Detection of Anti-Glomerular Basement Membrane Antibodies by a Radioimmunological Technique

CLINICAL APPLICATION IN HUMAN NEPHROPATHIES

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ABSTRACT A radioimmunoassay for detection of anti-glomerular basement membrane (GBM) antibody was set up with a 70,000 mol wt GBM antigen, labeled with Iodine-^{125}I and containing both types of oligosaccharidic chains present in the whole membrane.

Separation of free radioactive antigens from antigens bound to immunoglobulins was obtained by precipitation with polyethylene glycol (mol wt, 6,000), at a final concentration of 20%. In the presence of normal human or rabbit sera, less than 20% of labeled antigens were precipitated. In the presence of rabbit anti-human GBM antibodies, up to 82% of GBM antigens were precipitated, while in the presence of sera or of kidney eluate from a patient with Goodpasture's syndrome, the precipitation of GBM antigens reached 43%. The avidity of rabbit anti-GBM antibodies for human GBM antigens is higher than that of human anti-GBM antibodies. In the case of Goodpasture's syndrome, the binding of anti-GBM antibodies to labeled antigens was inhibited more efficiently by the disaccharide-containing glycopeptide than by the heteropolysaccharide-containing glycopeptide purified from whole GBM.

Anti-GBM antibodies were searched for in the serum of 300 normal blood donors, of 120 patients with glomerulonephritis (GN) and granular deposits, and of 14 patients with GN and linear deposits of immunoglobulins. After correction for the "nonspecific" precipitation, the average percentage (±1 SD) of labeled antigens precipitated in the serum of normal blood donors was 0.3±3.2%; 12 patients with GN and linear deposits exhibited high circulating anti-GBM antibody titers, while 8% of the patients with GN and granular deposits presented significant, albeit lower, anti-GBM activity in their sera.

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Received for publication 16 November 1973 and in revised form 18 March 1974.

INTRODUCTION

It is generally admitted that anti-glomerular basement membrane (GBM)[1] antibodies are involved in the pathogenesis of some cases of human and experimental glomerulonephritis (GN) (1–3). Several techniques have been used to study anti-GBM immunity in animals and in man. Immunofluorescence can be performed directly on glomerulonephritic kidneys or after transfer of circulating or eluted anti-GBM antibodies onto normal kidney slices (1–5). The existence of anti-GBM antibodies is then suggested by the presence of linear deposits along the GBM (1–5). Agar gel precipitation and passive hemagglutination tests have also been used in experimental (3) or in human (1, 6) GN for the detection of circulating anti-GBM antibodies. Recently, the leukocyte migration inhibition test has been applied to human GN, by the two-step (7) or the one-step method (6), in order to study cell-mediated anti-GBM immunity.

In this work, a direct binding test is described for detection of anti-GBM antibodies. The method is based on the fact that most of the immunoglobulin molecules are precipitated in 20% polyethylene glycol (PEG) solutions, while smaller antigens, like bovine serum albumin (BSA) remain soluble at this PEG concentration (8).

GBM antigens, with an average mol wt of 70,000, containing both types of oligosaccharide chains, have been purified from solubilized GBM (9). Anti-GBM

[1] *Abbreviations used in this paper:* Ag, antigen; BSA, bovine serum albumin; GBM, glomerular basement membrane; GN, glomerulonephritis; NHS, normal human serum; NRS, normal rabbit serum; PEG, polyethylene glycol.

antibodies were detected by using a technique similar to the Farr antigen binding test (10); however, a 20% PEG solution was used instead of ammonium sulfate in order to separate free GBM antigens from those bound to immunoglobulins, because these antigens remain soluble at this PEG concentration. The specificity and the avidity of rabbit and human anti-GBM antibodies for human GBM antigens have also been investigated.

Finally, anti-GBM antibodies were sought in the serum of 300 normal blood donors and of 134 patients with various nephropathies.

METHODS

Buffer. All serum dilutions or PEG solutions were made in borate buffer, ionic strength 0.1, pH 8.3–8.5.

Antigens. GBM were isolated from normal human kidneys obtained from autopsies, according to the method of Krakower and Greenspon (11). The morphological and biochemical criteria of purity were previously described (12). The GBM were solubilized by autoclaving a suspenson of membranes at 110°C for 3 h, in Tris-acetate buffer 0.1 M, pH 7.4 (9). GBM components, all of which contain both types of oligosaccharide chains, were isolated from the solubilized material by affinity chromatography and preparative polyacrylamide electrophoresis, in the presence of sodium dodecylsulfate. Their mol wt ranged from 70,000 to more than 300,000 (9). A soluble GBM fraction with an average mol wt of 70,000 was selected for radioimmunological studies (Fig. 1).

Glycopeptides containing either the disaccharide or heteropolysaccharide chains were isolated, after enzymatic digestion of GBM with pronase and collagenase, by gel filtration, ion exchange chromatography, and preparative paper electrophoresis (13, 14). Neutral sugars, hexosamines, and sialic acid were measured by previously described techniques (12, 14); amino-acid composition was determined in a Beckman model Unichrom amino-acid analyzer (Beckman Instruments, Inc., Fullerton, Calif.) (11). The protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall, with BSA as a standard (15) and the glycopeptide concentration by the ninhydrin reaction with leucine as a standard (16).

