody responses of the female baboon Monica to immunization with chimpanzee lymphocytes without adjuvant. See legend, Fig. 1, for further details.

week with a maximum level of 4 detected on the last (28th) week of the study.

*Baboons immunized with chimpanzee lymphocytes mixed with Freund's incomplete adjuvant.* The responses of the two baboons, Kate and Antoinette (Figs. 3 and 4) were very similar. Both had preexisting HA antibody titers which rapidly increased to peak levels of 4096 and 1536 by 5½ weeks. With continued biweekly immunizations, however, the HA levels gradually declined. LC responses were first observed 10 days after the first immunization and maximal LC titers of 1024–2048 were reached by 7½–9½ weeks. Antoinette's immunizations were stopped 9 weeks prior to the end of the study (Fig. 4). The results in Fig. 4 show that HA and LC titers persisted throughout this period. LA antibodies were first detected at 10 days (Antoinette) and 24 days (Kate), reached

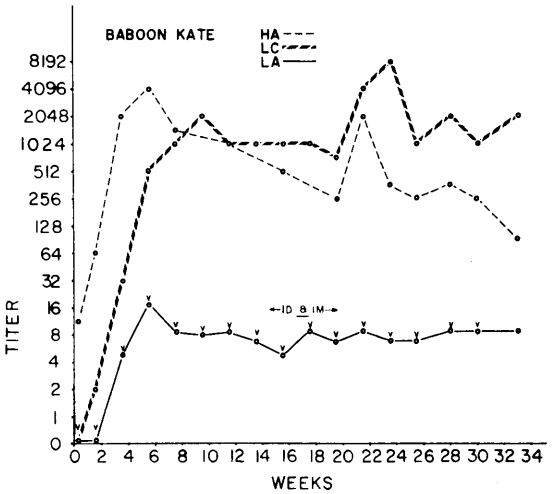


FIG. 3. Antibody responses of baboon Kate to immunization with chimpanzee lymphocytes in Freund's incomplete adjuvant. All doses were given by both intradermal (ID) and intramuscular (IM) routes. Titers are recorded as the reciprocal of the highest dilution of serum giving positive results. HA is hemagglutination, LC is lymphocytotoxicity and LA is leukoagglutination. "V" designates the time of each inoculation.

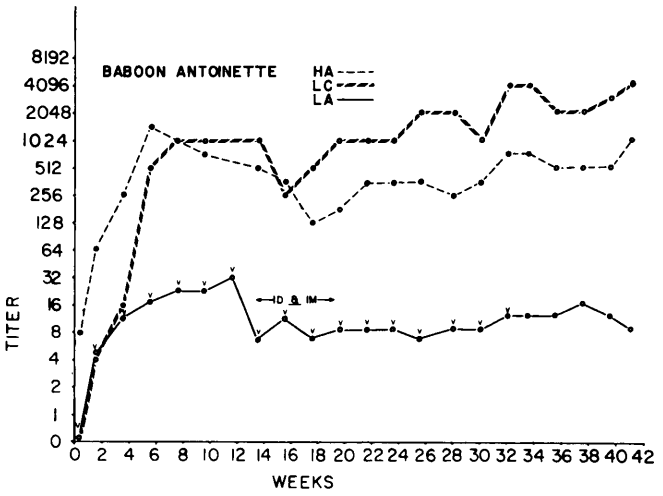


FIG. 4. Antibody responses of the baboon Antoinette to immunization with lymphocytes in Freund's incomplete adjuvant. See legend, Fig. 3, for further details.

maximum levels by 6–8 weeks, and remained very stable at titers of 8–16 thereafter.

