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Biological and Physicochemical Properties of Purified Anti-DNP Guinea-Pig Non-precipitating Antibodies

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Summary. Methods for isolation and purification of precipitating and non-precipitating guinea-pig antibodies are described.

The physicochemical properties of γ_1 and γ_2 non-precipitating antibodies are similar to γ_1 and γ_2 precipitating ones. Biological properties are also similar excepting the reverse Arthus reaction, which is positive with the precipitating and negative with the non-precipitating antibodies.

Bivalence of these antibodies was experimentally demonstrated. Precipitating antibodies K_0 do not differ greatly from those obtained with the corresponding non-precipitating ones. The incapacity to precipitates with the antigen may be a consequence of a steric impediment.

INTRODUCTION

Non-precipitating rabbit antibodies have been described by Heidelberger and Kendall (1935). When antibodies from an immune serum are precipitated by adding small quantities of antigen (1/10 to 1/20 of the total antigen determined by the quantitative precipitin technique), an antibody remains which combines with the antigen but is not capable of precipitating with it.

Few papers on this subject have been published (Heidelberger, Treffers and Mayer, 1940; Treffers, Heidelberger and Freund, 1947; Kabat and Benacerraf, 1949; Weigle and Maurer, 1957; Fiset, 1962; Klinman, Rockey and Karush, 1964; Klinman and Karush, 1967; Carter and Harris, 1967; Christian, 1970). In a previous communication (Margni and Binaghi, 1972), we have described the isolation and properties of anti-egg-albumin and anti-dinitrophenyl non-precipitating rabbit antibodies, specifically purified from anti-sera partially adsorbed with antigen to remove precipitating antibodies.

The present study has been developed to attempt the isolation, purification and behaviour of anti-DNP guinea-pig non-precipitating antibodies compared with the precipitating ones localized in the same immunoglobulin class.

MATERIAL AND METHODS

Antisera

A group of 150 random bred guinea-pigs weighing 350–450 g, were immunized with dinitrophenylated human-γ-globulin (DNP-HGG) highly conjugated (34 DNP/mol HGG), prepared as described by Russell Little and Eisen (1967).

The animals were injected s.c. in both hind legs with 1 mg of antigen in 0·5 ml saline emulsified with an equal amount of Freund's complete adjuvant. A week after the first antigen dose the animals received another injection and after 3 weeks of resting they were boosted and bled 7 days later.

Precipitating antigen

Dinitrophenylated bovine serum albumin (DNP-BSA) highly conjugated was used. It was prepared in the same way as DNP-HGG.

Precipitin analyses

The antibody content of the immune sera and their fractions was estimated by quantitative precipitin analyses in a diluent containing 0·005 M EDTA in 0·15 M NaCl, pH 7·4. The washed specific precipitates containing DNP protein antigens were dissolved in 0·1 N NaOH and analysed at 280 nm and 360 nm.

The antibody content of the precipitates was calculated from the values obtained after correction for the absorption at 280 nm contributed by the antigen.

Isolation and purification of precipitating and non-precipitating antibodies

The precipitating antibodies were precipitated by adding successively 1/20 of the total antigen determined by the precipitin curve, so many times as not to get a precipitin reaction.

After each addition the mixture were incubated 1 hour at 37° and 24 hours at 4°.

The precipitates from the several precipitations were collected, pooled, washed with saline-phosphate and taken in 0·1 M dinitrophenol pH 7·5 (1 ml/10 mg of antibody) for 1 hour at 37° and 24 hours in the cold. After centrifugation the supernatant was applied to a double-layered column of DEAE-cellulose and Amberlite IRA-400, according to Eisen, Gray, Russell Little and Sims (1967).

Saline phosphate was used as eluent.

The supernatant of the several precipitations containing the non-precipitating antibodies was passed through an immunoadsorbent column. As immunoadsorbent, polymerized DNP-BSA was used (Avrameas and Ternynck, 1969; Margni and Binaghi, 1972).

After passage of the supernatant the column was washed with saline–phosphate until it was completely free of protein. Dissociation was done with 0·1 M dinitrophenol and saline–phosphate was used as eluent. The effluent was then passed through an Amberlite IRA-400 column which bound the hapten and the purified non-precipitating antibodies emerged from the column.

The isolation of both precipitating and non-precipitating γ_1 and γ_2 antibodies were done in a similar way. Effluent tubes containing the antibodies were concentrated, dialysed extensively against 0·01 M phosphate pH 7·6 and passed through a DEAE-cellulose column equilibrated with the same buffer.

After the first protein peak was eluted, 0·1 M phosphate buffer pH 7·6 was applied.

Cellulose acetate electrophoresis

Gelatinized cellulose acetate (Cellogel) 2·5 × 17 cm was used. The technique was the one described by Margni *et al.* (1970a, b) and Heer and Margni (1971).

