RENAL TRANSPLANTATION IN THE INBRED RAT

III. A Study of Heterologous Anti-Thymocyte Sera*, ‡

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The work of Woodruff and associates established that the administration of heterologous antisera prepared by immunization with lymphocytes will delay allograft rejection when used in a system of skin transplantation in the rat (1, 2). Many subsequent reports in the recent literature have confirmed the immunosuppressive potency of such antisera in skin graft and renal allotransplant systems, in some instances across major histocompatibility barriers (3–11). At present, a large therapeutically successful series of patients exists having received renal allotransplants with horse anti-human lymphocyte globulin administered as an adjunct therapy to the usual immunosuppressive methods (11).

It is the purpose of this paper to report immunological studies of a heterologous antisera, made in rabbits to rat thymocytes, which have potent immunosuppressive properties as tested in the relatively invariable system of renal allotransplantation across a strong histocompatibility barrier in the inbred rat (12). Experimental data is presented that is relevant to an understanding of the distribution and mechanism of action of anti-lymphocyte serum. Additional data is presented which indicates the conditions under which nephritis might be produced by this reagent, since this complication may be the most limiting factor in its clinical application.

Materials and Methods

Animals.—All rats used were 250–350 g males of the inbred Lewis, (Lewis × BN)F_1 hybrid (LBN F_1), or (Lewis × F344)F_1 hybrid (LF344 F_1) strains (Microbiological Associates,

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Inc., Walkersville, Md.). They were housed in individual cages at the time of study or after renal transplantation and fed Purina Lab Chow and water ad lib. Prior to collection of 18 hr urines for protein determinations, they were deprived of food but not water, and animals receiving iodinated proteins were maintained on water containing 0.05% KI and 0.45% NaCl (13).

Preparation of Antisera.—Five lots of pooled sera from two New Zealand white rabbits each were obtained after immunization with 10^9 twice washed thymocytes in Hanks' balanced salt solution. The cells were obtained by mechanical homogenization, sieved through eight layers of gauze, finally incorporated into 1 ml of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.), and injected into the foot pads and multiple cervical sites. 10 and 17 days later, booster injections of an equivalent number of thymocytes without adjuvant were given to the animals in the same regions. The rabbits were bled out by cardiac puncture 7–10 days after the last injection. The sera were heated to 56°C for 45 min and one lot was stored unabsorbed. Most batches of antisera were absorbed, however, two times with 7.5% by volume of washed rat erythrocytes for 4 hr at 4°C and afterwards with 10% by volume of whole rat serum.

Isolation of the Immunoglobulin G Fraction (AT IgG)[1].—The IgG (7S, gamma G) fraction of the absorbed serum was obtained in the following way: One lot of whole serum was subjected to DEAE chromatography after dialysis and elution with 0.007 M Na phosphate buffer, pH = 6.3 (14, 15). Since this eluate sometimes contained small amounts of hemoglobin or transferrin, it was further purified by chromatography on Sephadex G-100 in saline phosphate buffer, pH 7.1, or by rechromatography on DEAE cellulose. Additional batches of absorbed rabbit anti-rat thymocyte serum were purified by precipitation at 18% Na_2SO_4, dialyzed against 0.007 M sodium phosphate buffer, and eluted as described above. The resulting IgG was immunochemically pure on immunoelectrophoretic and radioimmunoelectrophoretic analysis (16) when reacted against sheep anti-rabbit serum (Hyland Laboratories, Inc., Los Angeles, Calif.).

Preparation of the Pepsin Fragments.—The absorbed and chromatographically purified IgG fraction of the rabbit anti-rat thymocyte serum was digested with pepsin to the 5S divalent fragment, $F(ab')_2$, and the 3.5S univalent fragment, Fab', according to the method of Nisonoff et al. (17). After overnight dialysis at 4°C against 0.1 M Na acetate buffer, the pH was reduced to 4.5 and twice crystallized pepsin (Worthington Biochemical Corp., Freehold, N.J.) at pH 4.5 was added at a 1:50 pepsin:globulin ratio. The mixture was incubated at 37°C for 8 hr and after precipitation at 18% Na_2SO_4 concentration, the precipitate was dissolved in distilled water and dialyzed exhaustively against buffered saline (0.14 M NaCl, 0.01 M sodium phosphate buffer, pH 7.1) at 4°C. The univalent fragment, Fab', was obtained after dialysis of the $F(ab')_2$ preparation overnight at 4°C against 0.1 M sodium acetate. The pH was lowered to 5.0 and 2-mercaptoethanolamine HCl was added to a final molarity of 0.12. After flushing with nitrogen, this mixture was incubated at 37°C for 90 min, dialyzed against 0.02 M iodoacetamide in phosphate buffered saline for 8 hr, then exhaustively dialyzed against repeated changes of phosphate-buffered saline, and finally sterilized through a Millipore filter.

Assay of Antisera and Fragments.—All lots of crude or absorbed whole antisera, purified IgG fractions, or antibody fragments were evaluated for their ability to agglutinate rat thymocytes, and the lymphocytotoxicity titer was measured using a modification of the method of Gorer and O'Gorman (18). For agglutination, 0.025 ml rat thymocytes (5×10^6 cells/ml)

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[1] Abbreviations frequently used in this paper: Anti-thymocyte serum (ATS), anti-thymocyte immunoglobulin G (AT IgG), normal rabbit serum (NRS), normal rabbit immunoglobulin G (NR IgG). $F(ab')_2$ and Fab' refer to the bivalent and univalent antibody fragments obtained after pepsin digestion, recommended by the committee on nomenclature, World Health Organization Bulletin, 1964. Anti-Lymphocyte serum (ALS) is referred to as applied by other authors.

were added to 2-fold serial dilutions of antisera in lucite microtiter plates and incubated at room temperature for 90 min. A drop of the cell suspension was placed on a slide and was evaluated for agglutination under low power microscopy. Inhibition of agglutination by the Fab' preparation was tested by treating 50×10^6 rat lymphocytes with AT Fab' (10 mg/ml) for 90 min at room temperature, then by reacting these cells with serial dilutions of AT IgG. Lymphocytotoxic determinations were performed on samples similarly prepared for leukoagglutination. However, after incubation of serial dilutions of antisera and cells at room temperature for 30 min, 0.025 ml of a 1:10 dilution of freshly reconstituted lyophilized normal rabbit serum (Hyland Laboratories) as a source of complement was added, and this mixture was incubated at 37°C for 90 min. Slide preparations were examined under phase-contrast microscopy and the number of viable and nonviable cells determined. In addition, various test antisera from rabbits, normal rabbit sera, and globulins obtained from commercial sources were tested for their ability to agglutinate a 1% suspension of washed sterile sheep erythrocytes after serial dilution in microtiter plates. The immune response in rats to sheep erythrocytes was measured by hemagglutination 6 days after an intravenous injection of 0.5 ml of a 10% suspension of washed sheep red cells.

Other Preparations.—A rabbit antiserum to rat glomeruli was prepared by immunization with glomeruli isolated in the following manner: Rat renal cortex was passed through a 100 mesh sieve and washed 8–10 times after centrifugation at $1000 \times g$ until the suspension contained greater than 95% glomeruli (19). After incorporation into Freund's complete adjuvant, this material was injected into the foot pads and cervical regions of New Zealand white rabbits who were boosted 10 days later and bled 1 wk later. The serum was stored at −20°C until use. It was shown to have potent anti-kidney activity by its ability to produce an immediate proteinuria after intravenous injection. A more highly purified preparation of glomerular basement membrane was prepared from some of the isolated glomeruli by ultrasonic disruption (Raytheon model No. S102A) and differential centrifugation (19).

Study of Animals with Renal Transplants.—All animals studied for modification of rejection were treated with the various antisera prior to renal allotransplantation. No injections of antithymus serum or the IgG or antibody fragment preparations were given to animals subsequent to the renal transplant. The allotransplants were carried out in Lewis recipients receiving LBN F_1 hybrid kidneys and Lewis to Lewis isotransplants were done using a microvascular surgical technique (20). The functional and immunopathological characteristics of this system of rat renal allotransplantation have been previously described (12). Immunogenetically, rejection reflects strong histoincompatibility (21) including disparity at the Ag-B locus (22) and is host vs. graft. By day 7, the renal transplant no longer functions and a specific glomerular, interstitial, and tubular lesion is present. At this time as well, donor-specific hemagglutinating and lymphocytotoxic isoantibodies are present in the serum of the recipient. The day 7 animal in this system was thus chosen as the functional and morphological control for the experimental animals described in this study. At the time of transplantation, the recipient's right kidney was removed and, 4 days later, the left kidney was taken through a small left flank incision. Effective renal plasma flow (ERPF) was measured on day 7 after allotransplantation by a technique utilizing an analysis of the disappearance rate of ^{125}I orthoiodohippurate (Hippuran, E. R. Squibb & Sons, New York, N.Y.) (23). Anesthetized animals are injected with 10–20 μc of ^{125}I-Hippuran, and radioactivity was monitored for 60 min over the head by a collimated probe with a $1\frac{3}{4} \times 1\frac{3}{4}$ inches NaI crystal through a model 530A Baird-Atomic gamma spectrometer and a Texas Instruments Rectiriter recorder. ERPF is calculated using the equation of Sapirstein based on a two compartment system for the plasma disappearance of the isotope: $C = (Db_1b_2)/(Ab_2 + Bb_1)$ (24).