*Chimpanzees immunized with baboon lymphocytes and a splenic homogenate.* Neither animal had detectable preimmunization 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. 5. Rocky developed low levels of HA antibody 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

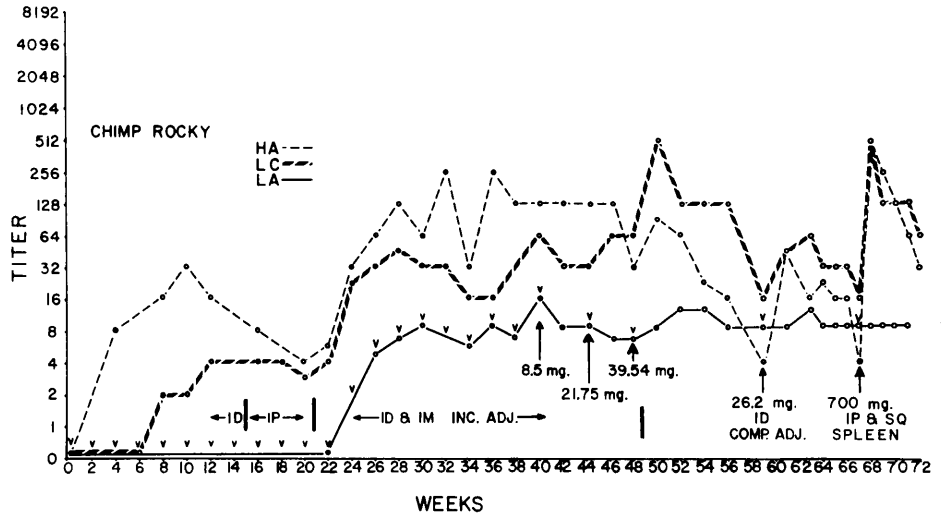


FIG. 5. Antibody responses of chimpanzee Rocky to immunization with baboon lymphocytes and spleen. HA is hemagglutination, LC is lymphocytotoxicity, and LA is leukoagglutination. Titers are recorded as the reciprocal of the highest serum dilution giving positive reaction. ID denotes use of the intradermal 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.

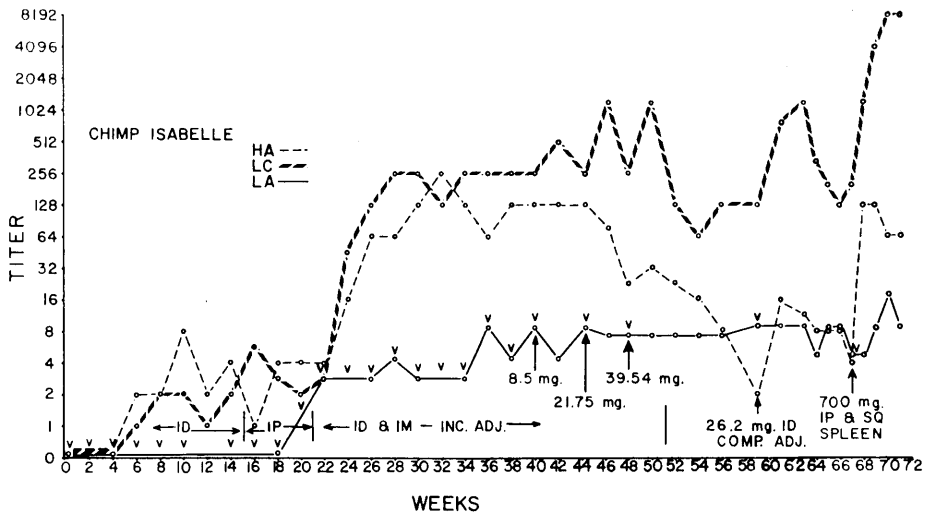


FIG. 6. Antibody responses of chimpanzee Isabelle to immunization with baboon lymphocytes and spleen. See legend, Fig. 5, for further details.

12 weeks. No LA antibodies could be detected in Rocky's sera during this period. Chimpanzee Isabelle's responses were very similar to those of Rocky for this period (Fig. 6), 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. 5 and 6), and after 4 weeks (the 26th week of the study), LA antibody activity appeared in Rocky's serum.

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 rise in antigen dose. Rocky's LC titer was elevated (8-fold) only at 2 weeks after the 30-fold increase 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 suspension (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. 6). Rocky's responses were similar (Fig. 5).

