Clin. exp. Immunol. (1976) 25, 185–190.

'Hidden' IgG antiglobulins in normal human serum

F. C. HAY, LYNN J. NINEHAM, G. TORRIGIANI* & I. M. ROITT *Department of Immunology, Middlesex Hospital Medical School, London*

(*Received* 24 *February* 1976)

SUMMARY

Hidden antiglobulins reacting with whole rabbit immunoglobulin were found by radioassay or indirect haemagglutination after gel filtration or ultracentrifugation of normal serum. Surprisingly, both the IgM and IgG antiglobulins were present in the same macroglobulin fractions. The IgG antiglobulins could be dissociated into 7S components by separating the serum under acid conditions. Antiglobulin activity was a function of the Fab region.

INTRODUCTION

Elevated levels of antiglobulins are a characteristic feature of rheumatoid arthritis and Still's disease (Torrigiani *et al.*, 1969). Complexes containing these antiglobulins have been found in both serum (Kunkel *et al.*, 1961) and synovial fluid (Hannestad, 1967) and are considered to be of importance in the pathogenesis of the synovial inflammatory response (Ziff, 1974).

However, sensitive techniques for both IgG and IgM antiglobulins have revealed that normal sera also contain these complexes in small amounts (Torrigiani & Roitt, 1967; Hay, Nineham & Roitt, 1975). In the course of a study on the nature of immune complexes and antiglobulins in rheumatoid arthritis, sera from patients and normal controls were fractionated on the basis of molecular weight. We were intrigued to observe that both IgM and IgG antiglobulins were revealed in our normal controls as a result of the separation procedure, and that this activity was restricted to the high molecular weight fractions.

The present report is concerned with the nature of these cryptic antiglobulins in normal sera.

MATERIALS AND METHODS

Sera. Serum was obtained from normal individuals working in the immunology laboratory. All had been apparently healthy for at least 4 months. The samples were frozen within 3 hr of collection, stored at −20°C and used within 1 month.

Antisera. Antihuman IgG and IgM were prepared by immunizing rabbits with purified Fcγ or IgM. The anti-IgM was rendered specific by adsorbing with a cyanogen bromide-activated Sepharose-4B (Hudson & Hay, 1976) immunosorbent of cord serum. The antisera were checked for specificity by immunoelectrophoresis and Ouchterlony immunodiffusion. Purified antibody was prepared by adsorbing the antisera on to cyanogen bromide-activated Sepharose-4B immunosorbents of human IgG or IgM followed by elution of the specific antibody with 0·1 M glycine–HCl buffer, pH 2·8. The purified antibodies were then radiolabelled with ^{125}I (Na ^{125}I, IMS 4, Radiochemical Centre, Amersham, Bucks) by a modification of Hunter & Greenwood's method (1962). Antibody, 1 mg in 1 ml of phosphate-buffered saline (PBS), 0·15 M, pH 7·2, was labelled with 500 μCi ^{125}I by adding 60 μg chloramine T. After 2 min incubation at room temperature 120 μg sodium metabisulphite was added to stop the reaction. Free iodine was removed on a Sephadex G-25 column. The labelled proteins were stored at −20°C.

Gel filtration. Sera were fractionated on columns (1·6 × 100 cm) of Ultrogel AcA 34. Samples of 1·5 ml were passed through the gel at a flow rate of 30 ml/hr. Sera were run at either pH 7·2 in PBS or at pH 4·0 in citrate–phosphate buffer 0·1 M. Acid fractions were neutralized immediately with 1 N sodium hydroxide.

* Present address: Immunology Unit, World Health Organisation, 1211, Geneva 27, Switzerland.

Correspondence: Dr F. C. Hay, Department of Immunology, Middlesex Hospital Medical School, Arthur Stanley House, 40–50 Tottenham Street, London W1P 9PG.

Ultracentrifugation. Serum was also separated into 7S and 19S fractions by rate zonal ultracentrifugation on a stepped gradient from 10 to 26% sucrose (Torrigiani & Roitt, 1965). Sera were spun in PBS pH 7·2 or in glycine–HCl buffer, 0·1 M, pH 2·5.

Immunoglobulin estimation. Total IgG and IgM in the separated fractions were estimated by single radial immunodiffusion (Mancini, Carbonara & Heremans, 1965).

