Glomerular Basement Membrane-Reactive Antibody in Anti-Lymphocyte Globulin

CURTIS B. WILSON, FRANK J. DIXON, JOSEPH G. FORTNER, and
G. JAMES CERILLI

From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, 476 Prospect Street, La Jolla, California 92037

ABSTRACT Equine immunoglobulin was detected along the glomerular basement membrane of three human homograft recipients who had been treated with equine anti-lymphocyte globulin. Anti-lymphocyte globulins, given these patients, were obtained by immunization of horses with lymphocytes from human spleens and/or lymph nodes and contained glomerular basement membrane-reactive antibodies. Quantitative paired-label isotope experiments (in rats) demonstrated that 30–170 $\mu\text{g}/\text{ml}$ of kidney-fixing antibodies were present in these preparations. The anti-lymphocyte globulins formed a line of identity with a sheep anti-human glomerular basement serum when reacted against collagenase-solubilized human glomerular basement membrane in double diffusion in agar. The renal fixation of these antibodies was blocked by absorption with human glomerular basement membrane, but not by buffy-coat leukocytes, indicating that they were directed specifically toward antigens in the basement membrane and were not cross-reacting anti-lymphocyte antibodies.

Anti-lymphocyte globulin preparations for human use were studied for glomerular basement membrane-reactive antibodies by a direct immunofluorescent assay in rats. Anti-lymphocyte globulin from 13 of 20 horses, and 7 of 10 serum pools from horses immunized with lymphocytes derived from solid lymphoid organs (spleen, thymus, lymph node, tonsil), contained glomerular basement membrane-reactive antibodies. Sera from 18 horses injected with thoracic duct cells or cultured lymphoblasts

had no glomerular basement membrane-reactive antibodies.

An equine anti-human thymus serum containing glomerular basement membrane-reactive antibodies, which produced fatal glomerulonephritis in monkeys, was shown to cause both immediate and delayed glomerular injury in monkeys after intravenous injection. The reaction of this antibody with glomerular basement membrane in vivo was associated with little complement deposition in spite of the fact that the antibody could fix complement. This lack of glomerular complement fixation resulted from almost complete in vivo decomplexation of the monkeys receiving this anti-lymphocyte globulin.

INTRODUCTION

Anti-lymphocyte globulin (ALG)¹ has come into widespread clinical use as an immunosuppressant in human organ transplantation. The effectiveness of this agent in prolonging skin grafts in rats (1) initiated its clinical use. Subsequent observations have revealed the nephritogenic potential of ALG. Lance reported nephritogenic effects of rabbit anti-mouse thymus sera in mice (2). Iwasaki et al. reported production of glomerulonephritis of the serum sickness type after administration of equine anti-canine lymphoid sera in dogs (3). Serum sickness has also been reported in mice (4) and man (5) after the administration of ALG. In a series of papers, Guttman, Carpenter, Lindquist, and Merrill reported glomerulonephritis in rats was produced by rabbit anti-rat thymus serum, presumably mediated in part by anti-

This is publication 465 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif., and was presented in part at the American Society of Nephrology Meeting, 23–24 November 1970, in Washington, D. C.

Dr. Fortner is from the Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, and Dr. Cerilli, from the Department of Surgery, Ohio State University Medical Center, Columbus, Ohio.

Received for publication 26 January 1971.

¹ *Abbreviations used in this paper:* ALG, anti-lymphocyte globulin; BUN, blood urea nitrogen; GBM, glomerular basement membrane; GBMRab, GBM reactive antibodies; Ig, immunoglobulin; PBS, phosphate buffered saline; PMN, polymorphonuclear leukocytes; SGOT, serum glutamic-oxaloacetic transaminase; SGPT, serum glutamic-pyruvic transaminase.

bodies capable of reacting with glomerular basement membrane (GBM) (6-8). Antibodies also have been found in equine anti-canine lymph node serum which fixed to dog glomeruli (9, 10).

To determine the nephritogenic potential of ALG in man, we studied renal tissues obtained from patients undergoing treatment with ALG in conjunction with renal or hepatic transplantation. Equine immunoglobulin (Ig) was demonstrated fixed in a linear fashion along the GBM in 3 of 35 patients in this group. Equine anti-human spleen and lymph-node lymphocyte sera administered to these patients were found to contain GBM reactive antibodies (GBMRab). These antibodies were quantitated and their specificity demonstrated. A survey of the incidence of GBMRab in ALG preparations produced for human use was done and correlated with the source of lymphocyte antigen used for immunization. The nephritogenic properties of an equine anti-human thymus antiserum in monkeys were defined.

METHODS

Immunofluorescent studies. Renal tissues from 35 patients (20 kidney and 15 liver homograft recipients) who had been treated with equine ALG were examined by the direct immunofluorescent technique (11). The reagents used were fluorescein isothiocyanate conjugated rabbit IgG shown to be specific for human immunoglobulin (IgG, IgA, IgM), complement (C) component C3, fibrinogen, albumin, and equine Ig by means of immunoelectrophoresis and double diffusion. The immunofluorescent microscopy techniques used in this laboratory have been described previously (12).