The sera of some animals receiving renal allotransplants were analyzed for the presence of donor-specific hemagglutinating antibody using the PVP method of Stimpfling (25) and donor specific lymphocytotoxic antibody (18).

Tissue Preparation and Examination.—Thin tissue slices of kidney, spleen, and thymus

from animals killed 7 days after transplantation were fixed in 2% cold glutaraldehyde, cold 95% ethanol, and 10% formalin. After overnight fixation at 4°C in glutaraldehyde, the tissue was washed in 0.25 M sucrose for a day or more (26), fixed secondarily in 1% osmium tetraoxide, and, after dehydration in cold graded ethanol, embedded in Epon 812 (27) for preparation of 1 μ sections that were stained with 1% toluidine blue O in borax (28). Formalin-fixed tissue was embedded in paraffin, and 5 μ sections were prepared and stained with hematoxylin and eosin and Schiff's leukofuchsin after periodic acid oxidation (PAS). For each transplanted animal, the transplanted kidney, the recipient's own kidneys, and the donor's nontransplanted kidney were available for microscopic study. The kidney, spleen, and thymus from control and experimental animals were examined first without knowledge of the experimental groups, and finally all of the histopathologic material was reexamined with knowledge of the experimental design. Based on previous experience with the sequential morphologic alterations developing during renal allograft rejection with this rat strain combination, the rejection process in the transplanted kidneys was quantitated by assigning a number corresponding to the posttransplant day during which similar morphologic features would be expected in unmodified animals (12). The immunosuppressive effect of anti-thymus sera was judged good, moderate, or poor if allografts removed 7 days after transplantation had the morphologic appearance of kidneys removed at 0–3 days, 4–5 days, and 6–7 days after transplantation, respectively. Such grading is highly reproducible.

Immunofluorescent examination of tissue and preparation of immunoreagents were performed as previously described (29) using the fluorescent antibody technique (30) on 95% cold ethanol-fixed, paraffin-embedded tissue (31). Fractions of rabbit or sheep antisera, obtained by DEAE-cellulose chromatography, to rat IgG (double chromatography on DEAE-cellulose), β_{1C} globulin (32), and sheep antisera to rabbit IgG (Hyland Laboratories) were conjugated with chromatographically purified fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratories, Baltimore, Md.) (33). Tissue sections were covered with labeled antiserum, incubated for 30–40 min, washed free of unbound antisera, and coverslips mounted with glycerin-saline. Fluorescein-blocking controls were done by exposing tissue sections to unlabeled antisera prior to "staining" with labeled antisera and absorbing labeled antisera with purified antigen, in the case of the anti-IgG, prior to "staining". Ultraviolet light was supplied to a Reichert microscope with fluorite dark-field objectives and condensers by an Osram HBO 200 watt mercury lamp and filtered with a KG-2 exciter filter. Sections were viewed with a W2B barrier filter in place.

Studies on Treated Rats.—White blood counts and total lymphocyte counts were done on blood obtained from the retroorbital plexus. β_{1C}-globulin (C'3) levels were determined by a radial diffusion immunoassay method (34) using 7 μl samples of rat plasma inoculated into agar plates made with the rabbit antiserum to rat β_{1C}-globulin described above. Urine protein excretion was measured by the method of Kingsbury (35), serum protein determinations were done by a biuret method (36), and double diffusion in agar gel was performed by the Ouchterlony method (37). Radioimmunoelectrophoresis of iodinated IgG fractions were performed by addition to whole rabbit serum and by development with a sheep anti-rabbit serum. Radioautographs were developed after exposure to Polaroid 3000 film.

Hemagglutination of IgG-Coated Red Cells.—Sheep erythrocytes coated with a subagglutinating amount of rabbit IgG in the form of amboceptor (Baltimore Biological Labs) were also employed as indicators of rat anti-rabbit IgG antibody. Sheep red cells plus amboceptor, 1:8000–1:16,000 dilution, were mixed and added in a 1% suspension into serial dilutions of test rat sera in microtiter plates. Mercaptan (DL-penicillamine) was added to dilutions of some sera to a final concentration of 0.1 M and incubated for 1 hr at 37°C before the red cells were added. Hemagglutination patterns were read after 4 and 18 hr (38, 39).

Iodination of Proteins.—AT IgG, normal rabbit IgG, (NR IgG), AT $F(ab')_2$, and F ab' fragments were iodinated with ^{125}I according to a modification (40) of the chloramine-T

method of Greenwood et al. (41) at a protein concentration of 1 mg/ml, followed by exhaustive dialysis at 4°C against phosphate-buffered saline, pH 7.1. The labeled proteins were used immediately, or after short periods of storage at 4°C, and centrifugation at 24,000 × *g* for 30 min was performed just prior to in vivo or in vitro use. For in vivo localization (42) or immune elimination studies (43), a volume of 0.4–0.5 ml containing 200–300 μg IgG was injected intravenously with serial bleedings from the retroorbital plexus into heparinized capillary hematocrit tubes and 20 μl of plasma were pipetted and counted with a Baird-Atomic No. 530A spectrometer.

Tissue Localization of Labeled Proteins.—In normal LF344 F_1 hybrid and Lewis rats, a paired label technique (44) using ^{131}I-labeled anti-thymocyte IgG and ^{125}I normal rabbit IgG, or vice versa, was employed in all in vivo and in vitro experiments. The two reagents were mixed and centrifuged at 24,000 × *g* for 30 min just prior to injection, and the animals were anesthetized with ether at various intervals thereafter. Prior to perfusion via the abdominal aorta with 6–10 blood volumes of 0.15 M NaCl, blood was obtained for counting so that the amount of specific antibody fixation could be determined from comparison of the isotope ratios in blood and various tissues according to the equation: ^{131}I-fixed = $^{131}I_{organ} - [(^{131}I_{plasma}/^{125}I_{plasma})^{125}I_{organ}]$.[2] The results were expressed as percentage of injected antibody or as specific activity. Some kidneys were fractionated into glomerular and nonglomerular fractions prior to counting (19) and selected kidneys were homogenized in a Sorvall omnimixer followed by precipitation with 20% trichloroacetic acid (TCA) and the amount of protein-bound radioactivity determined. Some of the homogenates were subjected to exhaustive dialysis against water to determine the per cent of dialyzable (free or peptide-bound) radioactivity. A few of the dialysates were concentrated by lyophilization and chromatographed on a Bio-Gel P-2 column (45) to determine the relative amounts of ^{131}I and ^{125}I bound to the peptide fraction. Radioautography of kidneys after formalin fixation and paraffin-imbedding was performed on 5 μ sections using Kodak NTB-3 nuclear track emulsion (46). For these experiments, ^{125}I AT IgG or ^{125}I NR IgG of the same specific activity were given to pairs of animals and the kidneys removed 10 min and 4 days later after perfusion. The paired label technique was also used to determine in vitro binding to purified glomerular basement membrane suspended in 10% bovine serum albumin in siliconized glassware. After incubation at 37°C with constant agitation, the sediment was washed once prior to counting.