Solid phase antiglobulin assay. 1 ml volumes of rabbit immunoglobulin solution (10 mg/1) in PBS were incubated in polystyrene tubes (LP3, Luckham Ltd, Sussex) for 1 hr at 37°C and then overnight at 4°C. After three washes with PBS the tubes were incubated at room temperature for 2 hr with 2 ml of 1% bovine serum albumin (Armour Pharmaceutical Co. Ltd, Eastbourne) in PBS (BSA/PBS) to block any remaining free sites. After three more washes with PBS the tubes were stored at 4°C.

The protein content of each fraction was estimated by measurement of the extinction at 280 nm. 100 μg aliquots were taken from selected fractions and added to the rabbit immunoglobulin-coated tubes. The volume in each tube was made up to 500 μl by adding PBS and 10% BSA to bring the final concentration of BSA to 1%. Coated tubes containing 500 μl BSA/PBS were used as background controls. The tubes were incubated at 37° for 1 hr and at 4°C for 30 min. Unbound proteins were then removed by washing three times with cold PBS. Human antiglobulins bound to the tubes coated with rabbit immunoglobulin were detected by incubating the tubes with 1 μg of purified radiolabelled anti-IgG or IgM in 1 ml BSA/PBS at 37°C for 1 hr and at 4°C for 30 min. Unbound labelled reagent was removed by three washes with cold PBS. The tubes were then counted in a gamma-ray spectrometer, the amount of radioactivity bound being a measure of the IgG or IgM antiglobulin in the fractions.

Agglutination assay for antiglobulins. Antiglobulins were also detected by a haemagglutination assay using pyruvic aldehyde treated sheep red cells coated with rabbit immunoglobulin. Indirect agglutination was performed by adding specific anti-human IgG.

Papain digestion. Immunoglobulin was prepared from pooled normal serum by ammonium sulphate precipitation at 45% saturation. The immunoglobulin was dialysed against PBS containing 0·002 M EDTA and 0·01 M cysteine and was then digested with 2 mg 100 ml papain for 4 hr at 37°C. Undigested immunoglobulin was removed by gel filtration on Sephadex G-100.

RESULTS

Antiglobulins in serum fractionated at pH 7·2

Normal sera were separated by ultracentrifugation. The gradient was divided into nineteen fractions and the total IgG estimated in each of them (Fig. 1, pH 7·2). Most of the IgG was found in the 7S region while only trace amounts were detected in the 19S peak. IgG antiglobulins measured by indirect haemagglutination were detectable in small amounts in the 7S fractions, but surprisingly, higher titres were now revealed in the 19S region.

We decided to investigate this unexpected finding of high molecular weight IgG antiglobulins using high resolution gel filtration. Sera were separated on AcA 34 at pH 7·2. Figs. 2(a) and 3(a) show typical gel filtration profiles obtained with two of the normal sera. Aliquots were examined from seven or eight representative fractions. As expected, IgM was present only in the excluded peak, but although most of

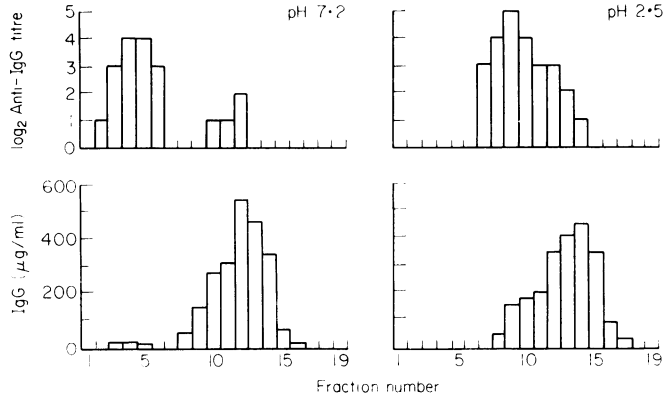

FIG. 1. IgG antiglobulins and total IgG in fractions of normal serum obtained after ultracentrifugation at either pH 7·2 or 2·5. The antiglobulins were determined by indirect haemagglutination.

