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DETERMINATION OF IgG ANTIGLOBULINS IN RHEUMATOID DISEASE USING INSOLUBILIZED HUMAN IgG

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SUMMARY

IgG antiglobulins in human sera were measured by single radial diffusion in agar after elution from ethyl chloroformate insolubilized immunosorbents of rabbit or human IgG. With both immunosorbents seronegative and seropositive rheumatoid sera contained significantly higher levels of IgG antiglobulins than did sera from healthy adults. Attempts to measure antiglobulins interacting specifically with the pFc′ half of the Fc region were not successful due to an unacceptably high degree of non-antibody binding.

INTRODUCTION

Conventional tests for rheumatoid factor depend on the agglutination of either red cells or latex particles which have been coated with IgG. Such tests mainly measure IgM antibodies since it is this class of immunoglobulin which is the most effective in agglutination reactions. In order to investigate the possible contribution of other immunoglobulin classes Torrigiani & Roitt (1967) developed a quantitative immunoadsorption procedure. In this method antiglobulins were adsorbed on to a matrix of insoluble rabbit IgG, subsequently eluted from the complex and finally estimated by single radial immunodiffusion using specific anti-immunoglobulin antisera. With this procedure it was shown that in addition to IgM antiglobulin factors, IgA and IgG antiglobulins were present in 50 and 100 per cent respectively of seropositive adult rheumatoid arthritis patients. In a later study Torrigiani *et al.* (1970) were able to demonstrate that sera from patients with seronegative rheumatoid arthritis have increased concentrations of IgG antiglobulins as shown by the immunoadsorption technique employing horse IgG as the substrate.

There is increasing evidence (Natvig, Munthe & Gaarder, 1971; Natvig, Gaarder & Turner, 1972) that rheumatoid factors have specificities for antigens present in autologous IgG and therefore data obtained using heterologous IgG may be difficult to interpret.

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Furthermore, it is now known that the antigenic sites of IgG which are reactive with classical rheumatoid factors are present in both the Cγ2 and Cγ3 homology regions of the Fc fragment (Natvig & Turner, 1970; Natvig, Gaarder & Turner, 1972). The availability of pFc′ fragment (≡Cγ3 homology region) suggested the possibility of investigating the reactivity of IgG antiglobulins with this half of the Fc region. We report here a modification of the immunosorbent technique in which insolubilized human IgG and pFc′ fragment were used for the adsorption of antiglobulins.

MATERIALS AND METHODS

Patients

Sera were obtained from nineteen patients with rheumatoid arthritis and having positive Waaler–Rose titres (ranging from 1 : 80 to 1 : 10,240).

A further group of twenty-two rheumatoid patients with a negative Waaler–Rose titre were also studied. These included twenty patients with juvenile rheumatoid arthritis and the age range of the group was 6–51 years.

The control group consisted of twenty-one healthy adults (thirteen male and eight female) ranging in age from 20 to 45 years.

Rheumatoid factor test

The sheep cell agglutination test (SCAT) was carried out using the method described by Anderson *et al.* (1970).

Insoluble cross-linked proteins

Rabbit IgG (prepared by DEAE-cellulose batch chromatography (Stanworth, 1960)) and human IgG (Cohn Fraction II, Kabi, Stockholm) were polymerized with ethyl chloroformate by a modification of the method of Avrameas & Ternynck (1967). One hundred milligrams of protein were dissolved in 2 ml of 0·1 M acetate buffer, pH 4·5. Then, over a period of 15 min, 120 μl of ethyl chloroformate were added with gentle stirring. The pH was maintained at 4·5 by the addition of N NaOH. The gel produced by the addition of ethyl chloroformate was allowed to stand at room temperature for 1 hr and was then dispersed in a tissue grinder. One hundred volumes of PBS, pH 7·4, were added, solid material allowed to settle and the supernatant removed. This step was repeated and then the insoluble material was washed with 0·1 M Na_2CO_3. After a further PBS wash the material was washed with 0·2 M glycine HCl, pH 2·2, until the OD 280 nm of the wash fluid was zero. Finally, the product was washed three times with PBS to give pH 7·4, adjusted to give 20 mg protein/ml and stored at +4°C with 1 : 1000 NaN_3.