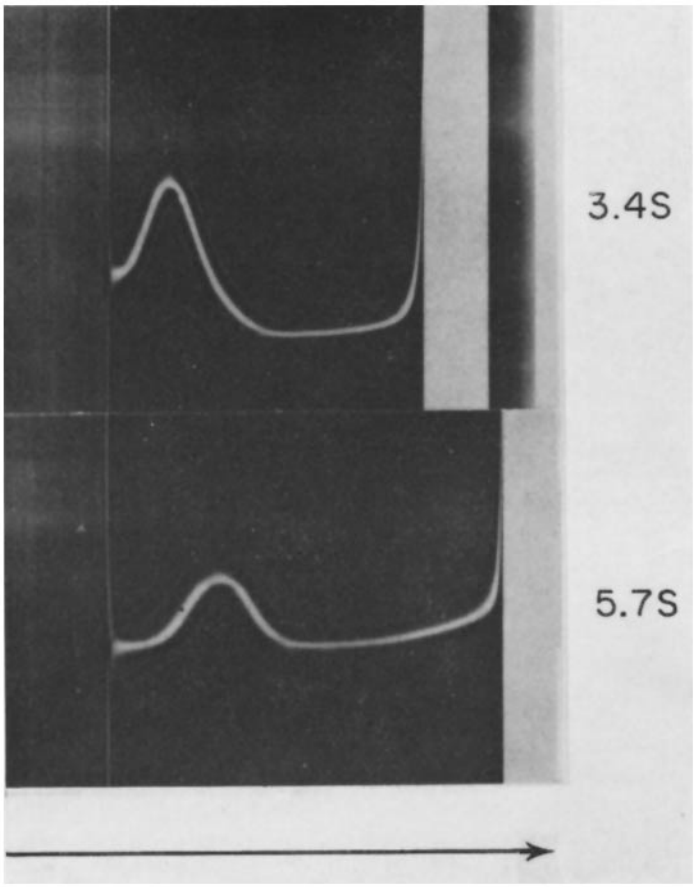
RESULTS

In Vitro Characteristics of the Heterologous Antisera and Suppression of Response to Sheep Erythrocytes.—The titers of the rabbit anti-rat thymocyte sera using the lymphoagglutination technique were from 1:384 to 1:786 with lymphocytotoxic titers in the same range. There was a 1–2 tube reduction in titer after purification of the antibody by DEAE-cellulose chromatography. The agglutination titer of the $F(ab')_2$ preparation was 1:192 at a protein concentration of 10 mg/ml, and it had no cytotoxic titer. The anti-glomerular serum had no lymphoagglutination titer but was cytotoxic at 1:16. The Fab' preparation inhibited agglutination by AT IgG at a dilution of 1:64. The $F(ab')_2$ and Fab' (1%) preparations showed s_{20} values of 5.7 and 3.4, respectively, by analysis in a Spinco model E ultracentrifuge[3] (Text-fig. 1).

[2] ^{125}I and ^{131}I were counted with a 3 inch thallium-activated NaI crystal by a Baird-Atomic model 540 gamma spectrometer with appropriate corrections for their gamma spectra. Samples did not exceed a 2×10^4 cpm rate.

[3] Kindly performed by Dr. T. J. Gill, III, Department of Pathology, Harvard Medical School, Boston, Mass.

By immunoelectrophoresis, the crude unabsorbed sera sometimes contained antibody to serum proteins, mainly α- and β-globulins, but these were not detected after absorption with rat serum. Also, anti-rat RBC antibody was demonstrated by hemagglutination in several of the preparations in titer of 1:16 to



TEXT-FIG. 1. Ultracentrifuge patterns of the pepsin-digested anti-thymus IgG (5.7S) and its reduced and alkylated fragment (3.4S). Both patterns shown are at 48 min at 48,000 rpm in a Spinco model E ultracentrifuge. Temperature is 20°C with 55° angle for the 5.7S and 65° angle for the 3.4S fragments.

1:64, and this was easily removed by absorption. All of the absorbed sera, as well as all but one of the IgG fractions and two of three normal rabbit serum preparations, contained agglutinins in low titer to sheep erythrocytes (Table I). Mercaptan treatment produced no decline in anti-sheep cell titer of the AT globulins, fraction (lot No. 6), indicating that the bulk of antibody was prob-

ably 7S. The anti-thymocyte agglutinating titer of this fraction was 1:256 and this specificity was not significantly affected by absorption with sheep red cells, although the latter reduced the anti-sheep red blood cell titer from 1:8 to less than 1:2 and had a profound effect on the antibody response to sheep erythrocytes. Whereas untreated rats, or rats pretreated with 10 mg normal rabbit IgG (NR IgG) containing no anti-sheep antibody, developed a hemagglutinating titer of 1:384, rats pretreated with 10 mg AT globulins (lot No. 6) over a 5 day period had titers of only 1:12 to 1:24. However, with absorption of the AT globulins (lot No. 6) as noted above, abolition of the immunodepressive effect

TABLE I

Antibody Activity in Various Rabbit Reagents with Sheep Erythrocytes

Preparation (lot No.)*	Agglutination titer
NRS (Hyland Labs., Los Angeles, Calif., No. 3042C1)	1:8
NR globulins (Mann Research Labs., New York, N.Y. No. S1869)	1:4
NR globulins (Pentex Inc., Kankakee, Ill. No. 51)	Negative
AT IgG (2)	1:4
AT IgG (3)	Negative
AT serum (4)	1:32
AT IgG (4)	1:4
AT IgG (5)	1:4
AT globulin (6)	1:8
Anti-glomeruli serum	1:8

* The protein concentration of the globulin fractions is 10 mg/ml.

on the response to sheep erythrocytes was observed. These results suggest that the passively transferred anti-sheep cell antibody (probably 7S) plays a role in suppressing the response to subsequently administered sheep erythrocytes, rather than the anti-lymphocyte effects of the antisera.

In Vivo Effects.—There was a profound lymphopenia with a 79 ± 12% (SD) decrease of peripheral lymphocytes and slight decrease of 25 ± 11.5% (SD) in serum β_{1c}-globulin 4 hr after a single intravenous injection of 1.2 mg of AT IgG. There was a decrease of 53 ± 10% (SD) in the peripheral lymphocyte count 4 hr after the intravenous injection of 1.2 mg of AT $(Fab')_2$ and a 26 ± 4% (SD) decrease after the injection of 1.2 mg AT Fab' fragment. Injection of 1.2 mg of NR IgG caused a decrease of 14 ± 5% (SD) in peripheral lymphocyte count. The net changes of β_{1c}-globulin level in these latter three groups were −7 ± 7%, −8 ± 7%, and +11 ± 5%, respectively.

Activity against Mast Cells.—In an in vitro cytotoxic assay for anti-mast cell activity,[4] it was demonstrated that the absorbed ATS caused a marked release

[4] Kindly performed by Doctors M. Valentine, R. Orange, and K. F. Austen, Robert Breck Brigham Hospital, Boston, Mass.

of histamine and hence either contained antibody to mast cells present in the thymus preparations or represents cross-reacting antibody.

The Immunosuppressive Effect of Anti-Thymus Serum.—To test the immunosuppressive effectiveness of all the preparations, animals were grouped into those that received the whole unabsorbed, crude ATS by intravenous injection, those that received the purified AT IgG fraction at two dosage levels, and those that received AT $F(ab')_2$, AT Fab', NR IgG, NR $F(ab')_2$, and rabbit anti-rat

TABLE II

*Effect of Pretreatment on Renal Allograft Rejection**

Rat No.	Pretreatment dose	Effective renal plasma flow	Morphologic evidence of immunosuppression
		ml/min/100 g‡	
1-02	4.0 ml AT serum	1.05	Good
1-82, 1-83	0.7 ml AT serum	0.99, 0.95	Good
2-13, 2-15, 2-17, 2-19	6 mg AT IgG	0.10, 0.32, 0.75, 1.30	Poor—good
2-48, 4-01, 4-02, 4-03, 4-09, 4-11	10 mg AT IgG	0.78, 1.08, 0.60, 0.67, 1.06, 1.18	Good
2-05, 2-06, 2-07	6 mg NR IgG	0.08, 0.06, 0.04	Poor (completely rejected)
2-93, 2-94, 2-95	6 mg AT $F(ab')_2$	0.13, 0.09, 0.21	Poor
3-21, 3-24	23–30 mg AT $F(ab')_2$	0.63, 0.74	Moderate—good
3-67, 3-68	23–30 mg NR $F(ab')_2$	0.07, 0.08	Poor (completely rejected)
3-56, 3-79, 3-86	23–30 mg AT Fab'	0.63, 0.30, 0.66	Moderate—good
3-62, 3-63	3.0 ml anti-glomeruli serum	0.08, 0.11	Poor (completely rejected)

* Day 7 animals studied for effective renal plasma flow and killed for histology.

‡ Based on the clearance of ^{125}I orthoiodohippurate. See text for description of method. Unilaterally nephrectomized rats have ERPF = 0.91 ± 0.23 (SD) and anephric rats have ERPF = 0.06 ± 0.03 (SD) measured by this method.

glomeruli serum (Table II). Allotransplanted kidneys removed 7 days after transplantation from control animals treated with NR IgG, NR $F(ab')_2$, or anti-glomeruli serum show the typical morphologic features seen in untreated 7 day renal allografts (Fig. 1). Numerous mononuclear cells with large vesicular nuclei and one or more prominent nucleoli diffusely infiltrated the cortical interstitial tissue and surrounded individual cortical tubules and glomeruli. Tubular necrosis was widespread in the cortex. Glomerular capillary loops were bloodless and occluded with fibrillar, PAS-positive material. Prominent large glomerular epithelial cells contrasted with decreased numbers of endothelial and mesangial cells which were frequently pyknotic and karyorrhexic. These animals had ERPF values in the range obtained in anephric rats.

