IMMUNOREACTIVE BASEMENT MEMBRANE ANTIGENS IN NORMAL HUMAN URINE AND SERUM*

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In studies of normal rabbit urine prior to the injection of heterologous nephrotoxic antiserum, Hawkins and Cochrane demonstrated traces of basement membrane material. However, during the phase of acute injury to rabbit glomerular basement membrane (GBM)[1] resulting from fixation of such antibody, significantly greater amounts of immunologically similar antigens were present in the urine (1). Subsequently, by immunizing rabbits with fractions of either autologous or homologous urine, Lerner and Dixon were able to induce an acute glomerulonephritis mediated by specific anti-GBM antibodies (2). The latter suggested more strongly that normal rabbit urine contained soluble antigens derived from or cross-reactive with homologous GBM.

Investigations reported here were undertaken to attempt detection of similar soluble antigens in urine of normal people and their isolation and characterization. Our results indicate that normal people excrete antigens immunologically related to GBM (3). Preliminary analyses suggest that these antigens are carbohydrate-rich and of high molecular weight. These antigens will react with homologous anti-GBM antibodies and can be demonstrated in trace amounts in normal human serum.

Materials and Methods

Antiserum.—Sheep antiserum to human glomerular basement membrane (SAHGBM) was raised by immunizing a sheep to particulate human GBM harvested by the method of Krakower and Greenspon (4), and was the gift of Dr. C. G. Cochrane. The serum was absorbed twice with half-volumes of pooled whole human blood, fractionated with ammonium sulfate at half saturation, dialyzed against phosphate buffered saline (PBS), and concentrated.

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; GBM, glomerular basement membrane; IEP, immunoelectrophoresis; NHS, normal human serum; PAS, periodic acid–Schiff; PBS, phosphate-buffered saline; PGT-GL, organically extracted and trypsinized GBM preparations; RAHIgG, fluorescein-conjugated rabbit 7S anti-human immunoglobulin G; SAHGBM, sheep antiserum to human glomerular basement membrane; SARIgG, fluorescein-conjugated sheep anti-rabbit IgG; SAS/60, ammonium sulfate (60% saturation).

Preparation of GBM Antigens.—Soluble GBM digests were prepared from 100 mg pools of particulate GBM isolated as above from human kidneys obtained at autopsy, using either highly purified collagenase[2] from *Clostridium histolyticum* or bovine pancreatic trypsin.[2] Digestions were done for 72 hr and 4 hr, respectively, as described by Spiro (5) and Cole (6). Solubilized GBM was then heated to 60°C for 30 min, desalted on Bio-Gel P-2 columns and lyophilized. Organically extracted and trypsinized GBM preparations (PGT-GL) used were the gift of A. S. Markowitz, Ph.D.

Preparation of Urinary Antigens.—

(*a*) *Separation procedures:* Forty-two 24-hr urine collections from 26 normal adults who voided into clean jars containing 1:1000 merthiolate as preservative were concentrated by ultrafiltration, dialyzed in the cold against distilled water, lyophilized, and stored separately. 500 mg lots of dry, nondialyzable urinary solid were then reconstituted in 5–6 ml of barbital buffer, pH 8.6, $\mu = 0.05$, and electrophoresed on Pevikon at 450 v for 20 hr by the method of Müller-Eberhard (7). Bovine serum albumin (BSA) trace-labeled with ^{125}I, was added to the starting material for a marker. Pevikon blocks were divided transversely into multiple segments and each segment was eluted individually with distilled water. The individual eluates were counted in a well-type sodium iodide crystal to determine the position of the marker, and then tested by double diffusion in agarose against specific antiserum, using the microtemplate method of Cochrane and Aikin (8). Individual eluates forming a precipitin line with SAHGBM were pooled, dialyzed against distilled water, and lyophilized. 25 mg lots of this material were subjected to electrofocusing in a glass column for 48–72 hr at 700 v using Ampholine carrier electrolytes, pH range 3–5, according to the method of Vesterberg and Svensson (9). Volumes of 2.5 ml were collected at the end of each run directly from the column, and their pH measured with a combination electrode, using a Beckman pH meter. These fractions likewise were tested individually, after dialysis against distilled water, and those containing antigenic material were pooled and lyophilized.