Quantitation of GBMRab in ALG preparations. GBMRab in seven ALG preparations were quantitated by the paired label technique (13) and included those (H-1c, H-11, and H-50a)³ given the three patients presented. 100 µg of ¹²⁵I or ¹³¹I-labeled ALG (14) with an equivalent amount of normal equine-Ig labeled with the other isotope were given intravenously to male Sprague-Dawley rats (150 g). The normal equine Ig was prepared by precipitation in a final concentration of 50% ammonium sulfate. Groups of four rats each were injected and bled out 18 hr later. The liver, spleen, lungs, heart, and kidneys were taken after extensive *in situ* perfusion with 0.01 M sodium phosphate buffered 0.15 M NaCl, pH 7.4 (PBS). The renal binding of ALG H-35 (P_{1a}) and H-56b was also quantitated in *Saimiri sciurea* monkeys (600 g). Pairs of monkeys were nephrectomized 18 hr after intravenous injection of 200 µg of ALG with an equivalent amount of paired label normal equine Ig. Because of inability to perfuse the nephrectomy specimens, washed kidney homogenates were used. Radioactivity was

determined in a NaI crystal dual channel scintillation counter. The specific amount of ALG bound to an organ was calculated by the formula:

Specific I*ALG organ bound = I*ALG organ bound

$$- \left[\frac{I^* \text{Eq Ig (organ)} \times I^* \text{ALG (blood)}}{I^* \text{Eq Ig (blood)}} \right]$$

The results are presented for each group of rats or pairs of monkeys as the mean per cent of ALG administered which was specifically bound to an organ.

Specificity of binding of I*ALG. Four of the I*ALG's that exhibited specific renal binding were absorbed with either human buffy coat leukocytes or human GBM to determine whether the kidney-fixing antibody might be a cross-reacting antileukocyte antibody. Antibodies to serum proteins detectable by gel diffusion were removed by prior absorption with pooled normal human serum. Four serial absorptions of 450 µg of I*ALG were done with washed human buffy coat leukocytes. The leukocytes were prepared by sedimentation in one part 3% dextran T500 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in PBS and two parts heparinized peripheral blood. The cells were washed four times in Hanks solution before absorption. The first three absorptions were done with 1×10^8 cells for 30 min at 37°C and the final absorption with a similar number of cells overnight at 4°C with constant shaking. Likewise, 450 µg of each I*ALG were absorbed with 25 mg human GBM (15) overnight at 4°C with constant shaking. The specific binding of the I*ALG to leukocytes or GBM used for absorption was calculated as described for organ binding.

The absorbed I*ALGs were used in *in vivo* paired label experiments identical with those used to quantitate the GBM reactivity of the original ALG, and the residual specific organ binding was determined. The effect of the absorptions on the binding of the I*ALG to buffy-coat leukocytes was also determined. 2 µg each of unabsorbed and absorbed I*ALG were incubated with 1.2×10^7 washed buffy-coat leukocytes for 1 hr at 37°C with frequent agitation. The specific binding of I*ALG to the leukocytes was calculated as described in the paired-label studies after four washes of the cells in Hanks solution.

***In vivo* determination and incidence of GBMRab in ALG.** ALG preparations for human use originating from horses, rabbits, or goats were assayed for GBMRab by direct immunofluorescence. Sprague-Dawley rats (150-200 g) were injected intravenously with 1.0-2.0 ml of an ALG preparation, housed in metabolic cages for urine collection, and bled out 18 hr after injection. Portions of the kidneys were frozen in liquid nitrogen for immunofluorescence and the remainder placed in Bouin's fixative for routine histologic examination. The presence of GBMRab was determined by direct immunofluorescence using fluorescein isothiocyanate conjugated rabbit anti-equine Ig, sheep anti-rabbit IgG, or rabbit anti-goat Ig. Positive staining was shown to be specific for Ig by absorption studies using homologous Ig. The ability of the GBMRab to activate rat C was determined by staining each positive tissue with fluorescein isothiocyanate conjugated rabbit anti-rat C3. The urine was studied for the presence of protein (12). Excretion of greater than 20 mg per 24 hr was considered abnormal.

Other measures of GBM reactivity in ALG preparations. The ALG preparations (H-1c, H-11, H-50a) were also studied for GBMRab by indirect immunofluorescence using cryostat sections of normal human kidney as a target. The sections were incubated with dilutions of the ALG for 30 min at room temperature. After washing, the sections were

³ The ALG preparations referred to throughout this paper will be designated as in Table III. The source is indicated by letter (H, horse; R, rabbit, and G, goat). Each animal is assigned a separate number. Different bleedings from the same animal are designated a, b, c, d. (ALG H-1a would mean the first bleeding studied from horse 1.) If the ALG was pooled sera from several animals, it is designated by P and the number of animals involved placed in parentheses.

stained with fluorescein isothiocyanate conjugated rabbit anti-equine Ig.

The three ALG preparations which reacted in vivo with patients' GBM were studied for GBMRAb by double diffusion in 0.7% ion agar in PBS containing 3% glycine. The human GBM antigen (a gift from Dr. Hans Marquardt) was prepared from glomeruli obtained by the method of Spiro (16) and solubilized by digestion with collagenase. Sheep anti-human GBM antibody (a gift from Dr. Charles Cochrane) was used for comparison. All precipitation lines to serum proteins were removed by prior absorption with pooled normal human serum.

Studies on the route of administration of the ALG preparations. To study the effect of local absorption of GBMRAb, the amounts of antibody fixed in the kidneys after intravenous and intramuscular administration were compared. One or more intramuscular injections not exceeding 0.1 ml were compared to intravenous injections in 150 g Sprague-Dawley rats. Fixation of Ig to the GBM was detected by direct immunofluorescence.

Studies on the production of glomerulonephritis in rats and monkeys with ALG preparations containing GBMRAb. Evidence for the immediate (heterologous) phase of nephrotoxic nephritis was sought in all rats used for the in vivo assay for GBMRAb. In addition, single intravenous injections of 2.5–5 ml of selected ALG preparations were made into Sprague-Dawley rats (150 g). One kidney was removed from each animal 2.5–4 hr after injection; the second was removed at sacrifice 24–72 hr later. These kidneys were studied by immunofluorescent and routine histologic means; the urinary excretion of protein was measured.

