THE IMMUNOSUPPRESSIVE ACTIVITY OF ANTILYMPHOCYTE ANTIBODY ISOLATED FROM A SPECIFIC IMMUNOADSORBENT

by

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To Dr. Malley and his staff without whose help this thesis would have been impossible

and

To my husband Jack and my son Mark who sacrificed so willingly.

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INTRODUCTION

The use of antilymphocyte sera (ALS) as an immunosuppressive agent has had widespread application, and some measure of success. However, the relatively large amounts of foreign protein necessary to prevent graft rejection often results in a variety of secondary complications. To overcome these difficulties many investigators have utilized the globulin fraction of ALS (1). Such preparations are enriched in immunosuppressive antibodies, but also contain large amounts of nonspecific globulins. Attempts to use the lymphocyte as an immunoadsorbent to specifically purify ALS antibody (2,3) have been successful, but have several limitations. The yield of antibody eluted from lymphocytes has been low (usually 1-5%), and the lymphocytes are not reusable for the selective removal of specific antibody from the ALS.

With this in mind an immunoadsorbent was prepared by chemically conjugating lymphocyte stroma to Sephadex G-100. This reusable immunoadsorbent provides a rapid, one-step method for the specific isolation of antilymphocyte antibody, in high yield. This thesis describes the method of preparation of the immunoadsorbent, the in vivo assays utilized in evaluation of the isolated fractions, and the immunosuppressive properties as evidenced by skin homograft survival in rhesus monkeys.

Review of Literature

Antilymphocyte sera (ALS) was first prepared by Metchnikoff by injecting the cells from guinea pigs and rats into rabbits (4). He reported both leukagglutination and cytotoxic properties of this sera. Shortly afterward, Besredka found that in vivo leukopenia could be induced by injection of Metchikoff's antisera (5). Almost all early workers reported that lymphopenia was followed by leukocytosis (5). A few years later, Papenheimer demonstrated that a hemolytic factor could be absorbed out and suggested that complement was involved in the in vitro cytotoxic effect (7). Almost 20 years later, Chew and Lawrence reported production of sera directed primarily against granulocytes or lymphocytes depending on the original sensitization (8). They maintained lymphopenia for 10 days in guinea pigs. Waksman demonstrated the suppression of humoral and cellular responses and extended graft survival by 2 days (9).

The next significant progress came with Woodruff and Anderson's experiments in which homograft survival was prolonged in rats (10). Much research followed in a variety of animals due to their success (11-20) in addition to early human studies (20-23).

Antilymphocyte serum has been prepared in a variety of ways in order to obtain high concentrations of the relevant antibody in small amounts of protein and to prevent formation of irrelevant or toxic antibodies (1,24-29).

The precise nature of the antigen or antigens essential for immunosuppression is not known, but they are widely spread over different

tissue and cell fractions. They are not all exclusively histocompatibility antigens since antisera produced in closely related species are poorly suppressive (31). Lymphatic cells such as thoracic duct lymphocytes, peripheral blood lymphocytes, spleen cells, lymph node cells, and thymus cells are suitable for immunization. Immunosuppressive sera has also been produced by immunizing with epidermal cells (31). Some cell fragments have been shown to raise immunosuppressive sera (32-33). A recent paper indicated that the most potent immunosuppressive sera was produced against nuclear and microsomal fractions while sera against the soluble fraction of lymphocytes had the strongest mitogenic effect (34). Thymic tissue may be the best source of cells for immunization since the effectiveness of the antisera is believed to be due to its ability to kill off long lived thymic dependent lymphocytes (34). The thymus may have antigens distinct from other cell types (36-38).

The mode of immunization has been as variable as the antigen. Both short and long term immunizations have been used with some success (1,30).

Many investigators have demonstrated the immunosuppressive properties of ammonium sulfate precipitated globulin prepared from antilymphocyte serum (13,39-40). It has also been demonstrated that the immunosuppressive activity is located in the IgG fraction from Sephadex G-200 (42).

Some attempts have been made to further purify the active component of antilymphocyte globulin. Woodruff reported isolation of

specific antilymphocyte antibody from the globulin fraction of ALS. He adsorbed the Al-IgG to whole cells, washed them, and then eluted the specific antibody at low pH at 37°. He reported recovery of 1-5% of the antibody which retained agglutinating activity but lost cytotoxic activity (43). The next relevant report was a more successful attempt at isolation of specific antibody by adsorbtion to acid washed membrane fractions of thymus membrane and subsequent elution at 0° at pH 2.5. The recovery was about 2% of the IgG present in the beginning sera. This method had both in vitro activity and in vivo immunosuppressive activity in mice (44). An additional report used isolated thymic membrane fractions to adsorb the antibody. Elution at low pH in the cold rendered an antibody with in vitro and in vivo activity. Yield was not reported (45).

In vitro, antilymphocyte sera will agglutinate lymphocytes, is cytotoxic in the presence of complement and stimulates lymphocyte proliferation in culture (1). The pepsin digest of the antibody agglutinates but it is neither cytotoxic nor immunosuppressive in vivo (46,47). This indicates that the Fc fragment of the antibody is required both for cytotoxicity and for in vivo immunosuppression (42). There is not a perfect correlation between the in vitro activity and immunosuppressive activity regardless of the method of measurement used. One recent report indicates that there is a good correlation between rising thymic agglutinin titers and immunosuppressive capacity in early antisera (29). Rosette inhibition also appears to correlate well with immunosuppressive capacity (48).

Survival of skin homografts remains the best test for immunosuppressive capacity of either the antisera or the globulin fraction. There has been a great deal of emphasis in recent years on evaluating sera to be used in human transplants in monkeys for immunosuppressive potency and toxicity (49,50).

The question of whether lymphopenia is required for suppression has been a matter of dispute. Evidence from experiments in which ALS was administered intravenously (51) and from experiments using radioactivity labeled lymphocytes (52), indicate that lymphocytes are destroyed in the course of treatment and that some lymphopenia, at least early in the course of treatment is required for adequate suppression of homograft rejection. There is evidence however that rejection can occur in a lymphopenic state (53) and that grafts can be retained after lymphocyte counts have returned to normal values (54).

A similar histological pattern is present in lymphatic organs. Nearly normal states (54-55) as well as complete depletion (12) have been observed during ALS therapy. One important histological finding is depletion of the paracortical areas of the lymph nodes after ALS treatment (33,52,56). These areas are normally occupied by the thymus dependent recirculating lymphocytes (57).

Antilymphocyte sera appears to be the most potent immunosuppressive agent in rodents and a good one in larger animals. Skin graft survival can be prolonged significantly by ALS treatment in rats and mice (11,13,32). Second set rejections can be inhibited and prior sensitization can be reversed (14,55,58). These results were obtained

when ALS was administered in nontoxic doses. To achieve this effect with immunosuppressive drugs would be difficult and the drug level required would be extremely toxic. An extended tolerance state against histocompatibility antigens has been reported when ALS was followed by administration of viable lymph cells (33).

Extended survival of organ graft with ALS therapy has been reported. In dogs, significant prolongation of survival times of kidney or liver homografts have been reported (17-18,20,49). The animals appeared to have a better clinical course with ALS than drugs (59). The suppression in rodents appears to be better than that achieved in dogs.

Many cellular hypersensitivity reactions have been tested under ALS suppression including graft versus host, bone marrow transplantation and lymphocyte transfer (14,60). ALS was found to be highly suppressive in all these cases. It is interesting that ALS treatment renders lymphocytes immunologically incompetent (14,55,60-62) even after the total number of circulating lymphocytes has returned to normal (54).

Although the reports are variable, it is well established that ALS can suppress the humoral response if it is given before or at the same time as antigen (63-64). The cellular immune response appears to be affected more, however (65). It is difficult to suppress secondary response with ALS (66) but previously established cellular immunity can be suppressed. The dose of ALS rendering satisfactory extension of graft survival ordinarily suppresses to some extent humoral immunity (13).

In artifically induced autoimmune systems, ALS is effective if applied at anytime (67).

Infections in which cell mediated immunity is important in control, such as viral infections, tend to be a problem in ALS suppressed animals. Specific side effects include antibody reactions against other cells and tissues and hypersensitivity reactions. Due to the antigenic relationship between lymphocytes and other tissues, cross reactions have been demonstrated against kidney and liver cells *in vitro* and kidney cells *in vivo*. Clinical signs of injury, however, have not been demonstrated (18,68). The ALS appears to act *in vivo* primarily with lymphocytes either because they are encountered more frequently or the antigen concentration recognized by the antibody on their surface is greater than other tissues, or both.

The hypersensitivity reaction to the foreign protein can be manifested as anaphylaxis, serum sickness with arthritis, vasculitis, and serum sickness nephritis. These dangers exist due to formation of antibodies against the foreign protein (19,69-70).

Antilymphocyte serum has proven to be very effective in extending skin homograft survival in the rhesus monkey (30,71-73). In addition, the rhesus monkey has proven to be a good test system for effectiveness and toxicity of antihuman lymphocyte antibody (49-50, 74).

Here, I would like to cover specifically the information available on immunosuppression in the rhesus with antirhesus lymphocyte serum.

Effective antirhesus lymphocyte sera has been produced in the horse, rabbit, and goat. Rabbit antirhesus sera appears to be the most effective in extension of skin homograft survival (30) but due to the quantities of sera required, the horse has been used more frequently. Toxicity is an interesting aspect of treatment with ALS in the monkey. It has been reported that rabbit antirhesus sera is toxic if administered intravenously (30,72) but not if given subcutaneously (72). One group reported excellent success with intravenous administration of horse antihuman globulin (72). They claimed that it was completely nonantigenic administered in this manner.

Experience with preparation of horse antirhesus globulin indicates a great deal of variability in toxicity from horse to horse (75). If the horse sera is nontoxic, the animal can produce effective sera for up to a year. Only one animal in the four reported made effective sera for that long, however.

Antirhesus lymphocyte antibody has been purified by ammonium sulfate precipitation followed by DEAE Sephadex chromatography (75).

The question of antigenicity of rabbit sera in the monkey is an interesting one. Balner reported that the monkey makes antibody against other rabbit protein but not gamma-globulin (30). Although he now concedes that the presence of antirabbit antibody in ALS treated animals is a major complication (75). Lance reports that antibody is made against rabbit antirhesus IgG but not against normal rabbit IgG (73). The complications of ALS treatment in the monkey are localized or generalized allergic reactions and a gradually

diminished effect of ALS treatment.

Other side effects of ALS therapy reported in the rhesus are toxicity and impaired resistance to infections. One type of toxicity manifested itself as a thrombopenia or anemia and could be resolved by adequate adsorbtions. The other type of toxicity is described by Balner as an unwell state resulting from ALS therapy in which monkeys sit in a hunched position, stop eating and become irritable. This may be caused by pain at the injection site (75). Lowering of host defenses to infection has not been a major complication in ALS therapy in the rhesus (75).

Tolerance induction has been attempted and one researcher reports success (73) and the other failure (76). Lance succeeded in extending the mean homograft survival by 15 days by injection of lymphoid cells and ALS (73). Balner failed by administering normal globulin before ALS (76).

In preliminary experiments, the ALS dosage schedule which extends skin graft survival does not significantly extend survival of orthoptically transplanted kidneys in the rhesus (75).

The amount of ALS and the optimal dosage schedule for extension of skin graft survival are still unsolved questions. Balner reports one animal of eleven retaining his graft for ninety-five days when ten weeks of treatment at 6 ml/kg/wk was given. One can only guess, but if each ml has 10 mg IgG, that would be a total of 600 mg/kg of IgG. This is the longest reported maintenance without tolerance induction of a skin graft in the rhesus. His data indicates that unless treatment is

continued at least eight days, a marginal effect is obtained (75). Both Cohen and Sell, however, reported effective suppression (32 day survival) with 150 mg/kg globulin from rabbit antirhesus sera given days 1, 2 and 3 after grafting (77). Sell further stated that the significant added extension of graft survival was the same obtained even if treatment was extended for several weeks.

In this same discussion Sell brought up the question of whether lymphopenia was required for continued suppression. He found that grafts survived several weeks after the lymphocyte count had returned to normal (77) which confirms Balner's earlier data (30).

An analysis of the suppression of humoral immunity correlated with the time of graft rejection has not been done in the monkey.

Recent evidence which has emerged has led most of the people in the field to believe that ALS works by a selective depletion of the recirculating pool of lymphocytes. Two excellent reviews are available (35,78). The major pieces of evidence to support this hypothesis will be briefly covered here.

Antilymphocyte serum is rapidly eliminated from the circulation. Lance demonstrated conclusively in an experiment with specifically purified antibody that 80% of the labeled antibody is cleared within 24 hours (3). Antilymphocyte serum penetrates lymphoid tissue very poorly (79). Antilymphocyte antibody coated lymphocytes are rapidly cleared by the liver, as demonstrated by ^{51}Cr-labeled cells (52,80) or with ^{131}I-labeled antibody (81). Treatment with ALS depletes lymphoid organs selectively of migrant lymphocytes. The paracortical areas

where the circulating pool localizes (56-57) is depleted during therapy with ALS. Treatment with ALS alters the labeling kinetics and migratory potential of lymphocytes. It appears to cause a shift in the lymphocyte population toward shorter lived cells (82-83). On transfer, these treated cells localize less in lymph nodes (52). Treated cells recover slowly from ALS exposure and are thymus dependent. This is consistent with depletion of the long lived lymphocyte (57,84-85) especially since thymectomy retards recovery (86-87). Antilymphocyte serum may act discriminately against cell mediated immunity (65,84) although recent evidence implicates thymus derived cells in humoral antibody formation (80).

To quote Mitchison (78), "ALS acts preferentially on the recirculating pool: it is simply the soldiers who stick their heads above the parapet who get shot."

MATERIALS AND METHODS

Cells: Cells used in preparation of stroma for immunization or for *in vitro* assay were obtained from the thoracic duct, the thymus, and peripheral blood of rhesus monkeys (*Macaca mulatta*). Peripheral lymphocytes were separated from the freshly drawn heparinized rhesus blood after one hour incubation at 37°C by adding an equal volume of 3% gelatin in saline. The suspended lymphocytes were drawn off, washed in saline at 37°C, counted, and diluted to the proper concentration. Rhesus thymus was obtained from animals between 155 days gestation and 2 years. Tissue was excised shortly after sacrifice of the animal. Thymic tissue was cut into 200 mg pieces and thymus cells were pressed through a fine wire mesh and collected in cold phosphate buffered saline (PBS), pH 7.2. The cell suspension was heparinized and filtered through cheesecloth. Thoracic duct lymphocytes were obtained by surgical cannulation (88). Thoracic duct lymphocytes and filtered thymocytes were washed three times in saline, counted and diluted to the proper concentration. To prepare rhesus erythrocytes for adsorption, freshly drawn heparinized rhesus blood was incubated at 37° in a plastic syringe from one to three hours. An equal volume of 3% gelatin in saline was added to the blood in a 100 ml plastic tube and the mixture was placed in a water bath at a 30° angle at 37°C for 45 minutes. The supernatant containing the lymphocytes was removed and the settled erythrocytes were

suspended in an equal volume of gelatin. This procedure was repeated at least five times. The red cell suspension was then diluted in warm saline and centrifuged in a 37°C centrifuge at 2,400 rpm for 30 minutes. The cells were then washed three times in saline. If not used immediately for absorption, they were preserved in Alsever's solution at 4°C for up to four weeks. Sheep red blood cells for immunization or agglutination assays were washed three times in PBS before diluting to the desired concentration.

Stroma: Stroma was prepared from pooled rhesus lymphocytes by slowly decreasing the ionic strength of the wash in three equal steps. After each washing in the hypotonic solution, the stroma and the remaining cells were pelleted at 15,000 rpm at 0° for 30 minutes. When lysis was complete the stroma was washed three times in distilled water, repelleted and suspended in PBS.

Preparation of Chromatographic resins: Sephadex G-100 or G-50 was prepared according to the method of Borsos and Rapp (89). Granular Sephadex G-100 or G-50 (Pharmacia, Uppsala, Sweden) was sieved through a 200 mesh sieve. Distilled water (50 ml/gm Sephadex) was added for each gram of Sephadex with stirring. After the material was dissolved, an additional 50 mls of distilled water per gram of Sephadex was added. The mixture was stirred for two hours and then allowed to settle for 10 minutes. The supernatant was suctioned off and the same volume of distilled water added. The mixture was then boiled 20 minutes on a magnetic stir plate. The resin was then allowed to settle for 15 minutes before the supernatant was removed by

suction and buffer was added.

Preparation of DEAE Cellulose: DEAE Cellulose-32 (Whatman-England) was prepared according to the Whatman Information leaflet except that double distilled deionized water was used. It was weighed and 15 volumes of 0.5 normal HCl was added and stirred for 30 minutes. The resin was placed on Buchner funnel with Whatman No. 1 filter paper and washed until the pH of the effluent was 4. Fifteen volumes of 0.5 N NaOH was added and the mixture was stirred for 30 minutes, filtered and washed until the pH reached 8. The resin was resuspended in .005 molar Tris, pH 7.8, degassed, and then poured into a chromatographic column and equilibrated with degassed buffer in the cold.

Preparation of Activated Sephadex: (90). Sephadex G-100 was allowed to settle and excess water was decanted so that an equal volume of settled Sephadex and water were present. Cyanogen bromide (100 mg/ml of water) was dissolved under the hood with magnetic stirring. CNBr (100 mg per ml of settled Sephadex) was added to the Sephadex with stirring. Immediately, 4 N NaOH was added drop wise to bring and maintain the pH at 11. The pH was monitored with a meter while addition of the NaOH was continued until the reaction stopped (10 min.). The mixture was then poured into a Buchner funnel and washed with 20 volumes of cold 0.1 molar $NaHCO_2$ with suction. The activated Sephadex was then suspended in one volume of sodium bicarbonate.

Conjugation of Stroma to Sephadex: Stroma was added to the activated Sephadex and stirred for 24 hours at $4^{o}C$. For each milli-

liter of activated Sephadex the stroma obtained from 10^8 lymphocytes was utilized in the preparation of the immunoadsorbent. The conjugate was washed in a Buchner funnel with twenty volumes of 0.1 molar sodium bicarbonate, five volumes of water, ten volumes of 0.1 molar glycine-HCl pH 2.5, ten volumes water and thirty volumes of 0.01 molar cacodylate saline buffer, pH 6.8. The conjugate was resuspended in one volume cacodylate saline and 2 volumes of unreacted Sephadex G-100 were added before pouring into a chromatographic column. The column was washed with buffer for at least 24 hours before addition of serum. The preparative procedure is summarized in Figure I.