Gel filtration was performed on Sephadex G-100 and G-200 columns, 2.5 × 90 cm, using 0.1 M Tris-HCl buffer, pH 8.1, 1 M with respect to NaCl, applying 15–30 mg pools of antigenic material from the electrofocusing step. Chromatographic peaks were read directly at 280 mμ wavelength on a LKB Uvicord scanner; the fractions comprising each peak were dialyzed, lyophilized, and tested against SAHGBM. To eliminate any neutral carbohydrate that might have eluted from the Sephadex columns, the fractions from that step were chromatographed separately on diethylaminoethyl cellulose (DEAE), using 0.0175 M phosphate buffer, pH 6.5; after the void volume from the column had passed, the antigen-containing fractions were then eluted with 1 M NaCl, dialyzed and retested against SAHGBM, prior to subsequent studies.

(*b*) *Analytic procedures:* Polyacrylamide gel electrophoresis was done by the method of Davis (10), using 7½% gels at pH 9.5 in Tris-glycine buffer. Gels were stained with amido Schwärz and periodic acid–Schiff (PAS) reagents, and duplicate gels were embedded in 1% agarose on 3 × 5 inch glass slides to allow double diffusion against antisera placed in parallel troughs cut in the agarose. Nitrogen was measured by an automated micro-Kjeldahl procedure, and neutral carbohydrate by the anthrone reaction of Roe (11).

Radiolabel Procedures.—Antigen isolates, and BSA[3], were labeled with ^{125}I or ^{131}I by the method of McConahey and Dixon (12).

Immunoelectrophoresis and Radioimmunoelectrophoresis.—Immunoelectrophoresis (IEP) of urinary antigens from the electrofocusing step and soluble GBM antigens, at concentra-

[2] Obtained from Worthington Biochemical Corp., Freehold, N.J.

[3] Armour Pharmaceutical Co., Kankakee, Ill.

tions of 10–20 mg/ml, was done at room temperature in 1% agarose in barbital buffer, pH 8.2 (μ = 0.04), for 35 min at 5.5 v/cm. Antiserum used was SAHGBM; precipitin lines were allowed to develop in a moist chamber at room temperature for 12–36 hr. Radio IEP was done under the same conditions on mixtures of radiolabeled and unlabeled antigens at concentrations of 20 mg/ml in PBS and in normal human serum (NHS) at 1:2 dilution.

Immunodiffusion Studies.—Ouchterlony analyses were carried out in 0.5% agarose in barbital buffer, pH 8.2; double diffusion studies from polyacrylamide gels were performed in 1% agarose, pH 8.2. Immunodiffusion studies were set up at soluble GBM antigen and urinary antigen concentrations from 1 to 20 mg/ml, depending on the particular study. (*a*) Analyses performed using SAHGBM compared concentrated urines from different individuals to: one another, to solubilized human GBM preparations, prepared as above, and to the PGT-GL of Markowitz (13). (*b*) In an effort to detect antigens in serum, double diffusion studies were undertaken, using dilutions of serum in the antigen wells against undiluted SAHGBM and in other plates undiluted sera against dilutions of SAHGBM. Sera tested were seven control sera from normal people and nine from patients with chronic kidney disease. The latter were picked from among patients whose clinical diagnosis was chronic glomerulonephritis and whose native kidneys, examined by direct immunofluorescence at time of nephrectomy, failed to show anti-GBM antibody fixation. Two bleedings were tested from each of the nine patients in the kidney disease group: serum drawn prior to nephrectomy and serum drawn from anephric patients awaiting renal allografts. Ouchterlony reactions were allowed to develop at room temperature and kept for 3–7 days before discarding.

Immunofluorescence Procedures.—Direct immunofluorescent examinations of rabbit kidneys and normal human kidney sections used for testing anti-GBM antibody-containing eluates were performed by the technique of Coons and Kaplan (14) as already described from this laboratory (15). Cryostat sections of flash-frozen tissues that had been stored at −20°C or −70°C were used; fluorescein-conjugated test reagents were prepared by the dialysis method of Clark and Shepard (16).

Absorption Experiments.—In order to test further the relationship of these soluble urinary antigens to human GBM, studies were carried out to attempt absorption of homologous antibodies with anti-GBM specificity. Samples of eluates prepared by acid-citrate elution (17), containing anti-GBM antibodies from kidneys of three patients with Goodpasture's syndrome and one not associated with the latter, were prepared separately at 1 mg/ml concentrations. Each of these was absorbed separately with each of the following at 10 mg/ml concentration: urinary material from the electrofocusing procedure, collagenase and trypsin-solubilized human GBM, solubilized sheep tropocollagen and BSA. All preparations were incubated at 37°C for 1 hr, held overnight at 4°C, centrifuged at 1800 *g* for 1 hr and then examined by direct immunofluorescence for abolition of fixation to frozen tissue sections of homologous kidney. When necessary, absorption experiments were carried out using 20 or 30 mg/ml added antigen until fixation, compared to unabsorbed controls, was blocked. Fluorescein-conjugated rabbit 7S anti-human immunoglobulin G (RAHIgG) was the indicator reagent.