A sufficient quantity of equine anti-human thymus ALG (H-35 (P₁₂)) was available for biologic studies in monkeys. Four 3–4 kg *Macaca nemestrina burmensis* monkeys were given 30 ml of this preparation intravenously and housed in metabolic cages for urine collection. Urinary protein was determined. Base line protein excretion (4 days) was found to average 18 mg/24 hr (range 5–32 mg). Renal biopsies were taken 4 hr after injection. In one monkey, hepatic and splenic biopsies were also obtained at 4 hr. In the two surviving monkeys, weekly renal biopsies were obtained. Snap frozen renal tissue was studied by direct immunofluorescence for equine Ig, monkey IgG, and C3. Bouin's fixed tissue was used for routine histologic examination. Anti-body responses to equine serum proteins were monitored by double diffusion in ion agar. Serum transaminase (glutamic-oxaloacetic and glutamic-pyruvic (SGOT, SGPT) determinations were performed by Dr. Thomas S. Edgington. One control monkey given 30 ml of normal equine Ig received comparable studies.

Serum C determinations (CH₅₀) (17) were done before injection and at 5 min, 10 min, 2 hr, 4 hr, and 24 hr after injection in two of the four monkeys receiving ALG. The ability of the glomerular-bound equine ALG to fix C was studied by overlaying cryostat sections of the kidney with dilutions of fresh human serum and incubating for 30 min. Bound C was determined by immunofluorescence using antisera specific for human C3. The ability of ALG (H-35 (P₁₂)) to inactivate C in normal serum in vitro was determined. Residual CH₅₀ levels in 1:2, 1:5, and 1:10 dilutions of normal human serum with either the native ALG given the monkeys or deaggregated (upper half of the tube after ultracentrifugation 100,000 *g* for 60 min) were measured. The CH₅₀ U remaining in the normal human serum after 60 min incubation (37°C) with the ALG dilutions (native and deaggregated) were compared to those found after in-

cubation with the same dilutions of heat-inactivated (56°C × 30 min), deaggregated, pooled normal equine serum.

RESULTS

Immunofluorescent studies. Equine Ig was found by direct immunofluorescence in 1 of 20 renal homografts and in the kidneys obtained at autopsy from 2 of 15 hepatic homograft patients. The staining was a continuous, smooth, linear pattern along the GBM. In addition to the linear equine Ig, the renal homograft contained trace amounts of human IgG and C3 in a linear pattern along the GBM. Kidneys obtained from the two patients receiving hepatic homografts also had small amounts of linear human IgG and C3 in the glomeruli. No deposits of human IgA, IgM, or fibrinogen were found in these tissues.

Histologic sections from the renal homograft revealed evidence of rejection with mononuclear infiltrates around venules and glomerular hypercellularity (Fig. 1). Histologic changes in the kidneys from the hepatic homograft patients consisted of only minimal glomerular hypercellularity.

Quantitation of GBMRAb in ALG preparations. The results of the paired-label quantitation studies are presented in Table I. Three of the ALG preparations (H-1c, H-11, and H-50a) exhibiting GBMRAb when administered to the renal or hepatic homograft patients reported in this paper showed specific binding of 0.43, 0.07, and 0.07% to the kidneys of rats. The GBMRAb in ALG H-35 (P₁₂), which was used for the biological studies in monkeys, was also quantitated in rats with a specific binding of 0.13%. The only ALG (H-56b) induced by inoculation of cultured lymphocytes having weak GBM reactivity with rat glomeruli on immunofluorescence was also studied quantitatively. It showed 0.01% and less than 0.01% binding to rat and monkey kidney, respectively. Two additional ALG preparations (H-12a, H-53a) without immunofluorescent-rat GBM reactivity failed to show specific renal binding. From the amount of protein contained in the original ALG it could be calculated that ALG H-1c, H-11, H-50a, and H-35 (P₁₂) contained 170, 45, 30, and 65 µg of kidney-fixing antibody/ml (in the rat). ALG H-35 (P₁₂) contained 345 µg of antibody/ml capable of binding to *Saimiri sciurea* monkey kidneys.

Striking amounts of ALG H-1c and H-35 (P₁₂) were also bound to liver and spleen as shown in Table I. The liver and spleen from rats and/or monkeys injected with these ALG preparations had deposits of equine Ig along the hepatic sinusoids and splenic reticulum when stained with fluoresceinated anti-equine Ig (Fig. 2).

*Specificity of binding of I*ALG.* The amount of ALG that bound to isolated GBM during the absorption was 4.9, 2.1, 1.3, and 1.0% of ALG H-1c, H-11, H-50a, and H-35 (P₁₂), respectively. The only ALG (H-56b)

FIGURE 1 Histologic alterations in the renal homograft from J. N. removed 19 wk after transplantation. *A*, a venule in the juxtamedullary region is shown surrounded by a mononuclear cell infiltrate. *B*, a glomerulus from this kidney shows moderate hypercellularity. Hematoxylin and eosin. $\times 250$.