Human procollagen was a gift from Prof. Ch. M. Lapiere, (Liège); streptococcal cell membranes Type 12 (T 12/126) and Type 5 (543/100) were obtained from Dr. J. Zabriskie (Rockefeller University, New York) and Clq was purified by the method of Yonemasu and Stroud (17).

Preparation of the labeled GBM antigen solution. Selected GBM antigens were labeled with ^{125}I according to the method of McConahey and Dixon (18). The radioactive antigens ([^{125}I]GBM Ag) exhibiting a specific activity of about 2.5 μCi/μg, were diluted in normal rabbit serum (NRS) or in normal human serum (NHS) 1:10. One volume of [^{125}I]GBM Ag solution (100 μg/ml) was mixed with 1 vol of 40% PEG. After an overnight incubation at 4°C, the mixture was centrifuged at 10,000 *g* for 20 min in a Sorvall refrigerated centrifuge (Ivan Sorvall, Inc., Newtown, Conn.). The precipitate, containing 25% of the starting radioactivity, was discarded. The supernate, used as [^{125}I]GBM Ag solution, was divided into 0.1-ml samples and then stored at −20°C. Before each testing, this solution was diluted with the borate buffer up to a protein concentration of 0.1 μg/ml.

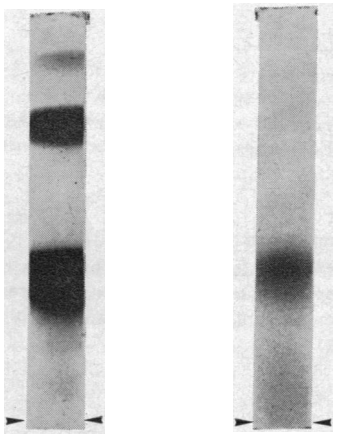

FIGURE 1 Acrylamide disc gel electrophoresis in dodecylsulfate of GBM antigens (37). Before running, samples were incubated with 4% sodium dodecylsulfate and 0.1 M mercaptoethanol for 1 h at 60°C: 7.5% gels were used and the proteins were stained with coomassie blue. Molecular weight of GBM antigens (gel B) was calculated by comparison with the relative mobility of albumin standards (gel A). The arrows denote the dye front.

To determine the relative amount of disaccharide and heteropolysaccharide units in the PEG supernate, 10 mg of unlabeled 70,000 mol wt GBM fractions, contained in 5 ml of borate buffer, was treated by the same procedure. The supernate was dialyzed against 20 liters of 0.05 M ammonium bicarbonate and lyophilized. This material and 0.1 μg of the [^{125}I]GBM Ag solution were submitted to the affinity chromatography (9). The carbohydrate and amino-acid composition of the proteins eluted with acidic buffer (9) was determined as previously described (12, 14). The molecular weight of labeled and unlabeled antigens eluted with the same buffer was evaluated by acrylamide disc gel electrophoresis in dodecylsulfate (9). Before running, the samples were incubated with 4% sodium dodecylsulfate and 0.1 M mercaptoethanol for 1 h at 60°C. The proteins were stained with coomassie blue; the gels were also sliced and the radioactivity of gel slices (0.4 cm in thickness) was counted in a double channel Picker nuclear spectrometer (Picker Corp., Cleveland, Ohio).

Antibodies. Rabbit anti-human GBM antibodies were a gift from Dr. J. P. Godon (Liège); their specificity was demonstrated by indirect immunofluorescence performed on normal human kidney slices, according to the method of Unanue and Dixon (3). Human anti-GBM antibodies were obtained from 16 sera of one patient with Goodpasture's syndrome. Rabbit and human IgG were prepared by ion exchange chromatography on DEAE-cellulose (19). Immunoglobulins were eluted with 0.02 M citrate buffer, pH 2.8, from kidneys of the same patient with Goodpasture's syndrome, according to a previously described method (20); IgG concentration in the eluate was determined by quantitative radial immunodiffusion (21).

Rabbit anti-whole human serum, anti-BSA, and anti-Clq antibodies were obtained by immunization of rabbits with the purified antigens mixed with complete Freund adjuvent.

Radioimmunological procedure. The solubility of [^{125}I]-GBM Ag was studied in the presence of various PEG

solutions. PEG, with an average mol wt of 6,000 (Siegfried, Zofingen, Switzerland), was dissolved in borate buffer at concentrations from 10 to 40% (wt/vol); 0.1 ml of the diluted GBM antigen solution was mixed with 0.4 ml of 1/10 NHS or NRS and further with 0.5 ml of the various PEG solutions. This mixture was left at 4°C for 18 h, then centrifuged at 1,000 *g* for 20 min. The supernate was discarded and the precipitate washed twice with the PEG solution at the final concentration used in the mixture. The percentage of [^{125}I]GBM Ag precipitated is calculated after radioactivity counting in a double-channel Picker Nuclear Spectrometer, with the radioactivity of [^{125}I]GBM Ag precipitated with 20% trichloroacetic acid as a 100% value.