Antibody Preparation: Antithymocyte sera (ATS) (.08 mls per ml of resin) was washed through the specific immunoabsorbent column with 0.01 molar cacodylate saline buffer (pH 6.8). Effluent was monitored at 254 mμ and fractions were collected at 20 minute intervals. Buffer wash was continued until all of the nonspecific material was eluted from the column. The absorbed antibody was eluted with 0.1 molar glycine-HCl, pH 2.5. A typical elution pattern is illustrated in Figure II. The column passage (CP) peaks 1, 2, and 3, were pooled as fraction I and peak 4 which contains specific antibody was pooled as fraction II. The arrow indicates where elution with 0.1 molar glycine-HCl was begun. Both fraction I (CP) and fraction II (ATAB) were concentrated and dialyzed against PBS by negative pressure dialysis.

Serum Preparation: Rabbit antirhesus thymocyte sera (ATS) was prepared by immunizing each rabbit with 5×10^8 fresh or frozen lympho-

Fig I

PREPARATION OF LYMPHOCYTE STROMA

IMMUNOADSORBENT

Sephadex

↓

Activated with CNBr at pH 11
100 mg CNBr/ml settled resin

↓

Activated Sephadex washed with 10 vol 1% $NaHCO_3$

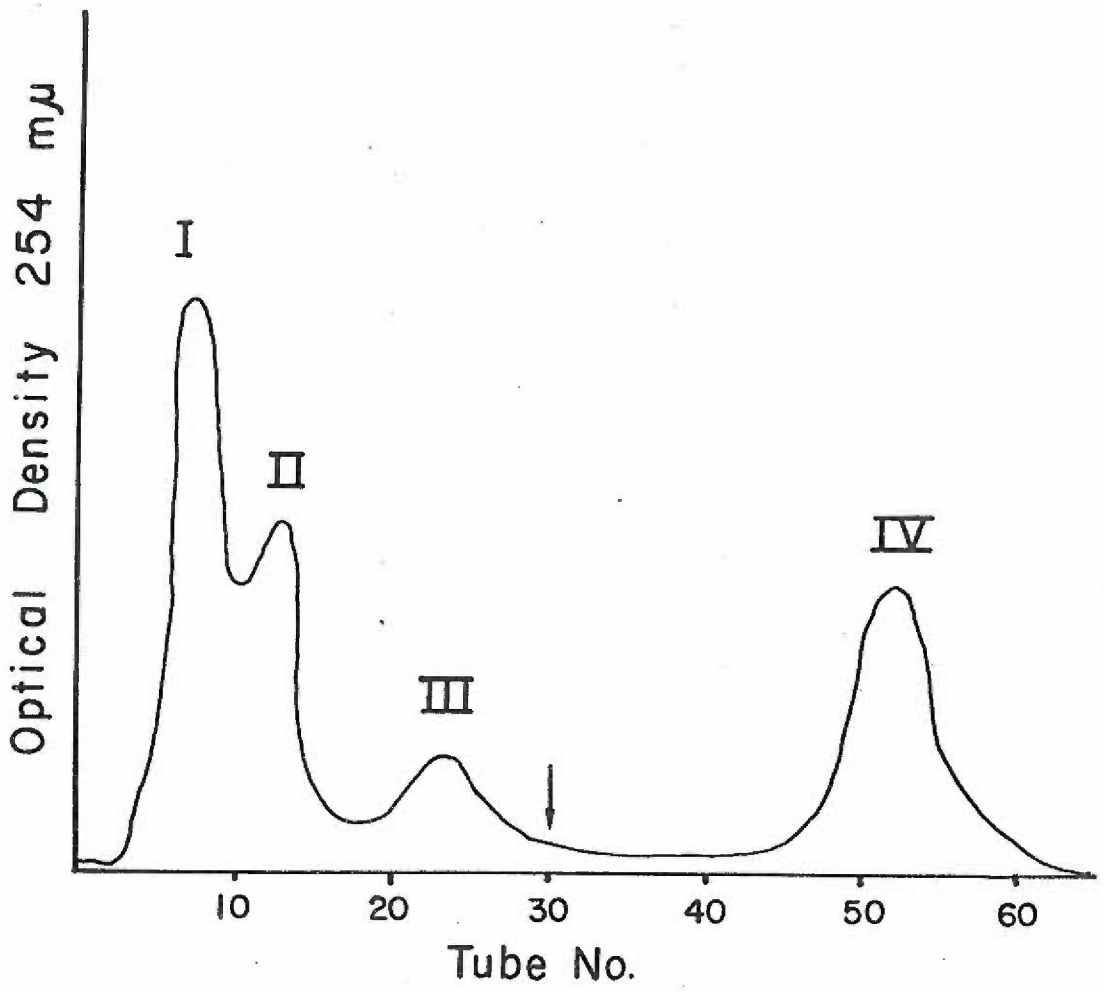
↓ Add Stroma and Stir 24 hrs at 4° C

Stroma-Sephadex washed successively with

(1) 20 vol 1% $NaHCO_3$
(2) 5 vol H_2O
(3) 10 vol 0.1M Glycine-HCl pH 2.5
(4) 10 vol H_2O
(5) 30 vol buffered saline

Fig II

Elution Pattern of Rabbit Anti-Lymphocyte Sera From the Specific Immunoadsorbent



The arrow indicates where elution with 0.1M Glycine HCl pH2.5 began

Peaks I, II, and III were pooled as column passage (C.P.)

Peak IV contained the antibody activity (ATAB)

cytes in complete Freund's adjuvant followed four weeks later with intravenous injection of 5×10^8 fresh thymocytes. One week later the rabbits were exsanguinated. Rabbit antirhesus lymphocyte sera (ALS) was prepared over the period of a year with subcutaneous injections of 10^6 peripheral or thoracic duct lymphocytes every two weeks. Both types of sera were inactivated 56° for 30 minutes and adsorbed with 1/10 volume of packed rhesus erythrocytes for two hours at 37°. Whole sera (ATS) or purified antibody (ATAB) or column passage (CP) were sterilized by Sietz filtration (.45 micron Millipore) and stored at -10°C until used.

Preparation of IgG from Normal Rabbit Sera: Normal rabbit sera (NRS) was dialyzed against .005 molar Tris buffer, pH 8 at 4°C. The sera was applied to a DEAE chromatographic column (1 ml sera to 6 ml of resin) and followed with .005 molar Tris. IgG was eluted with .05 molar sodium chloride in .005 molar Tris. This fraction was concentrated and dialyzed against PBS and analyzed by analytical ultracentrifugation.

Concentrations and Specific Activity Determinations: ATS, ATAB and CP were examined by analytical ultracentrifugation at 50,740 rpm at 20°C. Photographs were taken at 15 minute intervals for 60 minutes after the centrifuge reached maximum speed. Sedimentation coefficients were calculated and the protein concentration of the 7S peak was determined by planimetry from the 60 minute frames. Specific activities were determined by dividing the reciprocal of the leukagglutinin titer by the mg of protein or mg IgG per ml of assayed material.

Preparation of $F(AB)_2$ from IgG from Normal Rabbit Sera and Antithymocyte Antibody (91): IgG isolated from normal rabbit sera or antithymocyte antibody were dialyzed against 0.07 molar acetate buffer pH 4 with .05 molar sodium chloride and sterilized by Millipore filtration. Pepsin (3 X cryst, Act 1:6000 Pierce Chemicals) was added (3 mg/100 mg of IgG) and the mixture was incubated at 37°C for 18 hours. After incubation the pH was adjusted to 8 with 0.1 normal sodium hydroxide and the mixture was dialyzed and concentrated in the cold against several changes of PBS. The sample was resuspended in PBS and again analyzed on the analytical ultracentrifuge. Concentration was determined in the same manner as recorded above. Completeness of digestion was determined by immunodiffusion against donkey anti-whole rabbit serum.

Immunodiffusion: Three ml of 0.85% ion agar in cacodylate saline was added to dry slides which had been presoaked in 70% alcohol. The agar was allowed to harden before wells were cut. Antigen and antibody were added to the proper wells and the slides were incubated in a moist chamber at room temperature and examined at 4 and 24 hours. Photographs were taken as necessary.

Fluorescent Antibody Assay (92): Tissue from the sacrificed animal which might show deposition of antigen antibody complexes were selected for staining. These included thymic tissue, liver, kidney cross sections, spleen and lymph nodes. Fresh tissue was cut into 1.5 cm^2 by 3 mm pieces, wiped clean and placed on a filter paper. The tissue was placed in a tube of n-hexane which was precooled to -70°C in an

acetone-dry ice bath. After 10-15 minutes tissue was removed and placed in liquid nitrogen for storage. Sectioning of tissue was done by cryostat at 18-20° into thin sections not more than 20 microns. These were cut and fixed to slides and allowed to dry at room temperature. Tissue was fixed with 95% ethanol at room temperature for 5-10 minutes by immersion into solvent. This was followed by washing with PBS, and drying in an air stream. Antibody solution (Kallestad Laboratories, Minneapolis) was placed on the specimen and the slides were placed in a humid chamber and incubated overnight at 4°C. The slides were washed with a capillary pipet and then washed thoroughly with PBS. Slides were dried with a hair dryer. A drop of mounting media (buffered glycerol) was placed on the slide and the cover slip adhered. Slides were then observed for the presence of fluorescent antibody. Controls were 1) unstained sections, 2) unlabeled antibody, 3) heterologous globulin, 4) a mixture of labeled and unlabeled antibody. Positive control was tissue flooded with specific rabbit antirhesus antibody and washed before addition of labeled antibody.

Histological Examination of Biopsy Tissue: Skin grafts were excised weekly under light general anesthesia. The tissue sections were flattened on a filter paper and then placed in Bouin's fixative for up to 24 hours before embedding in paraffin. Sections were stained with hematoxylin, eosin and periodic acid Schiff's stain.

In Vitro Assay of Antibody Activity: Leukagglutinins were assayed by a micro modification of the method of Abaza and Woodruff (93). Whole sera (ATS) antibody (ATAB) or column passage (CP) were

diluted serially in 0.025 ml phosphate buffered saline (PBS). Thymocytes (0.05 ml of a suspension of 20 x 10^6 cells ml) were added to each dilution of serum in a microtiter plate (Cooke Engineering, Alexandria, Virginia) and incubated 1 hour at 37°C. Aliquots were removed from each well and read microscopically. Goat antihuman lymphocyte globulin prepared by ammonium sulfate precipitation was provided by Dr. B. Pirofsky at the University of Oregon Medical School. Cytotoxicity and leukagglutinin assays (93) on the original globulin and the antibody fraction were performed by Dr. Pirofsky's laboratory.

Transformation: Recovery of antilymphocyte activity from the immunoadsorbent was also assayed by transformation of rhesus peripheral lymphocytes (94). The original serum, purified antibody, and the column passage fraction were used as mitogens. Gelatin separated peripheral lymphocytes (2×10^6/ml) and mitogen (1-10 mg of IgG/tube) were cultured in roller tubes at 37°C for 48 hours before the addition of 2 μc of tritiated thymidine. Twelve hours later cells were harvested and the level of transformation measured as counts per minute of incorporated tritiated thymidine in the trichloracetic acid precipitable material by liquid scintillation (Packard Automatic Liquid Scintillation Counter).

In Vitro Assay of Rhesus Antisheep Red Blood Cell Antibody: Two fold dilutions of rhesus serum were made in micro titration plates. A 0.5% suspension of sheep red blood cells in PBS (.025 ml) was added to each well containing .025 ml of diluted sera. The plates were read macroscopically after a 1 hour incubation at 37°C for agglutination. Rabbit sera (0.025 ml) as a source of complement was added to each well

before returning the plates to the 37°C incubator for 1 hour. Plates were refrigerated overnight before reading macroscopically for hemolysins.

Formation of rhesus antirabbit antibody was assayed by passive cutaneous anaphylaxis in guinea pigs (4). Dilutions of monkey sera (0.1 ml) were injected into 6 separate sites on the shaved side of duplicate Hartley strain guinea pigs (250-350 g). Three hours later the guinea pigs were challenged by intravenous injection of a mixture of 0.25 ml normal rabbit sera and 0.25 ml 1% Evans blue dye. After 15 minutes reactions were read by measuring the diameter of the blued area. Positive reactions were graded as follows: 4 +, 20 mm or greater; 3 +, 15-20 mm; 2 +, 10-15 mm; 1+, 5-10 mm. The lowest dilution of sera giving a positive reaction was reported as the PCA titer.

Complete blood counts and urinalysis were performed by the clinical laboratory at the Primate Center. Blood electrolytes, BUNs, uric acid, cholesterol, total protein, albumin, bilirubin, alkaline phosphatase, transaminase and lactic dehydrogenase tests were performed on long term animals by Physicians Medical Laboratories in Portland, Oregon.

In Vivo Assay: In vivo assay of the immunosuppressive activity of antithymocyte sera, antithymocyte antibody, column passage, and normal rabbit sera was evaluated by extension of homograft survival in rhesus monkeys. Short term experiments in which animals were placed in plexiglas restraining chairs (developed at ORPRC) were designed to determine whether the antibody isolated from the specific immunoadsorbent column was immunosuppressive.. Skin grafted animals received treatment with either

ATS, ATAB, CP, or NRS on day + 1, + 2 and + 3 after surgery. Total dosage of ATS, CP and NRS was 150 mg of IgG per kg (60-60-30) while only 75 mg of IgG/kg (30-30-15) of ATAB was administered. Rejection was judged both by gross clinical changes and by histological examination of biopsied graft tissue. Biopsies were taken each week. Clinical rejection was recorded on the day all of the homografts were completely hemorrhagic. Animals were bled each week for sera and for CBC. The experimental plan is illustrated in Fig. III. The surgical setup and a newly grafted monkey's scalp are shown in Fig. V.

Long term experiments to determine how long grafts would survive if treatment was continued were performed using the ATS and ATAB as suppressive agents. Skin grafts were placed in the mid scapular area (2 homografts and 2 autografts) and animals were kept in cages rather than chairs. Animals received a total of 75 mg of IgG/kg (30-30-15) on day + 1, + 2, and + 3 and then starting on day 10, 10 mg of IgG/kg/week until rejection. At the time of rejection one animal from each group was sacrificed and complete gross and microscopic pathological examinations were performed. Animals were bled twice during the first week and then at weekly intervals for sera and CBC. Urine samples were obtained weekly. Complete blood chemistries were performed weekly. The experimental plan is illustrated in Fig. IV.

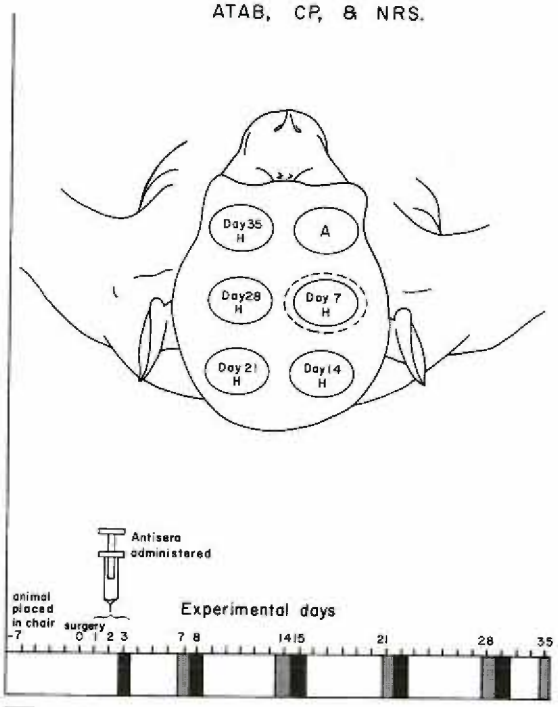
Dose Response Curve: In order to determine whether there was a dose dependent relationship in the *in vivo* suppression of the purified antibody, additional animals were grafted on the head in the manner previously described. Three animals received 35 mg of IgG/kg of purified

Fig. III

A. Experimental Design

B. Rhesus monkey in restraining chair. The hands are prevented from reaching scalp area where grafts are placed. Animals are hand fed.

Skin grafts in Rhesus monkeys treated with ATS, ATAB, CP, & NRS.



Biopsy

Bled for CBC & Sera for Ab assay.

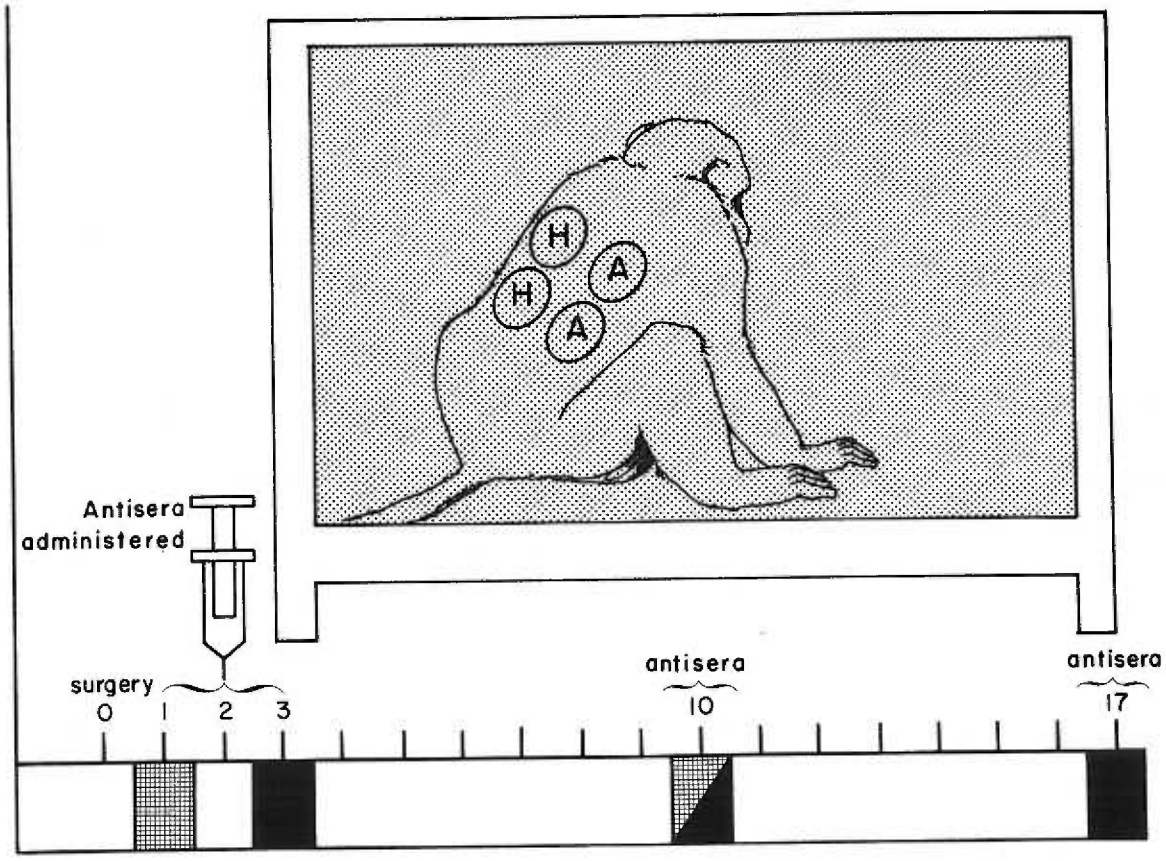
A=autograph-control

H=homograph-one removed every 7 days after placement.