Serum Fractionation.—To study whether NHS might contain antigenic material reactive with SAHGBM, bank blood was obtained, separated from red cells, and fractionated by dropwise addition of ammonium sulfate to 60% saturation (SAS/60) in the cold. The supernatant was discarded and the precipitate washed and reprecipitated, then dialyzed against water, and lyophilized. The lyophilized residue was then submitted to preparative electrophoresis on Pevikon as with the urinary procedures already described. When Ouchterlony analyses of eluates from repeated fractionations indicated that the immediate postalbumin zone contained traces of immunoreactive material detectable with SAHGBM, pools of such fractions were then chromatographed on DEAE columns, 1.0 × 30 cm, equilibrated with 0.0175 M phosphate buffer, pH 6.5. Stepwise elution of proteins was then performed by ad-

dition of NaCl to 0.1, 0.2, and 0.3 M concentrations, while recording continuously at 280 mμ. The separate peaks were then dialyzed against water, lyophilized, and tested by double diffusion analysis against SAHGBM.

Study of Urinary Antigens from Individual Normal People for Histocompatibility Antigen Activity and Blood Group Activity.—These were carried out separately by Dr. P. Terasaki, Dr. F. Rapaport, and Dr. G. M. Williams in lymphocytotoxic assay and mixed cell agglutination systems by absorption of standard typing sera. Study for blood group activity was done by serial absorption of test antisera using (*a*) lyophilized, crude urine residue, (*b*) the postalbumin fraction from Pevikon electrophoreses of urine, and (*c*) the antigen-containing fraction of urine from ampholyte columns from individuals with known blood types, testing for inhibition of A, B, H, Rh, MN, and Kell activity. These studies were performed by Dr. Thomas Edgington.

Biological Experiments.—

(*a*) *In vivo circulation:* To test whether isolated urinary antigens may circulate in vivo, 1 mg portions of separated urinary antigen fractions from G-200 were labeled with ^{131}I and injected intravenously into separate pairs of unimmunized 2.5 kg white rabbits, given potassium iodide in their drinking water. The latter were then bled at 5, 15, 60, and 120 min, and at 24 and 48 hr after injection. The trichloracetic acid precipitable and total counts per min (cpm) of 1 ml of serum were then plotted for each bleeding as a percentage of the amount at 5 min. Animals were housed in metabolic cages and urines collected. At 48 hr, animals were sacrificed, urinary bladders emptied to complete the collections, eviscerated, and total cpm of lungs, liver, heart, and kidneys measured in a whole body counter. Pooled urines from each animal pair were counted, dialyzed for 72 hr, and recounted. Where there was residual, nondialyzable radiolabeled ^{131}I, this was concentrated by lyophilization, submitted to preparative electrophoresis on Pevikon as above, with added BSA-^{125}I as marker, and eluted fractions counted in a dual-channel spectrometer. The eluates containing ^{131}I were then pooled and radio IEP done as before against SAHGBM to confirm presence of labeled antigen.

(*b*) *Immunization studies:* Immunization studies were undertaken in three previously unimmunized 2.5 kg male white rabbits injected at days 0, 8, and 15 with 1 mg antigen-containing material in incomplete Freund's adjuvant (IFA) from the electrofocusing procedure. First injections were into hind foot pads, the others subcutaneously. Two control animals were immunized with material also eluted from the electrofocusing column; the latter did not contain antigenic material when analyzed in double diffusion against SAHGBM, and eluted at a pH range above 3.5. Kidneys from both sets of animals were removed 7 days after the last injection and were processed for histological and immunofluorescent examinations using fluorescein-conjugated 7S sheep anti-rabbit IgG (SARIgG). Urines were examined by the sulfosalicylic acid method at 7-day intervals and prior to nephrectomy to detect proteinuria.

RESULTS

Electrophoresis of Concentrated Nondialyzable Urine Residue.—All human urines tested contained antigenic material reactive with SAHGBM, and double-diffusion studies characteristically demonstrated two precipitin lines (Fig. 1). These antigens migrated in the immediate postalbumin area during preparative electrophoresis on Pevikon at pH 8.6.