Human pFc′ fragment (prepared as previously described, Turner & Bennich, 1968) was insolubilized by a similar procedure.

Adsorption of antiglobulins from serum with insoluble immunosorbent

One millilitre of insoluble immunosorbent suspension (containing 20 mg protein) was incubated with 0·25 ml of serum for 1 hr at 37°C and then overnight at +4°C. Incubation at 37° was performed with constant rotation of the reactants. After incubation the mixture was spun (900 *g* for 5 min) and the supernatant discarded.

The insoluble antigen–antibody complex was washed five times with 2 ml cold saline,

resuspended in 0·5 ml of 0·1 M glycine-HCl, pH 2·5, and incubated at 4°C for 1 hr. The resuspended mixture was then centrifuged (900 *g* for 5 min) and the supernatant removed and brought to neutrality by the addition of 0·04 ml of 0·5 N sodium hydroxide. Insolubilized protein was washed three times after use with 2 ml of PBS, pH 7·4, and twice with 2 ml of physiological saline. It was re-used up to ten times.

Estimation of IgG antiglobulins in eluates from insoluble immunosorbents

IgG eluted from the insoluble matrix was measured by the single radial diffusion technique of Mancini, Carbonara & Heremans (1965) using a rabbit anti-human IgG serum absorbed with $F(ab')_2$ fragment. Rabbit anti-human IgG serum absorbed with both $F(ab')_2$ and pFc′ fragments was used to measure IgG antiglobulins eluted from insolubilized pFc′ fragment.

Eight dilutions of the International Reference Preparation for human serum immunoglobulins (Rowe, Anderson & Grab, 1970; Rowe, Grab & Anderson, 1972) were included on each plate. Previous estimates of immunoglobulin concentrations in terms of mg/ml of the reconstituted International Reference Preparation have shown a wide range of values (Rowe *et al.*, 1972) and it is recommended that concentrations of immunoglobulin be expressed in units per ml. However, in order to facilitate comparison with the data obtained by other groups we have used the conversion figure obtained by Rowe *et al.* (1972); i.e. 1 unit of activity of IgG corresponds to 80·4 μg of isolated IgG (with a 95 per cent confidence interval of 69·2–93·3 μg). The lower limit of detectability on our assay was 6 μg/ml. Diffusion was not allowed to proceed to completion and ring diameters were measured after 20–24 hr. Standard curves were plotted on log-linear graph paper and values for IgG antiglobulins read off directly.

Mean levels for each patient group were calculated ± 2 SD. Student's *t*-tests were performed as appropriate and *P* values calculated. *P* values $<0{\cdot}05$ were considered significant.

RESULTS

IgG antiglobulins reactive with rabbit IgG

IgG antiglobulins were detected in all rheumatoid sera and in eleven out of sixteen sera from healthy adults when insoluble rabbit IgG was used as a substrate (see Fig. 1). As shown in Table 1 the mean values for IgG antiglobulins in both groups of rheumatoid patients were significantly greater than the mean value for healthy adults ($0{\cdot}025 < P < 0{\cdot}050$).

IgG antiglobulins reactive with human IgG

When insoluble human IgG was used as a substrate, IgG antiglobulins were detected in all seronegative and seropositive rheumatoid sera and in eighteen out of twenty sera from healthy adults (see Fig. 2). The mean values for IgG antiglobulins in both groups of rheumatoid patients were again significantly greater than the mean value for healthy adults (Table 1).

The possibility that the higher levels of antiglobulins in the rheumatoid groups were a reflection of higher serum IgG levels was investigated by calculating the specific activities of the IgG antiglobulins reacting with human IgG (see Fig. 3). These values were obtained by dividing the levels of IgG antiglobulin by the total serum IgG for each individual. Considerable overlap is apparent but four out of eight seropositive rheumatoid patients have higher specific activities than any of the healthy adults or seronegative rheumatoid patients.

