ANTIGLOBULIN FACTORS IN SERA FROM PATIENTS WITH RHEUMATOID ARTHRITIS AND NORMAL SUBJECTS

QUANTITATIVE ESTIMATION IN DIFFERENT IMMUNOGLOBULIN CLASSES

BY

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The conventional tests for rheumatoid factor depend upon the agglutination of particles such as red cells or latex, coated with immunoglobulin-G. Since antibodies of the IgM class tend to be particularly effective in agglutination reactions, it is likely that the rheumatoid factor tests minimize any possible contribution from antiglobulin factors in other immunoglobulin classes. However, if the rheumatoid factors represent an immunological response of the patient to slightly altered autologous IgG, one would also expect to find antiglobulin factors in the IgG and IgA classes. In order to investigate this possibility we have developed a method which identifies the immunoglobulin class of the antiglobulin and also allows a quantitative assessment to be made. The method depends upon the absorption of antiglobulins on to insoluble cross-linked rabbit γ -globulin, their subsequent elution from this complex, and estimation by the immunoplate technique using specific anti-immunoglobulin antisera incorporated into the gel. A preliminary account of these studies has been reported (Torrighiani and Roitt, 1965a).

Material and Methods

Sera

27 sera from patients with rheumatoid arthritis all containing rheumatoid factors reacting with rabbit IgG in the sheep cell agglutination test (SCAT) were used. As controls, 21 sera from normal blood donors and four cord bloods were tested. All sera were stored at -20°C . and spun at 4,000 r.p.m. before use.

Rheumatoid Factor Tests

The SCAT was carried out using the method recommended by the Biological Standards Department of the National Institute of Medical Research, London, in

consultation with a number of laboratories (to be published). Experimental serum fractions were tested by the latex-fixation reaction, using rabbit and human FII (Burby and Behr, 1958). They were also tested for anti-rabbit IgG with pyruvic aldehyde-treated cells (Ling, 1961) coated with rabbit FII at a concentration of 0.8 mg./ml.

Insoluble Cross-linked Rabbit IgG

Rabbit Cohn fraction II (Hyland Laboratories) was polymerized with bis-diazotized benzidine by the method of DeCarvalho, Lewis, Rand, and Uhrick (1964). 15.8 ml. water was added to 360 mg. recrystallized benzidine in 3.75 ml. of 6 N HCl, and the solution cooled to 8°C . Over a period of 2 minutes, 7 ml. sodium nitrite solution (1.3 g. sodium nitrite in 32 ml. water) were added, and the resulting solution added with stirring to a precooled solution of 4.50 g. sodium acetate in 10 ml. water over a period of 1 minute. The polymerization of rabbit IgG was carried out, adding 7.2 ml. of tetrazotized benzidine solution to 1.6 g. fraction II in 100 ml. phosphate buffered saline pH 7.2 (PBS) over a period of 30 minutes. After a further 7 minutes, 1 ml. of 8 N potassium carbonate was added with continuous stirring. The insoluble IgG was dispersed in a Potter-Elvehjem homogenizer, washed five times with saline, and kept overnight at 4°C . in 1M glycine to reduce any subsequent non-specific absorption of proteins (cf. Williams and Kunkel, 1963). After a further wash with saline, the suspension was adjusted to contain 20 mg. protein/ml. and was stored at 4°C . The preparation was used within a week.

Absorption of Anti-globulins from Serum with Insoluble IgG

1 ml. of the insoluble rabbit IgG suspension was incubated with 0.5 ml. serum for 1 hr at 37°C . and then overnight at 4°C . After this procedure, tests for rheumatoid factor were consistently negative. The polymerized IgG was spun down and washed five times

with cold saline. The washed sediment was suspended in 0.5 ml. 0.1M glycine-HCl buffer pH 2.5 at 4° C. After 1 hr the mixture was spun and the sediment washed once with 1 ml. glycine-HCl; the combined supernatant fluids were rapidly concentrated to approximately 0.1 ml. in a small dialysis sac suspended vertically and surrounded with solid sucrose (Centeno, Shulman, Milgrom, and Witebsky, 1965). The dialysis sac was tied again to leave only a small air space between the knot and the liquid, and the contents dialysed against PBS, when the volume increased to about 0.15 ml. The fluid was transferred to a small plastic tube with a mark at 0.25 ml. and the dialysis sac washed with a few drops of PBS, the final volume of the concentrated eluate being made up to 0.25 ml. Treatment of the polymerized IgG-antiglobulin complex with acid for 3 hrs instead of 1 hr and washing three times instead of once did not release any further measurable immunoglobulins into the supernatant. Immunodiffusion studies showed that the molecular size of the eluted immunoglobulins was not altered by the acid treatment.

