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Enzyme-linked immunosorbent assay for circulating anti-glomerular basement membrane antibodies

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SUMMARY

An enzyme-linked immunosorbent assay for the quantitative determination of anti-glomerular basement membrane antibodies in human sera, which is both sensitive and reproducible, is described. The test detected circulating antibodies in each of seven patients with active anti-glomerular basement membrane disease, whilst sera from 42 patients, with a variety of other glomerulonephropathies, were negative by the test. It has also been possible to demonstrate a good correlation between the levels of circulating anti-glomerular basement membrane antibodies and the clinical course of disease in one patient with Goodpasture's syndrome.

INTRODUCTION

The identification of circulating anti-glomerular basement membrane (GBM) antibodies in patients with glomerulonephritis of unknown aetiology is an important diagnostic aid and provides a useful objective means for following the treatment of anti-GBM nephritis. Various methods are available, including gel diffusion (McPhaul & Dixon, 1969), passive haemagglutination (Macanovic, Evans & Peters, 1972; Mahieu, Dardenne & Bach, 1972), indirect immunofluorescence (McPhaul & Dixon, 1969; Wilson & Dixon, 1973) and radioimmunoassay (Mahieu, Lambert & Maghuin-Rogister, 1973; Wilson & Dixon, 1974; Buffaloe *et al.*, 1978), but none is entirely satisfactory. Gel diffusion is the least sensitive method and is only occasionally successful (McPhaul & Dixon, 1969), whilst haemagglutination techniques, although more sensitive, often give false-positive reactions in patients without other evidence of anti-GBM disease (Macanovic *et al.*, 1972; Mahieu *et al.*, 1972). Indirect immunofluorescence, with normal human or primate kidney as a target tissue, has been the standard technique for such antibody determinations but it is limited by the ready availability of normal kidneys and the variable reactivity between targets (McPhaul & Dixon, 1969; Wilson & Dixon, 1973). The most sensitive technique at present available is radioimmunoassay (Mahieu *et al.*, 1973; Wilson & Dixon, 1974; Buffaloe *et al.*, 1978), but this is limited by the short half-life of the reagents, the need for expensive equipment and the strict controls on the use of radioisotopes.

Engvall & Perlmann (1972) first showed that antibodies could be assayed by indirect enzyme-linked immunosorbent assay (ELISA), with enzyme-labelled antiglobulins as the indicator. This technique, which is inexpensive and simple to perform, has now been used successfully for the estimation of a wide variety of bacterial and viral antigens and antibodies (Voller, Bartlett & Bidwell, 1978). Apart from reports of the use of ELISA for the measurement of anti-DNA antibodies in systemic lupus erythematosus (SLE) (Pesce *et al.*, 1974; Engvall, 1976), this method has not hitherto been applied to the detection of autoantibodies. In this paper, an ELISA, which is both

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sensitive and reproducible, for the quantitative determination of anti-GBM antibodies is described, and its application in the management of patients with anti-GBM nephritis is considered.

MATERIALS AND METHODS

Enzymes. Highly purified, lyophilized *Clostridium histolyticum* collagenase was obtained from Cambrian Chemicals Ltd, Surrey.

Alkaline phosphatase was obtained from Sigma London Chemical Co. Ltd, Surrey, as a crystalline suspension in 3·2 M ammonium sulphate solution (5 mg protein/ml; 1,000 units/mg protein).

The enzymes were stored at 4°C.

Buffers. The following buffers were prepared according to Gomori (1955): 0·1 M tris-acetate, pH 7·4, containing 0·005 M calcium acetate; 0·1 M citrate phosphate, pH 3·0; 0·05 M tris–HCl, pH 8·0; 0·05 M carbonate buffer, pH 9·8.

Phosphate-buffered saline/Tween 20 (PBST), used for all washing procedures and certain dilutions in the ELISA, was 0·01 M phosphate-buffered saline, pH 7·1, containing 0·15% Tween 20.

Protein concentrations. These were determined by the Hartree (1972) modification of the Lowry method, with bovine serum albumin as a reference standard.

Antigen. Glomerular basement membranes (GBM) were prepared from human cadaver kidneys (Wheeler *et al.*, 1975) and were stored at −20°C as lyophilized preparations.