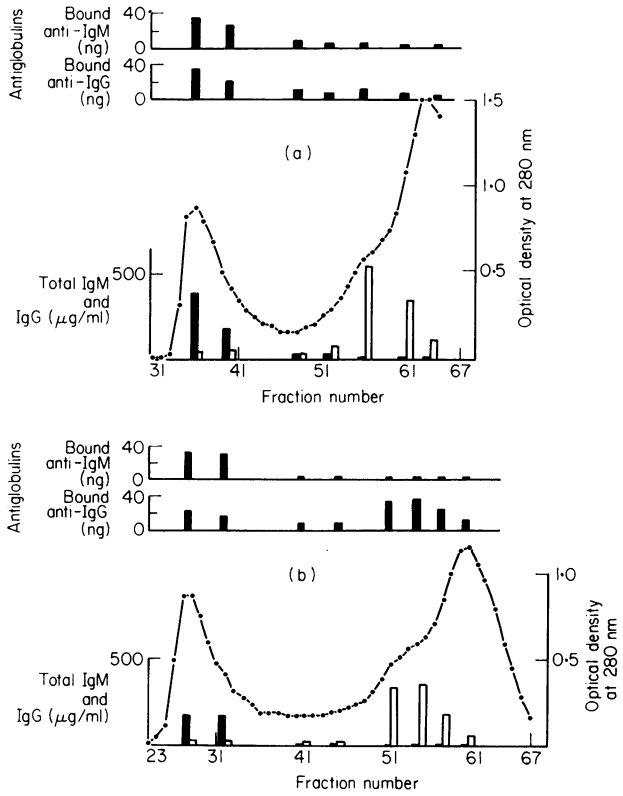

FIG. 2. Ultrogel AcA 34 separation of serum A showing high molecular weight IgG antiglobulin. The optical density profile (●) was recorded at 280 nm. Total IgM (solid columns) and IgG (open columns) were quantified by single radial immunodiffusion. Both IgM and IgG antiglobulins were determined by solid phase radioassay. (a) At pH 7·2; (b) at pH 4·0.

the IgG was in the 7S region, a small amount was found in the high molecular weight fractions. The same held true for each of the four sera studied.

IgM antiglobulins, determined by the radioassay, were only detectable in the heavy molecular weight fractions containing IgM. Traces of IgG antiglobulins were present in the 7S region but large amounts were always found in the high molecular weight fractions in agreement with our ultracentrifugation studies. It was possible that the IgM antiglobulins might attach to the rabbit immunoglobulin on the tube through only some of the Fab regions, leaving other antigen binding sites free. These could then combine with the Fc region of the radiolabelled rabbit anti-IgG used for detecting IgG antiglobulins, so giving falsely elevated values for the high molecular weight region. To test this hypothesis, radiolabelled purified rabbit anti-ovalbumin was added to serum fractions in parallel with anti-IgG and anti-IgM. Table 1 shows that only background levels of binding were obtained with anti-ovalbumin in all the fractions.

The far higher binding results seen with anti-IgG as compared with anti-IgM, and the low correlation between the two in this experiment provide further evidence for the specificity validity of the IgG antiglobulin findings and their independence from the IgM factors.

IgG antiglobulins were also estimated in whole serum before gel filtration so that the total antiglobulin activity recovered from the column could be calculated.

Following fractionation the IgG antiglobulins in the high molecular weight peak were assayed. Without allowing for any losses which might have occurred on the column, this peak alone contained on average 1·8 times the amount in the entire sample applied, showing that some of the IgG antiglobulin activity present in normal serum is hidden and only revealed on fractionation.