TABLE 1. IgG antiglobulins reactive with human and rabbit IgG. Statistical comparison of groups

Insoluble immuno-sorbent	Individuals studied	No. of cases studied	Mean level of IgG antiglobulins* (μg/ml $\pm$ 2 SD)	Student's t-test values
Human IgG	Healthy adults	20	11·9 ± 4·2	$t = 0{\cdot}8009$
Rabbit IgG	Healthy adults	16	10·2 ± 7·7	$0{\cdot}50 > P > 0{\cdot}40$
Human IgG	Healthy adults	20	11·9 ± 4·2	$t = 3{\cdot}26$
Human IgG	Seropositive R.A.	15	25·3 ± 17·1	$0{\cdot}005 > P > 0{\cdot}001$
Rabbit IgG	Healthy adults	16	10·2 ± 7·7	$t = 2{\cdot}07$
Rabbit IgG	Seropositive R.A.	13	16·9 ± 9·1	$0{\cdot}05 > P > 0{\cdot}025$
Human IgG	Healthy adults	20	11·9 ± 4·2	$t = 7{\cdot}404$
Human IgG	Seronegative R.A.	12	24·3 ± 10·5	$P < 0{\cdot}001$
Rabbit IgG	Healthy adults	16	10·2 ± 7·7	$t = 2{\cdot}134$
Rabbit IgG	Seronegative R.A.	8	17·2 ± 6·3	$0{\cdot}05 > P > 0{\cdot}025$
Human IgG	Seronegative R.A.	12	24·3 ± 10·5	$t = 0{\cdot}1541$
Human IgG	Seropositive R.A.	15	25·2 ± 17·1	$0{\cdot}90 > P > 0{\cdot}80$
Rabbit IgG	Seronegative R.A.	8	17·2 ± 6·3	$t = 0{\cdot}0857$
Rabbit IgG	Seropositive R.A.	13	16·9 ± 9·1	$0{\cdot}95 > P > 0{\cdot}90$

* Mean levels and ranges of serum IgG were: Healthy adults 8140 μg/ml (4920–10,820 μg/ml; $n = 19$). Seropositive R.A. 11,986μ g/ml (5900–24,270μg/ml; $n = 10$). Seronegative R.A. 10,098 μg/ml (3930–18,360 μg/ml; $n = 5$).

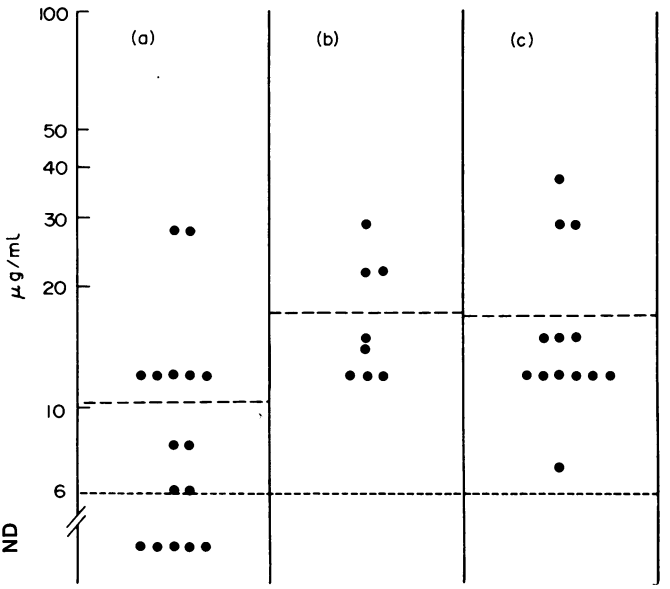

FIG. 1. IgG antiglobulins in (a) healthy adults and patients with (b) seronegative and (c) seropositive rheumatoid arthritis determined using insolubilized rabbit IgG. Each point represents a determination from an individual patient. Dashed lines indicate mean values for each group; the dotted line represents the lower limit of detectability for the method (6 μg/ml). ND = Not detectable.