Electrofocusing.—Pooled, salt-free, antigenically-active fractions eluted from Pevikon tested in three different Ampholine-carrier electrolyte systems, finally

at pH range 3–5 for 48–72 hr at 700 v, localized over a pH range 1.7–3.5. As tested in double diffusion, one antigen focused discretely at pH 1.7–1.8, indicating its isoelectric point. The other antigen localized over a pH range of 1.7–3.6, indicating by such a broad isoelectric zone that it had a heterogeneous composition and probably represented a family of antigens closely related physically.

Gel Filtration.—Chromatography on Sephadex G-200 at pH 8.1 allowed separation of the two major antigenic moieties characteristic of concentrated, normal human urine. The first component eluted off G-200 in the void volume, and henceforth is called fraction 1. It is represented in the precipitin line closer

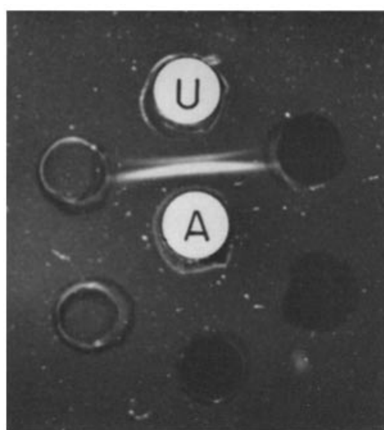

Fig. 1. Double diffusion study illustrating the characteristic precipitin lines developed between antiserum to human GBM (A) and urinary antigens (U) at 5 mg/ml.

to the antigen well in Fig. 1. The second component, fraction 2, was retarded on G-200. The relationship of these individual antigens to the mixture is illustrated in Fig. 2.

Analytic Procedures.—

(*a*) *Polyacrylamide gels:* Electrophoresis of pooled, antigen-containing eluates from the preparative electrophoretic step demonstrated several bands when stained with amido Schwärz reagent and PAS. When duplicate gels embedded in agarose were allowed to react in double diffusion against SAHGBM, a precipitin line formed which arched toward the major stainable band, which stained intensely with PAS and had a fast-moving, alpha mobility. Polyacrylamide disc electrophoresis of separated fractions 1 and 2 done in standard 7½% gels showed no staining with amido Schwärz, single lines with PAS, and precipitin lines in agar against SAHGBM when tested as above.

(*b*) *Carbohydrate analyses:* Analyses of fractions 1 and 2 by the anthrone

reaction for total neutral sugars indicated a content of approximately 50% for fraction 1 and 35% for fraction 2.

(*c*) *Nitrogen analyses* of fractions 1 and 2 showed that the former was approximately 1.9 and the latter 5.0% nitrogen by weight.

Immunoelectrophoresis.—As shown in Fig. 3, urinary antigens migrated in agarose at pH 8.2 with mobility of fast-moving alpha-globulins. Inasmuch as studies carried out with antigens dissolved in normal human serum showed no difference in their electrophoretic mobility from antigens in saline, there was

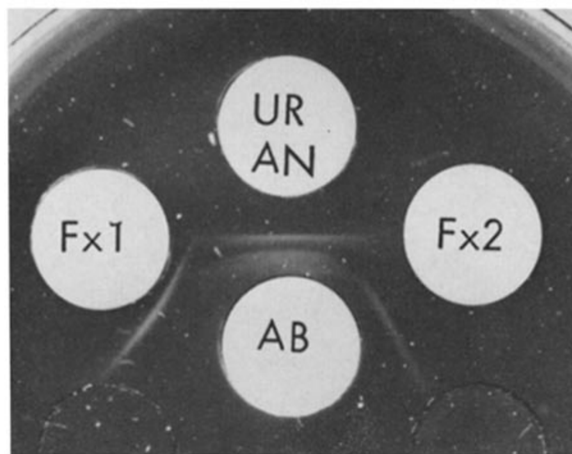

Fig. 2. Double diffusion study illustrating the relationship of the mixture of urinary antigens (UR AN) to fraction 1 antigens which void from G-200 and fraction 2 antigens which retard on G-200.

no indication of adventitious binding of the antigens to serum proteins under the conditions of these studies.