Estimation of Individual Immunoglobulins in Eluates from the Insoluble IgG

The immunoplate method of Fahey and McKelvey (1965) was used with specific anti-immunoglobulins (Red Cross Laboratory, Amsterdam), which were checked by immuno-electrophoresis. Standard curves were established, using doubling dilutions of a serum with known immunoglobulin concentrations. In a typical experiment, the lower limit of sensitivity for IgG in the eluate was 15 µg./ml., for IgA 5 µg./ml., and for IgM 22 µg./ml. These figures correspond with lower limits of sensitivity for the detection of antiglobulins in the original serum of 8 µg./ml. for IgG, 3 µg./ml. for IgA, and 11 µg./ml. for IgM.

Serum Fractionation

5 ml. serum were separated by chromatography on a 1 × 20 cm. column of DEAE-cellulose, using step-wise elution with the following buffers: 0.01M phosphate pH 7.8, 0.02M phosphate pH 6.2, 0.05M phosphate pH 4.4, and 0.5M phosphate pH 4.4 in 0.1M sodium chloride (Amante and Ancona, 1964). Certain of the

fractions obtained by chromatography were treated with an equal volume of 0.2M 2-mercapto-ethanol overnight at 4° C. and then dialysed against saline or 0.02M iodoacetamide in phosphate buffer pH 7.2. Samples treated with saline in place of 2-mercaptoethanol were used as controls. Separation of 7S and 19S fractions was effected by rate zonal ultracentrifugation over a discontinuous sucrose gradient (Torrighiani and Roitt, 1965b).

Results

Nature of the Antiglobulin Factors in the Serum of Patients with Rheumatoid Arthritis

Antiglobulin factors can be absorbed out from the serum of patients with rheumatoid arthritis using insoluble preparations of rabbit IgG. After elution from the complex with acid, the factors can be identified with specific antisera, as IgM, IgG, and also frequently as IgA immunoglobulins. Using a quantitative immunoplate method involving radial diffusion into an agar gel containing the specific anti-immunoglobulin serum, a quantitative assessment of antiglobulin in each immunoglobulin fraction in the eluate could be made.

Further characterization of the antiglobulin factors in the serum of a patient with rheumatoid arthritis was attempted by fractionation using ion exchange chromatography (Fig. 1, overleaf). The original serum gave the following antiglobulin values: IgG 15 µg./ml., IgA 24 µg./ml., and IgM 45 µg./ml.. Six fractions were taken as indicated and analyzed individually for the concentration of the individual immunoglobulins and also for rheumatoid factor-like activity using latex coated with either rabbit or human γ-globulin (Table I). The breakthrough peak fraction I contained only IgG and gave a titre of 1:32 when tested against latex-coated rabbit γ-globulin, although it was

TABLE I
RHEUMATOID FACTOR ACTIVITY, IMMUNOGLOBULIN COMPOSITION, AND ANTIGLOBULIN ANALYSIS OF FRACTIONS OBTAINED BY DEAE-CELLULOSE CHROMATOGRAPHY OF 5 ml. SERUM FROM A PATIENT WITH RHEUMATOID ARTHRITIS (cf. Fig. 1)

Fraction	Volume (ml.)	Immunoglobulin Concentration (µg./ml.)			Latex-fixation Titre		Antiglobulin Analysis*			
		IgG	IgA	IgM	Rabbit IgG	Human IgG	Volume Taken (ml.)	Concentration in Eluate (µg./ml.)		
								IgG	IgA	IgM
I ..	17	1900	—	—	32	—	2	45	—	—
II ..	15	300	200	—	8	—	4	—	—	—
III ..	33	15	22	—	1	8	8	—	—	—
IV ..	12	19	400	44	128	32	3	—	12	—
V ..	10	—	—	350	512	256	3	—	—	20
VI ..	8	110	—	44	16	—	2	—	—	—

*Vol. of fraction indicated absorbed with 20 mg. insoluble rabbit IgG; eluate made up to 0.25 ml.