Fig. IV

Experimental design of long-term experiments

Skin grafts in animals receiving extended treatment with ATS or ATAB.



SRBC

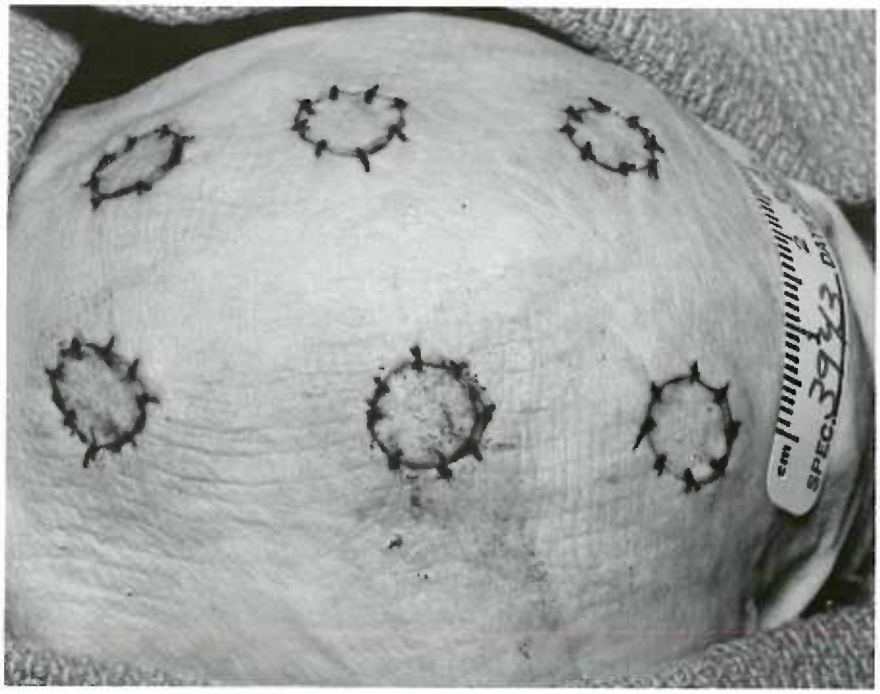
Bled-urine

Continued until rejection occurs.

Fig. V

A. Surgical set-up for skin transplants demonstrating the direct transfer of skin grafts.

B. Scalp immediately after completion of transplantation.



antibody and one received 100 mg/kg. Grafts were removed at weekly intervals as previously described. Blood samples were taken as described.

Surgical Procedures - Skin Grafting: Pairs of animals were anesthetized (2 liters of N_2O + 4 liters of O_2 at 0.7-1% Halothane by mask) and either the scalp in the short term experiments or the midscapular area of the back in the long term experiments were antiseptically prepared for surgery. Full thickness grafts (10 mm) from both animals were cut, debrided of adipose tissue, exchanged and sutured immediately into the analogous site on the paired animals. When grafts were placed on the scalp, 5 homografts were exchanged and one autograft was cut, rotated 180° and resutured into the same site. When grafts were placed on the back, 2 homografts and 2 autografts were performed. Bicillin CR (600,000 units) was administered intramuscularly routinely after surgery.

Biopsy: In the short term experiments, 1 homograft was removed each week until rejection occurred (+ 7, + 14, + 21, + 28, + 35) for histological evaluation. Biopsy was performed under light anesthesia. A clear margin of tissue was excised around the graft and the surgical site was secured with wound clips.

RESULTS

In vitro properties of the isolated antibody.

The percentage recovery of total protein and IgG of the two fractions (ATAB, CP) isolated from the immunoadsorbent from three separate experiments are shown in Table I. The left hand side of the table indicates the mg of IgG and total protein present in ATS applied to the immunoadsorbent. The right hand side of Table I indicates the recovery of IgG and protein obtained in both fractions. This procedure provided almost complete recovery of total protein. In experiment B, more than 100% recovery was calculated indicating a margin of error in this method of calculations based upon planimetry of 15%. The antibody fraction contained 18 to 26% of the total IgG and 6 to 9% of the total protein applied. The results of the three separate experiments demonstrate the reproducibility of this method.

The specific activities of both fractions from the immunoadsorbent column are recorded in Table II in terms of leukagglutinin activity (reciprocal of LA titer per mg of IgG). The experiments reported here are the same three reported in Table I. The specific activity of the antibody was higher than that of the column passage in all cases, and in each case was increased over the original serum.

Isolated antibody was applied to the immunoadsorbent column to determine whether the antibody retained its binding capacity for the adsorbent after concentration and dialysis. It bound and was eluted in the same manner as when whole sera was applied.

Various amounts of normal rabbit sera (NRS) were applied to the immuno-adsorbent to evaluate the degree of nonspecific binding of protein.

TABLE I

Recovery of Total Protein and IgG in Fractions Eluted from the Specific Immunoadsorbent

Exp	Total	Serum[1] mg	ATAB[2] mg	ATAB[2] %	CP[3] mg	CP[3] %	Total %
A	Protein	1484	87	6	1364	92	98
	IgG	435	80	18	269	62	80
B	Protein	1946	179	9	2085	108	116
	IgG	683	166	26	460	72	98
C	Protein	1484	120	8	1357	91	99
	IgG	435	103	23	264	60	84

(1) Concentration based upon planimetry

(2) Antibody fraction eluted with 0.1M Glycine-HCl, pH 2.5

(3) Pooled Peaks I, II and III eluted with initial buffer (C.S. pH 6.8)

TABLE II

Recovery of Specific Activity of Isolated Fractions from the Immunoadsorbent

Exp.	Fraction[1]	LA[2]	Protein	LA/mg Protein	IgG mg/ml	LA/mg IgG
A	ATAB	3072	17	173	16	191
	CP	384	160	2	32	12
B	ATAB	1536	36	43	33	46
	CP	192	190	1	41	5
C	ATAB	3072	24	128	21	149
	CP	384	90	4	18	22

(1) Specific activity (LA/mg) of ATS based upon mg of protein =8, on mg of IgG=26

(2) LA- reciprocal of the leukagglutinin titer

Less than 5% of the NRS was bound to the immunoadsorbent and eluted with 0.1M glycine-HCl, pH 2.5.

Maximum transformation by the three materials (ATS, ATAB, and CP) used as mitogens was obtained at a level of 6 mg IgG per culture tube. The maximum level of transformation (CPM - control CPM) is shown in Table III. There is a 10-fold difference in the mitogenic activity between the serum and antibody fraction which is not recovered in the CP fraction. Similar transformation experiments have been repeated several times with other preparations of these fractions, and the results have been essentially the same.

A "recombination" experiment was performed in which various quantities of antibody were mixed with column passage and added to culture tubes to determine whether the transforming capacity lost on separation of the two fractions could be recovered. The total concentration of IgG/culture tube was maintained at 6 mg/tube since that concentration gave the maximum amount of transformation in earlier experiments. The results reported in Table IV indicate that transforming activity is not recovered when fractions are recombined. This may indicate either denaturation or loss by dialysis of a potentiating factor necessary for transformation during the isolation procedure.

Goat antihuman lymphocyte globulin applied to the rhesus stroma immunoadsorbent column was found to bind and elute in the same manner as the antirhesus lymphocyte serum. The activity of the eluted antibody is shown in Table V. Specific activity is calculated based upon both the leukagglutinins per mg IgG and cytotoxicity per mg IgG. The specific activity based on the leukagglutinin titer increased

TABLE III

TRANSFORMATION OF RHESUS PERIPHERAL LYMPHOCYTES BY ANTI-THYMOCYTE SERUM AND THE TWO FRACTIONS SEPARATED BY IMMUNOADSORBENT

	mg IgG	CPM
ATS	6	32557
ATAB	6	3258
CP	6	202

TABLE IV

TRANSFORMATION BY RECOMBINED FRACTIONS OF ISOLATED ANTIBODY (ATAB) AND COLUMN PASSAGE (C.P.)

ATAB/tube (mg IgG)	CP/tube (mg IgG)	C.P.M.
6.0	-	114
5.4	0.6	705
4.8	1.2	86
4.2	1.8	46
3.6	2.4	28
3.0	3.0	37
2.4	3.6	34
1.8	4.2	42
1.2	4.8	22
.6	5.4	33
-	6.0	164

Controls: ATS 6mg IgG/tube 15,176 CPM
NRS 6 mg IgG/tube 49 CPM

TABLE V

RECOVERY OF SPECIFIC ACTIVITY OF GOAT ANTI-HUMAN LYMPHOCYTE ANTIBODY ISOLATED FROM IMMUNOADSORBENT CONJUGATE WITH RHESUS LYMPHOCYTE STROMA

	IgG mg/ml	LA[d]	Cyt[c]	LA/mg IgG	Cyt/mg IgG
Globulin[a]	12	500	5,000	42	410
Antibody[b]	10	5,00	10,000	515	1,030

[a] Goat antiglobulins prepared by ammonium sulfate precipitation

[b] Eluted with glycine HCl pH 2.5

[c] Cytotoxin

[d] Leukaglutinin

10-fold and the specific activity based on cytoxic titer greater than 2-fold.

Immunosuppressive properties of the purified antibody

Short term experiments

Graft survival in test animals in the short term experiments are reported in Table VI. The mean survival of control animals treated with normal rabbit serum (NRS) was 11 days confirming earlier observations (95). Rejection in one animal (3577) treated with ATS occurred at 21 days. Rejection had not occurred when the final homograft was removed for biopsy from animals 1390 and 399. The animals treated with the serum components which passed through the specific adsorbent column (CP) rejected at 15 and 18 days. Animals treated with the purified antibody showed significant extension of graft survival. Animal 89 rejected on day 25 while neither of the other two animals had rejected when the last graft was removed for biopsy. The results indicate that the purified antibody is as effective as whole sera in extension of homograft survival in rhesus monkeys at one half the IgG level.

Animals were observed daily for changes in skin grafts. In Figure VI, the daily changes in a control animal are shown. On the first two days after surgery some grafts show a slight erythema as seen in one graft on this animal while the remainder are pallid. On days 3 and 4, cyanosis edema and small hemorrhagic spots are present in most homografts. By the seventh day when the first biopsy was taken, grafts were darker and often a serous exudate was present. On the ninth or tenth day, a dramatic change occurred. Large hemorrhagic

TABLE VI

EXTENSION OF SKIN GRAFT SURVIVAL IN RHESUS MONKEYS TREATED WITH RABBIT ANTI-RHESUS THYMOCYTE ANTIBODY

Treatment	Dosage mg IgG/ kg	Animal No.	Day of Graft Rejection
NRS	150	3573	9
		1780	11
		3958	11
ATS	150	399	> 28
		1390	> 21
		3577	21
CP	150	2148	15
		400	18
ATAB	75	2706	> 28
		89	25
		86	> 35

NRS-normal rabbit sera; ATS-anti-thymocyte sera, CP-column passage; ATAB-antithymocyte antibody; > indicates when the last homograft was removed for histological examination before rejection occurred.

Fig. VI

Sequence of Graft Rejection in Control Animal

Day 1

All grafts are pallid including autograft (right front)

Day 3

Slight petechial hemorrhage and serous exudate are present

Day 7

One graft was removed for biopsy. The site was secured with wound clips

Day 8

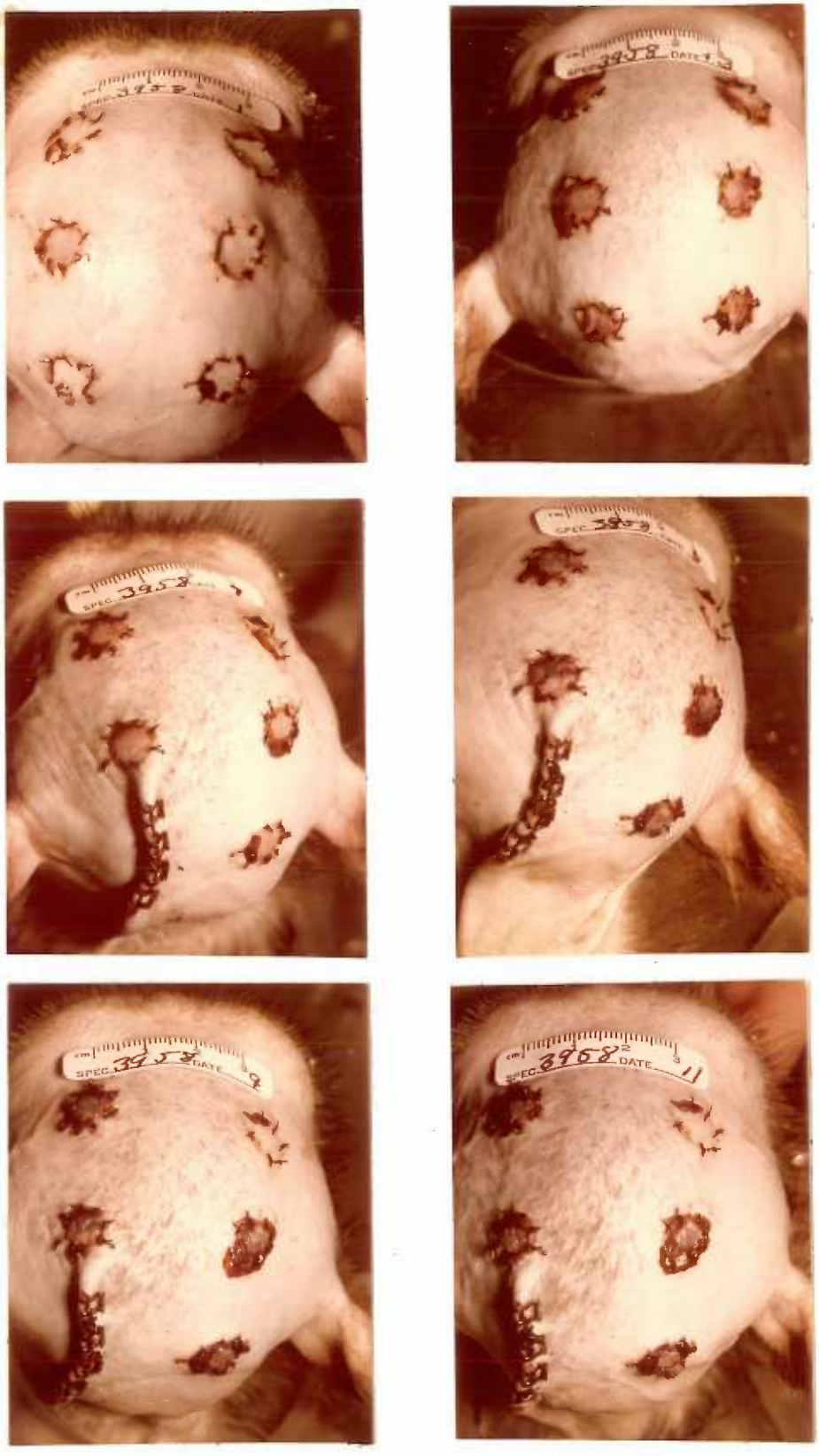
Rejection is beginning. Large petechial hemorrhages are present in all homografts. The autograft is clear

Day 9

More widespread hemorrhage

Day 11

Rejection is complete. All homografts are completely hemorrhagic



areas were present on all homografts. In this animal the autograft remained extremely clear. Rejection was complete by the eleventh day since all homografts were completely hemorrhagic. In suppressed animals, changes were delayed and rejection was more chronic. The first sign of rejection was large hemorrhagic areas followed by rejection about three days later.

Changes in the total white blood count are reported in Table VII. The count dropped initially in all animals, including the controls. In animals treated with either ATS or ATAB, the count remained in the low normal range for an extended period. This suggests that graft survival can be sustained without destroying host resistance to infection.

Clinically, the difference between the ATAB and the ATS suppressed animals was striking. Appetite was affected in all animals but the antibody treated animals continued eating even during treatment while the ATS treated animals did not eat for four days. The antibody treated animals also took fluids better than the ATS treated animals. Injection sites healed rapidly in ATAB treated animals while local edema followed by enduration was present for several days in ATS treated animals.

The level of anti-rabbit antibody in animals receiving treatment only during the first three days after surgery as measured by passive cutaneous anaphylaxis in guinea pigs is reported in Figure VII. In the NRS treated controls, anti-rabbit antibody was present by the eighth day and continued to increase through the twenty ninth day. The highest level of antibody measured was in the animals treated

TABLE VII

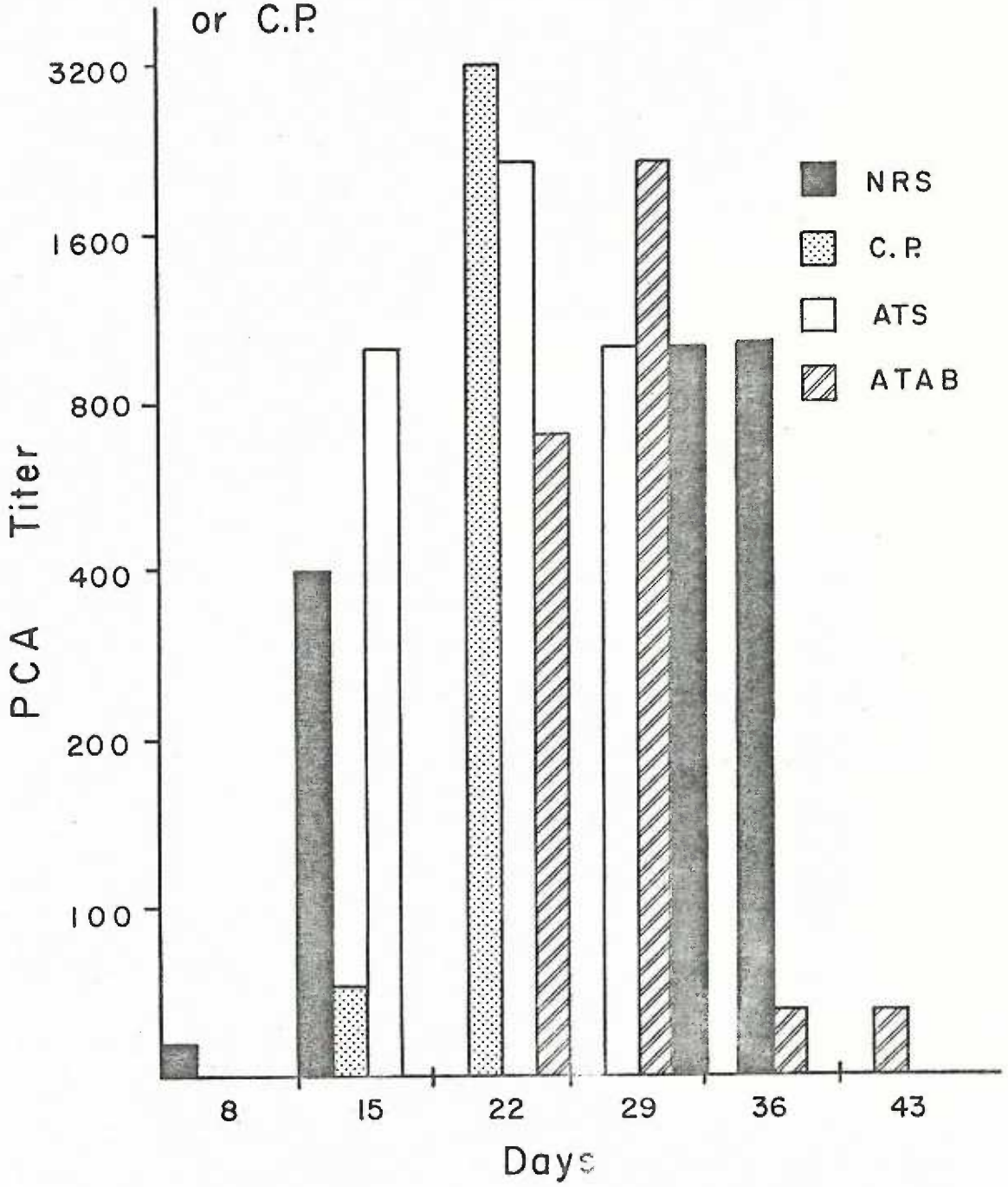
CHANGES IN CIRCULATING [1] WBC DURING TREATMENT WITH ATS, ATAB, CP, OR NRS

Treated with	Animal No.	WBC/mm^3 X 10^3 Experimental Day 0	3	8	15	22	29	36
ATS (150 mg IgG/kg)	399	7.4	3.8	3.4	2.9	7.2	6.5	
	1390	17.5	4.6	5.1	6.6	6.4		
	3577	13.3	6.4	4.0	6.0	3.8		
ATAB (75 mg IgG/kg)	2706	8.6	8.1	8.6	N.D.	8.1	5.7	
	89	15.2	4.6	4.4	5.7	5.2	4.5	6.3
	86	23.0	3.2	5.2	4.3	7.0	5.3	10.7
CP (150 mg IgG/kg)	2148	10.3	9.2	8.0	7.5	7.1		
	400	9.5	7.0	13.0	11.8	10.8		
NRS (150 mg IgG/kg	1780	23.3	10.6	8.3	10.3			
	3573	11.6	11.4	3.0	8.2			
	3958	6.5	5.2	7.2	4.9			

(1) Performed by Coulter Counter

Fig VII

Anti-Rabbit Antibody Formation in Rhesus Monkeys Treated With NRS, ATS, ATAB or C.P.