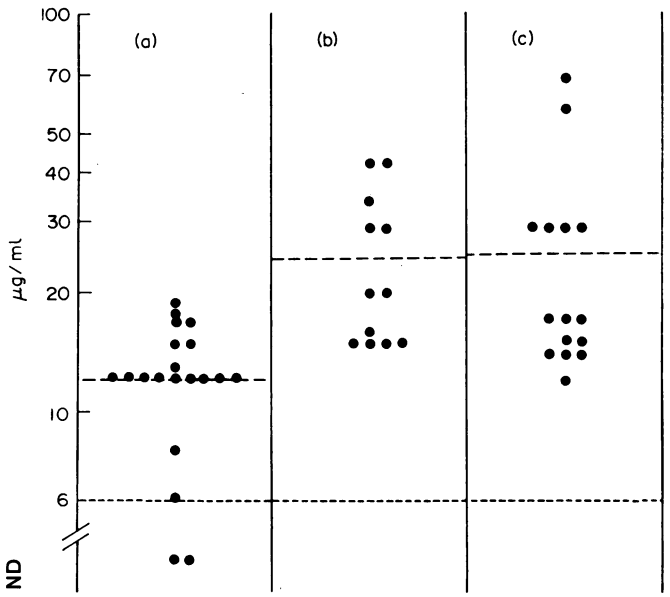

FIG. 2. IgG antiglobulins in (a) healthy adults and patients with (b) seronegative and (c) seropositive rheumatoid arthritis determined using insolubilized human IgG. Dashed lines indicate mean values for each group; the dotted line represents the lower limit of detectability for the method (6 μg/ml). ND = Not detectable.

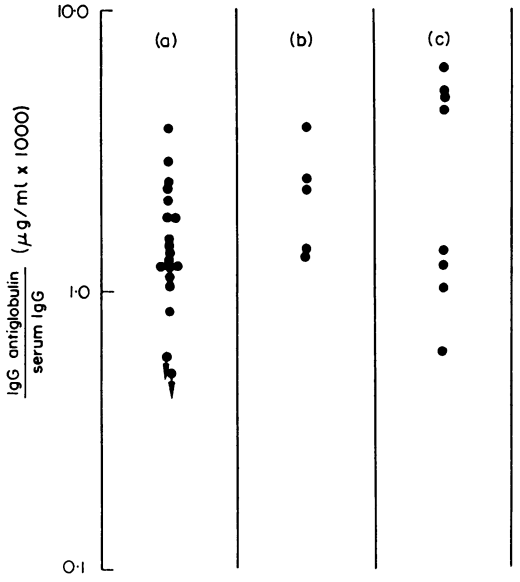

FIG. 3. Specific activity of IgG antiglobulins reacting with human IgG. Each point represents a determination from an individual patient. Two of the values for healthy adults represent the maximum possible levels since in both individuals IgG antiglobulins were not detectable. (a) Healthy adults. (b) Seronegative rheumatoid arthritis. (c) Seropositive rheumatoid arthritis.

Studies with insolubilized pFc′ fragment

When insolubilized human pFc′ fragment was used as a substrate extremely high levels of IgG 'antiglobulins' were found: 253 μg/ml for healthy adults (range 20–944 μg/ml) 388 μg/ml (range 96–1564 μg/ml) for seronegative rheumatoid patients and 369 μg/ml (range 59–1955 μg/ml) for seropositive rheumatoid patients. These findings suggested the possibility of random binding of IgG to the insoluble matrix through sites other than the specific antibody-binding sites. This was investigated by studying the binding of IgG and albumin from nine healthy adult sera to insolubilized pFc′ fragment. Both IgG and albumin were eluted from the matrix in every case. Furthermore, there was a correlation between the levels of albumin and IgG adsorbed from any given serum. There was no significant correlation between the serum IgG level and the level of eluted IgG 'antiglobulin'.

In another experiment an attempt was made to eliminate non-specific protein–protein interactions; the saline wash stage (prior to acid elution) was replaced by washing with a solution of 3 M urea in 0·85% saline. Adsorbed protein was then eluted in the usual manner and IgG and albumin levels determined. Neither IgG nor albumin were detectable in the eluates from insolubilized human IgG. IgG was detectable in all the eluates from insolubilized pFc′ fragment but albumin was not detectable. The levels of eluted IgG were within the same range as observed previously with pFc′ fragment but were all at the lower end of the range.

DISCUSSION

The use of insolubilized IgG for the quantitative determination of antiglobulins was developed by Torrigiani & Roitt (1967) and subsequently applied to a variety of patients with rheumatic diseases (Torrigiani *et al.*, 1969; Torrigiani *et al.*, 1970; Howell *et al.*, 1972).

The present investigation was initiated in order to establish, if possible, a method utilizing insolubilized *human* IgG. The technique used was able to detect, quantitatively, antiglobulins in sera from most healthy adults and all rheumatoid patients, whether adult or juvenile, seropositive or seronegative. Furthermore, insolubilized human IgG was able to discriminate between a group of rheumatoid patients and a group of healthy controls and was superior in this respect to insolubilized rabbit IgG.