TABLE I
*Binding of I*ALG to Rat Organs in Vivo*

ALG†	Per cent of 100 μ g ALG bound*				
	2 kidneys	Liver	Spleen	2 lungs	Heart
H-1c	0.43	1.85	0.31	0.06	0.04
Absorbed (cells)§	0.36	1.45	0.24	0.06	0.03
Absorbed (GBM)§	0.13	0.45	0.15	0.06	0.02
H-11	0.07	0.24	0.10	0.05	0.03
Absorbed (cells)	0.05	0.30	0.06	0.03	0.01
Absorbed (GBM)	0.00	0.21	0.04	0.02	0.01
H-50a	0.07	0.43	0.04	0.03	0.01
Absorbed (cells)	0.06	0.35	0.04	0.02	0.01
Absorbed (GBM)	0.04	0.39	0.03	0.01	0.01
H-35 (P ₁₂)	0.13	0.34	0.06	0.00	0.00
Absorbed (cells)	0.09	0.34	0.06	0.00	0.00
Absorbed (GBM)	0.05	0.10	0.01	0.00	0.00
H-12a	0.00	0.13	0.06	0.05	0.02
H-53a	0.00	0.00	0.00	0.04	0.02
H-56b	0.01	0.12	0.01	0.01	0.00

* The percentage represents the mean of four animals in each group.

† The ALG preparations are identified in accord with the descriptions in Table III.

§ The I*ALG was absorbed with either buffy coat leukocytes (cells) or isolated human GBM (GBM) before in vivo study.

produced by immunization with cultured lymphoblasts, which had weak in vivo fixation to rat GBM by immunofluorescence, had no specific binding to isolated human GBM. The percentage of I*ALG bound to 1×10^8 leukocytes during repeated absorptions is shown in Table II.

Repeated absorption with buffy-coat leukocytes resulted in an average reduction of 23% in the renal fixation of I*ALG, while a single absorption with GBM caused an average reduction of 69% (Table I). The binding of I*ALG to leukocytes was reduced 36% by prior absorption with GBM and 85% by absorption with peripheral leukocytes.

In vivo determination and incidence of GBMRAb in ALG. The incidence of GBMRAb in 81 ALG preparations for human use was determined by the fixation of Ig to rat GBM in vivo (Table III). The preparations studied consisted of 60 bleedings from 38 individual horses, and 10 pooled ALG preparations from 56 horses (24 of which were, in addition, studied individually). The remaining 11 preparations studied were individual and pooled sera from 126 rabbits and 15 goats.

FIGURE 2 Immunofluorescent pattern of equine Ig bound to the lining of the hepatic sinusoids (A) and to the splenic reticulum and vasculature (B) of a monkey 4 hr after intravenous injection of ALG H-35 (P₁₂). Fluorescein isothiocyanate conjugated rabbit anti-equine Ig. $\times 128$.

The results were divided according to the source of lymphocytes used as antigens. Inoculation with lymphocytes of splenic origin induced GBMRAb in 9 of 12 individual horses and in one pool. Inoculation with thy-

mocytes produced GBMRAb in one of five individual horses and in five of eight pools. Lymphocytes from lymph nodes were used for inoculation in two horses, from tonsils in one horse, and mixed lymphoid organs

TABLE II
*Binding of I*ALG to Peripheral Leukocytes during Absorption*

ALG†	Per cent of ALG bound to buffy coat cells*			
	1st absorption 1 $\times$ 10 ⁸ cells	2nd absorption 1 $\times$ 10 ⁸ cells	3rd absorption 1 $\times$ 10 ⁸ cells	4th absorption 1 $\times$ 10 ⁸ cells
	37°C 30 min	37°C 30 min	37°C 30 min	4 $\pm$ C overnight
H-1c	1.89	0.44	0.31	0.34
H-11	0.69	0.30	0.23	0.19
H-50a	1.01	0.70	0.38	0.35
H-35 (P ₁₂)	0.47	0.22	0.13	0.35

* 450 μ g of I*ALG with an equivalent amount of paired label normal equine Ig used in the in vivo quantitative experiments were absorbed.

† The ALG preparations are identified in accord with the descriptions in Table III.

TABLE III
Fixation of ALG to Rat GBM in vivo

ALG*	Lymphocyte source	Bleeding	GBM reactive antibody
		<i>days</i>	
H-1a, b, c	Spleen	69, 98, 163	+‡, +, +
H-2	Spleen	118	—
H-3	Spleen	237	—
H-4	Spleen	327	+
H-5	Spleen	71	+
H-6a, b, c	Spleen	53, 72, 120	+, +, —
H-7a, b	Spleen	74, 123	+, +
H-8a, b, c	Spleen	76, 124, 150	+, —, —
H-9a, b, c	Spleen	137, 143, 187	+, +, +
H-10	Spleen	75	—
H-11	Spleen	45	+
H-12a, b, c, d	Spleen	60, 120, 150, 180	—, +, —, —
H-13 (P ₃)	Spleen	—	+
H-14a, b, c	Thymus	90, 120, 150	—, —, —
H-15	Thymus§	730	+
H-16	Thymus	30	—‡
H-17	Thymus§	45	—‡
H-18	Thymus§ (membranes)	70	—‡
H-19 (P ₂)	Thymus§ (membranes)	65	—‡
H-20 (P ₄)	Thymus§	42	+
H-21 (P ₄)	Thymus§	42	+‡
H-22 (P ₄)	Thymus	90	+
H-23 (P ₁₂)	Thymus§	—	+
(H-23 → H-34¶)	Thymus§	44	+
H-35 (P ₁₂)	Thymus§	—	+
(H-35 → H-46¶)	Thymus§	21	+
H-47 (P ₃)	Thymus§	165	—
H-48 (P ₄)	Thymus§	180	—‡
R-1	Thymus	—	—
R-2 (P ₄₀)	Thymus§	28	—‡
R-3 (P ₄₀)	Thymus§	30	—‡
R-4 (P ₄₀)	Thymus§	43	—
H-49a, b, c	Lymph node	45, 60, 150	—, — , +
H-50a, b	Lymph node	330, 365	+, +
H-51	Tonsil	240	+‡
H-52 (P ₈)	Lymph node		
	Thymus,		
	Thoracic duct§	180	+
G-1	Spleen,		
	Thymus,		
	Peripheral blood	240	—
H-53a, b, c	Thoracic duct	45, 90, 210	—, —‡, —
H-54a, b, c	Thoracic duct§	150, 300, 365	—‡, —, —
H-55	Thoracic duct	90	—
H-56a, b	Cultured lymphoblasts§	35, 63	— , ?+
H-57, H-58	Cultured lymphoblasts	—	—
H-59	Cultured lymphoblasts§	60	—‡
H-60 → H-70	Cultured lymphoblasts§	60-212	—
R-5	Cultured lymphoblasts§	60	—‡
R-6 → R-9	Cultured lymphoblasts§	57	—
G-2 (P ₁₄)	Cultured lymphoblasts§	120	—