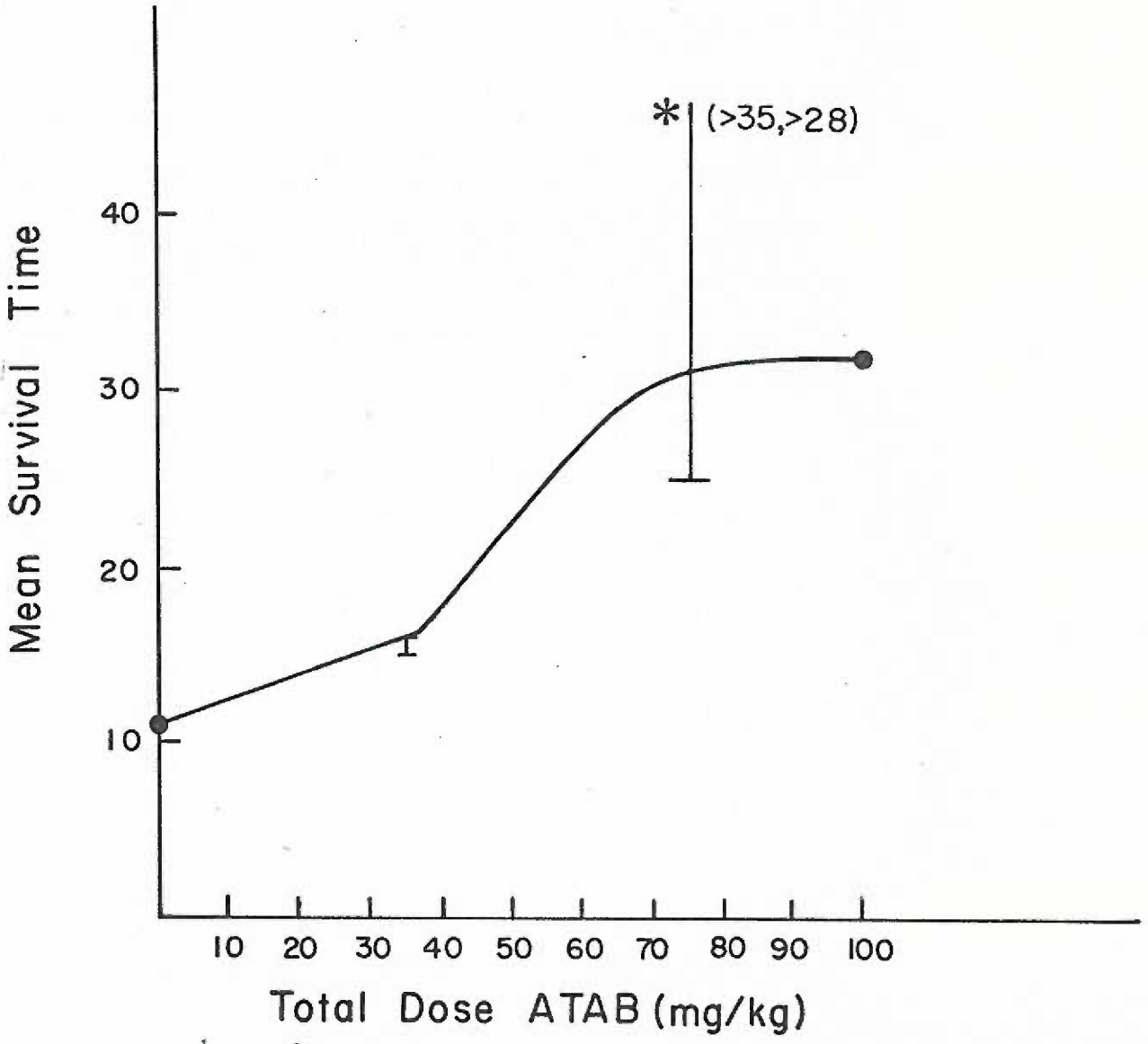
with CP. The total protein concentration was higher in this material than any of the other agents. The ATS treated animals had developed antibody to rabbit sera by the fifteenth day and it remained at a high level through the thirty-sixth day. No measurable anti-rabbit antibody was present in the ATAB treated monkeys until the twenty-second day. The antibody level peaked on the twenty-ninth day and then dropped off. The results indicate that the ATS and ATAB suppress antibody formation against themselves. This suppression is overcome by the fifteenth day in the ATS treated animals but not until the twenty second day in the ATAB treated animals. According to the literature, ATAB is cleared very rapidly but these results indicate that the immune response to it is only delayed, not prevented.

Skin homograft survival in rhesus monkeys treated with various doses of purified antibody is illustrated in Figure VIII. Three monkeys treated at a total dosage of 35 mg IgG/kg rejected their skin homografts at days 15, 15 and 14. One monkey treated with 75 mg IgG/kg rejected on the twenty-fifth day but the other two treated at this level did not reject. The one animal treated with 100 mg IgG/kg rejected on the thirty second day. The results indicate that the immunosuppression is dose dependent and that the best suppression occurs at 75 mg IgG/kg.

The levels of anti-rabbit antibody formation in these same animals is recorded in Figure IX. The animals treated with 35 mg IgG/kg had high levels of antibody by the fifteenth day. The level of antibody had dropped by the twenty-second day. In the animals treated with 75 mg IgG/kg, no antibody was measured until the day 22, when a low level was present. The maximum titer was present on day 29. By

Fig VIII

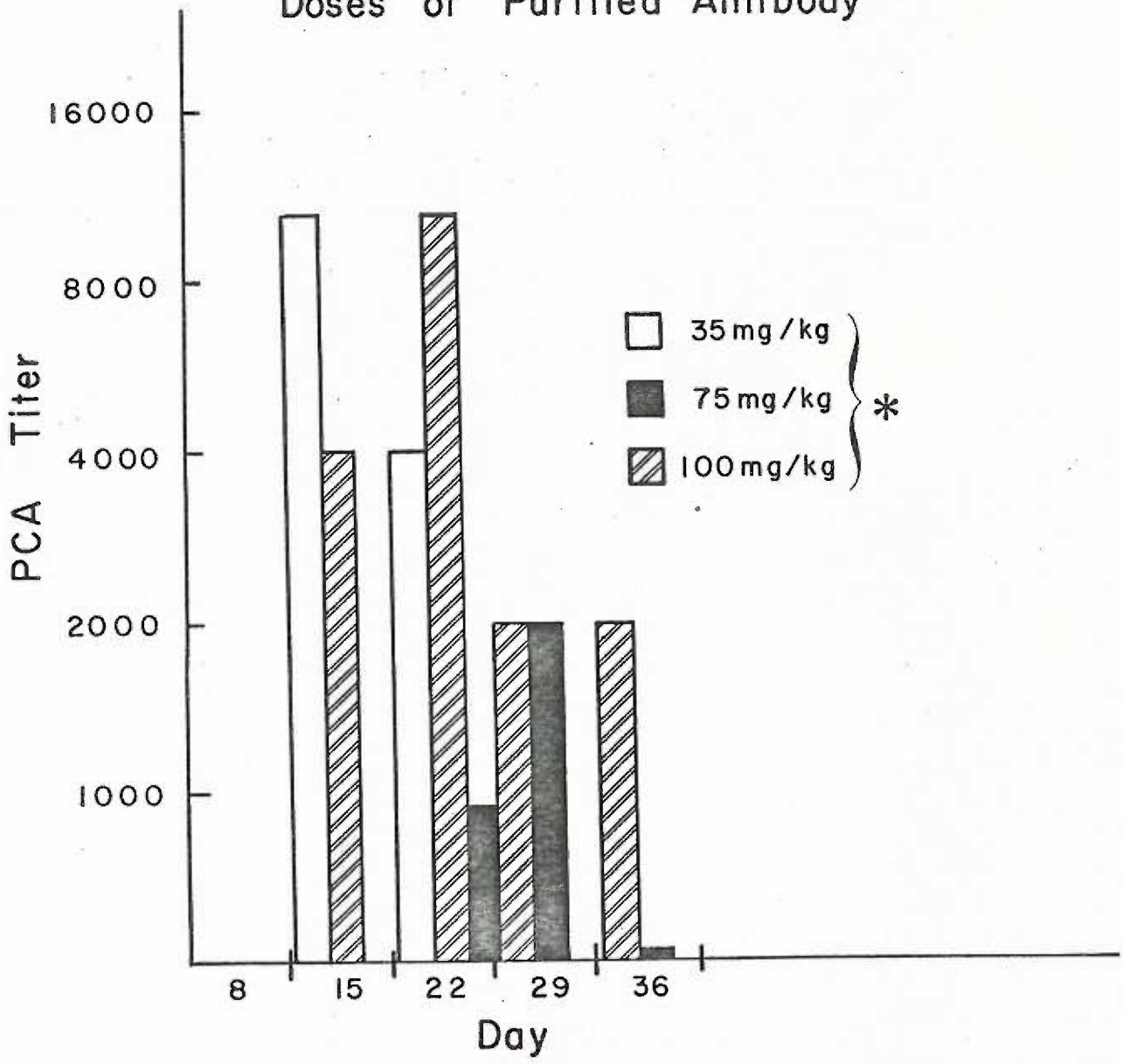
Skin Graft Survival in Rhesus Monkeys Treated With Various Doses of Purified Antibody



*Rejection did not occur before the last graft was biopsied.

Fig IX

Anti-rabbit Antibody Formation in Rhesus Monkeys Treated with Various Doses of Purified Antibody



* Total dosage administered during the first 3 days after surgery.

day 36, very little anti-rabbit antibody was measurable. In the animal treated with 100 mg IgG/kg, the peak titer was measured on the twenty-second day but the level did not drop off as rapidly as in the animals treated with 75 mg/kg. This again indicates that the 75 mg/kg is the best dosage since at 35 mg/kg, there is not enough ATAB present to suppress formation of antibody against itself. At 100 mg/kg, too much is present and again, antibody is formed and the titer decreases slowly.

The effect on the circulating white blood cell count is recorded in Table VIII. An initial decrease is present in all animals with little difference discernable.

As indicated in the experimental plan (Fig. III - Materials and methods section), grafts were removed each week after treatment for histological evaluation until all grafts were rejected. Photomicrographs were prepared to illustrate the histology of rejection in control and treated animals. In Figure X, PAS stains of a seven and a fourteen day biopsy are shown. The PAS stain is helpful in following the movement of the basement membrane. The first picture shows the new epidermis migrating under the grafted tissue. The second picture is a magnification of the migrating epidermis. By the fourteenth day, rejection had occurred. The epidermis is compact and dead. Again, the migrating epidermis is obvious and the front is enlarged in the second picture. Rejection is not as easy for the layman to recognize with the PAS stain as with the hematoxylin-eosin stain.

The next set of pictures, Fig. XI, were chosen because they

TABLE VIII

EFFECT ON THE CIRCULATING WBC OF VARIOUS DOSES OF PURIFIED ANTIBODY

Treated with	Animal No.	WBC/mm^3 X 10^3 Experimental Day 0	3	8	15	22	29	38
ATAB 35 mg/kg	3769	7.4	6.9	5.4	6.1	5.3		
	3196	9.0	6.2	3.5	19.2	19.8		
	3351	10.3	8.3	8.1	25.0	26.9		
ATAB 75 mg/kg	2706	8.6	8.1	8.6	N.D.	8.1	5.7	
	89	15.2	4.6	4.4	5.7	5.2	4.5	6.3
	86	23.0	3.2	5.2	4.3	7.0	5.3	10.7
ATAB 100 mg/kg	3975	9.3	4.4	4.8	7.6	7.7	9.1	7.6

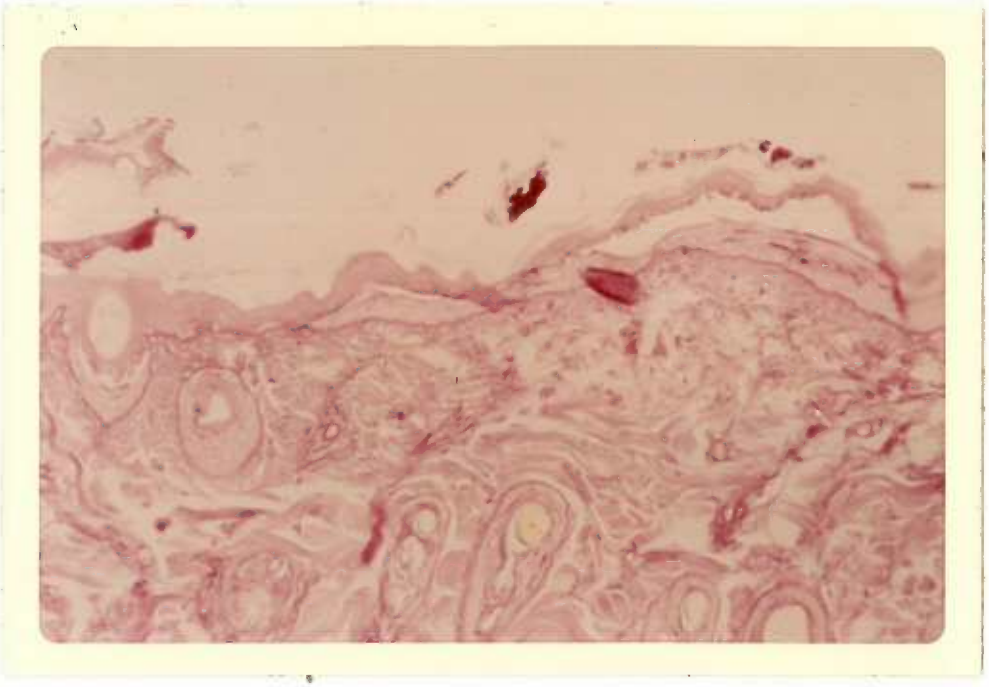
Fig. X

Sequence of Rejection in Normal Rabbit

Sera Treated Control Animal

A. PAS stain of a full thickness skin biopsy (day 7) from the scalp of Macaca mulatta 3573 treated with NRS (16X). The epidermis can be seen advancing under the grafted tissue.

B. As above (50X).



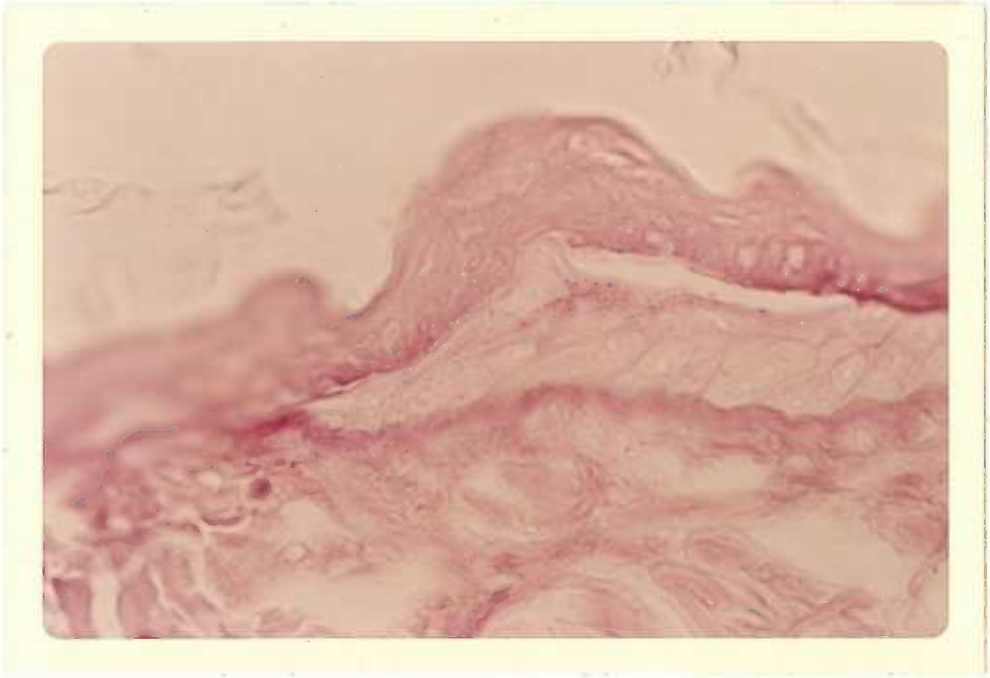
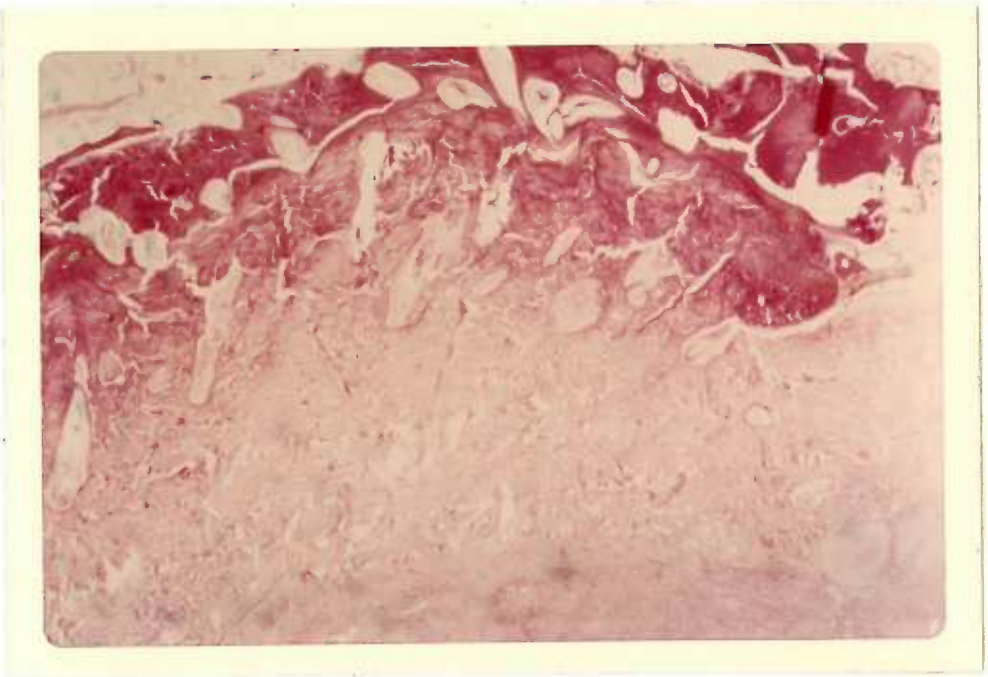


Fig. X

(Cont'd)

C. PAS stain of full thickness skin biopsy (day 14) from the scalp of Macaca mulatta 3573 treated with NRS (16X). Graft is compacted and has already rejected.