To prepare a soluble antigen for use in the ELISA, the lyophilized GBM was suspended in 0·1 M tris-acetate buffer, pH 7·4, containing 0·005 M calcium acetate, to a concentration of 25 mg/ml. Collagenase was added to 0·7% (w/w) of the GBM and the mixture was incubated at 37°C for 24 hr. More collagenase was then added, equal to 0·35% of the initial weight of the GBM, and incubation was continued for a further 24 hr. The mixture was then centrifuged at 3,000 ***g*** for 15 min and the soluble collagenase digest (CD) was stored in 0·1-ml aliquots at −20°C.

Rabbit anti-human IgG antiserum. This was kindly supplied by Dr S. H. Parry (Department of Microbiology, Newcastle upon Tyne). It was stored at −20°C.

Isolation of specific rabbit anti-human IgG antibodies. An immunoadsorbent column of Sepharose 4B–human IgG (Hudson & Hay 1976) was prepared and equilibrated with PBS, pH 7·1. Five millilitres of rabbit anti-human IgG antiserum were applied to the column and the effluent was monitored at 280 nm. When all the 'fall-through' had been collected, 0·1 M citrate-phosphate buffer, pH 3·0, was pumped through to elute the adsorbed specific rabbit anti-human IgG antibodies. The eluate was dialysed against PBS, concentrated to 5 mg/ml by ultrafiltration and stored at −70°C until used.

Conjugation of specific rabbit anti-human IgG antibodies to alkaline phosphatase. Alkaline phosphatase suspension (0·3 ml), containing 1·5 mg protein, was centrifuged at 1,000 r.p.m. for 10 min. The supernatant was discarded and the pellet mixed with 0·1 ml of the specific rabbit anti-human IgG at 5 mg/ml. After overnight dialysis against PBS, 10 μl of glutaraldehyde were added to give a final concentration of 0·2%. After 2 hr at room temperature, the solution was diluted to 1 ml and again dialysed against PBS. The solution was finally diluted to 10 ml with 5% BSA in 0·05 M tris–HCl, pH 8·0, containing 0·001 M $MgCl_2$ and 0·02% NaN_3. Conjugates were stored at 4°C and were found to be active for periods of more than 1 year.

Conjugate substrate. The conjugate substrate was *p*-nitrophenylphosphate (NPP), which was obtained in tablet form from Sigma London Chemical Company Ltd and stored at −20°C.

For use in the assay, NPP was dissolved in 0·05 M carbonate buffer, pH 9·8, containing 0·005 M $MgCl_2 \cdot 6\ H_2O$, to a concentration of 1 mg/ml. This solution was prepared fresh daily.

Patient sera. These were stored at −70°C in 0·2-ml aliquots and, wherever possible, a fresh aliquot was used for each assay.

ELISA method

(1) Collagenase digest was diluted with 0·05 M carbonate buffer, pH 9·8, containing 0·1% sodium azide, to a protein concentration of 10 μg/ml. One millilitre of this antigen

preparation was added to each polystyrene tube (65 × 10 mm; Sterilin Ltd, Middlesex) and the tubes were incubated in a water bath at 37°C for 3 hr. The tubes were stored overnight at room temperature for use on the following day.

(2) The antigen solution was removed and the tubes washed three times with PBST.

(3) Control and test sera were diluted with PBST and 1 ml of each serum dilution was added to a tube. The tubes were incubated for 5 hr in a water bath at 25°C and each serum dilution was assayed in duplicate.

(4) After three washes with PBST, 1 ml of conjugate, diluted 1 in 250 with PBST, was added to each tube and the tubes were incubated overnight in a water bath at 25°C.

(5) Excess conjugate was washed out and 1 ml of conjugate substrate was added to each tube.

(6) After incubation at 25°C for 1 hr, the reaction was terminated by the addition of 0·1 ml of 1 M NaOH solution to each tube.

(7) The absorbances of all solutions were read at 400 nm against distilled water.

In addition to known positive and negative sera, a direct conjugate control (DCC) and a substrate blank (SB) were included in each group of assays. The tubes for the DCC were filled with 1 ml of PBST instead of serum in step 3 and provided a control on the extent of non-specific conjugate binding. The substrate blank was obtained by substituting PBST for test serum in step 3 and conjugate in step 4, and provided a control on spontaneous hydrolysis of the conjugate substrate.