Immunodiffusion Studies.—

(*a*) *Urine:* Analyses of concentrated, dialyzed whole human urine specimens established that all contained antigenic material reactive with SAHGBM. Moreover, analyses of urines from different individuals showed immunologic identity between antigens from one person and those from another. When urinary antigens were studied alongside soluble preparations derived from enzymatic digestion of particulate human GBM, immunologic identity could be demonstrated between urinary antigens and some of the antigens present in the digests. The G-200–retarded antigen, fraction 2, showed immunologic identity with two antigens contained in both the collagenase and trypsin digests; the G-200–voiding antigen, fraction 1, showed immunologic identity with a third antigen of the trypsin-solubilized GBM, but formed a spur over a third

collagenase-soluble antigen with which it shared some, but not all, determinants. As shown in Fig. 4 fraction 2 urinary antigen formed a line of identity with the PGT-GL antigen of Markowitz when tested against the SAHGBM.

(*b*) *Study of serum from normal and nephritic patients by double diffusion:*

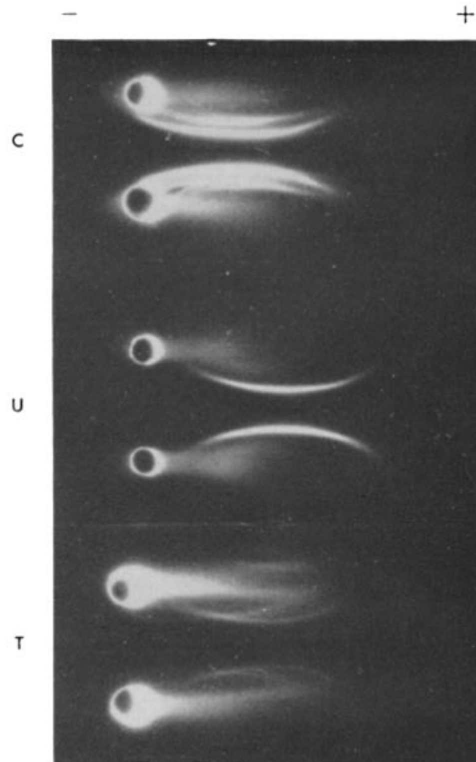

Fig. 3. Composite of radioimmunoelectrophoreses performed using collagenase-digested human GBM (C), urinary antigens from the electrofocusing step (U), and trypsin-digested human GBM (T). Upper well of each pair contained the antigens in a 1:2 dilution of normal human serum; the lower well contained antigens in saline. Antiserum used to develop precipitin lines was SAHGBM.

Equivocal precipitin lines developed in agarose opposite 1:8 and 1:16 dilutions of SAHGBM with four of the seven NHS which were allowed to react for as long as 7 days; the other three were negative. However, all of the nine sera tested from patients with terminal kidney disease developed precipitin lines at 1:4 and 1:8 dilutions of SAHGBM at 48 hr. The lines were heavier and appeared more quickly in the prenephrectomy sera in six of the nine tested and were of the same magnitude in pre- and postnephrectomy sera in samples from

one patient. All precipitin lines showed a common reaction of identity with one another.

Absorption Studies.—Urinary antigens harvested from the electrofocusing procedure were as effective as soluble antigens derived from collagenase and trypsin digestion of particulate human GBM and did block the fixation to glomeruli of homologous anti-GBM antibodies, as detected by direct immunofluorescence on normal human kidney sections. Two of these eluates required 10 mg/ml, and the other two, 30 mg/ml of added antigens; comparable con-

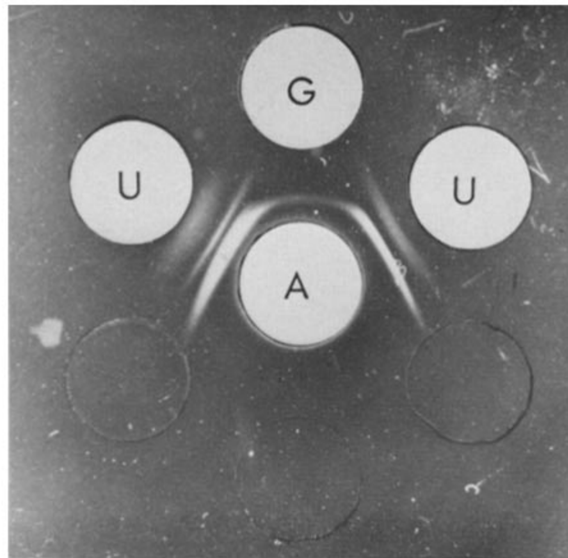

Fig. 4. Reaction of identity is shown between PGT-GL(G) soluble human GBM antigen and fraction 2 of mixed urinary antigens (U) at different concentrations. Antiserum (A) was SAHGBM.

centrations of a normal serum protein (BSA), and a solubilized fibrous protein, sheep tropocollagen, did not block this antibody fixation.