— = Not detected.

Fig. 1.—Chromatography of serum from a patient with rheumatoid arthritis on DEAE-cellulose, using step-wise elution with phosphate buffers. The fractions indicated were pooled and analysed for immunoglobulins and antiglobulin factors (see Table I).

negative using a human γ -globulin reagent. Fraction IV, containing predominantly IgA and relatively small amounts of IgG and IgM, had a titre of 1:128 in the latex test with rabbit γ -globulin. Fraction V, in which only IgM could be detected, had the highest rheumatoid factor activity. After storage in the deep freeze, the fractions were tested individually for their content of antiglobulin factors. As may be seen in Table I, IgG antiglobulins were detectable only in Fraction I, IgA antiglobulins in Fraction IV, and IgM antiglobulins in Fraction V, corresponding with the peak concentration of each immunoglobulin. The agglutinating activity of Fraction IV against red cells sensitized with rabbit γ -globulin was completely abolished by treatment with 2-mercaptoethanol. The titre of Fraction 1 containing IgG, was reduced from 1:8 to 1:2 by treatment with 2-mercaptoethanol, and to 1:1 by a combined treatment with 2-mercaptoethanol and iodoacetamine. Fraction IV, corresponding with the peak of IgA, was further analysed by centrifugation over a sucrose density gradient. The fractions most active in the SCAT sedimented between the 7S and 19S peaks (Fig. 2).

Further to exclude the possibility that the presence of IgM rheumatoid factor could give rise to the artefactual appearance of antiglobulins in the other immunoglobulin classes by co-precipitation, normal serum containing some IgG antiglobulin was mixed with Fraction V from the DEAE-cellulose column containing IgM rheumatoid factor and then analysed for antiglobulins, all eluates being made up in the

Fig. 2.—Density gradient centrifugation of Fraction IV (DEAE-cellulose column, Fig. 1) containing the peak concentration of IgA. The antiglobulin factors, determined by agglutination of sheep cells coated with rabbit IgG, sedimented between the 19S and 7S markers.

usual way to a final volume of 0.25 ml. The results presented in Table II show that the presence of

TABLE II
EFFECT OF IgM RHEUMATOID FACTOR ON RECOVERY OF ANTIGLOBULIN FACTORS IN OTHER IMMUNOGLOBULIN CLASSES

Serum Tested	Antiglobulin Concentration in Eluate (μ g./ml.)		
	IgG	IgA	IgM
0.5 ml. Normal serum	25	—	—
3.0 ml. Fraction V (Fig. 1)	—	—	20
0.5 ml. Normal serum + 3.0 ml. Fraction V	25	—	18

ence between these means was highly significant, with $P < 0.003$ (Student's t test).

In this small group of patients no correlation could be established between the values for these antiglobulin factors and the duration of the disease, presence of nodules, activity or grade of the disease, the haemoglobin concentration, erythrocyte sedimentation rate, or the presence of antinuclear factors or other autoantibodies.

Discussion

In our preliminary experiments we attempted to apply a method which had been used successfully for the estimation of antithyroglobulin antibodies (Torrighiani, 1965). In this method radio-labelled thyroglobulin was mixed with the serum to be tested and the complexes formed with antibodies of each immunoglobulin type precipitated by the addition of specific anti-immunoglobulin serum; the radioactivity of the precipitate gave a measure of the amount of anti-thyroglobulin in the particular immunoglobulin class. Attempts to apply this method to the study of antiglobulin factors, using as antigen aggregated rabbit IgG which had been iodinated, were unsuccessful possibly because of the coprecipitation of IgM rheumatoid factor complexed with radio-antigen,

with the complexes of IgA and IgG and their specific antisera generated in the second phase of the estimation. The present method of absorption and elution from aggregated rabbit IgG and quantification by gel diffusion avoided this complication, and it could be shown that the presence of IgM rheumatoid factor did not influence the estimation of antiglobulins in other immunoglobulin classes. The validity of the finding of antiglobulin factors of different types was confirmed by separation on DEAE-cellulose, when it could be shown that antiglobulins of each individual immunoglobulin class were demonstrable only in the chromatographic fraction containing the peak concentration of the relevant globulin.