* ALG. The source of the ALG is indicated by the letter (H, horse; R, rabbit; and G, goat); each animal is assigned a separate number. Different bleedings from the same animal are designated a, b, c, d (ALG H-1a would mean the first bleeding studied from horse 1). ALG prepared from pooled sera are designated P and the number of animals involved are placed in parentheses.

‡ and || indicate if the ALG preparation was effective (‡) or not (||) in prolonging skin grafts in monkeys (18).

§ Indicates the use of adjuvant for immunization.

¶ The individual horses (12 each) used in these pooled ALG preparations (H-23 (P₁₂) and H-35 (P₁₂)) were studied individually.

FIGURE 3 Gel diffusion pattern of ALG H-1c, H-11, H-50a with in vivo GBMRAb and H-53a which lacked in vivo GBMRAb reacted with collagenase-solubilized human GBM (center well). Sheep anti-human GBM sera (Sh-a-GBM) was used as a reference. Note the double line of identity which developed with ALG H-1c, H-11, and Sh-a-GBM.

in pooled sera of eight horses, and all were positive. Three groups of 40 rabbits each given thymic lymphocytes produced no detectable GBMRAb.

In contrast to the ALG prepared from lymphocytes derived from solid lymphoid organs, only ALG (H-56b) from 1 of 18 horses inoculated with lymphocytes collected from the thoracic duct or cultured lymphoblasts had a weak reaction with rat GBM by immunofluorescence. Sera from 5 individual rabbits and a serum pool from 14 goats inoculated with cultured lymphoblasts also had no GBMRAb. Irregular granular and patchy staining of the glomeruli seen in some rats could be easily differentiated from the smooth, continuous, linear staining characteristic of antibodies reacting with the GBM.

The presence of GBMRAb in the series of ALG preparations studied was limited to horses immunized with lymphocytes derived from solid lymphoid organs. In addition, there seemed to be a correlation between the crudeness of the inoculum and the development of GBMRAb. For instance, in one laboratory, 12 of 12 horses given thymic homogenate developed GBMRAb while 3 horses given purified thymocyte membranes did not. There was no relationship between the use of adjuvants and the development of GBMRAb. The GBMRAb appeared as early as the 21st day and persisted in most animals studied serially. There was no

correlation between the presence of GBMRAb and the in vivo immunosuppressive effect reported to us by the suppliers of 20 ALG's tested (Balner [18]) (Table III).

Other measures of GBMRAb in ALG preparations. By indirect immunofluorescence the ALG preparations (H-1c, H-11, H-50a) could be shown to fix to human GBM in vitro. When these ALG preparations were studied in an undiluted state, fixation to the GBM was obscured by intense staining of all cellular structures in the sections. If the ALG preparations were diluted, however, the diffuse staining diminished and the presence of linear staining along the GBM was evident.

ALG's H-1c, H-11, and H-50a were studied for the presence of anti-GBM antibody by double diffusion in agar. Double lines of identity developed between H-1c and H-11, and an antibody developed in sheep to human basement membrane (Fig. 3) when reacted with collagenase-solubilized human GBM. These ALG preparations contained 170 and 40 μ g, respectively, of kidney-fixing antibodies/ml (rat).

Studies on the route of administration of ALG preparations. Comparison of intramuscular and intravenous injections of the ALG preparation H-1c on in vivo glomerular fixation in rats showed that 5 to 6 times as much ALG was needed intramuscularly as was required intravenously to produce detectable fixation.

FIGURE 4 Smooth, continuous linear deposits of equine Ig are shown along the GBM of the glomerulus of a monkey 4 hr after intravenous ALG H-35 (P_{12}). Fluorescein isothiocyanate conjugated rabbit anti-equine Ig sera. $\times 250$.

Studies on the production of glomerulonephritis in rats and monkeys with ALG preparations containing GBMRAb. No evidence of the heterologous phase of nephrotoxic nephritis was seen in the rats used for the in vivo assay of GBMRAb as manifested by protein excretion or histologic alterations. The intravenous administration of 2.5–5 ml of ALG preparations H-1c, H-15, and H-52 (P_8) failed to elicit proteinuria or polymorphonuclear accumulation in the glomeruli of rats studied at either 2.5–4 hr or 24–72 hr after injection. Heavy deposits of equine Ig found in these specimens at 2.5, 4, and 24 hr disappeared by 72 hr. Rat C was not identified in the glomeruli at any time.

ALG H-23 (P_{12}) and H-35 (P_{12}) produced a progressive and fatal glomerulonephritis when administered to monkeys in doses of 120 mg/kg, three times per week (19). Frozen tissues from these monkeys were studied in our laboratory and revealed linear fixation of equine Ig, monkey IgG, and C3 along the GBM. To study the mechanism of this glomerulonephritis, four monkeys were given single intravenous injections of H-35 (P_{12}). Two monkeys died 6 and 12 hr after injection, apparently from the toxic effects of the ALG.