Fractionation of Serum.—

Several experiments demonstrated that the pooled postalbumin eluates from preparative electrophoresis of the SAS/60-precipitated fraction of NHS contained trace amounts of antigenic material reactive with SAHGBM. DEAE chromatography of this postalbumin fraction indicated that the peak of protein eluting at 0.2 M NaCl concentration contained two antigens reacting with SAHGBM. Subsequent double-diffusion studies carried out with urinary antigens and this serum isolate in adjacent wells showed immunological identity

between them (Fig. 5). These two antigens of the serum fraction separated on Sephadex G-200, one voiding off G-200, and the other retarded. Electrofocusing experiments with the antigen-containing fraction eluted from DEAE showed, however, that they localized in an isoelectric zone between pH 4 and 5, substantially different from the characteristic localization of the urinary antigens.

Histocompatibility and Blood Group Antigen Absorption Studies.—Absorption studies carried out with selected antisera demonstrated no significant histocompatibility antigen or blood group antigen activity associated with the basement membrane antigens from individual normal urines.

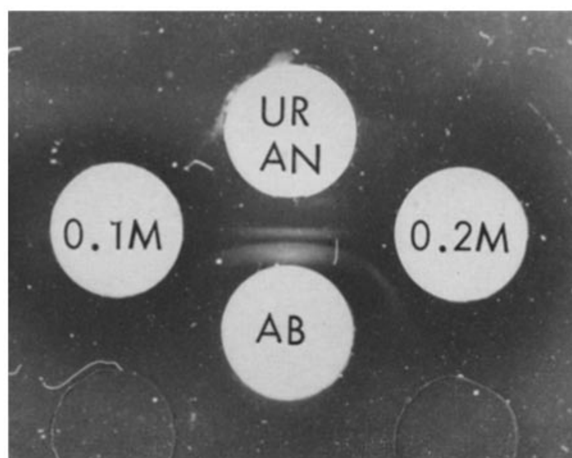

Fig. 5. Double diffusion study showing line of identity between antigen contained in fraction of normal human serum which eluted from DEAE at 0.2 M phosphate buffer, pH 6.5 and fraction 2 of the mixture of urinary antigens (UR AN).

Biological Experiments.—

(*a*) *In vivo circulatory studies:* Radiolabeled fractions 1 and 2 disappeared promptly from the circulation, although the slope of decay for fraction 1 was somewhat different than that of fraction 2 (Fig. 6). Only 1.2% of the radiolabel of fraction 2 measured in the circulation 5 min after injection persisted in the circulation at 48 hr, whereas an average of 5.5% of counts persisted at 48 hr in the animals injected with fraction 1. Differences in the fate of these labeled antigens are summarized in Table I, and indicate that the larger antigen (fraction 1) was altered, and dialyzable label only was excreted in the urine. On the other hand, 25% of excreted label in urine of animals injected with the smaller antigen was nondialyzable, and preparative electrophoresis and radio IEP indicated that the label was attached to apparently unaltered antigen. Approximately 49% of the injected radiolabel of fraction 1 antigen was unaccounted for

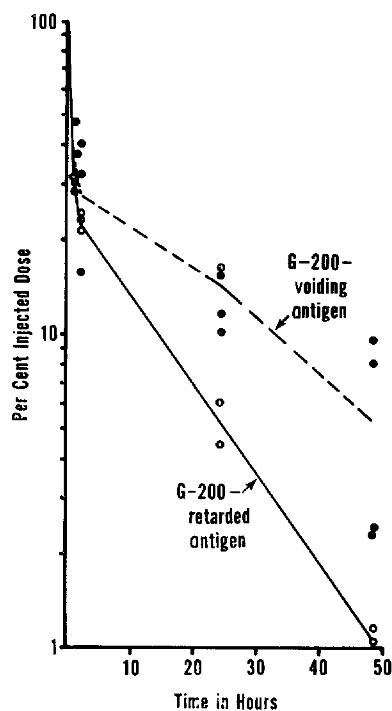

Fig. 6. Graphic display of disappearance curves of total radioactivity from serum of previously unimmunized rabbits injected intravenously with radiolabeled preparations of separated urinary antigens. The total counts per minute in circulation 5 min after injection is plotted as the 100% value for injected dose on this graph.