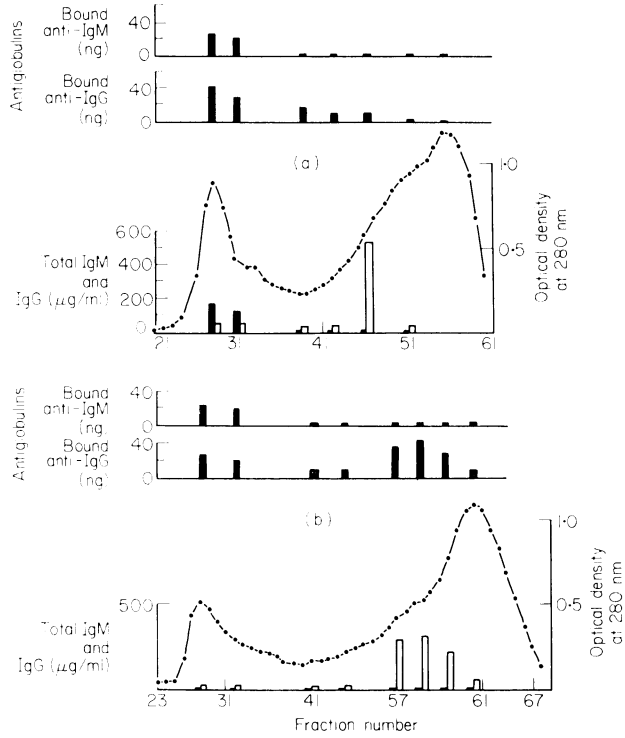

FIG. 3. Ultrogel AcA 34 separation of serum B. Symbols as in Fig. 2. (a) At pH 7·2; (b) at pH 4·0.

Antiglobulins in serum fractionated at acid pH

Ultracentrifugation was carried out at pH 2·5 and the nineteen fractions were neutralized, and immediately tested as before. Under these conditions high titres of IgG antiglobulins were found in the 7S area of the gradient (Fig. 1, pH 2·5). Neither IgG nor IgG antiglobulins were detectable in the 19S region.

Similarly, separation by gel filtration at pH 4·0 revealed that most of the IgG antiglobulins had transferred to the 7S region (Figs 2b and 3b). By contrast, the behaviour of the IgM antiglobulins was unaltered by acid treatment.

TABLE 1. Validation of IgG antiglobulin results. Fractions from gel filtration of a normal serum were tested for antiglobulins by the radioassay using different rabbit antibodies for the final stage

Fraction no.	Anti-IgG	Anti-IgM	Anti-ovalbumin
28	77*	27	0·33
31	83	16	0·34
38	38	3·5	0·32
42	23	2·8	0·33
45	43	2·1	0·39
48	49	2·2	0·34
53	21	1·9	0·45
57	17	2·1	0·36

* Nanograms of antibody bound per tube.

Site of antiglobulin activity

The possibility existed that the binding of IgG reflected a property of a particular type of Fc region, rather than antibody activity associated with the Fab region. Table 2 shows that with whole IgG, antiglobulins could be found by radioassay with both anti-light chain and anti-Fc sera. However, with the papain digest of normal IgG the activity was detected only with anti-light chain reagent, demonstrating that the binding of antiglobulins was solely dependent on the Fab portion of the molecule.

TABLE 2. Localization of the binding site for rabbit IgG in human IgG antiglobulin assayed by solid phase radioassay

	Radiolabelled second antibody	
Antiglobulin preparation	Anti-light chain	Anti-Fc
Whole IgG	90*	188
Papain digest	26	0·3

* Nanograms of antibody bound per tube.

DISCUSSION

In normal sera we have always detected low levels of IgG and IgM antiglobulins. In the present study, however, we have shown that much of the antiglobulin is hidden in whole serum, since much greater total activity is revealed after separation of the proteins by gel filtration or ultracentrifugation. Furthermore, both the IgM and IgG antiglobulins were of high molecular weight. On fractionating serum in acid, large amounts of IgG antiglobulin activity appeared in the 7S peak whereas all the IgM antiglobulin remained of high molecular weight. It was possible that the anti-IgG reagent was detecting, not true IgG antiglobulins, but non-specific IgG bound by the IgM antiglobulin. However, the release of 7S IgG antiglobulin activity under acid conditions demonstrates that the high molecular weight IgG was truly antiglobulin in nature.

It was also possible that the IgM antiglobulin had free Fab regions able to bind radiolabelled rabbit anti-IgG but this could be discounted since only background levels of binding were obtained when labelled rabbit anti-ovalbumin was used in its place.

Thus it seems likely that the high molecular weight IgG is composed of self-associated IgG antiglobulins similar to those found in large amounts in rheumatoid arthritis (Pope, Teller & Mannik, 1974). On the other hand, they may represent 7S antiglobulins bound to small amounts of circulating immune complexes.