Anti-GBM antibodies were titrated as follows: 0.1 ml of [^{125}I]GBM Ag solution was mixed with 0.4 ml of tested serum serially diluted in 1/10 NHS or NRS, then left at 37°C for 1 h; 0.5 ml of 40% PEG was added and the samples were washed and counted as for solubility studies, after an incubation at 4°C. Anti-GBM IgG antibodies were titrated by the same procedure, but a starting IgG solution of 100 μg/ml in NHS or NRS was used; in this case, control experiments were performed with similar concentrations of normal IgG in NHS or NRS.

Calculation of the "specific" precipitation. The specific precipitation of [^{125}I]GBM Ag was calculated after correction for the non-specific precipitation obtained in the presence of NHS or NRS pools. The formulas described by Farr (10) were used for this correction.

$$\text{Correction factor (CF)} = \frac{\%\ \text{Ag ppt. NS}}{100 - \%\ \text{Ag ppt. NS}}$$

Percentage of specific precipitation = % Ag ppt. TS − [(100 − % Ag ppt. TS) CF] in which, "% Ag ppt. NS" represents the percentage of precipitation observed with a pool of normal human or rabbit sera, and "% Ag ppt. TS," the percentage of precipitation observed with the tested serum.

Control of reproducibility. The accuracy of measurement of anti-GBM antibodies was evaluated by calculating the coefficient of variation (CV) of results of duplicate determinations around their means, by the following formula (22):

$$\text{CV} = \sqrt{\frac{\Sigma d^2}{2n}}$$

where

$$d = \frac{\text{higher value of each pair}}{\text{lower value of each pair}} - 1 \times 100$$

Sensitivity of the radioimmunoassay. The sensitivity of the radioimmunoassay was determined by correlating the anti-GBM antibody titers obtained by this technique with those obtained by indirect immunofluorescence and passive hemagglutination. Indirect immunofluoresence was performed according to the method of Unanue and Dixon (3) and passive hemagglutination according to a previously described method (6). Rabbit anti-human GBM antibodies and anti-GBM antibodies found in six sera of one patient with Goodpasture's syndrome were titrated by the three methods.

Selection of subjects. Anti-GBM antibodies were sought in the serum of 300 normal blood donors and later in the serum of 134 patients with various nephropathies. Two groups of patients were selected on the basis of immunofluorescent microscopy data: the first was composed of 14 patients with linear deposits of immunoglobulins; the second was formed of 120 patients with granular deposits. Patients of the former group presented in 13 cases severe endo- and extracapillary proliferative GN and in one case, a polyarteritis nodosa; patients of the second group exhibited either "non-proliferative" GN, i.e., membranous GN (19 cases) and GN with segmental and focal hyalinosis (13 cases) or "proliferative" GN, i.e., pure endocapillary proliferative GN (14 cases), membrano-proliferative GN (15 cases), lobular GN (4 cases), endo- and extracapillary proliferative GN (14 cases), focal GN with extracapillary proliferation (17 cases) or intercapillary fibrinoid deposits (13 cases), and lupus nephritis (11 cases). Before use, all sera were heated to 56°C for 30 min and then stored at −20°C.

TABLE I

Carbohydrate Composition of GBM Antigens Used for the Immunological Studies

Component	Whole membrane	Soluble antigens	
		A*	B‡
	mg/100 mg of protein		
Glucose	2.6	3.10	2.90
Galactose	2.7	3.13	3.05
Mannose	1.1	0.80	0.90
Fucose	0.45	0.36	0.35
Hexosamine	1.8	1.06	1.04
Sialic acid	1.05	0.71	0.65

* GBM antigens purified by affinity chromatography and preparative polyacrylamide electrophoresis (9).
‡ GBM antigens obtained by PEG precipitation followed by affinity chromatography.

RESULTS

Carbohydrate and amino acid composition of GBM fractions used for the immunological studies. The carbohydrate composition of GBM antigens purified by affinity chromatography is given in Table I. Although the membrane antigens contained the same carbohydrates as the whole basement membrane, a higher proportion of glucose and galactose was found, thus indicating an enrichment in disaccharide units. Similarly, the soluble membrane material recovered after the PEG precipitation exhibited the same carbohydrates as the whole basement membrane, suggesting that both types of oligosaccharide chains were not separated by the treatment of GBM antigens with PEG. Moreover, it should be stressed that the molecular weight of unlabeled and labeled GBM antigens obtained after the PEG precipitation was quite comparable to that of the soluble GBM fraction selected for radioimmunological studies, i.e., 70,000 (Fig. 1). The amino acid composition of GBM antigens purified by affinity chromatography is given in

TABLE II

Amino Acid Composition of GBM Antigens Used for the Immunological Studies

Amino acid	Whole membrane	Soluble antigens A*	Soluble antigens B‡
	residue/1,000 amino acid residue		
Cysteic acid	22	21.9	20.4
3-hydroxyproline	3	9.1	8.3
4-hydroxyproline	67	70.0	72.1
Aspartic acid	67	74.2	69.5
Threonine	36	39.1	37.2
Serine	52	42.3	40.2
Glutamic acid	95	97.3	99.1
Proline	73	69.4	71.2
Glycine	224	220.7	224.9
Alanine	65	40.0	36.9
Valine	38	40.4	41.2
Methionine	14	19.4	18.8
Isoleucine	29	37.6	39.4
Leucine	59	73.6	76.5
Tyrosine	18	16.1	14.2
Phenylalanine	28	21.3	20.9
Hydroxylysine	22	26.5	25.1
Lysine	25	21.2	21.9
Histidine	16	16.9	18.1
Arginine	47	43.0	44.1

,‡ For A and B‡, see the footnote of Table I.