The finding of antiglobulin factors in each of the immunoglobulin classes has also been described by other investigators. Positive latex-fixation tests of low titre were associated with the IgG fraction of serum from two patients with rheumatoid arthritis (Heimer and Levin, 1966), while studies on complexes with sedimentation coefficients between 7S and 19S led Chodirker and Tomasi (1963) to infer the existence of low molecular weight rheumatoid factor in some sera. IgA antiglobulins were identified immunologically in a number of sera by Williams and Kunkel (1963) and Heimer and Levin (1966) using the technique of absorption and elution from solid globulin preparations. The distribution of reactivity towards aggregated rabbit globulin among the major immunoglobulin classes provides further support for the view that the rheumatoid factors arise as an antibody response to the host's own IgG, which through modification becomes immunogenic and exposes new determinants responsible for the species cross-reactions (Milgrom and Witebsky, 1960a). The affinity of rheumatoid factors for IgG bound in an immune complex and the experimental production of antiglobulins by hyperimmunization (Eyquem, Guyot-Jeannin, and Podliachouck, 1959; Milgrom and Witebsky, 1960b, Aho and Wager, 1961; Abruzzo and Christian, 1961) make it likely that the modification of host IgG comes about through combination as antibody with an unidentified antigen. The preponderance of IgM factors found, may be indicative of a continuing antigenic stimulus by newly-formed modified γ -globulin. The physical nature of the antigen (? micro-organism) with which the host's IgG is initially complexed might be important, since it has been shown that the IgM antibody response is enhanced when an antigen is in a particulate rather than a soluble form (Torrighiani and Roitt, 1965b). For instance, the production of macroglobulin rheumatoid factor-like substances by hyper-immuni-

zation of rabbits with *E. coli* is a response to the circulating complexes of bacteria coated with autologous antibody (Abruzzo and Christian, 1961)

The present finding of antiglobulin factors of IgM type in all the rheumatoid patients with positive SCAT, and in the IgA class in approximately 50 per cent. of these patients, contrasts sharply with the observation made in the control group where the methods used failed to detect these factors at the level of sensitivity attained. The control group consisted largely of relatively young individuals, usually blood donors, not matched with the rheumatoid group for age and sex; these results should thus be considered rather as a demonstration of the specificity of the method used to detect the antiglobulin factors, than as a measure of their true incidence in the population.

An unexpected result was the finding of IgG antiglobulins in all sera tested whether from rheumatoid patients or from normal individuals, although the concentrations were higher in the pathological sera. There is some evidence that the binding of these globulins with the rabbit IgG substrate is of an immunological nature. Thus IgG isolated from a patient's serum was capable of agglutinating latex particles coated with rabbit but not with human IgG. Furthermore, preliminary experiments have indicated that the antiglobulin activity of normal human IgG is not increased by heat aggregation and is present in the Fab rather than the Fc papain fragment. IgG factors reacting with determinants exposed on the $F(ab')_2$ and Fab fragments of different anti-D preparations are frequently demonstrable in normal human sera (Osterland, Harboe and Kunkel, 1963; Mandy, 1966); however their relationship to the IgG anti-rabbit globulins described in the present investigation has not been established. Studies (Watson and Collins, 1963; Mandy, Fudenberg and Lewis, 1965) also suggest that antiglobulins with a range of specificities are present normally in the rabbit. The IgG antiglobulin which we detected in human cord blood presumably reflects transplacental passage of maternal immunoglobulin, and it will be of interest to see whether the level declines until immunological events stimulate the production of the infant's own antiglobulin factors; the Gm grouping of the antiglobulins from cord serum would clearly be of relevance in this context.

With the appropriate specific antisera it should be possible to determine the distribution of the IgG antiglobulins amongst the four main subclasses; $\gamma 2a$, $\gamma 2b$, $\gamma 2c$, and $\gamma 2d$. With the present method

the reactivity against self IgG could also be determined, although the complication that the substrate may undergo some decomposition under the acid eluting conditions thereby vitiating the result, should be taken into account; it was for this reason that a rabbit IgG substrate was chosen as the basis for the method described in the present communication. However, using radiolabelled self IgG would make it possible to correct for non-specific release by acid. The importance of determining such reactions with self globulin are underlined by the observation of Restifo, Lussier, Rawson, Rockey, and Hollander (1965) that injection of purified autologous IgG into the uninvolved joint of a patient with sero-positive rheumatoid arthritis produces an acute inflammatory response. They further noted that IgG preparations from other rheumatoid patients could only produce a comparable response when they shared some Gm groups with the recipient (Hollander, Fudenberg, Rawson, Abelson, and Torralba, 1966). This strongly suggests that antiglobulins are implicated in at least one phase in the pathogenesis of the joint lesions. This phase may come into play only after the injected IgG has undergone modification, possibly by reacting immunologically with a constituent present in the joint.