Hidden 19S antiglobulin of undefined class has previously been reported in rheumatoid arthritis patients (Allen & Kunkel, 1966) but the only antiglobulins previously described in unfractionated normal serum have been those directed against pepsin digested immunoglobulin ('pepsin agglutinators'; Osterland, Harboe & Kunkel, 1963). It has previously been suggested (Turner, 1974) that at least part of the antiglobulins detected in solid phase assays might be due to a non-specific binding of immunoglobulin. We have not found this to be so in our radioassay (Hay *et al.*, 1975) and the studies presented here confirm the specificity of the method in that IgG antiglobulin activity was mostly in the fractions excluded from the gel but virtually no binding occurred in the 7S peak where the bulk of the IgG was present.

We wished to check that the binding of IgG antiglobulin to rabbit IgG on the tubes represented a true antigen–antibody reaction rather than non-specific sticking through the Fc region. Examination of the papain digest of normal IgG verified that only the Fab portion of the molecule was involved in the binding.

It is interesting to speculate on the role of these antiglobulins in normal sera. Immune complexes are probably continually being formed as a result of minor subclinical infection, and those complexes too

small to be readily removed by the reticulo-endothelial system might provoke hypersensitivity reactions were they to become trapped in the parts of the vascular bed such as the glomerular capillaries. Pre-existing IgG antiglobulins may aid removal of these complexes by enhancing their binding to macrophages.

It must be concluded that the pathogenic mechanism in rheumatoid arthritis cannot be related solely to the presence of antiglobulins; factors such as the site of synthesis, alterations in quantity and avidity or specificity may be of critical importance.

This work was supported by the Medical Research Council of Great Britain and the Arthritis and Rheumatism Council. We thank Miss Christine Meats for preparation of the manuscript.

REFERENCES

ALLEN, J.C. & KUNKEL, H.G. (1966) Hidden rheumatoid factors with specificity for native γ-globulins. *Arthr. Rheum.* **9**, 758.

HANNESTAD, K. (1967) Presence of aggregated γG-globulin in certain rheumatoid synovial effusions. *Clin. exp. Immunol.* **2**, 511.

HAY, F.C., NINEHAM, L.J. & ROITT, I.M. (1975) Routine assay for the detection of IgG and IgM antiglobulins in seronegative and seropositive rheumatoid arthritis. *Brit. med. J.* **iii**, 203.

HUDSON, L. & HAY, F.C. (1976) *Practical Immunology*, chap. 8. Blackwell Scientific Publications, Oxford.

HUNTER, W.M. & GREENWOOD, F.C. (1962) Preparation of ^{131}I labelled human growth hormone of high specific activity. *Nature (Lond.)*, **194**, 495.

KUNKEL, H.G., MÜLLER-EBERHARD, H.M., FUDENBERG, H.H. & TOMASI, T.B. (1961) Gamma globulin complexes in rheumatoid arthritis and certain other conditions. *J. clin. Invest.* **40**, 117.

MANCINI, G., CARBONARA, A.O. & HEREMANS, J.F. (1965) Immunochemical quantitation of antisera by single radial immunodiffusion. *Immunochemistry*, **2**, 235.

OSTERLAND, C.K., HARBOE, M. & KUNKEL, H.G. (1963) Anti γ-globulin factors in human sera revealed by enzymatic splitting of anti-Rh antibodies. *Vox Sang. (Basel)*, **8**, 133.

POPE, R.M., TELLER, D.C. & MANNIK, M. (1974) The molecular basis of self-association of antibodies to IgG (rheumatoid factors) in rheumatoid arthritis. *Proc. nat. Acad. Sci. (Wash.)*, **71**, 517.

TORRIGIANI, G., ANSELL, B.M., CHOWN, E.E.A. & ROITT, I.M. (1969) Raised IgG antiglobulin factors in Still's Disease. *Ann. rheum. Dis.* **28**, 424.

TORRIGIANI, G. & ROITT, I.M. (1965) The enhancement of 19S antibody production by particulate antigen. *J. exp. Med.* **122**, 181.

TORRIGIANI, G. & ROITT, I.M. (1967) Antiglobulin factors in sera from patients with rheumatoid arthritis and normal subjects. *Ann. rheum. Dis.* **26**, 334.

TURNER, M. (1974) In: Workshop Report, Antiglobulins including rheumatoid factors. *Progress in Immunology* II, **5**, 307. North-Holland, Amsterdam.

ZIFF, M. (1974) Autoimmune processes in rheumatoid arthritis. *Progress in Immunology* II, **5**, 37. North-Holland, Amsterdam.