Table II. They contained more 3-OH proline and hydroxylysine residues than the original insoluble membrane; there was no major difference in the amino acid composition of GBM fractions obtained with or without PEG precipitation.

The amino acid and carbohydrate composition of the tested disaccharide-containing glycopeptide is given in Table III. It should be stressed that this glycopeptide contained glucose, galactose, and hydroxylysine in equimolecular amounts.[2]

The carbohydrate and amino acid composition of the tested heteropolysaccharide-containing glycopeptide is given in Table IV. Aspartic acid, threonine, serine, glutamic acid, proline, glycine, and alanine were the most abundant amino acids of this glycopeptide. The heteropolysaccharide chain was formed of fucose, hexosamines, mannose, galactose, and sialic acid. No hydroxylysine or glucose were found.

Detection of rabbit anti-human GBM antibodies. The solubility of labeled membrane antigens was studied in the presence of various PEG concentrations. It was found that the addition of 0.5 ml of 40% PEG to 10 ng

[2] The amino acid sequence of one of these glycopeptides is Hyl-Gly-Glu-Asp-Gly, the disaccharide prosthetic group being linked with the hydroxylysine residue (23).

TABLE III

Carbohydrate and Amino Acid Composition of the Disaccharide-Containing Glycopeptide Used for the Immunological Studies

Component*	Composition
	mol/mol glucose
Glucose	1.0
Galactose	1.0
Hxdroxylysine	0.98
Glycine	2.02
Aspartic acid	1.01
Glutamic acid	0.89

* In addition to these amino acids, smaller amounts of hxdroxyproline, proline, and serine were also present.

of [^{125}I]GBM Ag, contained in 0.5 ml of NRS 1/10, precipitated less than 20% of the radioactive antigens (Fig. 2). GBM antigens remain, therefore, quite soluble in PEG at the final concentration of 20%, which provides a total precipitation of the immunoglobulin molecules (8). In the presence of rabbit anti-human GBM IgG antibodies, the precipitation of labeled antigens in 20% PEG went up to 82% (Fig. 2). Titration of rabbit anti-GBM antibodies was done by mixing serial dilutions of the whole serum or IgG solutions with 10 ng of [^{125}I]GBM Ag and precipitating immunoglobulin-bound antigens with PEG at a final concentration of 20%. It has been considered arbitrarily that the antibody titer is the inverse of the serum dilution at which 10% of radio-

TABLE IV

Carbohydrate and Amino Acid Composition of the Heteropolysaccharide-Containing Glycopeptide Used for the Immunological Studies

Component*	Composition
	mol/mol fucose
Fucose	1.0
Hexosamines	5.3
Galactose	3.9
Mannose	3.5
Sialic acid	2.5
Aspartic acid	1.5
Threonine	0.6
Serine	0.8
Glutamic acid	0.7
Proline	0.9
Glycine	1.2
Alanine	0.8
Valine	0.4

* In addition to these amino acids, smaller amounts of leucine, isoleucine, and lysine were also present.

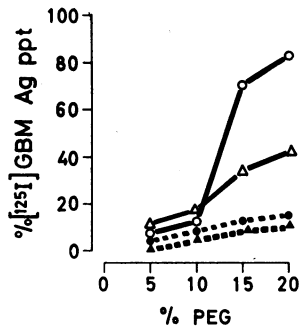

FIGURE 2 Solubility of [^{125}I]GBM Ag at various PEG concentrations, in presence of NRS 1/10 (●---●), of NHS 1/10 (▲---▲), of rabbit anti-GBM IgG solution diluted 10 times (○——○), or of Goodpasture's syndrome serum 1/10 (△——△). 10 ng of labeled GBM Ag were mixed with 0.4 ml of the various diluted sera, then with 0.5 ml of the different PEG solutions. After an incubation at 4°C overnight, the precipitates obtained by centrifugation at 1,000 *g* were washed twice with the PEG solution at the final concentration used in the mixture; the radioactivity was counted in a Picker Nuclear Spectrometer.

active antigens are specifically precipitated. Examples of such titration curves, obtained with either whole rabbit anti-serum or purified rabbit anti-GBM IgG, are given in Fig. 3. In these cases, the anti-GBM antibody titers averaged 10^{-4} and 6.10^{-4}, respectively.

Detection of anti-GBM antibodies in sera and kidney eluate from a patient with a Goodpasture's syndrome. As in NRS, the nonspecific precipitation of [^{125}I]GBM Ag in NHS was lower than 20%, with 20% PEG. However, in the presence of a similarily diluted serum of the patient with severe Goodpasture's syndrome, 43% of the same antigens were precipitated (Fig. 2).