Panush, Bianco & Schur (1971) and Bianco *et al.* (1971) have recently published results of an extensive investigation of antigammaglobulins in rheumatoid arthritis, using human IgG insolubilized by glutaraldehyde. Comparison of the IgG antiglobulins detected by these and other investigators using different insoluble immunosorbents is presented in Table 2. The levels of IgG antiglobulins reactive with rabbit IgG were very similar whether the IgG was insolubilized with BDB or ethyl chloroformate. However, comparison of the levels of IgG antiglobulins reactive with human IgG insolubilized by either ethyl chloroformate or glutaraldehyde showed considerably higher values with the latter. Furthermore, the ethyl chloroformate procedure failed to differentiate seropositive and seronegative rheumatoid groups, whereas the glutaraldehyde procedure gave essentially similar levels for the healthy controls and the seronegative rheumatoids but significantly elevated levels for the seropositive rheumatoids. It is also of interest that Torrigiani *et al.* (1970) observed that horse and rabbit IgG insolubilized by BDB differed markedly in their ability to discriminate between seronegative R.A. and healthy controls.

The elevated levels of IgG antiglobulins observed in the serum of patients with rheumatoid arthritis might arise, in part, from elevations in the total serum IgG of these patients. This possibility was investigated by calculating, where possible, the specific activities of IgG antiglobulins in the three patient groups. Half of the seropositive rheumatoid patients were found to have higher specific activities of IgG antiglobulins than any of the healthy controls or seronegative rheumatoid patients. This suggests that the levels of IgG antiglobulins are probably not related to the levels of total serum IgG.

The presence of IgG antiglobulins in the serum of most healthy individuals is in agreement with the observations of Torrigiani & Roitt (1967), Torrigiani *et al.* (1970), Panush *et al.* (1971) and Bianco *et al.* (1971). The significance of this observation is by no means clear. It

TABLE 2. Comparison of mean IgG antiglobulin levels obtained by different procedures

		This report (ethyl chloroformate insolubilization) (μg/ml)	Torrigiani & Roitt (1967) (bisdiazotized benzidine insolubilization) (μg/ml)
	Healthy controls	10	11
Rabbit	Seronegative R.A.	17	ND
IgG	Seropositive R.A.	17	19

		This report (ethyl chloroformate insolubilization) (μg/ml)	Panush *et al.* (1971) (glutaraldehyde insolubilization) (μg/ml)
	Healthy controls	12	98
Human	Seronegative R.A.	24	113
IgG	Seropositive R.A.	25	210

ND = Not determined.

may indicate a normal antibody response to host IgG undergoing catabolism and bearing previously hidden antigens on its surface. Alternatively such antiglobulins may be synthesized following failure to remove complexes of certain antigens with IgG antibody and so may represent one end of the true rheumatoid factor spectrum.

From the evidence presented in this report it is clear that ethyl chloroformate insolubilized pFc′ fragment is able to bind low levels of albumin and this raises the possibility that some of the immunoglobulin eluted may have become bound through interactions with sites other than the antibody-binding site. Attempts to reduce such interactions by using urea during the wash stages were successful but it was not established that the residual bound IgG was all antiglobulin. In this context it is of interest that Steward *et al.* (1973) have recently shown that most IgM rheumatoid factors with specificities for pFc′ antigens have low binding affinity. Other investigators (Stone & Metzger, 1968; Cerottini & Grey, 1969; Normansell, 1971; Abraham, Clark & Vaughan, 1972) have also reported low binding affinities for antiglobulins. If IgG antiglobulins have similar binding constants to the IgM antiglobulins the use

of a dissociating solvent such as urea is likely to displace the antiglobulin with the rest of the IgG and the procedure may be of limited value.

This investigation suggests that the IgG of several species (including man) may be insolubilized by one of several different procedures and used as an immunosorbent for antiglobulins. Which of these methods is the most appropriate for routine application in the field of clinical rheumatology is not yet established. However, earlier fears that the insolubilized human IgG matrix may be degraded and mistakenly estimated as antibody were probably groundless and there is evidence to favour the view that homospecific antigen is preferable to cross-reacting heterospecific antigen.

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