Transplanted kindeys removed 7 days after transplantation from animals receiving unabosrbed ATS or 10 mg of AT IgG showed morphologic evidence of good immunosuppression. Infiltrating mononuclear cells were confined to perivascular areas. Tubules and glomeruli showed no evidence of rejection or

TABLE III

Donor Pretreatment with Anti-Thymocyte Antibody: Transfer of the Immunosuppressive Effect with Kidney-Bound Antibody

Rat No.	Pretreatment dose	Effective renal plasma flow*	Recipient titer to NR IgG‡	
			Whole titer	Mercaptan-resistant
		*ml/min/100 g**		
2-71	6–15 mg AT IgG (4)	0.33	Not done	
2-72		0.32		
2-78		0.23		
2-79		0.33		
2-84	16 mg NR IgG	0.06	Not done	
2-96		0.11		
2-97		0.15		
3-25	25 mg AT IgG (5)§	0.17	1:96	0
3-26		0.22	1:24	0
3-27		0.29	1:24	1:6
3-29	25 mg AT IgG (5)	0.05	1:12	0
3-30	Absorbed with Lewis thymocytes	0.04	1:6	0

* Renal function 7 days after allotransplantation.

‡ Hemagglutinin titer of sheep erythrocytes coated with NR IgG reacted with the serum of the recipient receiving the donor-pretreated kidney 7 days after allotransplantation.

§ This lot of antisera was prepared by immunizing rabbits with LBN_{F1} thymocytes. Absorption with Lewis thymocytes was incomplete and reduced the lymphoagglutinin and lymphocytotoxic titer 4-fold. Theoretically, any antibody directed against LBN_{F1} transplant antigens should still be present after absorption.

ischemia (Fig. 2). These rats had day 7 ERPF values in the normal range for rats with one kidney, while the rats pretreated with 6 mg of AT IgG showed poor to good immunosuppression with variable function correlating well with the histology. All pretreated animals had normal peripheral blood lymphocyte counts 7 days after transplantation. Day 7 sera from rats treated with antithymocyte serum did not have donor-specific isoantibody by the hemagglutination and lymphocytotoxic tests. These antibodies are usually found at this time in the sera of recipients with completely rejected kidneys. Moderate to

good suppression of renal allograft rejection was seen in animals treated with the AT $F(ab')_2$ and AT Fab', with renal function from near normal to normal.

Severe nephritis developed in the host kidney of all animals pretreated with unabsorbed ATS. Most glomeruli, but not all, showed hypercellularity and mesangial matrix increase which not infrequently involved only a portion of the glomerular tuft. Endothelial, mesangial, and epithelial cells participated in the glomerular hypercellularity. Occasional adhesions between capillary loops and Bowman's capsule occurred and proliferation of capsular epithelium formed occasional "crescents." The kidneys of recipient animals treated with NR IgG and AT IgG and removed at the time of allotransplantation showed slight evidence of nephritis characterized by mild focal hypercellularity.

Large and medium-sized thymocytes were depleted from the thymic cortex of most animals receiving ATS (Fig. 4), but the majority of the thymuses from AT IgG or antibody fragment-treated rats did not show consistent alterations and, when examined "blind" without knowledge of the experiment, they could not be consistently separated from control animal thymuses (Fig. 3). Similarly, only some spleens showed small follicles with large lymphocyte depletion.

The Immune Response to the Foreign Protein.—After an intravenous course of five daily injections of 1.2 mg of AT IgG, agar gel diffusion analysis showed circulating foreign protein in the recipient animals at day 7 after the first injection, but by day 14 this was no longer measurable. At day 14 and day 21 when foreign protein was not circulating, there was no precipitating antibody to the rabbit protein, even though there was a very high titer of hemagglutinating antibodies and a very rapid immune elimination of the foreign protein. Table IV shows that all groups of rats which received AT IgG, the antibody fragments, or NR IgG had immune elimination of ^{125}I-labeled NR IgG administered intravenously 2 wk after their first injection, which in these cases corresponded to the first day of the allotransplant. It is noteworthy that the animals treated with the AT antibody showed a more rapid immune elimination of the foreign protein than their respective NR IgG injected controls. Control uninjected rats showed no evidence of immune elimination of this test dose of ^{125}I NR IgG during a 3 wk period, and the plasma radioactivity declined as a single exponential with a half-life ($t_{1/2}$) of 6.5 days. Hemagglutination titers obtained using rabbit IgG-coated sheep RBC's correlated well with the immune elimination results. Animals receiving the anti-thymus antibody or antibody fragment had a significantly higher titer than animals receiving the NR IgG or NR $F(ab')_2$. Thus, the immune response to the foreign protein under the "immunosuppressive" influence of AT IgG was increased. Most of the antibody was mercaptan-sensitive, i.e., 19S. A single large dose of the AT IgG or a series of five subcutaneous injections resulted in titers as high as five small intravenous doses.

Immunofluorescent Tracing.—AT IgG conjugated to FITC was used in both

TABLE IV

Antibody Response to Rabbit IgG

Rat No.	Pretreatment dosage	Immune elimination*	Hemagglutination titer‡	
			Whole	Mercaptan-resistant
2-01	6 mg NR IgG	++	1:12	
2-02		+	1:24	
2-03		++		
2-08	None	−	0	
2-09		−	0	
2-10		−		
2-11		−		
2-20	6 mg AT IgG	+++	1:192	
2-21		+++	1:384	
2-22		+++		
2-23		+++		
2-68		+++	1:192	1:24
2-81		+++	1:384	1:48
2-69		+++		
2-70		+++		
2-40		+++		
2-41		+++		
3-39	6 mg AT IgG (single injection)		1:384	1:96
3-57	6 mg AT IgG (s.c.)§		1:768	1:96
3-58			1:384	1:48
2-73	6 mg AT $F(ab')_2$	+++	1:384	1:96
2-74		+++	1:192	1:96
3-19	6 mg NR $F(ab')_2$		1:3	0
3-20			0	0
3-56	23–30 mg AT Fab'		1:384	48
3-79			1:192	24
3-62	3.0 ml anti-glomeruli serum		1:24	0
3-63			1:96	0

* Immune elimination of 200–300 μg ^{125}I NR IgG 14–16 days after the first injection and 7 days after the last injection. Scored according to the time of >99% disappearance of plasma radioactivity. − Negative; + 4 days; ++ 1 day; +++ 10 min.

‡ Sheep erythrocytes coated with 1:8000–1:16,000 dilution of amboceptor and titered using serum obtained from rats 14 days after their first injection.

§ Subcutaneous injection in the cervical region. All other injections were by the intravenous route.

in vivo and in vitro tracing experiments. 5 mg of the conjugate were injected into rats, and 2½ hr later the animals were killed, and the spleen, thymus, lymph nodes, lung, heart, and kidneys obtained for immunofluorescent study. No localization was found in this way at this dosage level. To gain more sensitivity, sections of normal spleen, thymus, lungs, heart, lymph node, liver, and kidney, which were stained in vitro with FITC-AT IgG, showed cytoplasmic localization of the fluorescent antibody in cells of the spleen and thymus with no preferential distribution. Direct staining of the other organs was negative. 2½ hr after a single injection of 0.5 ml of the crude unabsorbed ATS, the kidney contained a considerable amount of rabbit IgG, rat IgG, and rat β_{1c}-globulin

TABLE V

*Anti-Thymocyte Organ Localization after a Single Intravenous Injection in Normal Rats of ^{131}I AT IgG (4)/^{125}I NR IgG As a Paired Label**

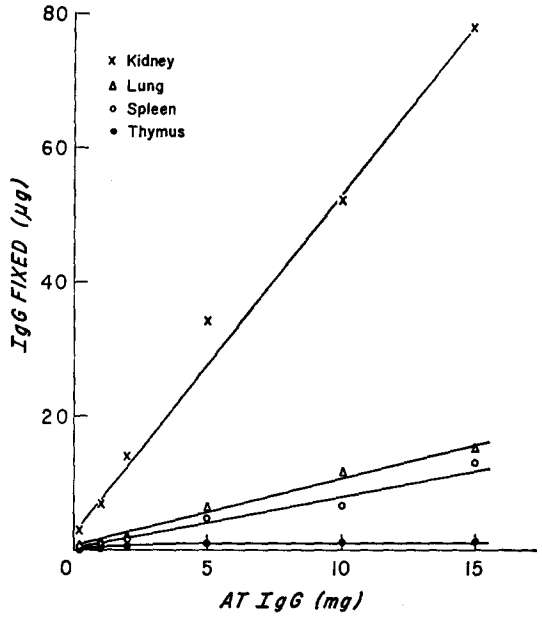
No. Rat	Time	Kidney	Liver	Spleen	Lung	Thymus	Lymph node	Heart	Muscle	Skin
		%	%	%	%	%	%	%	%	%
2-89	10 min	1.30	2.00	0.19	0.31	0.01	<0.01	0.03	—	—
2-90	10 min	0.92	1.92	0.21	0.25	0.02	<0.01	0.07	—	—
2-54	2.5 hr	0.57	2.74	0.78	0.69	0.02	—	—	—	—
2-53	4th day	0.33	<0.01	0.04	0.13	<0.01	<0.01	—	—	—
2-91	4th day	0.12	<0.01	<0.01	0.07	<0.01	<0.01	<0.01	<0.01	<0.01
3-11	4th day	0.16	0.12	0.02	0.04	0.02	<0.01	<0.01	<0.01	<0.01
2-92	7th day	0.06	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
3-12	4th day‡	0.04	0.04	<0.01	0.02	<0.01	<0.01	<0.01	0.01	<0.01

* Expressed as per cent fixed of injected $^{131}I = {}^{131}I_{organ} - [({}^{131}I_{plasma}/{}^{125}I_{plasma}){}^{125}I_{organ}]$.
‡ Injected with paired label subcutaneously in cervical region.

deposited in granules in the mesangium and along capillary walls of glomeruli, suggesting the presence of immune complexes by in vitro tracing.