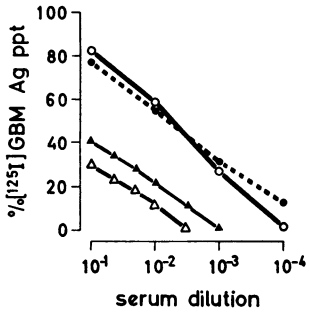

FIGURE 3 Titration of antibodies to human GBM by precipitation of immunoglobulin-bound antigen with PEG at a final concentration of 20%. Titration curves obtained with whole rabbit serum (●---●), with purified rabbit IgG (○——○), with the serum of a patient with Goodpasture's syndrome (△——△), or with immunoglobulins eluted from the kidneys of the same patient (▲——▲) were represented. It has been considered arbitrarily that the antibody titer is the inverse of the serum dilution at which 10% of labeled antigens are specifically precipitated.

Titration of human antibodies to GBM antigens was therefore performed by precipitation of immunoglobulin-bound antigens with PEG at a final concentration of 20%. Examples of titration curves obtained either with anti-GBM serum or with eluted anti-GBM antibodies are given in Fig. 3. With the same IgG concentration, it was shown that the anti-GBM antibody titer was higher with eluted than with circulating IgG. The precipitation of [^{125}I]GBM Ag was followed in 16 sera of the same patient obtained before and after bilateral nephrectomy. A significant increase of the precipitation was observed immediately after the bilateral nephrectomy with a maximal value of specific precipitation of 42%.

Specificity and avidity of human and rabbit anti-GBM antibodies for membrane antigens. To control the specificity of the radioimmunoassay, rabbit anti-whole human serum or anti-Clq antibodies were incubated with [^{125}I]GBM Ag. No significant specific precipitation was observed. Inhibition experiments were then performed. Increasing amounts (20–100 ng) of unlabeled GBM antigens, human procollagen, Clq, or streptococcal cell membrane antigens were added to a solution of human anti-GBM antibodies binding specifically 20% of [^{125}I]GBM Ag. 100 ng of unlabeled GBM antigens strongly inhibited the binding of 10 ng of radioactive antigens with anti-GBM antibodies, while 100 ng of procollagen, Clq, or streptococcal antigens slightly reduced the specific precipitation (Fig. 4). Increasing amounts of both types of GBM glycopeptides were also added to a similar solution of human anti-GBM antibodies eluted from the kidneys of a patient with Goodpasture's syndrome. Whereas 100 ng of the disaccharide-containing glycopeptide inhibited 60% of the binding of labeled GBM antigens with anti-GBM antibodies,

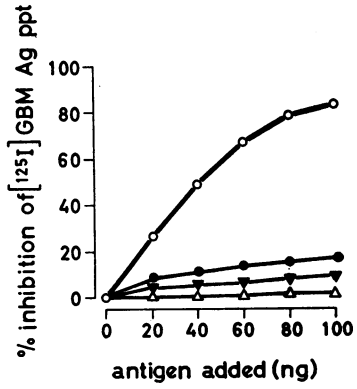

FIGURE 4 Inhibition of binding of labeled GBM Ag with anti-GBM antibodies, by various unlabeled antigens. Increasing amounts (20–100 ng) of GBM antigen (○——○), of procollagen (●——●), of streptococcal cell membranes (▼——▼), and of Clq (△——△) were added to a solution of human anti-GBM antibodies binding specifically 20% of [^{125}I]GBM Ag (10 ng).

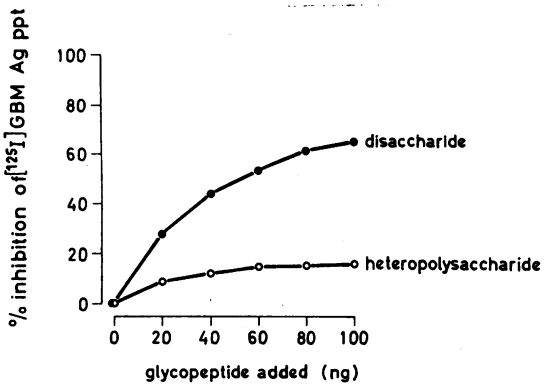

FIGURE 5 Inhibition of binding of [^{125}I]GBM Ag (10 ng) with anti-GBM antibodies by both types of glycopeptides isolated from GBM. Increasing amounts (20–100 ng) of disaccharide-containing glycopeptide (●——●) or heteropolysaccharide-containing glycopeptide (○——○) were added to immunoglobulins eluted from Goodpasture's syndrome kidneys. A solution of anti-GBM antibodies binding specifically 20% of labeled GBM Ag was used.

100 ng of the heteropolysaccharide-containing glycopeptide reduced the specific precipitation by only 20% (Fig. 5). Comparable inhibition data were obtained with all anti-GBM antibodies studied so far, such as those found in the serum of seven patients with GN and linear deposits of immunoglobulins (23). In control experiments, it was found that similar amounts of the disaccharide-containing glycopeptide did not affect the binding of BSA to anti-BSA antibodies.

The dissociation rate of rabbit or human GBM-anti-GBM antibody complexes was studied by the following experimental procedure: 10 ng of [^{125}I]GBM Ag were first mixed with 0.4 ml of the tested serum during 90 min at 37°C; 1 μg of cold GBM antigen was then added to the mixture, and after 5 min, 1, or 4 h, the immunoglobulin-bound antigens were precipitated with 0.5 ml of 40% PEG. Control experiments were performed by the same experimental methodology, but borate buffer was added to the mixture instead of cold GBM Ag. Fig. 6 shows that the dissociation rate of human GBM-anti-GBM antibody complexes was much faster than that of rabbit GBM-anti-GBM antibody complexes. These results are probably representative of the difference of avidity between human and rabbit anti-GBM antibodies for GBM antigens.