D. As above (50X).



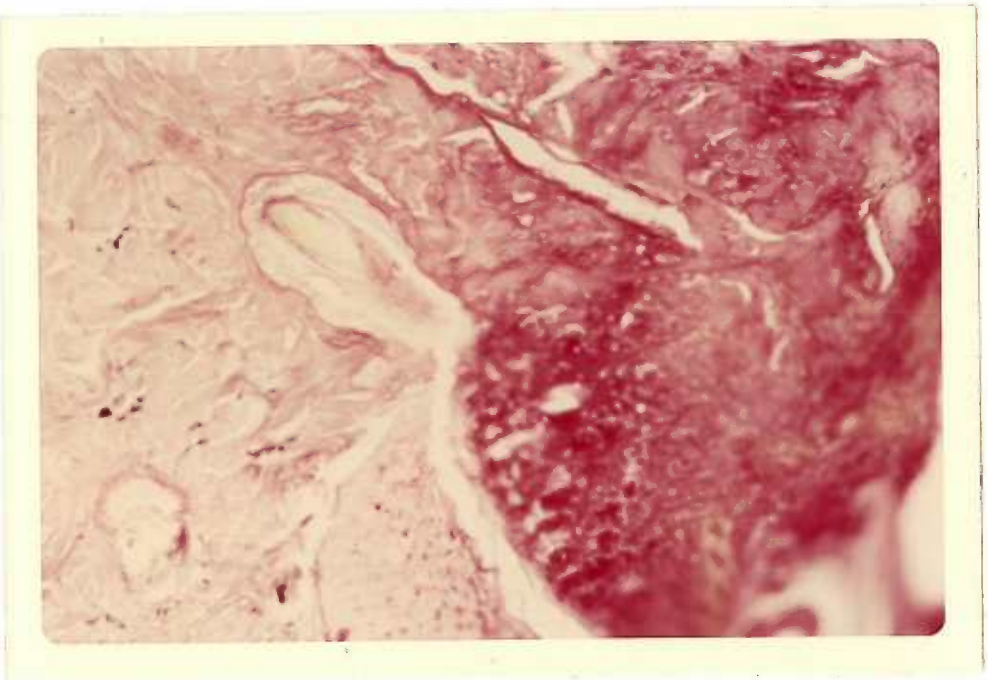
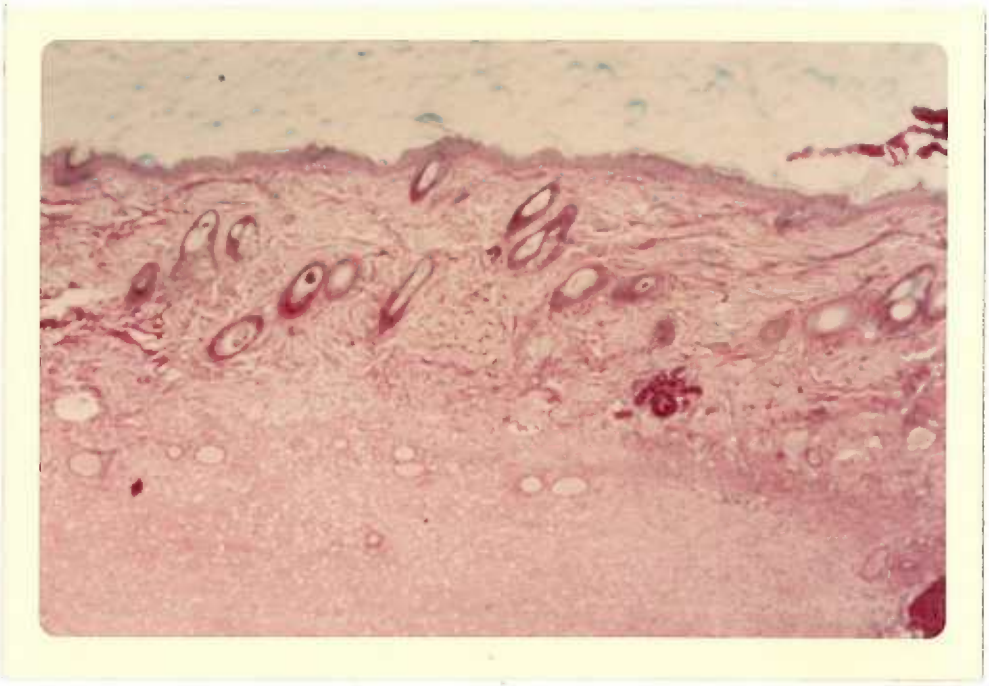


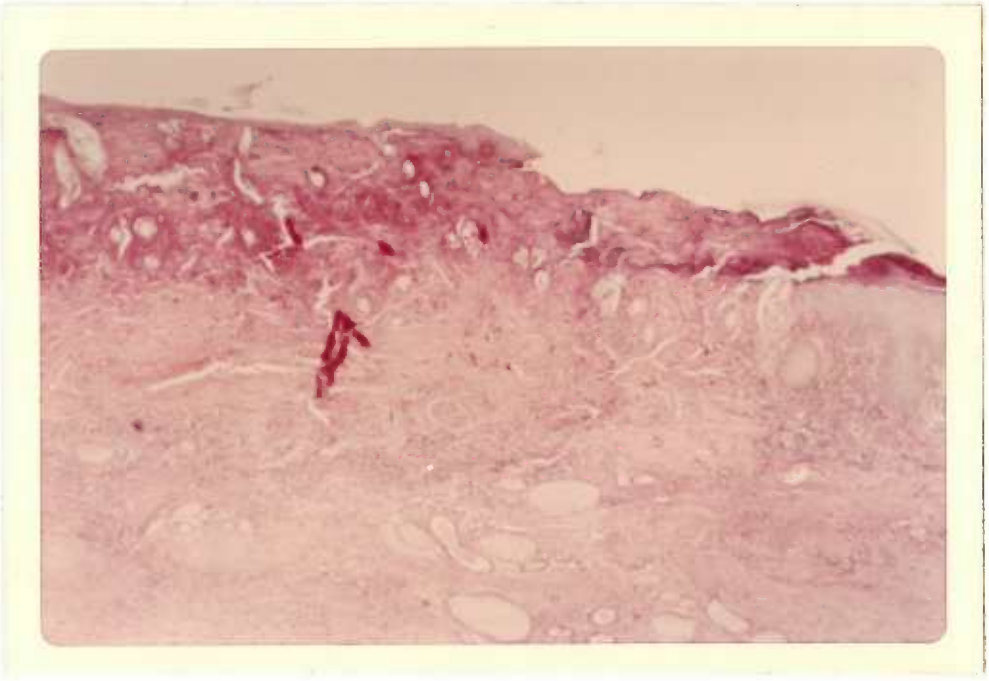
Fig. XI

Sequence of Graft Rejection in Rhesus Monkey Treated with a Low Dose of Purified Antibody

A. Hematoxylin-eosin stain of a full thickness skin biopsy (day 7) from the scalp of Macaca mulatta 3769 who had been treated with 35 mg/kg ATAB (16X). The skin looks normal.

B. Hematoxylin-eosin stain of a full thickness skin biopsy (day 14) from the scalp of Macaca mulatta 3769 who had been treated with 35 mg/kg ATAB (16X). Rejection has begun. Notice the compact nature of the grafted tissue, and the lifting of the advancing epidermis.





taken from animal 3769 which was treated with 35 mg/kg ATAB and rejected on the sixteenth day after surgery. Grafts were taken at day 7, 14, and 21 and illustrate the sequence of events during rejection. In the first picture, the skin is normal with no indication of graft rejection. By the fourteenth day, the rejection process had started as evidenced by the lifting of the grafted tissue by the new epidermis. By the twenty-first day, rejection was complete. The rejected skin is compact and raised from the graft bed. The new epidermis is advancing rapidly.

The next picture, Fig. XII, shows non-rejection on the thirty-fifth day in animal 86 who was treated with ATAB. The stain is a little dark but the skin graft is definitely viable.

The next set of pictures, Fig. XIII, the sequence on non-rejection in an ATS suppressed animal is shown. The first picture taken at 14 days shows normal grafted tissue. In the next picture (day 21) a lymphocytic infiltrate is present but the graft had not rejected. The epidermis can be seen beginning to grow under the graft. The next picture from the same day illustrates the lymphocytic infiltrate. On day 28, as shown in the last picture, rejection still has not occurred.

Long term experiments

The experimental plan is illustrated in Fig. IV of the Materials and Methods section. The three animals treated with 75 mg IgG/kg of ATS rejected at the same time control animals had in the short term experiments. This was expected since 150 mg/kg was used in the short term experiments. One of the animals from this group,

Fig. XI

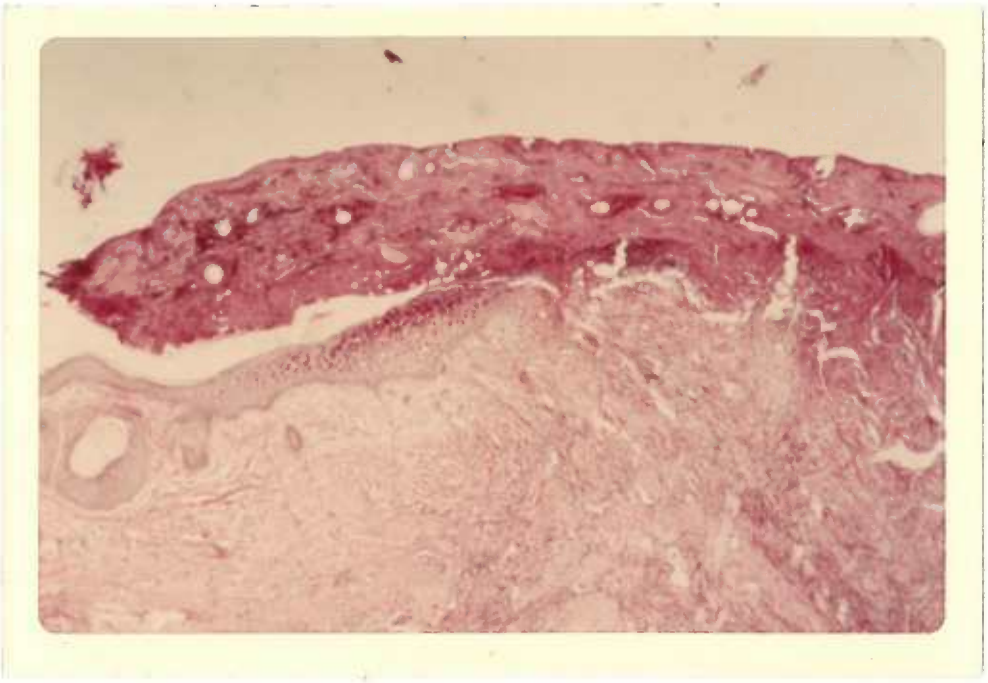
(Cont'd)

C. Hematoxylin-eosin stain of a full thickness skin biopsy (day 21) from the scalp of Macaca mulatta 3769 who had been treated with 35 mg/kg ATAB (16X). Rejection was scored on day 15. This is a good example of how the graft is lifted out by the advancing epidermis.

Fig. XII

Nonrejection in a Rhesus Monkey who Received a Suppressive Dose of Purified Antibody

Hematoxylin-eosin stain of full thickness skin biopsy (day 35) from the scalp of Macaca mulatta 86 treated with 75 mg/kg ATAB (16X). Rejection has not occurred and the graft looks like normal skin.



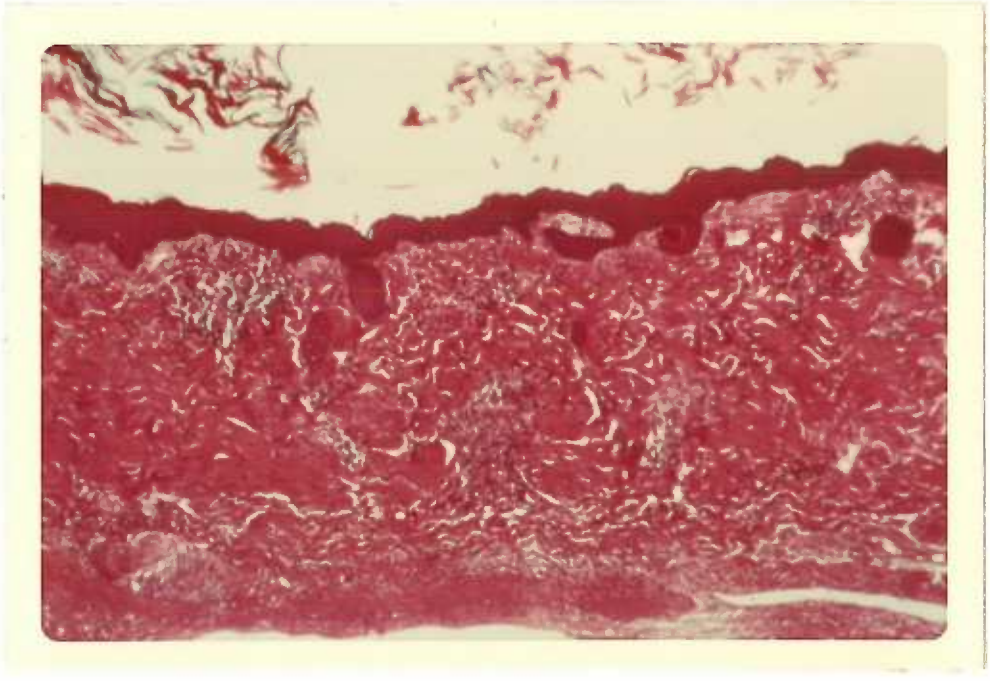


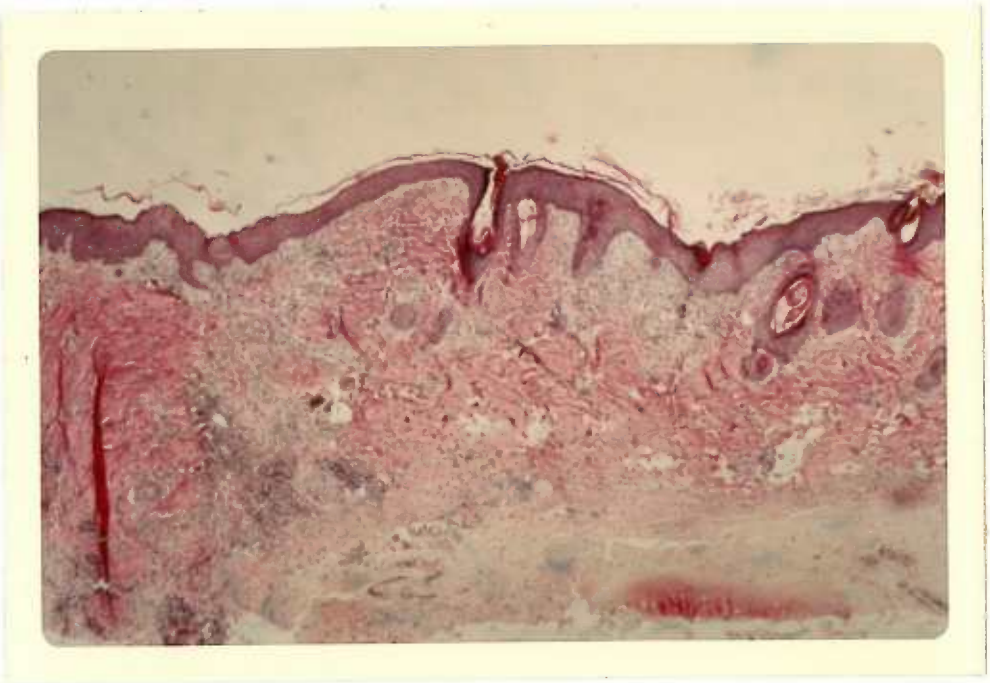
Fig. XIII

Sequence of Histological Events in Rhesus Monkeys

Treated with Antithymocyte Sera

A. Hematoxylin-eosin stain of a full thickness skin biopsy (day 14) from the scalp of Macaca mulatta 399 treated with 150 mg/kg ATS (16X). The skin looks normal.

B. Hematoxylin-eosin stain of a full thickness skin biopsy (day 21) from the scalp of Macaca mulatta 399 treated with 150 mg/kg ATS (16X). Lymphocytic infiltrate is apparent. The epidermal front is beginning to advance.