To calculate the results, the mean absorbance of the duplicate DCC reading was subtracted from the mean absorbance of each serum dilution.

Method of ELISA quantitation. A standard curve was constructed with a known high-titre anti-GBM antibody-containing serum (AB), which was assayed at dilutions ranging from 1 in 1,000 to 1 in 100. This dilution range was calibrated to represent 10 to 100 arbitrary antibody units (a.a.u.) of activity and thus provided a standard curve of absorbance against antibody activity.

Test sera and normal human serum (negative control) were assayed at a dilution of 1 in 100 and the absorbances obtained were read off on the standard curve to give an estimate of anti-GBM antibody activity.

RESULTS

Standardization of ELISA

The effect of the amount of antigen added during the coating procedure on the uptake of specific antibodies is illustrated in Fig. 1. Above 10 μg/ml, the increase in absorbance was small and 10 μg/ml was therefore selected as the standard antigen-coating concentration.

The effect of the duration of antibody incubation is shown in Fig. 2. Approximately 5 hr of incubation gave optimal binding of enzyme to the tubes.

The optimal duration of conjugate incubation is illustrated in Fig. 3. For practical purposes, overnight incubation with conjugate was selected.

With the optimal conditions of antigen concentration and antibody and conjugate incubation

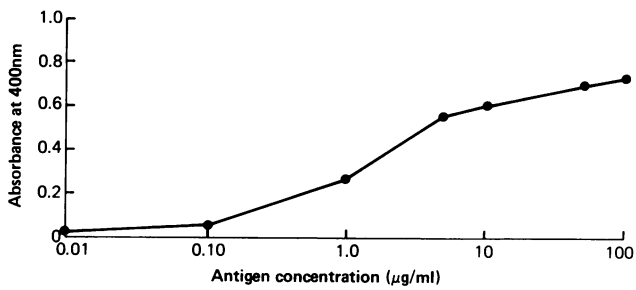

Fig. 1. Optimal antigen adsorption. Tubes were coated with 1 ml of antigen, concentrations ranging from 0·01 to 100 μg/ml. Antibody and conjugate dilutions were 1:100 and 1:250 and incubation periods were 5 and 18 hr respectively.

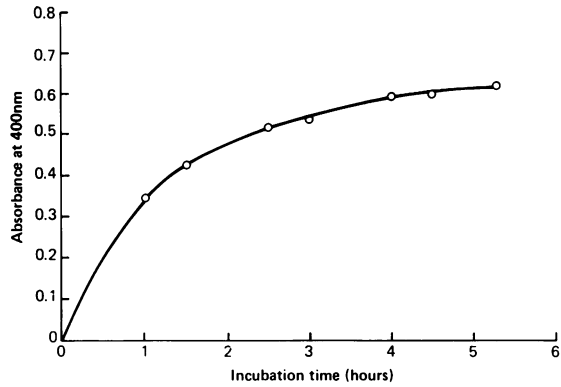

Fig. 2. Optimal antibody binding. Tubes were coated with 1 ml of antigen, concentration 10 μg/ml. Antibody dilution was 1:100 and incubation period ranged from 1 to 5·25 hr. Conjugate dilution and incubation period were 1:250 and 18 hr respectively.

times, a standard curve was obtained with serum AB, as detailed under Materials and Methods. A representative standard curve is illustrated in Fig. 4.

Assay of test sera

With the standard method, 76 sera from 50 patients with various forms of renal disease were examined, including acute and chronic glomerulonephritis, Henoch–Schönlein purpura, polyarteritis nodosa, systemic lupus erythematosus, Buerger's disease, pyelonephritis, hypertension and anti-GBM disease. In addition, sera from normal control individuals, with no history of renal disease, were also assayed. The results are shown in Fig. 5. When two or more sera from one patient were assayed, the highest result only is illustrated and when one serum was assayed several times, the mean result is shown. The results show that normal controls and all groups of patients, except those with active anti-GBM disease, had anti-GBM activities of less than 7 a.a.u. In marked contrast, those patients with active anti-GBM disease, had activities ranging from 32 to 191 a.a.u.