Reproducibility and sensitivity of the GBM radioimmunoassay. The accuracy of measurement of rabbit anti-GBM antibodies was evaluated by calculating the CV of results of duplicate determinations around their means. In an assay in which the final dilution of anti-GBM antibody was 10^{-3}, the CV was 7.74%, calculated on the basis of values ranging between 65 and 36% obtained from 11 samples.

The sensitivity of the radioimmunoassay was evaluated by correlating the anti-GBM antibody titers obtained in this technique with those obtained by indirect immunofluorescence or passive hemagglutination. The seven sera exhibting anti-GBM antibodies in the radioimmunoassay all demonstrate the presence of anti-GBM antibodies by indirect immunofluorescence method or passive hemagglutination. However, the mean anti-GBM antibody titer obtained with this new assay technique was five times higher than that obtained by passive hemagglutination and 20 times higher than that obtained by indirect immunofluorescence.

Anti-GBM antibody levels in nephropathies. After correction for the non-specific precipitation of [^{125}I]GBM Ag obtained in a pool of 92 normal blood donor's sera, the percentage of labeled GBM Ag precipitated in the serum of 300 normal blood donors was 0.3±3.2%. It has been arbitrarily considered that patients with at least 10% of labeled GBM Ag precipitated exhibited a significant anti-GBM activity in their serum. Results of anti-GBM antibody levels in nephropathies are given in Fig. 7.

It should be stressed that (*a*) most of the patients with GN and linear deposits exhibited high anti-GBM antibody titers in their serum; only one patient with polyarteritis nodosa and one with rapidly progressive GN exhibited negative results; (*b*) 8.3% of patients with GN and granular deposits presented significant, but lower, anti-GBM activity in their serum; amongst these patients with positive binding, two presented

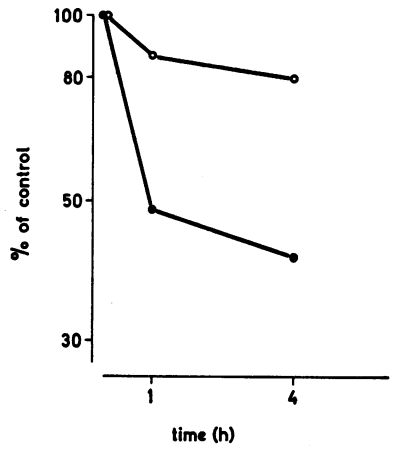

FIGURE 6 Dissociation curves of rabbit (○——○) and human (●——●) GBM-anti-GBM antibody complexes. [^{125}I]GBM Ag (10 ng) was mixed with 0.4 ml of tested sera for 90 min at 37°C; then 1 μg of cold GBM Ag was added, and after 5 min, or 1 or 4 h, the GBM-anti-GBM antibody complexes were precipitated with 0.5 ml of 40% PEG. In control experiments, the same procedure was used, but borate buffer was added to the mixture instead of cold GBM Ag.