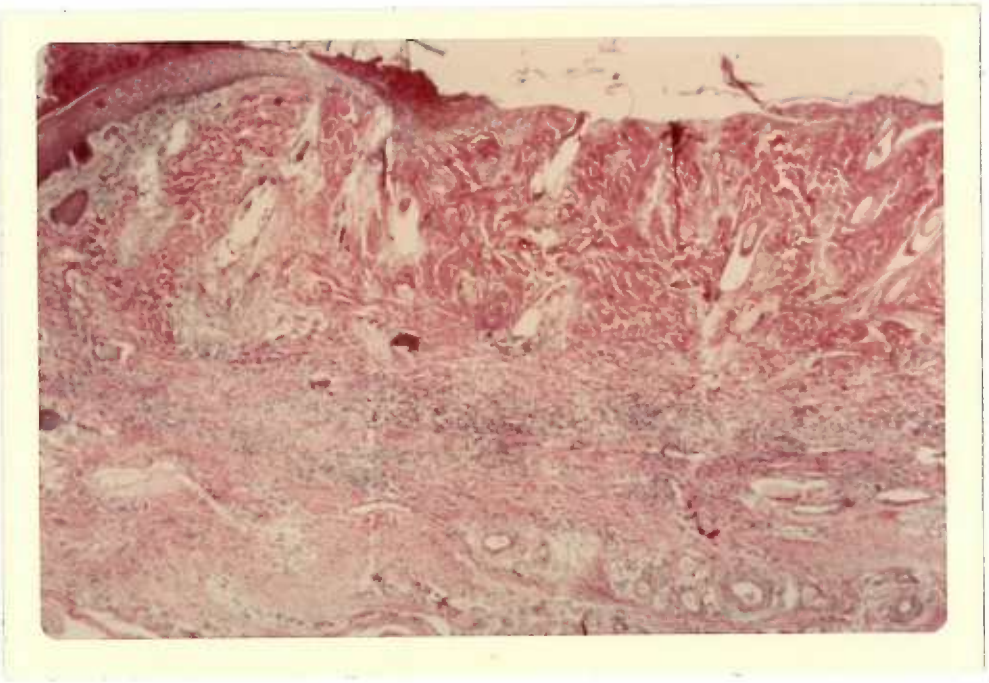
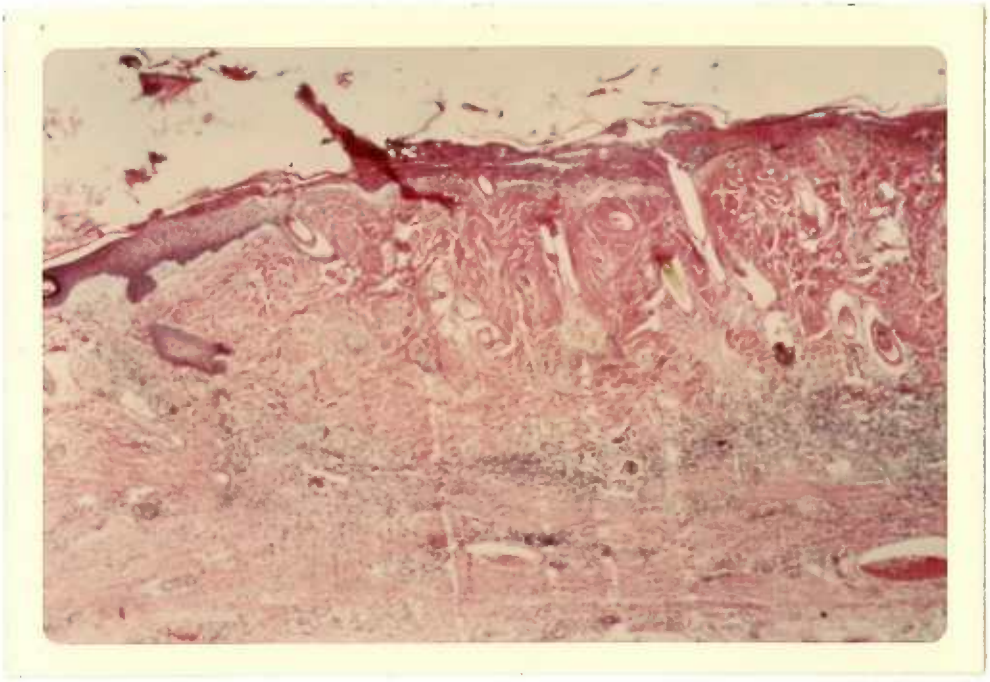


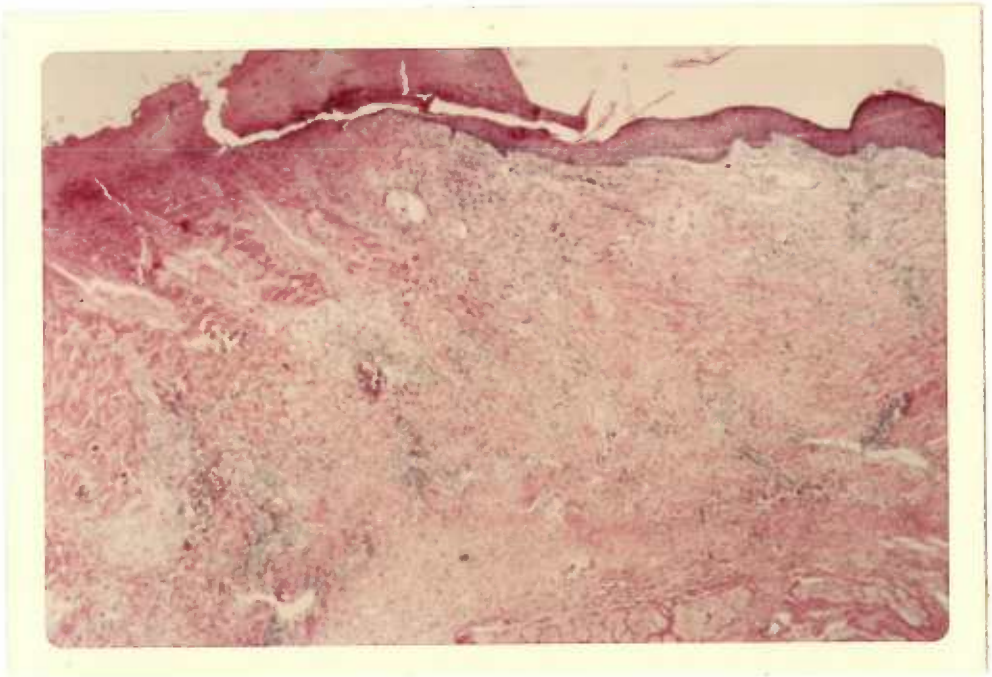
Fig. XIII

(Cont'd)

C. Hematoxylin-eosin stain of a full thickness skin biopsy (day 21) from the scalp of Macaca mulatta 399 treated with 150 mg/kg ATS (16X). The skin graft has not rejected. Lymphocytic infiltrate is apparent.

D. Hematoxylin-eosin stain of a full thickness skin biopsy (day 28) from the scalp of Macaca mulatta 399 treated with 150 mg/kg ATS (16X). The tissue has not rejected. Lymphocytic infiltrate is apparent.





3305, was sacrificed on the fourteenth day after surgery since rejection had occurred. A complete pathological examination was performed and no deviation from normal was found either by gross or microscopic examination. At autopsy, samples of spleen, nodes, liver, kidney, and thymus were examined by fluorescent microscopy for the presence of antigen-antibody complexes and none were found.

In the animals treated with 75 mg/kg ATAB initially followed by 10 mg/kg each week, recognizable rejection did not occur. After day 35, it was very difficult to recognize rejection. The grafts remained flat and readable up to that time but then, both the homografts and autografts shrunk as in wound healing. In the first animal, 3952, on day 38 both the homograft and autografts had a tiny scab in the center. On day 44, one homograft and one autograft were removed for histological examination to determine whether rejection had occurred. Histologically the homograft and autograft were identical, and it did not appear that rejection had occurred. In Fig. XIV, photomicrographs of the tissue are included. The homograft is shown in the first picture and two pictures of the autograft are shown. Treatment was continued to day 45 on this animal and no further changes in the remaining grafts occurred. The results of the pathological examination were negative. No changes in any tissue were found. The kidneys were examined very closely for changes and none had occurred. The fluorescent antibody examination of the kidney, spleen, nodes, thymus and liver were also negative. This indicates that even after extensive treatment, no detectable antigen antibody complexes have been deposited in the above tissues. Weekly urinalysis examinations confirmed that

Fig. XIV

Appearance of Skin Homografts and Autografts in Rhesus Monkeys after Extended Treatment with Purified Antibody

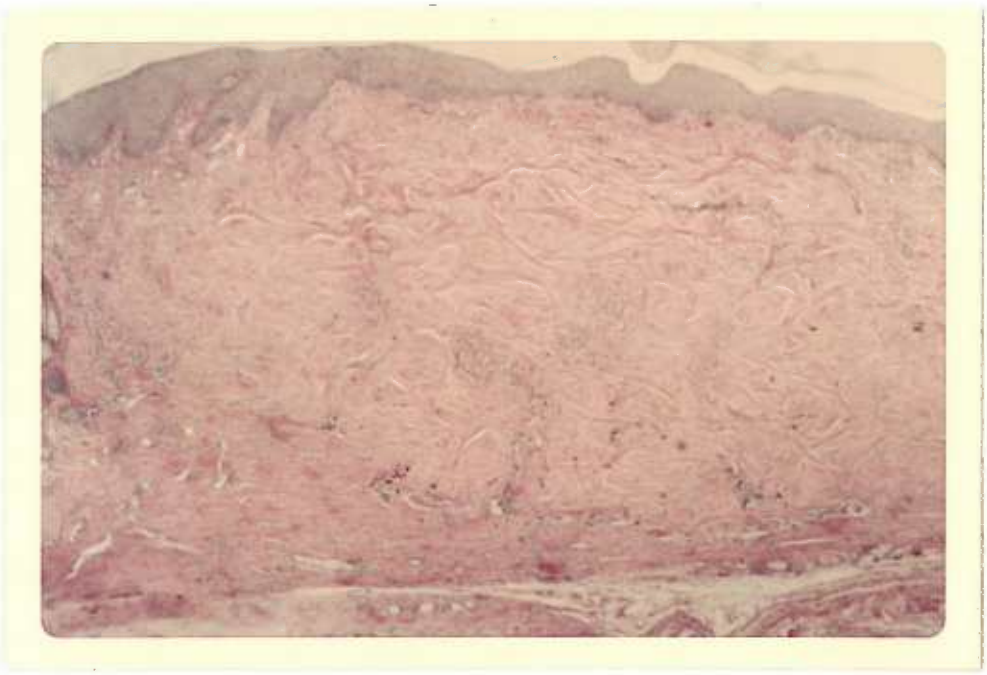
A. Hematoxylin-eosin stain of a full thickness skin homograft biopsy (day 44) from the mid-scapular area of Macaca mulatta 3952. The animal was treated with ATAB for an extended period. The skin looks normal indicating that no rejection had occurred (16X).

Fig. XIV

(Cont'd)

B. Hematoxylin-eosin stain of a full thickness skin autograft (day 44) biopsy from the mid-scapular area of Macaca mulatta 3952. The animal was treated with ATAB for an extended period. The tissue looks just like the homograft (16X).

C. As above.



no complexes were deposited in the kidneys since protein was never excreted in the urine during any of the long term animal experiments.

Animal 4535 had a similar course. On day 27, a tiny scab was present on both homografts. By day 45, both the homograft and autograft sites looked the same. One homograft and one autograft were removed for biopsy. Treatment was stopped after day 38 to see if there would be any change in the remaining graft. None has occurred for 80 days.

The final long term animal, 3191 was frought with difficulty. One homograft was a technical failure and the second graft was knocked off by an animal handler on day +24. It had not rejected at that time. Treatment was continued on the animal to assess at least the antigenicity of the suppressing antibody. To attempt to clarify the question of whether rejection was occurring at the time the slight scabbing was present in the other animals, a second graft from the original donor was placed on the head of 3191 on day 36. He received his last antibody treatment on day 38. If he was sensitized, he should have rejected the graft in a second set type of rejection in four to six days (95). The graft did not reject until the ninth day. It was not a white graft as has been described (95) but looked exactly like a first set rejection. Three days after the second graft rejected we placed a third transplant from the original donor. One of the homografts was incubated in ATAB for 1 hour before placement and the second was incubated for the same period in PBS. The graft incubated in PBS was rejected 9 days later while the antibody treated graft rejected 14 days later. This indicates that antibody can work

at the graft site. The absence of a second set rejection indicates that the animal was not sensitized to the donor tissue.

In the course of the long term experiments the circulating blood picture was monitored weekly. The total white blood cell counts are recorded in Table IX. As in the short term experiments, after an initial decline, the counts return to normal values.

Electrolytes, glucose, inorganic phosphates, creatinine, blood urea, nitrogen, uric acid, cholesterol, total protein, albumin, bilirubin, alkaline phosphatase, lactic dehydrogenase, and transaminase levels were also monitored weekly. On one animal, 3952, the potassium level was increased on the third treatment day but then dropped down to normal levels and remained there. There were no other significant changes in any of the other animals studied.

The levels of antirabbit antibody formation in animals treated for extended periods with ATAB are recorded in Fig. XV. In animal 3952, the early response pattern is the same as seen in the short term animals receiving a single set of treatments. The response was slow to develop and the maximum titer occurred a week later. It was sustained at a high level and then dropped to 2000 and remained there through termination. Treatment was continued through day 45 on this animal. The level of antibody at that time was higher than the peak titer in animals receiving only initial treatment (Fig. VII).

In animal 4535, the levels are quite similar. Sera was checked on day 76 and the level of antibody was still significant. Treatment was stopped on day 38. A fifty percent drop in titer occurred by the following week and the decline was sustained.

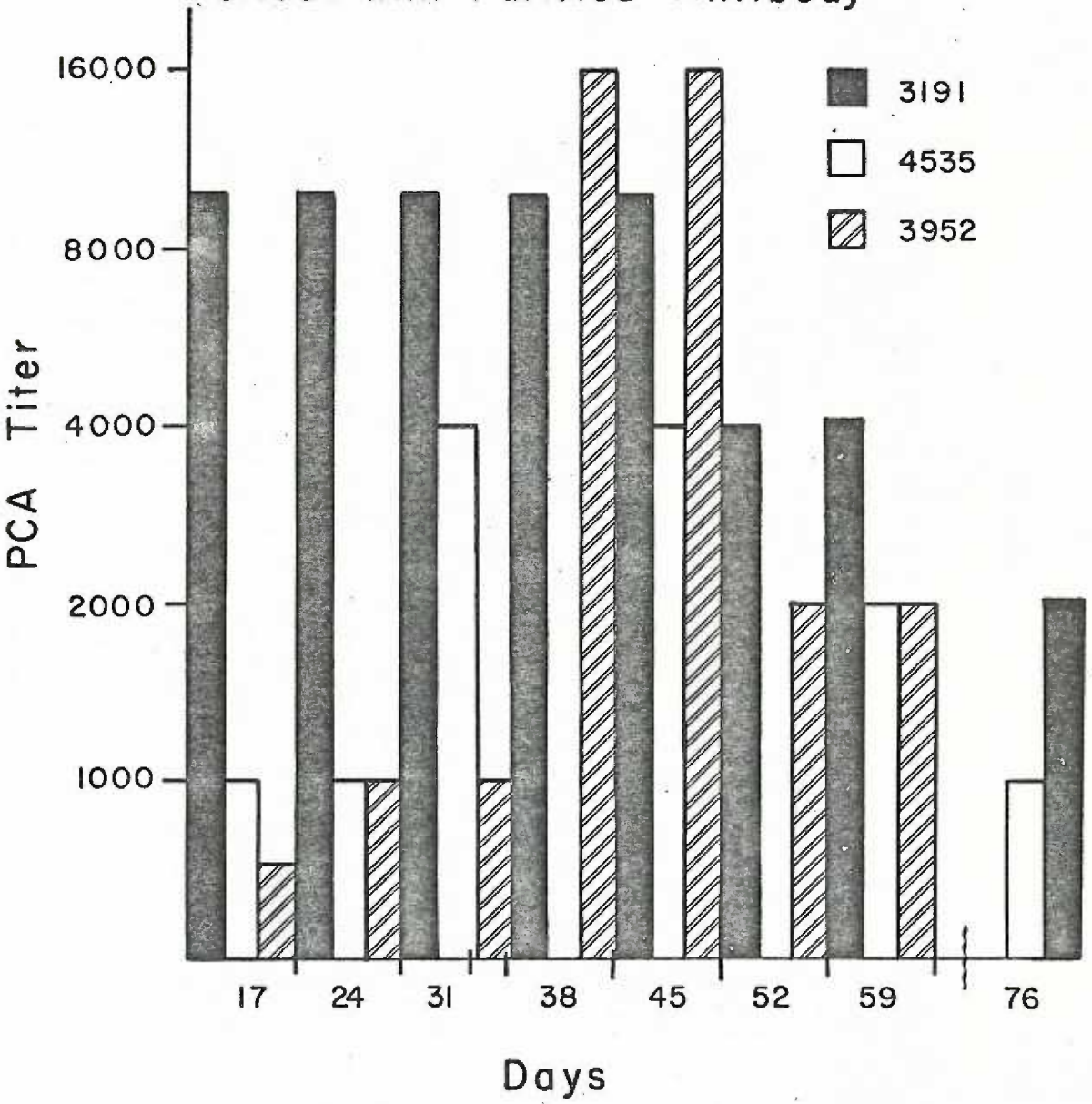
TABLE IX

EFFECT OF LONG TERM TREATMENT WITH ATS
AND ATAB ON THE CIRCULATING WHITE BLOOD COUNT

Treatment	Animal No.	WBC/mm^3 X 10^3 Experimental Day									
		0	3	10	17	24	31	38	45	52	59
ATS 75mg/kg	4557	5.5	8.5	6.1	6.2						
	3597	7.7	4.7	5.9	6.6						
	3305	8.0	13.3	9.6							
ATAB 75mg/kg + 10mg/kg per week	3952	ND	5.3	5.7	8.3	9.3	9.0	12.5	14.6	12.4	11.8
	4535	17.3	7.7	9.3	12.3	11.5	11.1	12.1	11.1		
	3191	11.6	5.6	15.4	10.4	9.2	8.1	13.0			

Fig XV

Anti-rabbit Antibody Formation in Rhesus Monkeys Treated for Extended Periods with Purified Antibody



In these two animals, the antibody response to rabbit sera was suppressed early but developed later at an eight to ten fold higher level than in short term animals. This may indicate that the maintenance dose was not adequate to hold down anti-rabbit antibody production.

Animal 3191 does not fit the pattern established by the other two. He produced high levels of anti-rabbit antibody by day 17 and continued at that level as long as treatment was continued. His humoral responses did not appear to be suppressed at all. His graft was not rejected on day 24 and he did not appear to be able to mount a second set rejection so his cellular immunity was definitely effected.