In total, 32 sera from eight patients in various stages of anti-GBM disease were assayed. Twenty-three were positive, with activities ranging from 7 to 191 a.a.u. The remaining nine sera were negative (< 7 a.a.u.) and were all taken either during remission or after nephrectomy.

In order to assess the reproducibility of the ELISA technique, 16 positive sera were tested weekly against the standard AB serum. The results are illustrated in Fig. 6 and show that, for antibody

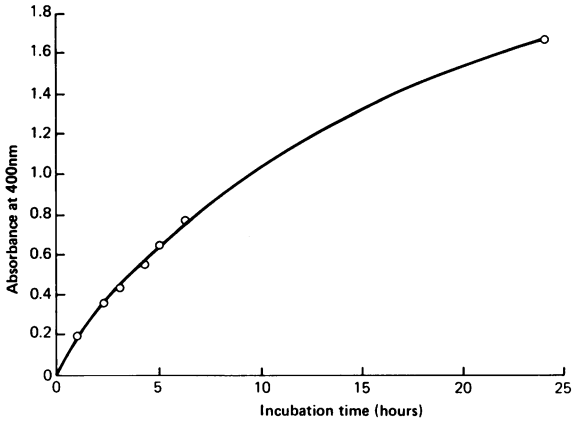

Fig. 3. Optimal conjugate binding. Tubes were coated with 1 ml of antigen, concentration 10 μg/ml. Antibody dilution and incubation period were 1:100 and 18 hr respectively. Conjugate dilution was 1:250 and incubation period ranged from 1 to 24 hr.

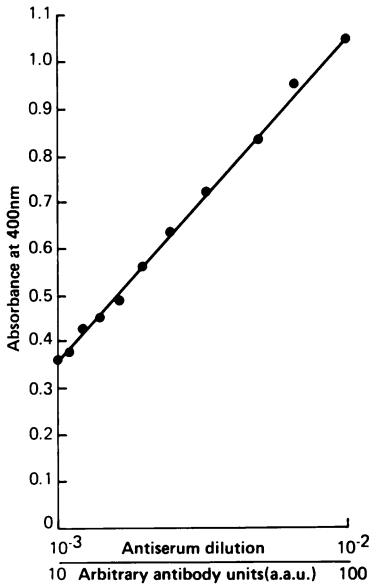

Fig. 4. Calibration curve for measurement of anti-GBM antibodies. Tubes were coated with 1 ml of antigen, concentration 10 μg/ml. Antibody dilutions ranged from 1:100 to 1:1,000 and incubation period was 5 hr. Conjugate dilution and incubation period were 1:250 and overnight respectively.