Heterologous or immediate phase of nephrotoxic nephritis manifested by immediate proteinuria occurred in all four monkeys, with the two that survived having 393 and 821 mg in the first 24 hr. Microscopic hematuria

with large numbers of casts was also present. Immunofluorescent study of the 4 hr renal biopsies revealed heavy linear deposits of equine Ig along the GBM in all four monkeys (Fig. 4) with minimal C3 deposits in the same distribution in only one. Spleen and liver biopsies at 4 hr and autopsy specimens at 6 and 12 hr revealed heavy accumulation of equine Ig along the splenic reticulum and hepatic sinusoids (Fig. 2). The levels of SGOT and SGPT were elevated during the first 24 hr after administration of this ALG in two of three monkeys studied.

Hemolytic C activity in the two monkeys studied was reduced 70% within 10 min of injection and reached even lower levels at 2, 4, and 24 hr. To explain this decrease in circulating C activity with virtual absence of detectable C3 tissue localization, the ALG was mixed in varying dilutions with normal human serum. Native and deaggregated ALG H-35 (P_{12}) diminished hemolytic C activity 75 and 70% respectively in a 1:5 dilution in normal serum, the same dilution as was achieved in the monkeys. The glomerular-bound GBMRAb from this ALG could be shown to fix C in tissue sections by indirect immunofluorescence. These findings suggested that the ALG might produce immediate damage to the GBM without significant C localization. The lack of C fixation was not because equine antiserum was unable to fix C but apparently was caused by a reduction of available C by another effect of the ALG.

Histologic observations within the first 12 hr after ALG administration revealed a variable accumulation of polymorphonuclear (PMN) leukocytes in the glomeruli; a maximum of 10 per glomerulus was seen in the only monkey having trace amounts of glomerular C3. PMN leukocytes were also present in hepatic sinusoids, splenic pulp, and alveolar septa. All these areas contained equine Ig by immunofluorescence.

Kidney biopsies, taken after circulating monkey anti-equine Ig antibody was detectable, revealed linear deposits of monkey IgG and C3 in addition to equine Ig along the GBM. Histologic sections taken at this time revealed a proliferative glomerular lesion with apparent thickening of the GBM. PMN leukocyte accumulation in the glomeruli was variable. Proteinuria, which was continuously present, declined initially but rose again coincident with the accumulation of monkey IgG and C3 and corresponding to the autologous (delayed) phase of the nephrotoxic nephritis.

At autopsy of the two monkeys, 17 and 58 days after injection, equine Ig was found on the GBM, hepatic sinusoids and splenic reticulum.

DISCUSSION

The identification of potentially nephritogenic GBMRAB in human ALG preparations similar to that responsible for anti-GBM antibody mediated glomerulonephritis (20) is not surprising and is consistent with observations of GBMRAB in rabbit anti-rat thymus serum studied by Guttman, et al. (6). Binding of equine Ig to human homograft glomeruli has been reported (21, 22). Vascular elements in many tissues are capable of inducing nephrotoxic antibodies when injected into animals (23). Katz, Unanue, and Dixon reported the nephritogenic properties of rabbit antibody produced by injections of rat splenic homogenate (24). This anti-spleen antibody possessed properties similar to classic Masugi nephritogenic antibodies and was capable of inducing both the heterologous and autologous phases of nephrotoxic nephritis in rats. The anti-spleen antibody had 90% cross-reactivity with kidney but differed from anti-kidney antibody by disappearing from the kidney twice as rapidly.

The three GBMRAB positive ALG preparations administered to the patients reported here were induced by injection of cells obtained from solid lymphoid organs. Of 20 horses injected with these lymphocytes, 13 made antibodies capable of binding to the GBM. In four preparations studied in detail, fixation to GBM was blocked by prior absorption with human GBM but not by lymphocytes, suggesting that the GBMRAB in these preparations were directed toward basement membrane antigens contaminating the inoculum and not a cross-reacting anti-lymphocyte antibody. The lack of GBMRAB

in ALG induced by injection of thoracic duct cells or cultured lymphoblasts supported this assumption. The GBMRAB-positive ALG preparations formed a precipitin line of identity with a sheep anti-human GBM antibody when reacted with collagenase-solubilized GBM demonstrating further the specificity of the reaction. Finally, two rabbit anti-human GBM sera did not possess detectable lymphocytotoxicity. (D. Kallestad, Kallestad Laboratories, Minneapolis, Minn.)

GBMRAB in equine anti-human thymic serum (H-35 (P₁₂)) were shown to produce progressive and fatal glomerulonephritis in monkeys after repeated injections (19). These antibodies were also capable of causing immediate glomerular injury accompanied 7–10 days later by increased proteinuria at the onset of the autologous phase of nephrotoxic nephritis in monkeys. The immediate injury was produced in the virtual absence of detectable C localization in the glomeruli presumably because of the decomplexation caused by the ALG rather than inability of the equine Ig to fix C. The means of this decomplexation is the subject of another investigation and may involve an anti-complement antibody.

The pathologic significance of the GBMRAB given patients is difficult to assess. The renal homograft of J.N. lost function after ALG administration (intramuscularly) containing 19 mg of GBMRAB (as quantitated in rats) to treat a third rejection episode. The glomerular pathology might have been associated with either rejection or GBMRAB. Any nephritogenic effect of this GBMRAB was presumably reduced by virtue of the intramuscular route of administration. Local absorption of GBMRAB given intramuscularly may explain both the infrequent observation of GBM-bound foreign Ig (25) and the severe local reactions (5, 26).