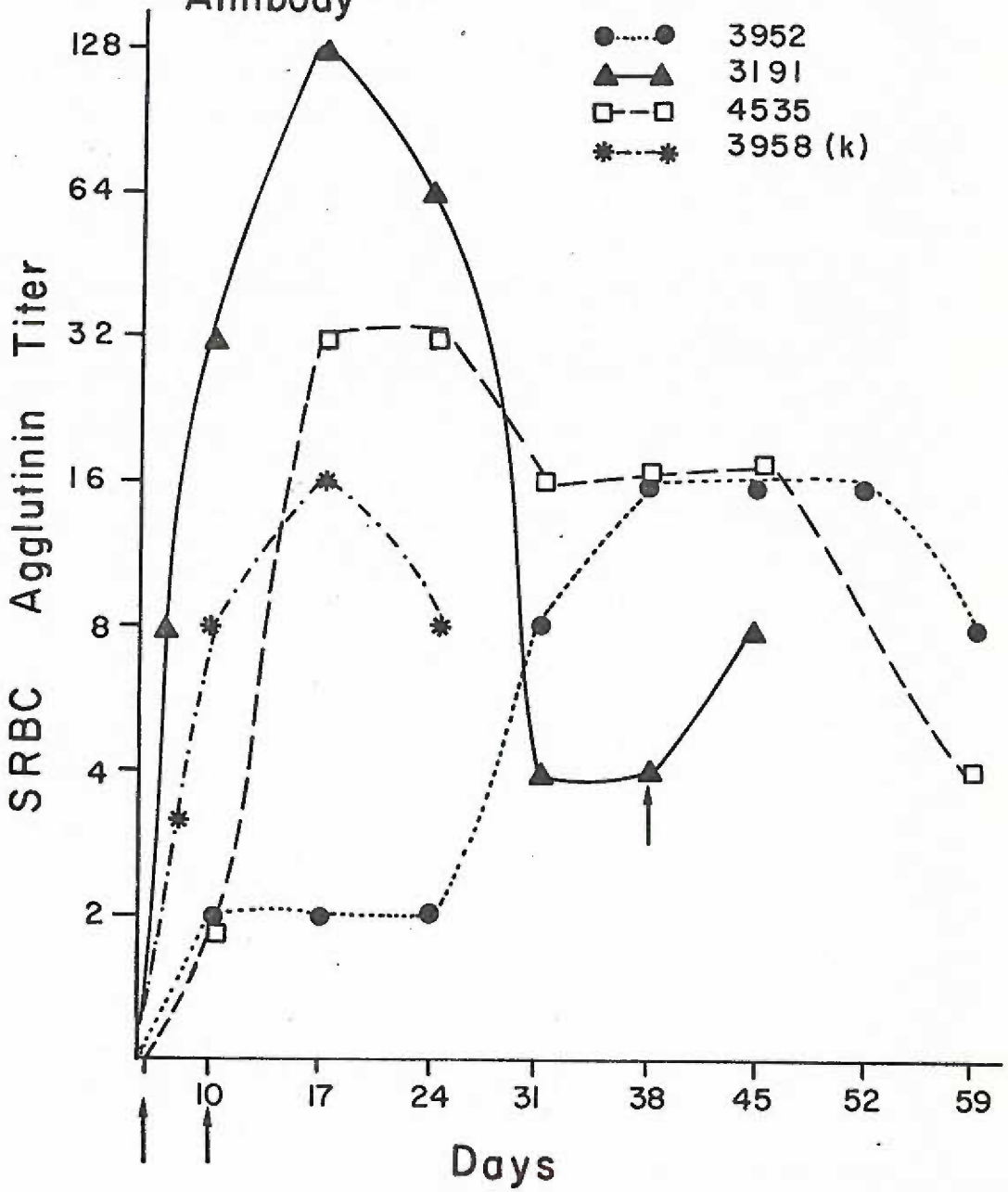
Sheep red blood cells were used as an additional antigen to determine the affect of ATAB and ATS on humoral immunity. There was a great deal of variability in the results of the agglutinin assay as reported in Figure XVI. In 3952, the response was delayed but rose after day 24. In 4535, the increase was more rapid and fell off slowly. In 3191, the titer rose rapidly and peaked on day 17 but it fell off by day 31. On day 38, a booster injection was administered to test the animal's ability to respond late in the suppressed state. There was an increase in titer indicating an ability to respond to a secondary stimulation.

In animals treated with 75 mg/kg ATS, there was little difference from control values (Fig. XVII).

The hemolysis assay in animals treated with either ATAB (Fig. XVIII) or ATS (Fig. XIX) were not strikingly different from the control. The response was biphasic in 3952 and 4535. It dropped off after day 31 and then increased again. The response of 3191 might have

Fig XVI

SRBC Agglutinin Titers in Rhesus Monkeys Suppressed with Purified Antibody

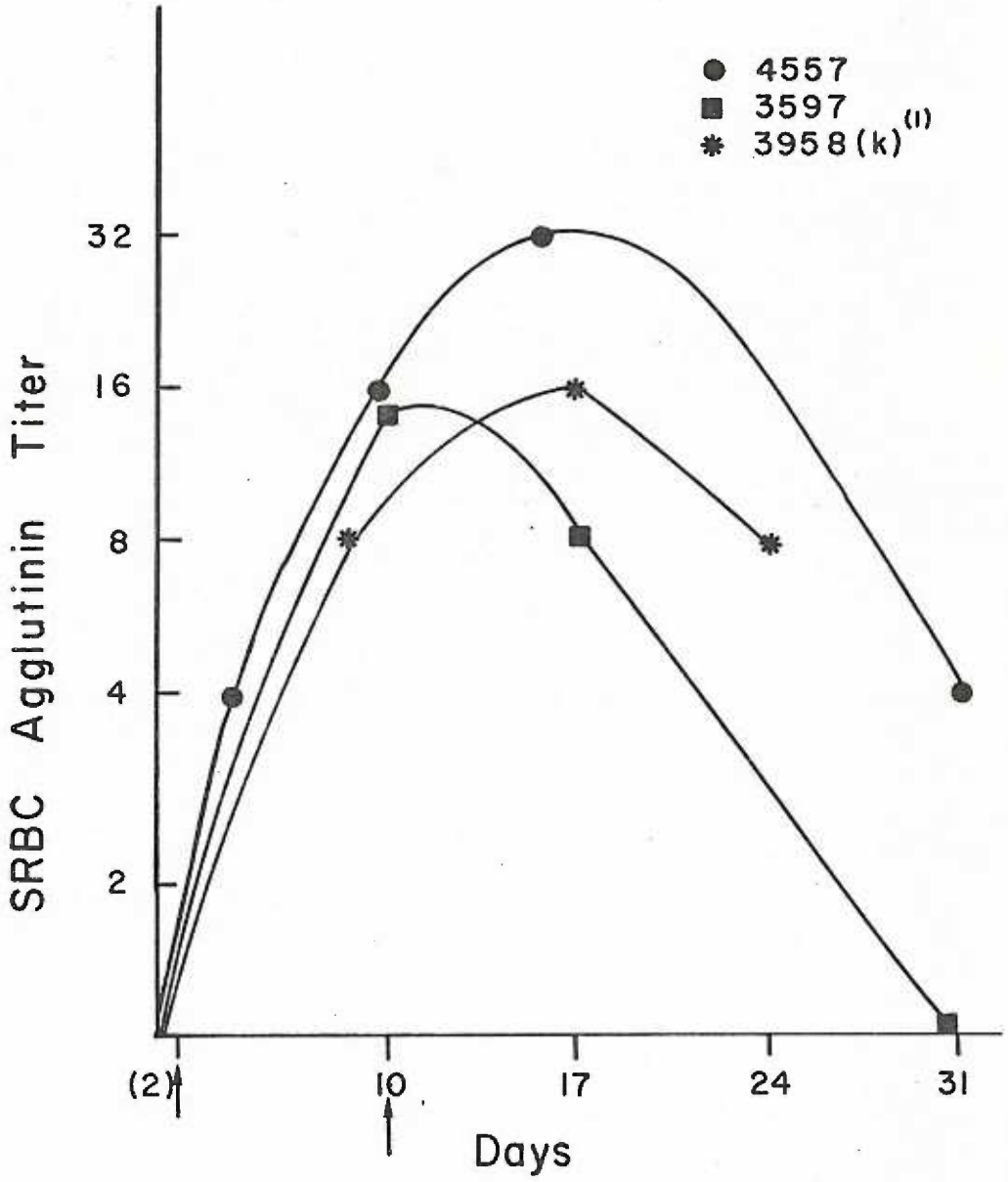


↑ Indicates administration of SRBC on day 38, only animal 3191 received SRBC.

k = Treated with NRS + SRBC

Fig XVII

SRBC Agglutinin Titers in Rhesus Monkeys Suppressed with ATS

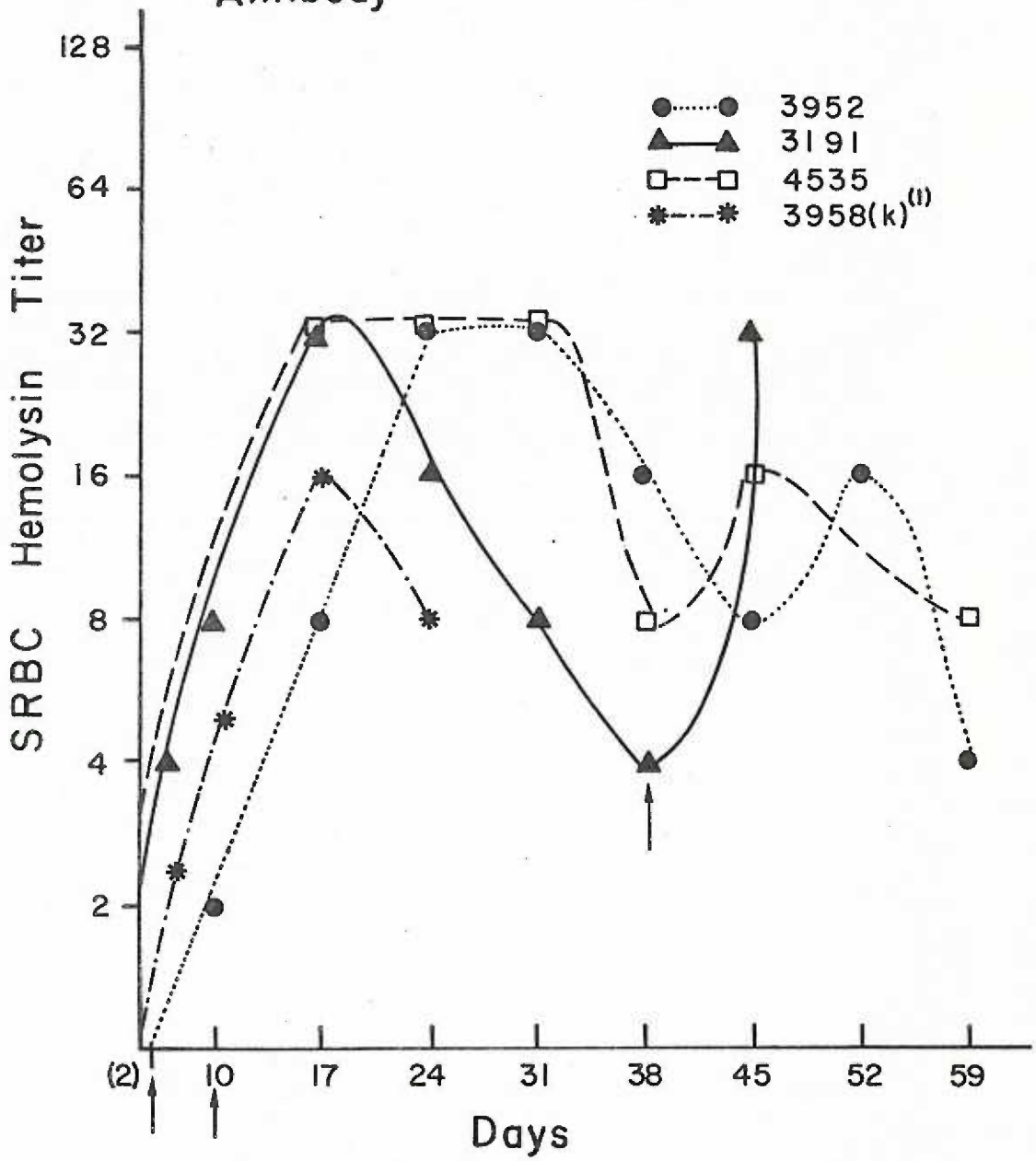


(1) k = SRBC + NRS

(2) ↑ = SRBC administered

Fig XVIII

SRBC Hemolysin Titers in Rhesus Monkeys Suppressed with Purified Antibody

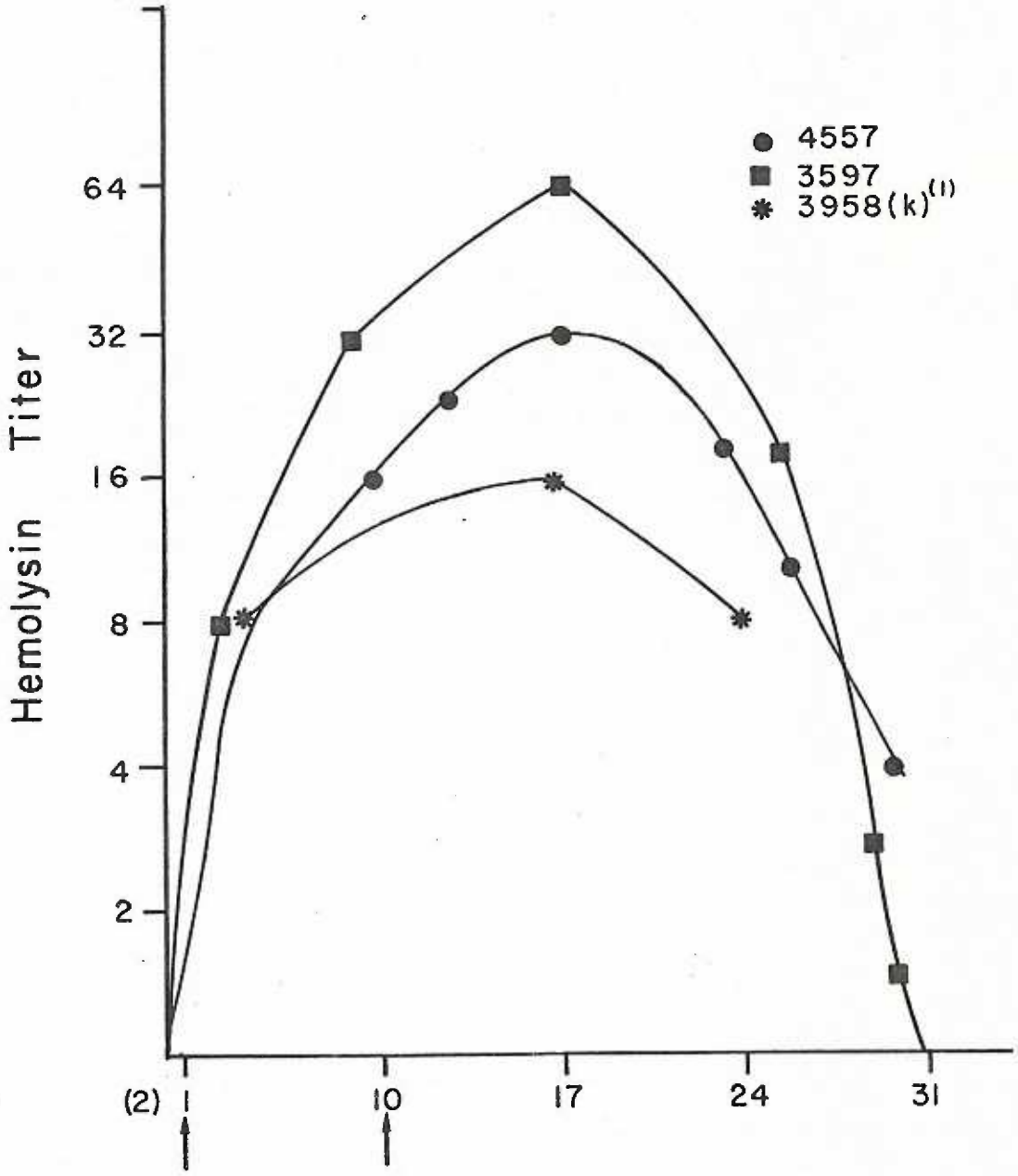


(1) k = NRS + SRBC

(2) ↑ = SRBC administered on day 38, only 3191 received SRBC.

Fig XIX

SRBC Hemolysin Titers in Rhesus Monkeys Suppressed with ATS



(1) k = NRS + SRBC

(2) ↑ = SRBC administered

showed the same effect but it was masked by a secondary stimulation on day 38 prior to which it mounted a significant response.

In summary, the effect of ATS or ATAB treatment on the humoral immune response to the protein antigen and SRBC appears to be a delay rather than a suppression.

Fluorescent antibody assay

A portion of the biopsied graft tissue (day 7) removed from animal 3975 which was treated with 100 mg IgG/kg was assayed for the presence of antibody at the graft site. The sectioned tissue was treated with fluorescein labeled goat anti-rabbit globulin (as described in the Materials and Methods section), and examined by fluorescent microscopy. In the positive control the fluorescence was diffused throughout the tissue as would be expected. The negative control tissue showed no fluorescence present. In the antibody treated animal a line of fluorescence was present along the bottom of the graft (Figure XX). This indicates that antibody does penetrate to the graft site and may cover the histocompatibility determinate of the graft delaying or preventing rejection.

In vivo and in vitro activity of the $F(AB)_2$, fragment of ATAB

Due to the antigenicity of the ATAB, the question of the activity of the pepsin digested fragment of the purified antibody was explored. Since the Fc fragment is the antigenic portion of the molecule, if the $F(AB)_2$ fragment was immunosuppressive, it might be more useful in treatment.

The $F(AB)_2$ fragment was assayed in vitro for leukagglutinating activity, cytotoxicity and transformation. The specific activity (LA/mg $F(AB)_2$) is compared to that of intact antibody in Table X.

Fig. XX

Localization of Antibody at the Graft Site

Demonstrated by Fluorescence

Full thickness skin homograft from the scalp of Macaca mulatta 3975 (day 7). The animal had been treated with 100 mg/kg ATAB. The 10 micron section was stained with fluorescein labeled goat antirabbit globulin. Notice localization of the fluorescent label along the bottom of the graft. This indicates presence of antibody at the graft site. The graft is on the right side of the picture while the host tissue is on the left (500X).