Finally, inoculation of both chimpanzees with baboon spleen homogenate 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 HA and LC titers rose to 512 (128-fold and 32-fold, respectively) within 1 week but decreased sharply thereafter, dropping to 32 and 64, respectively.

*Discussion.* 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 lymphocytotoxic (LC) antibody even when stimulated biweekly for as long as 20 weeks. Essentially no leukoagglutinating (LA) activity was observed. The incorporation of adjuvant (FIA) into a similar dose of antigen led to a significant rise in HA and LC antibody in the same

chimpanzees as well as moderate levels of LA activity. Further stimulation with larger doses of antigen in adjuvant increased the response in chimpanzees 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/inoculum without adjuvant produced high titered sera in rabbits (7–9) and horses (10, 11). These doses were approximately 40 times the number of cells used in our study (Table I). Metzgar *et al.* (1), however, made monthly intravenous injections of chimpanzees for as long as 8 months with mixed human cell preparations containing $10^9$ leukocytes and obtained LA titers of 4–32 which were similar to those of our animals when adjuvants were not used. The results obtained by these workers may not be directly comparable with our studies because their antisera were absorbed with erythrocytes prior to titration. Johanssen and Seiler (12) described one chimpanzee immunized repeatedly with human peripheral blood lymphocytes without adjuvant first by the intravenous and then the subcutaneous route. Nine inoculations of $10^6$–$10^8$ cells were given over a period of 30 weeks resulting in maximum LC titers of 2–8. Although the difference between primates and rabbits in their response to heterologous stimulation could be attributed to the dose of antigen per kilogram of body weight, this could not be the explanation for the difference between primates and horses. Perhaps the lower response level in our interprimate model as well as human to primate systems 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 antibody 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 (13), and are relatively easy to isolate from the peripheral blood with 90% or greater purity (5). 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 prior to bleeding and immunization and, therefore, we selected a biweekly injection schedule for fear that the animals might not do well in 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°) until used for immunization. Although the majority of investigators preparing ALS have employed living cells, it is not established that frozen and thawed cells are less immunogenic.

As the immunizations continued HA antibody titers tended to decrease gradually, while LC antibody levels became higher. Isabelle's HA titer began to fall in the face of prolonged immunization (Fig. 6). 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, however, resulted in an increase in HA titer (Fig. 6).

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 the 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, he also developed a persistent neutrophilic leukocytosis with total peripheral white cell counts elevating to between 20,000 and 57,000/mm$^3$. Moor-Jankowski (personal communication) has suggested that by dividing the inoculum among several deep intramuscular sites, and decreasing the total FCA in half to 1.0 ml, such untoward sequellae might not have occurred, and perhaps would have resulted in higher levels of response.

*Summary.* Two chimpanzees and 4 baboons were cross immunized biweekly with peripheral blood lymphocyte preparations, with and without Freund's adjuvant and their humoral immune responses followed by hemagglutination (HA), leukoagglutination (LA), and lymphocytotoxicity (LC). Chimpanzees and baboons responded poorly or not at all to immunization with lymphocytes from one another at dosages of less than about $10^6$ lymphocytes/kg when adjuvant was not admixed. When Freund's incomplete adjuvant (FIA) was added to preparations containing a similar amount of antigen, baboons developed LC titers of 4096 to 8192, and LC titers of chimpanzees (4–8), rose to 256. HA antibody levels increased similarly after the incorporation of adjuvant, while LA levels increased, but remained low.

When the antigen-FIA dose was increased from $10^7$ to $3 \times 10^7$ cells/kg of body weight ($42 \times 10^7$ and $124 \times 10^7$ total cells, respectively), peak titers of 512–1024 appeared in the sera of chimpanzees 2 weeks later. Administration of approximately $1.7 \times 10^7$ cells/kg ($69 \times 10^7$ total cells) with Freund's complete adjuvant (FCA) resulted in little change in LC titer over that observed with FIA. Intraperitoneal inoculation of chimpanzees using spleen homogenate from baboon resulted in a substantial elevation of both LC and HA antibody titers.

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