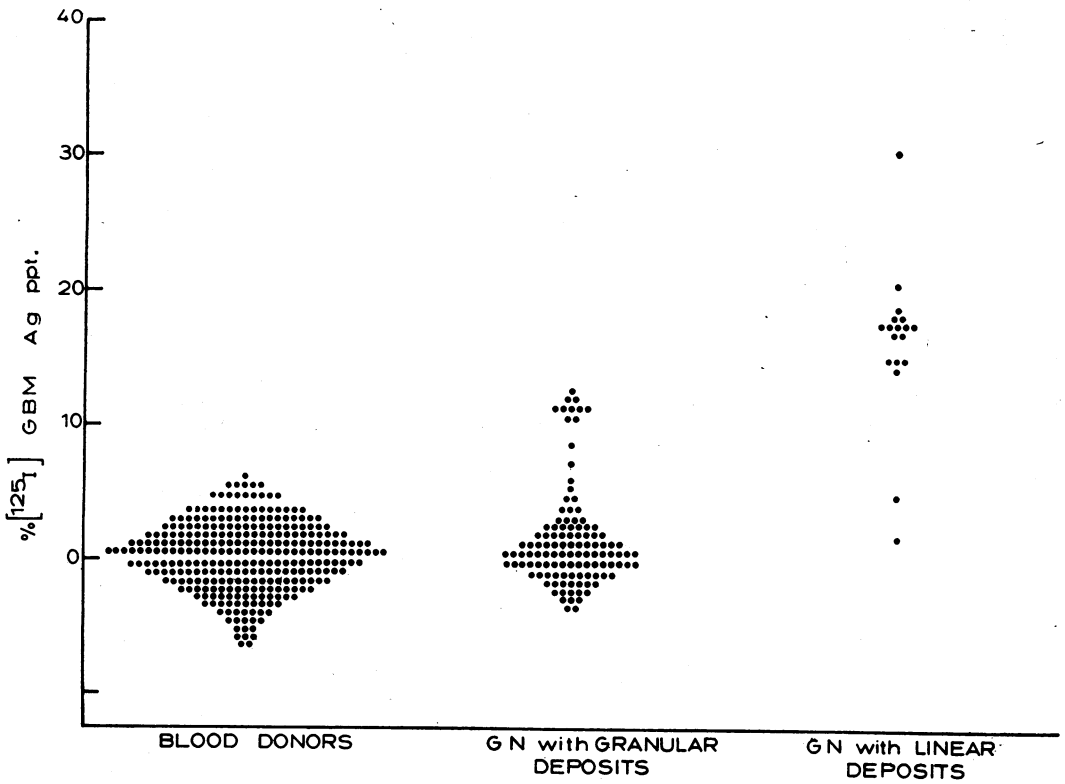

FIGURE 7 Anti-GBM antibody levels in human sera. Anti-GBM antibodies have been looked for by precipitation with PEG at a final concentration of 20% in the serum of 300 blood donors, of 14 patients with GN and linear deposits, and of 120 patients with GN and granular deposits. The mean percentage (±1 SD) of specific precipitation of [^{125}I]GBM Ag in NHS was 0.3±3.2%. Only patients exhibiting at least 10% of radioactive GBM Ag precipitated were considered to present a significant binding of GBM antigens.

membrano-proliferative GN, two presented focal GN with extracapillary proliferation, three endo- and extracapillary proliferative GN, and the three others lupus nephritis; (*c*) all patients with non-proliferative GN exhibited negative results. In four of these "positive" patients, IgG fractions were isolated from serum and were shown to contain a significant binding activity.

DISCUSSION

It is well known (24, 25) that GBM are mostly glycoproteins containing two different oligosaccharidic chains; one is a disaccharide formed of equimolecular amounts of glucose and galactose, while the other is a heteropolysaccharide composed of mannose, fucose, galactose, sialic acid, and hexosamines (13, 14). However, the basement membrane structure responsible for the production of anti-GBM antibodies remains a matter of controversy. GBM contains at least three major antigenic components: one of these components is the "collagen-like" material with the disaccharide chains, while the two others, differing in their molecular weights, contain the heteropolysaccharide units (26). A first group of investigators, using fractions of collagenase or tryptic digests of GBM, have induced nephrotoxic antibodies into heterologous species (27, 28); these fractions contain the sialoprotein material of the GBM (27) or do not appear related to the collagen-like material, as they contain high amounts of glucose and galactose but lack hydroxylysine (28). Moreover, these latter fractions, of relatively high molecular weight, exhibit large amounts of glycine residues without hydroxyproline (28). Other investigators (29), using antibodies eluted from kidneys of a patient with Goodpasture's syndrome, suggest that the collagen-like glycoproteins are indeed the antigens responsible for this autoimmune GN. These discrepancies justify the choice of GBM antigens containing both types of oligosaccharide chains for radioimmunoassay. The 70,000 mol wt fraction obtained by heating basement membrane to 110°C does not necessarily represent an intact structural subunit of the whole GBM; it might result from the cleavage of one or more covalent bonds. However, since these antigens have been isolated from GBM glycoproteins by affinity chromatography, using Sepharose cyanogen bromide coupled

with specific rabbit anti-human GBM IgG (9), one may assume that they contain some of the major antigenic determinants of the whole GBM structure, regardless of the fact that these antigenic determinants may be present on either a continuous or an adjacent peptide chain.

In this work, it was shown that a synthetic polymer, PEG, could be used in a direct binding test for detection of circulating anti-GBM antibodies; the addition of this polymer to the reaction medium is used to exclude from the solvent most of the immunoglobulin molecules and GBM antigen bound to immunoglobulins, while the GBM antigen could not be excluded in a free form. The mechanism of action of PEG is not known, but might be similar to that of dextran on the precipitation of immune complexes, i.e., a steric exclusion of the antigen-antibody complexes from the domain of the polymer (30). The principle of the PEG technique used is therefore similar to that used for the antigen binding test previously described by Farr (10). The Farr technique cannot be applied to the detection of anti-GBM antibodies, since GBM antigens are insoluble in 50% saturated ammonium sulfate.[3] However, it has been shown (8) that titration curves of antibody to other antigens, such as BSA using the PEG technique, are quite comparable to those obtained by precipitation with 50% saturated ammonium sulfate. One may thus assume that PEG at a final concentration of 20% precipitates all immunoglobulin-bound antigens. The [^{125}I]-GBM Ag solution used was prepared by precipitation of radioactive GBM antigen with PEG at a final concentration of 20%. The precipitated material is also radioactive and could correspond to aggregated GBM antigens or to a contamination of 70,000-mol wt GBM glycoproteins by higher mol wt GBM components (9). This precipitation with 20% PEG is therefore a prerequisite for the radioimmunoassay, since it reduces the nonspecific precipitation in NHS or NRS of the [^{125}I]GBM Ag. It is likely that the GBM antigen does not bind nonspecifically to normal serum macromolecules since it remains quite soluble after incubation in presence of normal serum and precipitation with 20% PEG.