Paired Label Antibody Organ Localization of Anti-Thymus IgG.—Table V presents the data obtained in one of the experiments in which localization in various organs of the AT IgG was determined using the paired label technique. ^{131}I AT IgG and ^{125}I NR IgG were injected into groups of rats which were killed 10 min, 2.5 hr, 4 days, and 7 days later. As can be seen, there was a significant amount of AT IgG radioactivity in the kidney at various times after intravenous injection of the paired label. The amount of radioactivity in spleen, lung, and liver was significantly greater at 10 min and 2.5 hr than at 4 days, and probably reflects the early deposition of immune complexes or denatured protein. In a saturation experiment where 200 μg of the AT IgG/NR IgG–paired iodine label were injected with increasing amounts of unlabeled AT IgG, there was progressive increase of fixation in kidney, lung, and spleen measured 4 days later, as shown in Text-fig. 2. Saturation of kidney did not occur at a level of 80 μg, contained in 15 mg AT IgG, the maximum amount

that could be administered without toxic effects to the animals. Since Katz et al (47) found that rabbit anti-rat spleen antibody saturated kidney at a level of 220 μg, the lack of a plateau with anti-thymus antibody at this level is not surprising. Although the thymus seemed to be saturated at the 5 mg level, Text-fig. 3 shows that because of the decreasing weight of thymus and increas-

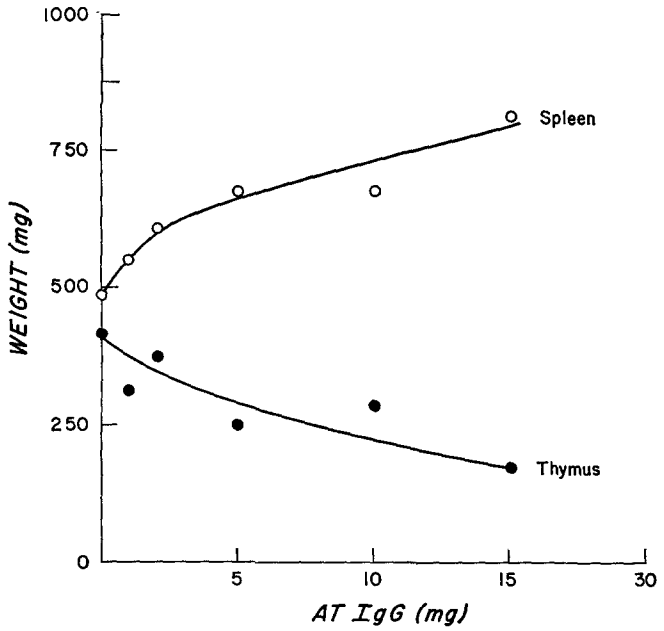


TEXT-FIG. 2. Organ localization of anti-thymus IgG: Saturation experiment in which increasing amounts of unlabeled AT IgG were given as a single intravenous dose with a fixed dose (300 μg) of paired label ^{125}I AT IgG/^{131}I NR IgG to normal rats and specific radioactivity determined 4 days later. Assuming identical distribution of labeled and unlabeled AT IgG, the absolute amount in μg fixed per organ was calculated and the relationships are shown above. Since doses of AT IgG greater than 15 mg were usually quite toxic, complete saturation of kidney was not possible.

ing weight of spleen, saturation may actually occur per unit weight at a slightly higher dose for thymus and lower dose for spleen. A second lot of AT IgG gave qualitatively similar values for all organs, with 1.14% in kidneys at 2.5 hr and 0.66% at 4 days, while liver contained 2.74% and <0.01%, respectively.

From the ratio of the two isotopes in plasma 10 min after injection, it could be calculated that between 20.2 and 33.6% of the radioactivity of AT IgG was cleared from plasma, suggesting a high percentage of anti-rat tissue antibodies. However, since the sums of the localization values for the organs measured (Table V) were less than 4%, 20–30% of the injected AT IgG must have been diffusely distributed elsewhere, either as anti-rat IgG, or because the AT

IgG was partially denatured. Since two lots of AT IgG and NR IgG used in separate experiments with the iodine labels reversed gave similar results, and since injection of the paired label into a rabbit produced no significant difference in the plasma ratio at 10 min, denaturation of the antibody IgG seemed unlikely. Nevertheless, an increased susceptibility of immune IgG to denaturation during labeling is still a possible explanation, and could also explain the greater degree of renal tubular localization shown below. The serum



TEXT-FIG. 3. Alterations in weights of spleen and thymus with increasing doses of AT IgG; same rats as in Text-fig. 2.

clearance of the ^{125}I AT $F(ab')_2$ and ^{125}I Fab' showed a multiexponential disappearance curve with an approximated half-life of less than 0.5 days. Within the first 24 hr, 47% of the injected AT $F(ab')_2$ radioactivity was recoverable in the urine, showing the rapid renal clearance and catabolism of this fragment.

Table VI presents the localization data in the kidney when ^{125}I AT IgG and ^{131}I NR IgG was used as a paired label and the kidneys fractionated into glomerular, tubular, and medullary fractions. With the exception of the animal injected subcutaneously, there was a constant amount of radioactivity (10%) in the glomerular fraction of rats killed at various times after injection. Although the major proportion of total radioactivity was not in the glomerular fraction, on a weight basis the glomeruli had 19.6 times the specific ac-

tivity of the tubular fraction. Radioautography clearly demonstrated the specific glomerular localization of AT IgG compared to the low background of radioactivity from NR IgG injected controls. (Figs. 5 and 6).

Text-fig. 4 shows the half-disappearance time of the radioactivity from the

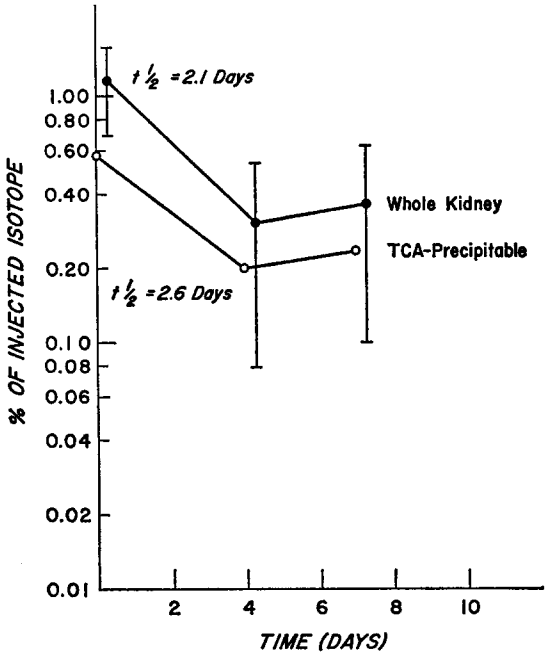
TABLE VI

Anti-Thymocyte Localization in Kidney

Rat No.	Time	Medullary	Tubular	Glomerular
		%	%	%
2-89	10 min	6.9	82.1	11.0
2-90	10 "	19.3	71.6	9.1
2-91	4 days	11.3	74.8	13.9
3-74	4 "	5.0	87.0	8.0
2-92	7 "	5.0	82.4	12.6
3-78*	4 "	16.0	84.0	<0.3

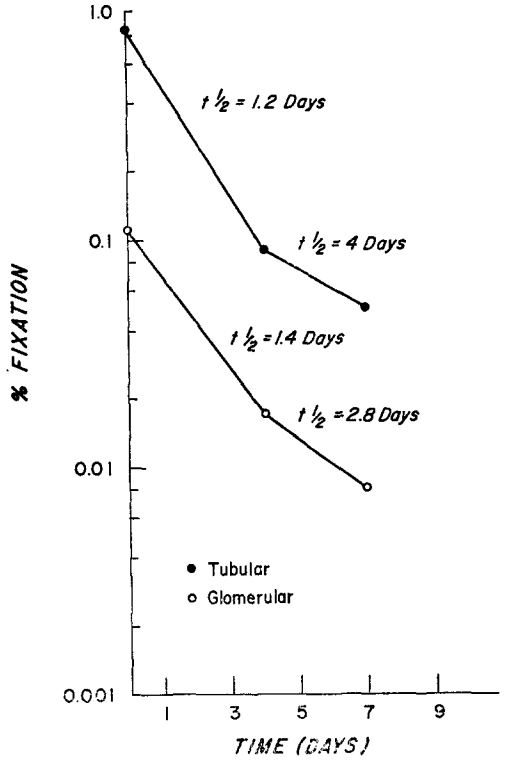
Per cent distribution of specific antibody radioactivity using paired label technique.