TABLE I

Fate of ^{131}I-Labeled Urinary Antigens Injected Intravenously into Rabbits

Counts per minute	Fraction 1 animals 1	Fraction 1 animals 2	Fraction 2 animals 3	Fraction 2 animals 4
Injected Dose	10×10^6	10×10^6	14×10^6	14×10^6
	Percentage of injected dose detected at sacrifice			
In circulation*	0.9	2.9	0.9	0.9
Liver	5.2	4.6	1.2	1.2
Spleen	0.1	0.1		
Lungs	0.1	0.3	0.1	0.1
Kidneys	0.4	0.6	0.5	0.4
Urine	57.0	32.0	81.4	86.4
Dialyzed urine	2.0	1.7	28.1	24.8

Radio-activity reflects counts at time of sacrifice, 48 hours after injection. All counts are corrected for decay of standards.

* Derived from formula: Plasma cpm/ml × 40 ml/kg body weight = counts in plasma volume of rabbit.

in urine, liver, spleen, lungs, or kidneys, and presumably was sequestered elsewhere in the rabbit carcasses.

(*b*) *Immunization studies:* Rabbits immunized with mixed human urinary antigens from the electrofocusing procedure produced antibodies which fixed to their own glomerular basement membrane. This could be demonstrated by direct immunofluorescent examination of the immunized rabbits' kidneys with fluorescein-conjugated anti-rabbit IgG, where autologous immunoglobulin G was fixed in a continuous, linear manner to host GBM. Histological examination by light microscopy indicated presence of scattered polymorphonuclear leukocytes in glomeruli of these same kidneys; however, no significant proteinuria was detectable. The control rabbits had no renal abnormality by light or immunofluorescent microscopy.

DISCUSSION

These studies demonstrate the presence of antigens immunologically related to soluble extracts of human glomerular basement membranes in normal human urine and serum. Their recognition at each step of isolation resulted from use of an antiserum produced in sheep immunized with particulate GBM. The indication of immunologic identity between these urinary antigens and normal human GBM is inferred from double diffusion analyses of individual and pooled concentrates of urinary fractions next to extracts of human GBM prepared by use of trypsin, collagenase, and combined trypsin-solvent extraction (13).

This inference regarding immunological relationship is strengthened by two lines of evidence. First, absorption experiments performed with the antigens from normal human urine demonstrated their capacity in blocking fixation of homologous anti-GBM antibody to human kidney sections as detected by direct immunofluorescence. The potency and restricted specificity of antibodies contained in these eluates have been demonstrated convincingly (17–19). The inability of comparable concentrations of a crystallized serum protein and a fibrillar tissue protein to block the reactions emphasizes that it is a specific reaction not dependent on protein concentration. Secondly, immunization with a mixture of these urinary antigens was capable of evoking production of anti-GBM antibodies, with fixation of autologous IgG on the host animals' kidneys in the characteristic linear pattern demonstrable by direct immunofluorescence. Further indication that urinary basement membrane antigens as detected and described herein might be derived, at least in part, from native GBM may be drawn from the studies of Hawkins and Cochrane who demonstrated small amounts of comparable antigens in normal rabbit urine, and an increase in amount and alteration in physical characteristics in response to acute anti-GBM antibody-induced glomerulonephritis (1). Similarly, the potency of these rabbit antigens in evoking an homologous anti-GBM antibody-mediated, acute glomerulonephritis bespeaks an unmistakable immunological cross-relationship in the rabbit.

In spite of the immunologic relationship established already between native GBM and urinary basement membrane antigens, the structure(s) from which they are derived is not clear. In an effort to determine whether the antigens in urine might be found also in serum and their presence in urine explained partially by passive excretion by the kidneys, three principal studies were performed. (*a*) IEP and radio IEP were done, and these showed no indication of significant binding in vitro to serum proteins. (*b*) Studies of isolated, radiolabeled antigen preparation injected into rabbits to find evidence that they might circulate in vivo indicated their prompt disappearance from the circulation and excretion of one antigen intact into rabbit urine. (*c*) Double diffusion analyses undertaken with sera from patients with terminal renal disease with kidneys intact and after nephrectomy showed the presence of trace amounts of antigenic material reactive with SAHGBM. For this reason, further experiments were undertaken to find basement membrane antigens in normal human serum. Fractionation of different pools of blood bank serum consistently indicated the presence of trace amounts of antigenic material. Moreover, there appeared to be two such antigens, separable on G-200, which showed a reaction of identity with the urinary basement membrane antigens in gel diffusion.