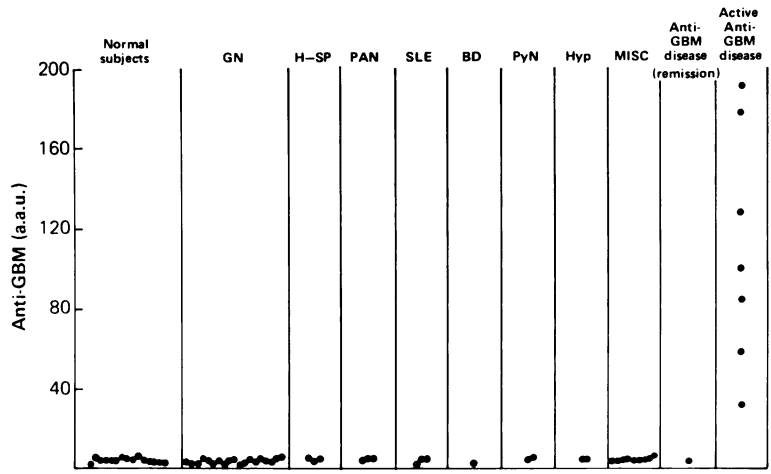

Fig. 5. Titres of anti-GBM antibodies in various forms of renal disease by ELISA. GN includes chronic glomerulonephritis (5); proliferative glomerulonephritis (5); membranous glomerulonephritis (3); chronic proliferative glomerulonephritis (1); membranoproliferative glomerulonephritis (2); mesangiocapillary glomerulonephritis (1); mesangioproliferative glomerulonephritis (1); acute segmental glomerulonephritis (1). H-SP = Henoch–Schönlein purpura; PAN = polyarteritis nodosa, including one case of Wegener's granulomatosis with renal involvement; SLE = systemic lupus erythematosus; BD = Buerger's disease; PyN = chronic and acute pyelonephritis, including one case post-transplant (rejected); Hyp = hypertension; MISC = miscellaneous, including malabsorption (1), rheumatic fever (1), acute renal failure post-prostatectomy (1), alcoholic cirrhosis (2), acute renal failure post-duodenal ulcer and myocardial infarct (1), acute glomerulonephritis with pulmonary haemosiderosis (1), rapidly progressive glomerulonephritis confused by severe asthma and chronic bronchitis (1), pulmonary interstitial fibrosis and nephritis (1).

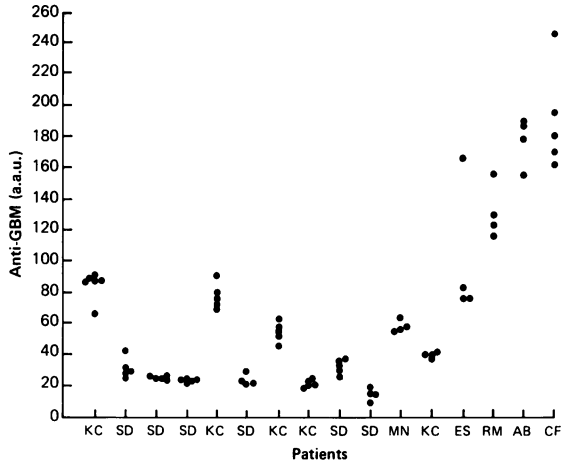

Fig. 6. Reproducibility of ELISA for anti-GBM antibodies. Sixteen positive sera were assayed weekly against standard serum.

levels between 7 and 100 a.a.u., the reproducibility of the technique is good. Above 100 a.a.u., more variability is apparent, reflecting extrapolation beyond the limit of the standard curve. With such high-titre sera, a more reliable result can be obtained by repeating the assay with a higher dilution of the test serum.

To assess the clinical usefulness of the ELISA for anti-GBM antibody, assays were available on serial serum samples from two patients (K.C. and S.D.) with anti-GBM disease. Eight sera from patient K.C. and 10 sera from patient S.D. were assayed over a period of approximately 6 months. The results are shown in Fig. 7. When a serum sample was assayed several times, the mean result ± 2 standard deviations is expressed.

Patient K.C. This patient showed a marked rise in anti-GBM antibody activity from 21 to 85 a.a.u. between days 1 and 28. The antibody activity remained high until day 65 (78 a.a.u.) and then fell steadily to 11 a.a.u. during the remainder of the period of study. These results correlate well with the clinical course of the disease in this patient. He presented on day 1 with a 3-week history of haemoptysis and peripheral oedema. At that time, his renal function was only very slightly impaired, with a serum creatinine of 151 μmol/l and a creatinine clearance of 71 l/24 hr. A renal biopsy confirmed anti-GBM disease but, as the renal function remained normal, he was not treated. On day 28, following a further week of haemoptysis, the serum creatinine level had risen to 700 μmol/l and corticosteroid therapy was started. The patient's renal function deteriorated further and he was started on maintenance haemodialysis.

Patient S.D. In this patient, the anti-GBM antibody levels fell steadily from 32 to 8 a.a.u. during

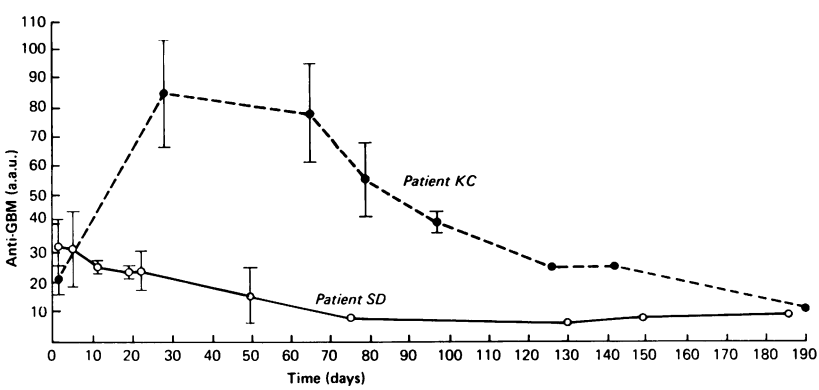

Fig. 7. Serial study of anti-GBM antibody titres in two patients, K.C. and S.D., with active anti-GBM disease.