* Isotope injected subcutaneously in the cervical region. All other injections of 200 μg of the paired label were given intravenously.



TEXT-FIG. 4. Localization of anti-thymus IgG in kidney at intervals after a single intravenous injection of the paired iodine labels. The whole kidney values are shown as the mean and range obtained from two to four animals. The amounts of radioactivity which are precipitable with TCA are proportional to the total amount and show a similar half-time disappearance rate.

kidney on the basis of the organ localization data. It is seen that a portion of the AT IgG radioactivity disappeared from the kidney in the first 4 days very rapidly with a half-disappearance time of 2.5 days, while there was very little change in radioactivity between the 4th and 7th days, suggesting the presence of a population of antibody fixed in kidney (Text-fig. 5). In another



TEXT-FIG. 5. Localization of anti-thymus IgG in kidney: comparison of tubular and glomerular fractions at intervals after a single intravenous injection of the paired iodine labels. The disappearance rates in this experiment are the same for the two fractions.

experiment, the disappearance rate of radioactivity leaving an isotransplanted kidney from a donor injected with the paired label and the disappearance rate of the paired label from an injected recipient grafted with a "cold" isotransplanted kidney showed a half-disappearance time of 0.8 and 1.1 days, respectively. The nature of tubular radioactivity was demonstrated when radioautographs 10 min and 4 days after injection of ^{125}I AT IgG or ^{125} I NR IgG revealed predominantly proximal tubular cytoplasmic localization, present in the NR IgG–injected controls, but in greater concentration in the antibody-injected animals. At 4 days, the grains appeared to coalesce, consistent

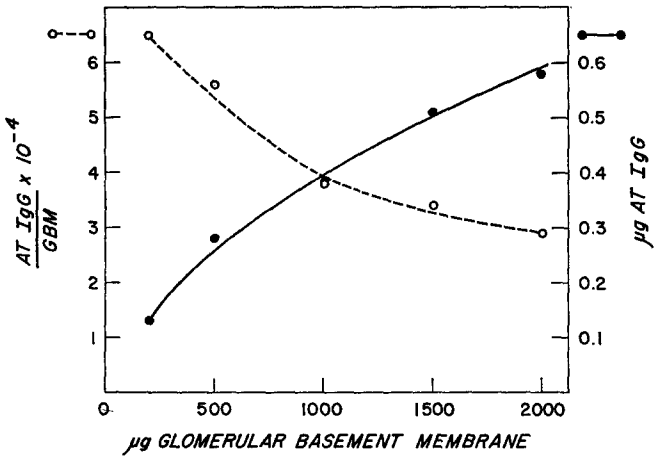
with the concept that such tubular localization represents a catabolic site for the reabsorbed and perhaps altered IgG reacting at distant sites. There was no apparent localization along blood vessels or tubular basement membranes. Chromatography of dialyzed kidney homogenates of both NR IgG and AT IgG–injected rats on Bio-Gel P-2, revealed the presence of peptide-bound radioactivity in both instances.

Since the kidney is an organ of excretion for iodide and small peptides, localization of radioiodide per se is not proof of antibody fixation. Data on TCA-precipitable (proteins and large polypeptides) radioactivity (Fig. 4) gave similar kinetics when compared to the whole kidney radioactivity, and since 49–61% of total radioactivity was TCA-precipitable and a similar percentage was nondialyzable in these experiments, the contained radioactivity in kidney was at least partly bound to protein and, hence, must have been due to specific antibody. In another experiment, the renal pedicle of a rat was clamped prior to and 1 hr after the injection of the paired label. 4 days later, the clamped kidney contained about one-third the amount of TCA-precipitable radioactivity as did the unclamped kidney, probably reflecting the acquisition of dissociating antibody.

The Effect of Donor Pretreatment on the 7 Day Allotrransplant.—When it became apparent that there was a significant amount of renal binding by antibody, it seemed possible that a locus of action could be on the kidney (target organ) itself. To test this hypothesis, one group of LBN F_1 hybrid donors was pretreated with a course of 6–15 mg of lot No. 4 of AT IgG over a 5–9 day period, and kidneys were transplanted into unmodified Lewis recipients. Another group of donors were pretreated with 16 mg of NR IgG and subsequently transplanted. A subsequent group of LBN F_1 donors were pretreated with 25 mg of lot No. 5 of AT IgG, obtained by immunizing rabbits with LBN F_1 thymocytes, and then transplanted while a control group was given an equivalent amount of AT IgG (lot No. 5), which was absorbed with 10^9 Lewis thymocytes for 4 hr at 4°C, resulting in a 4-fold decrease in titer. These two latter groups were done to rule out the possibility that antibody to genetically specific histocompatibility antigens was being made by the rabbits, and that the donor pretreatment phenomenon would be a form of "heterologous" enhancement.

Donor pretreatment with 6–15 mg of AT IgG (Table III) was effective in slightly reducing the morphologic and functional characteristics of typical allograft destruction in the day 7 animal, and the animals so treated had ERPF values of about one-third normal, while the animals injected with 16 mg of normal rabbit IgG had nonfunctioning kidneys. In the group of animals treated with 25 mg of anti–LBN F_1 thymus IgG (lot No. 5), there was also significant renal function, compared to the group treated with antisera incompletely absorbed with Lewis thymocytes which had completely

rejected kidneys. This would indicate that the antibody transferred on the donor kidney was not of histocompatibility antigen specificity, but of anti-lymphocytic or "immunosuppressive" specificity and reactive to lymphocytes of both rat strains. At the time of study, 7 days after allotransplantation, the recipients had detectable antibody to the rabbit IgG, further proof of antibody transfer on kidney and its subsequent dissociation to immunize the new host. On the basis of organ localization data obtained by the paired label method, the amount of IgG transferred in such a donor pretreatment experiment could



TEXT-FIG. 6. In vitro binding of anti-thymus IgG to purified rat glomerular basement membranes (GBM), performed with the paired iodine label technique. When a fixed amount of iodine-labeled AT IgG and NR IgG was mixed with increasing amounts of GBM, increasing amounts of AT IgG was bound (closed circles), amounting to 2% of the AT IgG at the highest antigen level. Only 0.3% of the NR IgG remained with the washed GBM at each of the antigen concentrations. The open circle plot shows the ratio of bound AT IgG at each concentration of GBM and indicates a progressive saturation of binding sites.

not exceed 15–25 μg of antibody protein, and 80% of this would be within the proximal tubular cells. Since 200–300 μg NR IgG given intravenously for the immune elimination studies failed to induce an immune response over a course of 3 wk, the renal bound and transferred antibody IgG was considerably more immunogenic than normal IgG, probably because its binding site directed it to a critical area in the lymphoid system, demonstrating in another way the ability of anti-lymphocyte antibody to augment the immune response to itself.

In Vitro Localization of Anti-thymocyte Antibody on Glomerular Basement Membrane.—In an in vitro glomerular basement membrane-binding experiment using the paired label technique (Text-fig. 6), some antibody activity was demonstrated towards purified glomerular basement membrane (GBM)

antigens. 2% of the AT IgG bound to GBM while only 0.3% of NR IgG bound nonspecifically.