The specificity of the radioimmunoassay has been demonstrated by the absence of significant precipitation of radioactive GBM antigens in the presence of rabbit anti-whole human serum antibodies. This observation rules out the possibility of a significant serum protein contamination in the [^{125}I]GBM Ag. Moreover, the cross-reactivity of [^{125}I]GBM Ag with other collagen-like glycoproteins has been studied. At the concentrations used, Clq, which exhibits a collagen-like biochemical composition (17), and procollagen do not inhibit the specific precipitation of GBM–anti-GBM antibody complexes in the presence of 20% PEG. The specificity of anti-GBM antibodies has been further investigated in one case of Goodpasture's syndrome. It has been shown that the disaccharide-containing glycopeptide inhibits more efficiently the binding of anti-GBM antibodies with GBM antigens than does the heteropolysaccharide-containing glycopeptide. Similar data have been recently obtained with anti-GBM antibodies found in the serum of seven patients with GN and linear deposits of immunoglobulins (23). Cross-antigenicity between GBM and streptococcal cell membranes has been reported (31). Such cross-reactions have not been observed by radioimmunoassay with the antisera available and in presence of a 10-fold antigen excess.

It has been demonstrated by the direct binding test that anti-GBM antibodies can be detected in the serum or in IgG fractions from immunized rabbits, and in sera IgG fractions or kidney eluate from Goodpasture's syndrome. In the best conditions, the specific precipitation of [^{125}I]GBM Ag obtained with human anti-GBM antibodies was 42% and was therefore not as high as for rabbit anti-human GBM antibodies. Several facts could explain this difference: (*a*) the absolute anti-GBM antibody concentration in the serum of rabbits immunized with heterologous GBM is much higher than that in the serum of human autoimmune GN. These low human anti-GBM antibody titers could be due to the fixation of antibodies on the "target" organ. The increase in [^{125}I]GBM Ag precipitated immediately after the bilateral nephrectomy of the Goodpasture's syndrome patient is in agreement with such a hypothesis and confirms similar observations of other workers (1, 32); (*b*) GBM are very complex glycoproteic structures and therefore the GBM antigens used might not involve all the antigenic determinants of the whole GBM, despite the fact that these antigens have been purified by affinity chromatography with anti-whole human GBM IgG; (*c*) the avidity of human anti-GBM antibodies for GBM antigens is lower than that of rabbit anti-human GBM antibodies. The difference in the dissociation rate between rabbit and human GBM-anti-GBM antibody complexes supports this explanation; (*d*) GBM antigens eventually present in circulating blood could interfere with the binding of labeled GBM antigens with anti-GBM antibodies. The demonstration of GBM cross-reactive antigens in normal human serum or urine is in agreement with this hypothesis (32, 33).

Preliminary results obtained with patients exhibiting various nephropathies are of interest. Indeed, most of the patients with GN and linear deposits of immunoglobulins present high GBM binding activity in their serum. McPhaul and Dixon (32) have demonstrated that only 40% of people known to have anti-GBM antibody–mediated GN were shown to exhibit circulating anti-GBM antibodies by indirect immunofluorescence.

[3] P. Mahieu, and P. H. Lambert. Unpublished data.

By radioimmunoassay, only 2 out of 14 patients with GN and linear deposits did not present circulating anti-GBM antibody. These two negative results could be explained by the fact that some extramembranous immune complex deposits may give a false linear pattern of immunofluorescence (34). Nonspecific binding of [^{125}I]-GBM Ag on molecules other than immunoglobulins is unlikely to occur, since IgG fractions purified from positive sera of GN patients bind similar amounts of labeled GBM antigens to anti-GBM serum antibodies. The method of extraction and purification of [^{125}I]GBM Ag might modify some of the antigenic determinants present in intact GBM. However, GBM antigens used always carry antigenic determinants accessible in vivo on the whole GBM structure, since immunoglobulins eluted from Goodpasture's syndrome kidneys bind these [^{125}I]GBM Ag. Finally, the reproducibility of the radioimmunoassay is quite comparable to that reported for other radioimmunoassay systems (35), and it is more sensitive than indirect immunofluorescence or passive hemagglutination. It appears, therefore, that this direct binding test is a sensitive and quantitative method for detection of circulating anti-GBM antibodies in patients with GN and linear deposits of immunoglobulins.

The existence of circulating anti-GBM antibodies in a few cases of proliferative GN and granular deposits of immunoglobulins confirms previous results obtained with the passive hemagglutination test (6). By radioimmunoassay, it has been further demonstrated that IgG fractions isolated from these positive sera bind the GBM antigens. Binding tests performed in patients without proliferative GN are negative. The GBM binding activity observed in the serum of some cases of proliferative GN with granular deposits is always lower than that observed in GN with linear deposits. The role of circulating anti-GBM antibodies detected in proliferative GN without linear deposits remains questionable. The presence of granular IgG deposits and the low concentration of these antibodies are not in favor of a major role of such antibodies in the pathogenesis of these types of nephritis. However, it is possible that these antibodies react in vivo with GBM antigenic determinants normally hidden inside the GBM structure. Such complexes might be formed in situ on altered GBM or might result from a release of such antigens in the circulation, as in the autologous immune complex nephritis (36).

ACKNOWLEDGMENTS

This work was supported by Grant Sr 3.617.71 of the Swiss National Fund, by the Annette Kade Foundation, and by the Belgian National Research Foundation.

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