the course of observation. It was not possible to establish a correlation between the anti-GBM antibody activity and the clinical course of the disease, as this patient had been maintained on dialysis prior to the start of the experiment.

DISCUSSION

Several methods have been described for the diagnosis of anti-GBM antibody-induced nephritis, but none is entirely satisfactory. The first of these depends on the direct demonstration of linear deposits of anti-GBM antibody in renal biopsy material, by means of fluorescein-labelled anti-Ig reagents (Wilson & Dixon, 1973). The non-specific accumulation of serum proteins, in particular IgG and albumin, may, however, give rise to 'pseudo-linear' appearances by this technique (Wilson & Dixon, 1979).

Other diagnostic methods depend on the demonstration of circulating anti-GBM antibodies and, of these, radioimmunoassay (RIA) is the most sensitive technique available at present. A number of RIA procedures have been reported (Mahieu *et al.*, 1973; Wilson & Dixon, 1974, 1976; Buffaloe *et al.*, 1978). Wilson & Dixon (1974, 1976) studied sera from a large group of patients with well-characterized renal diseases. The test detected anti-GBM antibodies in 76 of 78 patients with Goodpasture's syndrome (97%) and in 43 of 52 patients with anti-GBM antibody-induced nephritis. Furthermore, a study of sera from 447 patients with evidence, by immunofluorescence, of immune complex-induced glomerulonephritis revealed only six with evidence of circulating anti-GBM antibodies. Although RIA procedures are particularly suitable for large-scale operations, they suffer from a number of limitations. The requirement for expensive counting equipment, the acknowledged hazards of radioactive materials and the instability of the radioactive label on prolonged storage have tended to exclude RIA from many laboratories.

During the last decade, enzyme-linked immunosorbent assays (ELISA) have been found to be as sensitive as the corresponding RIA and with the added advantage of fewer requirements for specialized equipment. However, the main advantage of the ELISA is in the stability of the enzyme conjugate compared with isotopic reagents. Enzyme activity and immune reactivity of conjugates have been shown to remain stable for periods of more than 1 year at 4°C (Engvall, Jonsson & Perlmann, 1971).

In this paper, we have described an ELISA for the detection and quantitation of circulating anti-GBM antibodies, which is both sensitive and reproducible. The test was successful in detecting circulating anti-GBM antibodies in 23 of 32 sera from eight patients with anti-GBM disease. Sera from 42 patients with a variety of other glomerulonephropathies revealed no false-positive results. Furthermore, with this assay, it has been possible to demonstrate a good correlation between the levels of circulating anti-GBM antibodies and the clinical course of disease in a patient with Goodpasture's syndrome. Too few patients have been studied to date to demonstrate any significant difference in the levels of anti-GBM antibodies in patients with Goodpasture's syndrome as compared with those with renal involvement only.

In this ELISA, the antigen employed was a simple collagenase digest of human GBM. However, it is planned to fractionate this antigenic preparation into its known glycopeptides. With these glycopeptides as antigens in the ELISA, it may be possible to refine the assay and highlight possible differences in the autoantibody specificities of patients with different clinical presentations of anti-GBM disease.

If this ELISA is to find widespread application, certain problems of calibration remain to be solved. Because of the differing slopes of the dose–response curves of various sera used as standards, it has so far not been possible to define amounts of circulating antibody in precise units. Nevertheless, evidence is accumulating that this assay is valuable in following the progress of plasmaphoresis and immunosuppression therapy in patients with anti-GBM disease.

Finally, we wish to emphasize the potential value of ELISA, particularly in the diagnosis and follow-up of organ-specific autoimmune diseases.

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