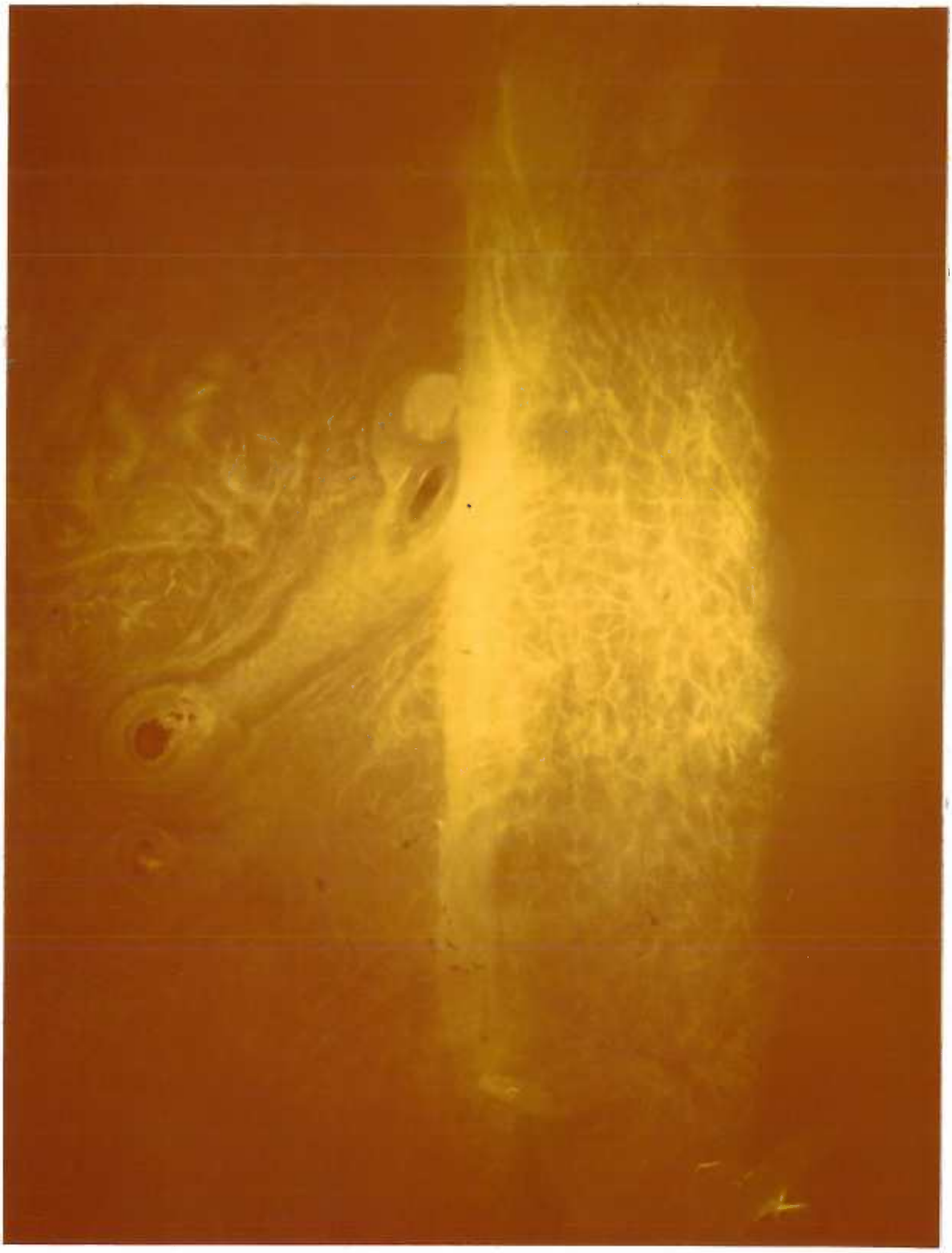


TABLE X

SPECIFIC ACTIVITY OF $F(AB)_2$ FRAGMENT FROM PURIFIED ANTIBODY

	LA/mg IgG	LA/mg $F(AB)_2$
ATS	26	
ATAB	165	
AT $F(AB)_2$		96
NRS	---	
NR IgG	---	
NR $F(AB)_2$		---

AT $F(AB)_2$ = Pepsin digest of ATAB
NRIgG = IgG isolated from NRS by DEAE
NR $F(AB)_2$ = Pepsin digest of NRIgG

It does agglutinate and was found to be non-cytotoxic for peripheral rhesus lymphocytes. The transforming activity is reported in Table XI. The $F(AB)_2$ appears to transform better than the purified antibody but it was administered at a higher level if the differences in molecular weight are considered.

The $F(AB)_2$ fragment was analyzed on the analytical ultracentrifuge and found to have a sedimentation coefficient of 5S. The material was tested for the presence of Fc by gel diffusion against donkey anti-whole rabbit serum. In Fig. XXI, a comparison between the purified antibody and the $F(AB)_2$ fragment is shown. The $F(AB)_2$ did not precipitate.

To determine whether the $F(AB)_2$ fragment was immunosuppressive, its ability to extend skin graft survival in a rhesus monkey was tested. The short term treatment schedule was used. The animal was treated with a total of 50 mg $F(AB)_2$/kg which is equivalent to the level of antibody found to be suppressive. The animal (3943) rejected the grafts on day 10 so there was no extension of graft survival at all. The animal produced no anti-rabbit antibody as measured by PCA.

TABLE XI

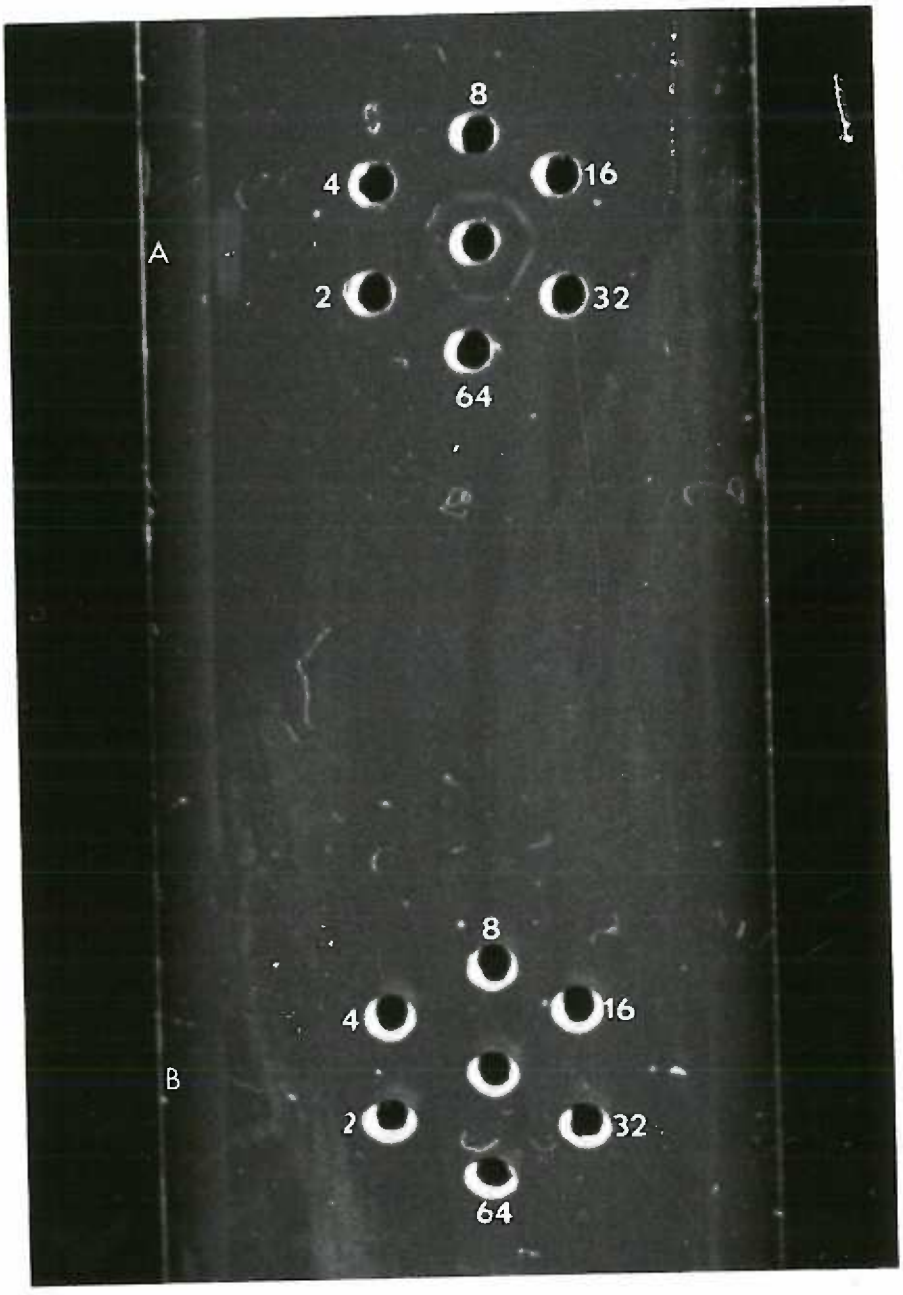
TRANSFORMATION OF RHESUS LYMPHOCYTES BY THE $F(AB)_2$ FRAGMENT OF ATAB

Mitogen	CPM
ATS (6 mg IgG/tube)	15,176
ATAB (6 mg IgG/tube)	114
$ATF(AB)_2$ (6 mg $F(AB)_2$/tube)	865
NRS (6 mg IgG/tube)	49

Fig. XXI

Gel Diffusion of ATAB and the $F(AB)_2$ Fragment Against Goat Anti-whole Rabbit Sera

Goat antirabbit whole sera is in both center wells. In A, two-fold dilutions of rabbit antirhesus thymocyte antibody are in the outside wells. In B, two-fold dilutions of the $F(AB)_2$ fragment of ATAB are in the outside wells. No lines of precipitation with the $F(AB)_2$ indicate that it is free of F_c fragment.


8
4
16
A
2
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64
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B
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64

DISCUSSION

The development of a specific immunological adsorbent for isolation of anti-lymphocyte antibody provides an improved tool for transplantation research. Anti-lymphocyte antibody can be isolated in a one-step procedure from a reusable adsorbent. Since the adsorbent is reusable, it is readily adaptable to large scale production required for a major transplantation program. In addition, the methodology developed will allow widespread use of stroma conjugates for isolation of other materials which bind to a specific cell type. The immunoadsorbent currently in use was prepared by conjugating hyptonically lysed rhesus lymphocytes to Sephadex G-100 over two years ago. It still functions efficiently and constantly with no protein loss. The antibody isolated from the immunoadsorbent represents 6-9% of the total protein and 18-26% of the IgG present in rabbit anti-rhesus lymphocyte sera. The specific activity (leukagglutinins/mgIgG) of the isolated antibody fraction increased approximately 10 fold over that of the original sera.

An attempt was made to correlate transformation of rhesus peripheral lymphocytes with the purification of rabbit anti-rhesus lymphocyte antibody. The ability of anti-rhesus thymocyte sera to stimulate rhesus peripheral lymphocytes is evident (Table III). Although the antibody fraction retains significant mitogenic activity, approximately 90% of the activity present in the original ATS is lost on passage of the sera through the immunoadsorbent.

The results of a mixing experiment in which increasing concentrations of antibody were mixed with decreasing amounts of the nonspecific material (CP) indicated that the material responsible for transformation could be denatured in the course of the isolation procedure, or that potentiation factors were lost.

Recent reports (26,34) indicate that the major mitogenic factor of lymphocytes is not associated with the stroma, but was found with antigenic determinates located in the soluble fraction of a lymphocyte preparation. The phylogenetic relationship between man and the rhesus monkey suggested utilization of the rhesus stroma immunoadsorbent for the purification of anti-human antibody. Table V illustrated that the recovery of specific anti-human lymphocyte antibody activity is in the same range as found for the purification of anti-rhesus lymphocyte antibody. This introduces the possibility of a practical application for this procedure in the preparation of purified antibody to be used as an immunosuppressant in humans. The human antibody could be isolated either from a rhesus stroma column or from a human stroma preparation.

In the short term experiments, the immunosuppressive properties of the purified antibody (ATAB) whole antithymocyte sera (ATS), column passage and normal rabbit sera were compared. The results indicate that the antibody is as effective as whole sera in extension of graft survival at one-half the IgG level. In addition, the ATAB treated animals had a better appetite and fewer fluid maintenance problems than those treated with the whole sera. No enduration at

the injection site was present in the animals receiving purified antibody. The extension of graft survival obtained in this experiment with both the whole sera and the purified antibody compares favorably with reports in the literature (75, 78). Balner considers an extension successful if it survives as long as treatment is continued. Cohen and Sell reported 32 day survival in one animal using the same regime we adopted (77). Our "early" antisera preparation method is very good. Since we did not have toxicity problems even with our whole sera, the thymus in the rhesus is probably the best source of tissue for immunization.

The limited changes in the circulating blood count during treatment indicate that adequate suppression can be achieved without lowering host defenses to infection. Although infections are a common complication in other species, they do not appear to be in the rhesus.

The antigenicity of the various treatment materials answers a basic question that has remained confusing in the literature. Contrary to Lance's report, antibody is made against normal rabbit sera (75). Since the antibody and the ATS are about equally immunogenic in these experiments and since the protein concentration of the antibody is lower, the purified antibody is more immunogenic than the whole sera. Perhaps this is due to the lymphocytes rendering the antibody bound to them more immunogenic. More molecules of specific antibody capable of binding to and killing lymphocytes are present in the antibody treated animal than in the ATS treated animal. The dead lymphocytes may persist in the animal with the antibody bound to their surface. According to Lance (3) specific

antibody is cleared rapidly from the circulation but, in these animals treated for three days, antibody is not formed until day 22. It must still be present somewhere to stimulate antibody formation. This raises the question of whether the majority of researchers doing classical immunosuppression studies are looking late enough for an immune response.

The dose dependency of the extension of graft survival poses an interesting problem. Clearly 35 mg/kg is not enough and 100 mg/kg appears to be too much. The antigenicity data gives a clue to why. The 35 mg/kg dosage is enough to be immunogenic, but not enough to extend graft survival. The 75 mg/kg dose extends graft survival and delays the production of anti-rabbit antibody. At 100 mg/kg so much antibody is present that the humoral immune response against it cannot be suppressed. The anti-rabbit antibody formed early probably binds to the remaining free ATAB rendering it non suppressive.

The histological studies on biopsied tissue confirmed our clinical judgement of when rejection had occurred. These were blind studies since the histologist was given the tissue without knowing how the animal was treated. He scored the tissue simply as rejected or non-rejected.

The short term experiments during which animals were confined in restraining chairs were very valuable in determining whether the antibody isolated from the specific immunoadsorbent was suppressive. The scalp is a good non-mobile site for grafts and provides easy visualization. The scalp site also permitted weekly biopsy of

grafts. The method provides remarkable freedom from technical failures. We lost a few grafts due to animals rubbing their drinking tube or sneaking their hand up through the neck hole, but with proper adjustment the chairs are satisfactory. The major drawbacks to this type of experiment are the time limitation and the shortage of animals which will adapt to the chair. The upper limit in keeping an animal in the chair is about 45 days. Even the best chair animal starts losing his appetite and tends toward dehydration. They do much better in cages where they can exercise freely. Usually, one animal in three will not adapt to the chair. They refuse to eat or drink and struggle constantly. With the shortage of animals anyway, this is a major problem.

In the long term experiments, we attempted to overcome these problems by placing grafts in midscapular area of the back and keeping the animals in cages. The percentage of technical failures was a definite problem. About one animal in four damaged their grafts by scratching or rubbing against the cage. Balner cages his grafted animals in plexiglas cages to avoid this complication.

The long term experiments did not tell us much about ATS but a great deal about the purified antibody. The first major finding was that our antibody was so effective that by the time rejection occurred, if it did occur, we could not recognize it. On the back, the grafts healed as in wound healing. For about 35 days the grafts remained flat and were readable, but after that there was shrinkage and healing over. The autographs healed in the same way. When

we examined an autograft and homograft histologically which were removed on day 45 there was no difference. When a second graft was placed on an animal in this series, accelerated rejection did not occur indicating that the animal was not sensitized to donor tissue and due to depletion of his long lived lymphocytes, did not have the capacity to mount an accelerated rejection. It was in this same animal during the third grafting that we were able to show that incubation of a skin graft with purified antibody could extend graft survival for five days over that of untreated tissue. This is good evidence that the antibody can work at the graft site covering the foreign determinates and delaying rejection.

The presence of antibody at the graft site was demonstrated in our fluorescent antibody experiments also. This strongly indicates that part of the mode of action of the antibody involves covering of foreign histocompatibility determinates at the graft site. This type of experiment could not have been done without purified antibody.

The absence of pathological changes in the animal treated for extended periods with the purified antibody indicate that it is safe for use in human transplants. The absence of changes in kidney is especially encouraging since the levels of anti-rabbit antibody were high and the possibility of immune complex deposition was very real. The fluorescent antibody experiments did not detect immune complexes in kidney tissue either so although the complexes must be present, they do not appear to be a major problem. The accounts in the literature of detection of immune complexes in the kidney involved intravenous administration of the suppressant. Our subcutaneous administration may have circumvented this possible

complication.

The increased level of anti-rabbit antibody formation in the long term treatment animals compared to the levels in the short term animals may indicate that our maintenance dosage was not high enough. The usual procedure is to continue the beginning dosage each week for at least eight weeks. We tried a much lower maintenance level which was about one seventh our original dosage. Perhaps it would be preferable to maintain at about one half the initial dosage. The other alternatives in view of the antigenicity and the effectiveness of the original dosage is to give only that amount and then treat rejection crisis with that same dosage when they occur. By day 36 in the short-term experiments, the antibody level had dropped to a very low level. Since the initial dosage can prevent rejection to that time, treatment could be repeated when rejection threatens.

The effect of the purified antibody on the humoral response to sheep red blood cells is complicated and difficult to assess, since it is so variable from animal to animal. In animal 3952, there was definitely a delay in the onset of the agglutinin response but this was not true in the other two animals. In the hemolysin response, there appears to be no difference from control values. One interesting aspect is that the suppressed animal can respond to a secondary stimulation as evidenced by animal 3191 when a third injection of SRBC was administered on day 38.

The experiments with the $F(AB)_2$ fragment of the purified antibody confirm prior observations in the literature in which the whole globulin fraction was pepsin digested. The purified antibody fragment had not been tested for immunosuppressive properties prior to this report. This indicates that the Fc portion of the antibody is necessary for immunosuppression and lends support to the currently accepted mechanism of action of the antibody. Certainly killing of long-lived lymphocytes is part of the mechanism of action of the antibody, although antibody demonstrated at the graft site argues that it may also act to cover histocompatibility differences at the graft site. If masking or local enhancement were the only mechanisms of action, $F(AB)_2$ could suppress graft rejection.

The purified antibody will be useful in further exploring the mode of action of ALS.

SUMMARY AND CONCLUSIONS

A reusable specific immunoadsorbent was prepared for one-step isolation of anti-lymphocyte antibody. The adsorbent was prepared by conjugating hypotonically lysed rhesus lymphocyte to a modified dextran.

The specific antibody isolated from the immunoadsorbent had increased in vitro and in vivo activity. No pathological changes resulted from extensive treatment of rhesus monkeys with purified antibody. The humoral immune response was delayed rather than suppressed by the antibody. Antibody was detected at the graft site by flourescent antibody assay.

The purified antibody will represent a major advance in treatment of transplant recipients as well as acting as a valuable tool for probing the mechanism of suppression of transplant rejection. The methodology of the isolation of specific material from stroma conjugates will have widespread applications.

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