With the wide variety of cross-linking reagents now available, it is possible to prepare insoluble aggregates from the majority of soluble protein antigens. Thus the present method for quantifying antiglobulin factors may be applied more generally to the determination of antibodies directed against any antigen which can be prepared in an insoluble form.

Summary

- (1) Antiglobulin factors in different immunoglobulin classes in human sera can be identified and measured by a technique involving absorption to and elution from insoluble preparations of rabbit IgG. The eluted antiglobulins are assayed by radial immunodiffusion in agar containing the specific anti-immunoglobulin.
- (2) IgM antiglobulins were present in all and IgA antiglobulins in fourteen out of 27 sera from rheumatoid patients with positive SCAT; neither were detectable in 25 normal sera tested. All sera contained IgG antiglobulins, the level in the patients' sera being significantly higher than that in the normal subjects.
- (3) The distribution of antiglobulins among the different immunoglobulins supports the view that rheumatoid factors arise as an antibody response, presumably to modified host IgG.
- (4) The method employed can be applied generally to the quantitative estimation of antibodies in different immunoglobulin classes (and even in subclasses), provided the antigen can be prepared in an insoluble form.

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Les facteurs antiglobuline dans les sérums des malades atteints d'arthrite rhumatismale et des sujets normaux

RÉSUMÉ

(1) Les facteurs antiglobuline dans de différentes classes d'immunoglobuline du sérum humain peuvent être identifiés et mesurés par un procédé comprenant l'absorption aux, et la décantation des préparations insolubles de l'IgG du lapin. On titre les antiglobulines par l'immunodiffusion radiale sur agar contenant l'anti-immunoglobuline spécifique.

(2) Les antiglobulines IgM furent présentes dans tous, et les IgA dans 14 sur 27 sérums des malades rhumatisants dont la réaction de Waaler-Rose fut positive, mais elles furent absentes dans les 25 sérums normaux étudiés. Tous les sérums contenaient des antiglobulines contre l'IgG et leur taux était appréciablement plus élevé dans le sérum des malades que dans celui des sujets normaux.

(3) La distribution des antiglobulines parmi les différentes immunoglobulines vient à l'appui de la théorie selon laquelle les facteurs rhumatoïdes représentent des anticorps qui se forment, on présume, contre l'IgG modifiée de l'hôte.

(4) La méthode employée peut être appliquée en général à la détermination des anticorps dans de différentes classes d'immunoglobuline (et même des sous-classes), pourvu que l'on prépare l'antigène sous une forme insoluble.

Los factores antiglobulina en sueros de enfermos con artritis reumatoide y en los de sujetos sanos

SUMARIO

(1) Los factores antiglobulina en diferentes clases de inmunoglobulina del suero humano pueden identificarse y medirse por un método que comprende la absorción a, y la decantación de preparaciones insolubles de la IgG del conejo. La titulación de las antiglobulinas se hace por inmunodifusión radial sobre agar conteniendo la anti-inmunoglobulina específica.

(2) Las antiglobulinas IgM se encontraron en todos, y las IgA en 14 de los 27 sueros de enfermos con artritis reumatoide y con una reacción de Waaler-Rose positiva; no se notó ninguna en los 25 sueros normales investigados. Todos los sueros contuvieron antiglobulina contra la IgG y su nivel fué apreciadamente más alto en el suero de los enfermos que en el de los sujetos normales.

(3) La distribución de las antiglobulinas entre las diferentes inmunoglobulinas soporta el punto de vista de que los factores reumatoïdes representan anticuerpos que se forman presumiblemente contra la IgG modificada del huésped.

(4) Se puede aplicar el método descrito a la determinación en general de anticuerpos en diferentes clases (y hasta subclases) de inmunoglobulina, provisto que se prepare el antígeno en forma insoluble.