Development of Immune Complex and Autologous Phase-Nephrotoxic Nephritis.—The crude unabsorbed anti-thymus serum which contained antibody to serum proteins caused severe immediate glomerular lesions in the recipients which were dose-related and only mild focal glomerular alterations in rats treated with the absorbed and purified AT IgG or fragments. No evidence for complex-induced nephritis was noted in the transplanted kidney in pretreated recipients, and it may be that the greatly augmented anti-IgG response of the host prevented the formation of soluble complexes which would have resulted in nephritis. However, the in vivo and in vitro evidence that cross-reacting antibody with glomerular basement membrane specificity was present in these preparations made possible the development of nephrotoxic nephritis. In rats given a series of five intravenous injections of the AT IgG or the AT $F(ab')_2$, there was no significant immediate proteinuria (greater than 15 mg/day). However, 6–7 wk after this series of injections, the animals showed an increase in their protein excretion from 40 mg to as high as 110 mg/24 hr with gradual clearing over the next 3 wk. Tissue sections of kidney examined by immunofluorescence at this time showed a linear deposition of host IgG along basement membrane, confirming the presence of an autologous phase-nephrotoxic nephritis (48). Furthermore, on the basis of the quantitative data obtained with localization of this lot of AT IgG using the paired label method, it can be estimated that during the standard series of the 5–8 injections of 6–10 mg of AT IgG, approximately 1–2 μg of kidney-fixing antibody (glomerular) was delivered to each animal, a dose sufficient to induce autologous phase-nephrotoxic nephritis if all were fixed to glomerular basement membrane, and if an adequate host response to the foreign protein occurs (48). The abundant antibody response in these anti-thymocyte–treated animals has been shown above. A second series of animals, followed for 3 months after intravenous injection of a different lot of AT IgG, developed only borderline and transient increases in protein excretion to 15 mg/day at 6–8 wk.

DISCUSSION

The characteristics of the heterologous rabbit antisera reported here in terms of titer against lymphocytes and lymphopenic effects (in vivo) are in agreement with the work of others (4, 8, 49–54). $F(ab')_2$ and $F(ab')$ pepsin fragments of AT IgG are capable of suppressing the allograft response to kidney allotransplants in rats to a degree nearly as effective as administration of the IgG (7S) fraction, while normal rabbit serum or its fractions, as well as an antiglomerular antibody, has no immunosuppressive effect. Further evidence of the potency of our AT IgG preparation was obtained when a 10 mg pretreatment dose prolonged survival in a group of three rats allowed to live beyond

the 7 day study period. They are alive at present with normal urea nitrogen and ERPF 28 days posttransplant.

Several lines of evidence have been presented to show that the sera raised against thymocytes contain antibodies of different specificities or cross-reactivities. Basement membrane specificity has been demonstrated by in vivo and in vitro experiments which show that an antibody against glomerular basement membrane is present which most likely arises from the presence of stromal contamination in the immunizing preparation. A side effect of the use of antithymus IgG, related to the presence of anti–basement membrane antibody in some lots, was the development of an autologous phase-nephrotoxic nephritis. Since a second lot of antisera tested for this potential produced only borderline changes in protein excretion, it is apparent that variations among rabbits in their response to basement membrane antigens must exist. Once a small amount (less than 2 μg) of rabbit antibody is fixed to glomeruli, the greatly augmented host antibody response to the foreign protein will assure the development of proteinuria with deposition of immunoglobulins and complement along the basement membranes. It is noteworthy that only 10% of the radioactivity in kidneys was in the glomeruli, and the bulk of the remainder was within the cytoplasm of proximal tubular cells, an important site of gamma globulin catabolism in the rat (55). Therefore, such localization could be a result of reabsorption of filtered normal IgG, or could represent catabolism of IgG denatured prior to injection, or could be a result of immune interaction elsewhere in the body. Either possibility would explain the increased tubular localization of AT IgG as compared to NR IgG, since localization of the former in glomeruli could produce an increased permeability to plasma proteins. Antibodies to various serum proteins were noted in some batches of antisera and, if not removed, induced a glomerular lesion characteristic of immune complex deposit disease. Thus, it is apparent that these heterologous antisera are potentially nephritogenic by two different mechanisms: formation of soluble antigen-antibody complexes, or the presence of anti-kidney antibodies (56).

Another specificity of the ATS revealed by the mast cell assay for histamine release in vitro is best explained by the fact that the thymus contains mast cells which were probably present in the immunizing suspensions. It has been noted that intravenous administration of ATS in high dosages produces a toxic reaction (10, 11) which could be related to a pharmacologic mechanism such as histamine release, although the presence of endotoxin in the various preparations has not been excluded.

Many of the rabbit antisera and fractions contained anti–sheep red cell agglutinating activity and some of them were entirely mercaptan-resistant (7S). Since it has been reported that ALS will depress the immune response to sheep erythrocytes (3, 5, 54, 57, 58), and since it is not stated whether or

not the preparations used contained anti-sheep red cell antibody, the results cited cannot be attributed solely to a specific effect of ALS but could have been due in part to the passive transfer of antibody to sheep RBC's (59). This is supported by our finding that pretreatment with 10 mg of the globulin fraction of ATS with a titer of 1:8 (all 7S) to sheep erythrocytes suppressed agglutinin formation when tested 6 days after an intravenous challenge with sheep cells. Absorption, however, of the anti-thymus globulin with sheep cells prior to administration of the same dose did not suppress agglutinin formation. Although it is not clear whether the anti-sheep erythrocyte titers found in the anti-thymocyte preparations are a reflection of a cross-reacting antibody to rat thymocytes, absorption of the AT globulin with sheep red cells did not significantly alter the titer to rat thymocytes. The failure to suppress antibody formation to sheep cells with antibody fragments of ALS in another study (58) may have been due to the species involved or the low dosages employed but absence of anti-sheep red cell antibody may have played a role.

It has been stated that the immunosuppressive properties of ALS may prevent an immune response to itself (7) based on the evidence of Monaco et al. (5) that mice treated with ALS failed to make such a response to the foreign protein. The data of the present study fail to support this finding since augmented responses to the foreign protein were the rule. One reason for this discrepancy may be that our studies were done with a lower dosage of antibody. Monaco et al. injected their mice with several plasma volumes of ALS over a 3 wk period and then looked for antibody to rabbit IgG 1 wk after the last injection. The failure to detect an antibody response may have been due to not having allowed sufficient time for the clearance and metabolism of the foreign protein, which may cause immunological paralysis or prevent detection of free antibody in the circulation. On the other hand, the immune response may have been truly altered because of a massive lymphocytotoxic effect, or because of the use of whole serum which may produce some degree of antigen competition to IgG (60). However, administration of several proteins in the form of anti-lymphocyte globulin to a large series of human patients did not suppress the development of anti-horse precipitins (11). It seems reasonable to explain the higher antibody titers to IgG in the AT IgG–treated rats compared to normal IgG–treated controls by assuming that more antigen, i.e. rabbit IgG, is delivered to the immune system by virtue of its anti-lymphocyte specificity. Supporting evidence for this concept is found in the isotope localization data which show that more specific antibody fixes in lymphoid tissue than NR IgG. Such localization in lymphoid tissue and in kidney by anti-rat lymph node antibody has been previously observed (61). The higher titers in the experimental animals probably do not reflect a type of enhancement of antibody formation similar to the effect of X-ray or cytotoxic drugs (62, 63) but alternatively could be due to an adjuvant effect of circu-

lating particulate material (destroyed lymphocytes) (64), or in the case of the antibody fragments, be due to cell-bound antigen delivery producing an adjuvant effect. The mechanism of the observation of Frenkel et al. (57) that AL IgG–treated rats make more antibody than NR IgG–treated controls when subsequently immunized with keyhole limpet hemocyanin may also be explained by an adjuvant hypothesis.

The exact mechanisms of action of ALS in suppressing the immune response to tissue allografts remain obscure although several important factors emerge. It has been stressed by Monaco et al. (5) that the lymphocytotoxic effect as evidenced by lymphopenia and cell depletion in lymphoid organs in chronically treated animals may be responsible for the immunosuppressive ability of the antisera. Waksman et al. (50) previously demonstrated, as well, that the lymphopenia induced by ALS correlated with the absence of the cellular response in delayed hypersensitivity, and Turk and Willoughby (65) have pointed out that both central and peripheral effects of the antisera on cellular inflammatory reactions may be operative. Although it is true that sustained lymphopenia and depletion of cells from lymphoid organs can occur with the administration of massive amounts of ALS, this is not a necessary condition for immunosuppression to renal allografts, since pretreatment with AT IgG will effectively cause significant prolongation of graft survival even though lymphopenia may be only transient. Furthermore, the fact that AT $(Fab')_2$ and AT Fab′ fragments produced prolongation of renal allograft survival is evidence against lymphocytotoxicity as the necessary condition for immunosuppression. The plasma clearance rates of the $F(ab')_2$ and Fab′ are similar to the values obtained for gamma globulin fragments of heterologous proteins in various other species combinations (66). The rapid renal clearance of these fragments probably accounts for the finding that increased dosage is required to demonstrate an immunosuppressive effect when the intravenous route is used, since not all of the specific antibodies would have a chance to fix in vivo prior to their presentation to the kidney. Although the Fab′ is less effective than the IgG on a dose basis, it would lend itself well to a study of its effect on cells and membranes in vitro since agglutination or cytotoxicity need not complicate the system. Levey and Medawar (7) have postulated that "sterile activation" of lymphocytes may be the immunosuppressive mode of action of ALS. They have noted many blast forms in the peripheral blood and tissues of treated animals. Moreover, it has been shown that ALS in mixed leukocyte culture is very effective in inducing blast transformation (67). Reasoning from the data we have obtained in our system, it would seem that observations of increased blastogenesis in vivo and in vitro can be explained on the basis of the marked immunogenicity of the AT IgG, and its effectiveness in this regard is due to the delivery of the AT antibody as an antigen to the cell membrane where the transformation is induced. Thus the possibility of a form of antigen competition (60) at the cell membrane as an operative mechanism

in the immunosuppressive ability of ALS is suggested by our studies. For the reasons presented above, the binding site and affinity of the antibody molecule for lymphoid cells renders it a much more efficient competitor than circulating antigen which would rely on a more random type of interaction with the immune system.