Three independent investigations tend to substantiate the data regarding soluble immunoreactive basement membrane antigens in the circulation. (*a*) The report by Tan and Kaplan (20) described a mouse serum protein that cross-reacted with homologous glomeruli. (*b*) Soluble collagen-like material has been found in serum by chemical (21), as well as by immunological techniques (22), although the relevance of observations regarding collagen to basement membrane antigens is tenuous. (*c*) Willoughby has found antigens in the serum and urine of rats which cross-react with native rat lung basement membranes (23).

The presence of basement membrane antigens in normal human serum as well as the serum of nephrectomized patients indicates that the kidneys are not their unique source and perhaps not their major source. Our information regarding the metabolism in the rabbit of the radiolabeled, smaller, G-200–retarded fraction 2 antigen, readily excreted intact into rabbit urine, suggests that it might be derived in the human from either renal or nonrenal basement membranes. On the other hand, the deviant curve of disappearance of the radiolabel of the larger, G-200–voiding fraction 1 antigen from the rabbit circulation, cleavage of label from the antigen, and persistence of radioactivity in livers of animals injected with it all suggest that this antigen as found in the urine probably is released *de novo* from some renal source such as GBM. The presence of more total antigenic material in prenephrectomy compared to postnephrectomy sera from six of nine terminal nephritic patients is compatible with a bidirectional release of antigen(s) by glomerulonephritic kidneys into the circulation, as well as into the urine.

Although two precipitin lines are seen usually in double diffusion between

urinary extracts and SAHGBM, three or more have been noted in some instances. Such variability should be anticipated in light of the electrofocusing experiments where one antigen (fraction 2) localized over a very broad isoelectric zone, from pH 1.7 to 3.5, indicating a heterogeneous physicochemical composition. The chemical group responsible for the marked electropositivity of these antigens reflected in their isoelectric points is not known. The true carbohydrate content of these antigens also must be considered an unsettled point. In spite of the single lines seen in analytic polyacrylamide gel electrophoresis experiments with the separated fractions 1 and 2, preliminary experiments utilizing agarose gel filtration and polymerized immune absorbent suggest that much of the neutral carbohydrate may not be an integral part of the antigens themselves. Hence, we consider it likely that our preparations contain a sizeable chemical contaminant. These antigenic preparations do not contain blood group substance or recognizable histocompatibility antigens, as demonstrated by their failure to inhibit significantly any typing sera against commonly characterized antigens.

Irrespective of the anatomic source(s) from which they are derived and the present state of their chemical purity, the observations on these antigens are important for several reasons. (*a*) Isolation from human biologic fluids of soluble antigens which are reactive with homologous anti-GBM antibody may afford better understanding of the specificity of these antibodies and the biologically-active antigenic fragments of basement membrane. Suggestions have been made already that the glycoprotein portion of solubilized GBM is the important antigenic part in nephrotoxic nephritis (24, 25). (*b*) Demonstration of basement membrane antigens normally present in serum allows a more reasonable insight into the potential events which may precede sensitization to one's own basement membrane, an idea implicit in the concept of specific anti-GBM antibody-mediated glomerulonephritis (26). (*c*) Such antigens may afford ultimately a rational basis on which to approach the reestablishment of tolerance to homologous immunoreactive basement membrane antigens in this autoimmune disorder. (*d*) Studies carried out on well characterized and chemically-defined basement membrane subunits may yield more precise formulation of concepts regarding basement membrane synthesis and repair in normal and pathologic circumstances.

SUMMARY

Using a sheep antiserum to human glomerular basement membrane (GBM), studies of urine from healthy adults showed the presence of two cross-reactive antigens. These antigens were purified partially by preparative electrophoresis and electrofocusing, and separated on G-200; both appeared to be acidic, of high molecular weight, and carbohydrate rich. Their immunologic relationship to human GBM solubilized by several techniques was deduced from lines of iden-

tity with the native GBM digests in double diffusion analyses. These antigens will combine with homologous anti-GBM antibodies and block their fixation to human kidney sections, and will evoke heterologous anti-GBM antibody production in the rabbit.

Fractionation studies of normal human serum indicated the presence of trace amounts of basement membrane antigens in the circulation. Although the serum antigens appear immunologically identical to the urinary antigens, the precise anatomic structures from which both are derived is not certain.

Demonstration of immunoreactive basement membrane antigens in the circulation provides a plausible source of immunogen for the potential development of anti-GBM antibody-mediated glomerulonephritis as well as a clue to a mode for reestablishment of tolerance in such an autoimmune disorder.

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