Amounts of GBMRAB in the ALG given intravenously to the two liver homograft recipients (27) were estimated from the rat isotopic studies. Patient G. B. received a total of 2.8 mg of GBMRAB 20 days before death; patient C. V. received 2.0 mg, 165 days and 0.5 mg, 4 days before death. Only minimal glomerular alterations were observed in the kidneys of these patients at autopsy. Any nephritogenic effects of the GBMRAB might have been modified by the concomitant use of chemical immunosuppressive regimens in both patients. The rather broad basement membrane reactivity of some ALG preparations with quantitatively greater hepatic than renal fixation should be considered in evaluating hepatic injury as was seen in the above two cases.

APPENDIX

Case G. B. This 42 yr old Caucasian male underwent total hepatectomy and hepatic homotransplantation for unresectable bile duct carcinoma. His initial immunosuppression program consisted of azathioprine, prednisolone, and ALG, 15–

20 ml daily intravenously. Hepatic dysfunction persisted with elevations in SGOT, alkaline phosphatase, and total and conjugated bilirubin in spite of increased amounts of all the above immunosuppressive agents in addition to local radiation therapy (100 R daily for 5 days). The patient expired 37 days after transplantation from bilateral Gram-negative pneumonia. No proteinuria or elevation in serum BUN or creatinine were observed. During the posttransplantation period, the patient received the following amounts of ALG: H-12a-72.5 ml, H-17-285.0 ml, H-50a-92.5 ml, and H-54a-55.0 ml.

Case C. V. This 27 yr old Caucasian female underwent hepatectomy and hepatic homotransplantation for a primary hepatoma. The patient was preterminal at time of transplantation after chemotherapeutic and radiation therapy for the tumor. Transplantation immunosuppressive therapy consisted of azathioprine, prednisolone, and intravenous ALG daily for 6 wk supplanted by varying doses and schedules. Persistent elevations of total and conjugated bilirubin, SGOT, and alkaline phosphatase were noted after transplantation. Her condition stabilized sufficiently 3 months after transplantation to allow discharge on azathioprine, prednisolone, and ALG, until her death occurred in hepatic coma and terminal bronchopneumonia 170 days after transplantation. No proteinuria or elevation in serum BUN or creatinine were observed in the postoperative period. During the posttransplantation period, the patient received the following amounts of ALG: H-11-45 ml, H-12a-22.5 ml, H-16-6.25 g, H-17-100 mg, H-50a-15 ml, H-53a-183 ml, H-53b-125 ml, and H-58-11.8 g. Two additional ALG preparations not available for study were also given in 15 and 52.5 ml amounts.

Case J. N. This 23 yr old Caucasian female had a 10 yr history of glomerulonephritis culminating in renal failure requiring hemodialysis for 2 months. She underwent bilateral nephrectomy with concurrent insertion of a renal homograft obtained from her brother (Teraski C minus match). The kidney functioned promptly with a creatinine clearance of 81 ml/min on the 2nd postoperative day. Her immunosuppressive regimen consisted of azathioprine, prednisone, and intramuscular ALG. The dosage of ALG was 2 ml daily for 5 days pre- and post-transplantation and then on alternate days. Rejection episodes occurred on the 3rd and 28th postoperative days and responded to increased steroids, local radiation and, actinomycin C. A 3rd rejection episode with increased proteinuria (500 mg/24 hr) occurred on the 63rd posttransplantation day. It was elected not to increase the prednisone but to treat the patient with high doses of intramuscular ALG H-1c-4 ml, plus 2-4 ml of ALG R-1 daily for 4 wk. Renal function improved over the next 10 days and then deteriorated. Proteinuria remained unchanged. The kidney was removed 19 wk after transplantation at which time the creatinine clearance had fallen to 8 ml/min. The patient received ALG: H-1c-190 ml and approximately 100 ml of ALG R-1.

ACKNOWLEDGMENTS

We wish to acknowledge the kind cooperation of the following investigators for providing the ALG preparations used in this study: Dr. G. Gray, Hypersensitivity Diseases Research, The Upjohn Company, Kalamazoo, Mich.; Doctors K. Heide and F. Seiler, Behringwerke AG, Marburg, Germany; Mr. Donald Kallestad, Kallestad Laboratories, Inc., Minneapolis, Minn.; Dr. D. E. Kayhoe, National Institute of Allergy and Infectious Diseases, National Institutes of

Health, Bethesda, Md.; Dr. G. S. LaFontaine, Bionetics Research Laboratories, Bethesda, Md.; Dr. J. S. Najarian, Department of Surgery, University of Minnesota Medical School, Minneapolis, Minn.; Dr. T. E. Starzl, Department of Surgery, University of Colorado Medical Center, Denver, Colo.; Dr. H. E. Taylor, Medical Research Council, Ottawa, Canada; and Dr. E. R. Unanue, Department of Pathology, Harvard Medical School, Boston, Mass.

This research was supported by United States Public Health Service Grant AI-07007, United States Public Health Service Contract PH 43-68-621, and United States Atomic Energy Commission Contract AT (04-3)-410.