The present data also show that the antibodies which fix to kidney and dissociate with a half-life of 0.8–2.6 days are biologically effective as immunosuppressive antibodies since donor pretreatment produces some prolongation of kidney survival after transplantation in to an unmodified recipient. The significant amount of renal binding is in part due to the route of administration of the AT IgG, since subcutaneous administration produces very little kidney localization. Since antibody specifically made against isolated glomeruli was not immunosuppressive, target organ specificity per se cannot be a critical requirement. Absorption experiments showed that the kidney-bound antibody was of anti-lymphocyte specificity and not directed against histocompatibility antigens; therefore a degree of true cross-reactivity probably exists, and these experiments are not an example of enhancement in the usual context (68). However, it cannot be ascertained for certain, given the dissociation kinetics of this antibody, whether there may be a local immunosuppressive effect by a masking of histocompatibility antigens thereby preventing the release of antigen or by interfering with lymphocyte sensitization or function in the destruction of the allograft. Furthermore, the fact that the recipients of donor pretreated kidneys had abundant antibody to the foreign protein, although a subimmunogenic dose in terms of NR IgG was transferred, indicates that the dissociating AT antibody is a potent immunogen, and therefore probably has some lymphoid cell membrane specificity as a part of its binding site.

SUMMARY

Heterologous rabbit anti–rat thymocyte sera, its immunoglobulin G fraction, and the bivalent and univalent antibody fragments obtained by pepsin digestion are potent immunosuppressive reagents when tested in a system of renal allotransplantation between the LBN F_1 hybrid and Lewis rat strains.

The AT $F(ab')_2$ is not lymphocytotoxic in vitro but has agglutinating ability, while the AT Fab′ neither agglutinates nor is cytotoxic to rat lymphocytes, but will inhibit the in vitro reaction. The AT IgG and the $F(ab')_2$ are more immunogenic in their host than normal rabbit IgG and $F(ab')_2$, probably due to increased delivery of the antibody to the immune system.

Donor pretreatment studies demonstrate that a cross-reacting, highly immunogenic antibody with anti-lymphocyte specificity may bind to renal sites and be transferred to the new host after transplantation. In addition, the crude unabsorbed anti-thymocyte antisera may induce a nephritis characteristic of immune complex disease which can be eliminated by complete absorption with serum proteins. Further in vivo and in vitro evidence is pre-

sented that the AT IgG contains small amounts of antibody to glomerular basement membrane antigens and may induce an autologous phase-nephrotoxic nephritis. The amount of in vivo binding by AT IgG to GBM was reduced by subcutaneous rather than intravenous administration.

Most of the rabbit antisera tested contain antibody in low titer to sheep erythrocytes and in vivo experiments indicate that the nature of the immunodepressive effect of AT globulin to sheep erythrocytes is due in part to the passive transfer of antibody and is not necessarily due to a specific antilymphocyte effect.

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EXPLANATION OF PLATE 86

FIG. 1. Control 7 day renal allograft. Recipient was pretreated with normal rabbit IgG. The graft shows morphologic evidence of complete rejection. Hematoxylin and eosin. × 125.

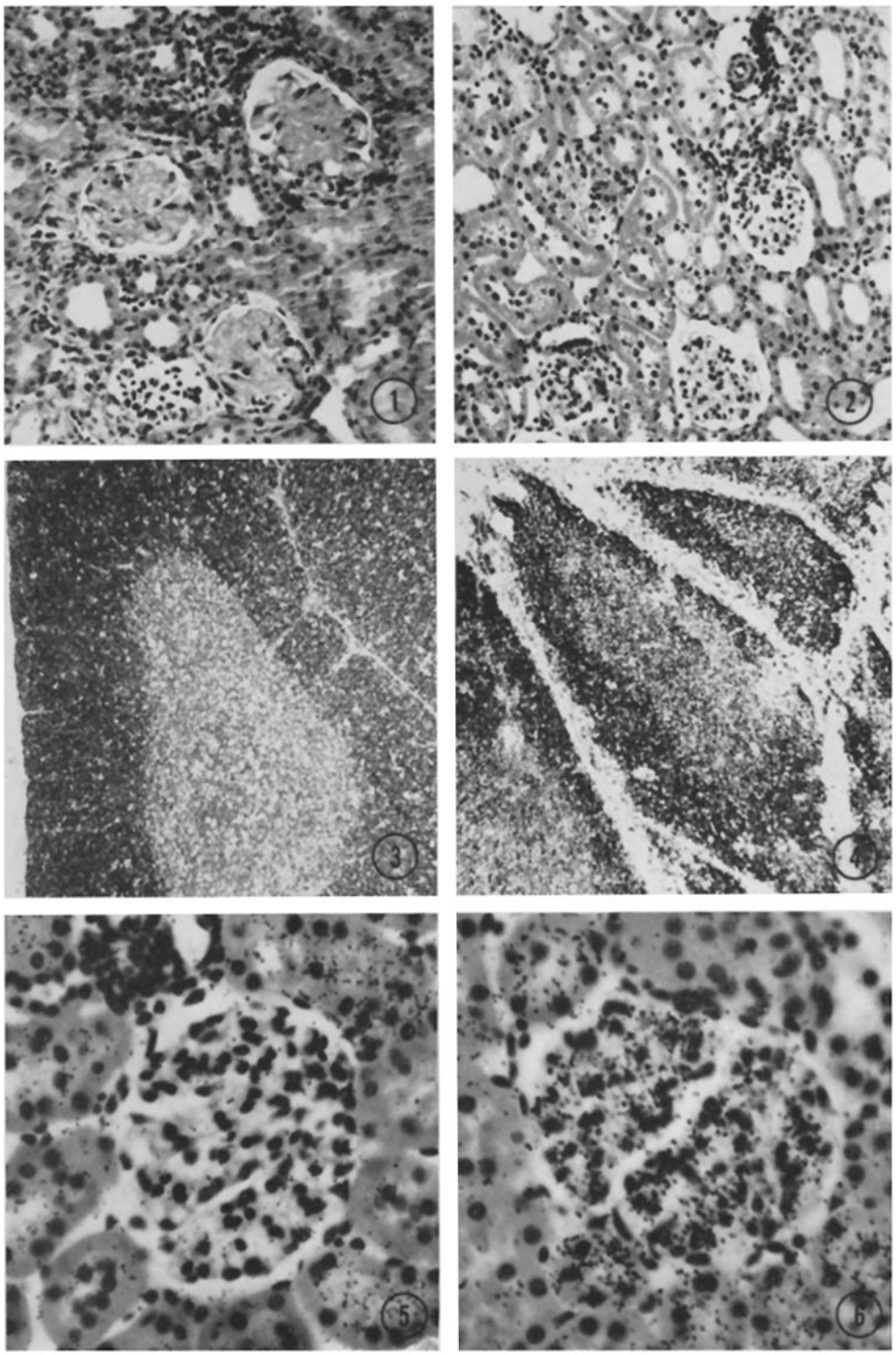
FIG. 2. 7 day renal allograft from a recipient that was pretreated with rabbit anti-rat thymocyte IgG. The only morphologic evidence of allograft rejection consists of a small perivascular collection of mononuclear cells (upper right). Hematoxylin and eosin. × 125.

FIG. 3. Normal rat thymus. The wide cortex contains medium and large sized thymocytes. Many of the treated rats had thymuses indistinguishable from this. Hematoxylin and eosin. × 45.

FIG. 4. Thymus from rat treated with 10 mg rabbit anti-rat thymocyte IgG. The cortex is thinned and depleted of small, medium, and large-sized thymocytes. The medulla of the thymus also shows depletion of thymocytes. Hematoxylin and eosin. × 45.

FIG. 5. Radioautograph of kidney from normal rat receiving ^{125}I normal rabbit IgG and killed 4 days later. Hematoxylin and eosin. × 250.

FIG. 6. Radioautograph of kidney from normal rat receiving ^{125}I anti-thymocyte IgG and killed 4 days later. Hematoxylin and eosin. × 250.



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