REFERENCES

1. Woodruff, M. F. A., and N. A. Anderson. 1962. Effect of lymphocyte depletion by thoracic duct fistula and administration of anti-lymphocyte serum on the survival of skin homografts in rats. *Nature (London)*. 200: 702.
2. Lance, E. M. 1968. The effects of chronic ALS administration in mice. In *Advance in Transplantation Proceedings of the First International Congress of the Transplantation Society*. J. Dausset, J. Hamburger, and G. Mathé, editors. Munksgaard, Copenhagen S. 107.
3. Iwasaki, Y., K. A. Porter, J. R. Amend, T. L. Marchioro, V. Zuhlke, and T. E. Starzl. 1967. The preparation and testing of horse anti-dog and anti-human anti-lymphoid plasma or serum and its protein fractions. *Surg. Gynecol. Obstet. Int. Abstr. Surg.* 124: 1.
4. Cohen, B. J., M. J. de Vries, M. J. van Noord, and F. H. Lubbe. 1970. Strain-specific renal toxicity of heterologous anti-lymphocyte γ -globulin in mice. *Transplantation*. 10: 1.
5. Monaco, A. P., M. L. Wood, and P. S. Russell. 1967. Some effects of purified heterologous anti-human lymphocyte serum in man. *Transplantation*. 5: 1106.
6. Guttman, R. D., C. B. Carpenter, R. R. Lindquist, and J. P. Merrill. 1967. Renal transplantation in the inbred rat. III. A study of heterologous antithymocyte sera. *J. Exp. Med.* 126: 1099.
7. Guttman, R. D., C. B. Carpenter, R. R. Lindquist, and J. P. Merrill. 1967. Treatment with heterologous antithymus sera: nephritis associated with modification of renal allograft rejection and the immune status of the host to the foreign protein. *Transplantation*. 5: 1115.
8. Lindquist, R. R., R. D. Guttman, C. B. Carpenter, and J. P. Merrill. 1969. Nephritis induced by anti-lymphocyte serum. *Transplantation*. 8: 545.
9. Diethelm, A. G., G. J. Busch, J. M. Dubernard, W. McN. Orr, R. J. Glasscock, A. G. Birtch, and J. E. Murray. 1969. Prolongation of canine renal allografts by horse anti-dog lymphocyte serum and globulin. *Ann. Surg.* 169: 569.
10. Orr, W. McN., A. G. Birtch, A. G. Diethelm, J. M. Dubernard, J. Duquella, and R. J. Glasscock. 1970. A study of the potential nephrotoxicity of heterologous anti-lymphocyte serum. *Clin. Exp. Immunol.* 6: 305.
11. Coons, A. H., and M. H. Kaplan. 1950. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exp. Med.* 91: 1.
12. Wilson, C. B., and F. J. Dixon. 1970. Antigen quantitation in experimental immune complex glomerulonephritis. I. Acute serum sickness. *J. Immunol.* 105: 279.
13. Pressman, D., E. D. Day, and M. Blau. 1957. The use of paired labeling in the determination of tumor-localizing antibodies. *Cancer. Res.* 17: 845.

14. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29: 185.
15. Krakower, C. A., and S. A. Greenspon. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* 51: 629.
16. Spiro, R. G. 1967. Studies on the renal glomerular basement membrane. Preparation and chemical composition. *J. Biol. Chem.* 242: 1915.
17. Mayer, M. M. 1967. Complement and complement fixation. In *Experimental Immunochemistry*. E. A. Kabat and M. M. Mayer, editors. Charles C. Thomas, Springfield, Ill. 2nd edition. 133.
18. Balner, H., U. P. Eysvoegel, and F. J. Cleton. 1968. Testing of anti-human lymphocyte sera in chimpanzees and lower primates. *Lancet*. 1: 19.
19. Taylor, H. E. 1970. The occurrence of glomerular basement membrane reactive antibodies in anti-human antithymocyte globulin. *Transplant Proc.* 2: 413.
20. Lerner, R. A., R. J. Glasscock, and F. J. Dixon. 1967. The role of antiglomerular basement membrane antibody in the pathogenesis of human glomerulonephritis. *J. Exp. Med.* 126: 989.
21. Carpenter, C. B., A. Rashid, A. I. Katz, G. J. Busch, A. L. Sheffer, M. D. Valentine, A. G. Birtch, R. E. Kay, K. F. Austen, and J. P. Merrill. 1968. Adverse reactions to the use of horse anti-human lymphocyte globulin (ALG) in man. American Society Nephrology Meeting, Washington, D. C., 25-26 November 1968.
22. Thiel, G., J. Moppert, J. Mahlich, F. Buhler, T. Vischer, F. Enderlin, H. Weber and H. U. Zollinger. 1971. Glomerular damage after intravenous administration of antilymphocyte globulin (ALG) in man and rhesus monkey. *Transplant Proc.* 3: 741.
23. Unanue, E. R., and F. J. Dixon. 1967. Experimental glomerulonephritis: Immunological events and pathogenetic mechanism. *Advan. Immunol.* 6: 1.
24. Katz, D. H., E. R. Unanue, and F. J. Dixon. 1967. Nephritogenic properties of cross-reacting kidney-fixing antibodies to heart, spleen and muscle. *J. Immunol.* 98: 260.
25. Traeger, J., D. Fries, J. P. Revillard, J. Brochier, and N. Brunat-Blanc. 1969. Antilymphocyte globulins in kidney transplantation: effects on glomerular disease. *Transplant Proc.* 1: 1006.
26. Kashiwagi, N. B. A. B., L. Brettschneider, C. G. Groth, and T. E. Starzl. 1968. Clinical reaction and serologic changes after the administration of heterologous antilymphocyte globulin to human recipients of renal homografts. *Ann. Intern. Med.* 68: 275.
27. Fortner, J. G., M. H. Shiu, H. Balner, C. B. Wilson, G. Sichuk, N. Kawano, J. T. Holmes, and E. J. Beattie, Jr. 1971. Observations on prolonged immune suppression for human liver homografts. *Transplant Proc.* 3: 383.