Immunodiffusion

Double diffusion in agar was performed by the method described by Ouchterlony (1958).

Immunoelectrophoresis

Scheidegger's method (1955) was employed using an LKB equipment. 1·25 per cent agar in Veronal buffer 0·025 M, pH 8·2 was used.

The rabbit anti-guinea-pig serum was obtained in the same way as indicated in antisera, using 1 ml of guinea-pig serum mixed with equal parts of Freund's complete adjuvant for each inoculation.

Sedimentation coefficients

These were done by Dr J. Dellacha using a Spinco ultracentrifuge model E, with temperature automatic regulator.

Comparison of molecular weights

Ten milligrams of precipitating antibody and 25 μg of non-precipitating antibody of the same class labelled with ^{125}I according to McConahey and Dixon (1966), were passed simultaneously through a 1·8 × 100-cm Sephadex G-200 column, using an LKB fraction collector. The precipitating antibody exclusion volume was determined by reading the ultraviolet absorbance at 280 nm in a Zeiss MPQII spectrophotometer, while the non-precipitating one was determined by counts per minute (cpm), using a scintillation spectrometer.

Binding properties of purified precipitating and non-precipitating antibodies

These were determined by equilibrium dialysis, as described by Eisen and Karush (1949).

Tritiated dinitrophenyl γ-amino butyric acid (^{3}HDNP-GABA) was used as hapten.

Passive haemagglutination

Dinitrophenylated sheep red cells were used according to Bullock and Kantor (1965).

Complement fixation

This was done by reading 50 per cent haemolysis using DNP-BSA as antigen, 3 CH_{50} of complement, incubation at 4° for 18 hours and spectrophotometric reading at 530nm (Lelchuk, Vacs and Margni, 1969).

Reverse Arthus reaction

Guinea-pigs were inoculated intracutaneously with 500 μg antibody in 0·2 ml saline, followed by 10 mg/ml antigen intravenously. The reactions were read after 24 and 48 hours.

Passive cutaneous anaphylaxis (PCA)

The PCA reaction were assayed in homologous (guinea-pig) and heterologous (mouse) skin, according to Ovary (1952). Challenge with antigen was made after a 3-hour latent period.

Coprecipitation assay

This was performed by quantitative precipitin analyses using precipitating antibodies alone and precipitating antibodies mixed with non-precipitating ones. After incubation for 1 hour at 37° and 24 hours at 4° the precipitates were analysed. The amount of non-precipitating antibody was calculated from the difference of precipitate between the two series.

Control series were made using 2 mg/ml of precipitating guinea-pig antibody of another specificity (anti-egg-albumin) and the same antibody mixed with 400 μg/ml of anti-DNP non-precipitating antibody. Egg albumin was used as antigen.

Pepsin digestion of antibodies

The method described by Nisonoff, Wissler, Lipman and Woernley (1960) was used to obtain $F(ab')_2$ fragments.

Reduction and alkylation of non-precipitating antibodies

Performed according to a modification of Porter's method (Margni *et al.*, 1970a). The purified proteins (20 mg/ml), previously dialysed against 0·5 M Tris-HCl buffer, pH 8·2, were treated with 0·1 M mercaptoethanol for 1 hour at room temperature and then cooled at 0° in an ice bath. A 25 per cent M excess of 0·1 M iodoacetamide in 0·5 M Tris-HCl, pH 8·2, cooled at 0°, was immediately added. The mixtures were kept at that temperature for 1 hour and then dialysed with constant stirring against 100 vol. of 0·15 M sodium chloride during 24 hours, with three changes.

RESULTS

PRECIPITATING AND NON-PRECIPITATING ANTIBODIES

It was possible to isolate and purify both antibodies by the methods described above. From 650 ml of pooled guinea-pig anti-DNP serum, containing 3·7 mg Ab/ml, 1050 mg of precipitating antibodies and 137 mg of non-precipitating antibodies were recovered. Cellulose acetate electrophoresis and immunoelectrophoresis showed that non-precipitating antibodies were localized in the γ_1 and γ_2 immunoglobulin fractions, with the same electrophoretic mobility as γ_1 and γ_2 precipitating antibodies (Fig. 1).

When the non-precipitating antibodies were passed through a DEAE-cellulose column, the fraction eluted with 0·01 M phosphate buffer pH 7·6 was pure γ_2 globulin, thus γ_1 eluted with 0·1 M phosphate buffer pH 7·6. The same occurred with the precipitating antibodies. All of them were essentially free of contamination with hapten determined by spectrophotometric reading at 360 nm.

The antibodies activity of the purified preparations were determined by measuring the amount of protein retained by polymerized DNP-BSA, as described by Margni and Binaghi (1972). The results indicated that the binding activity for γ_1 and γ_2 precipitating antibodies were 91, 84, 81, and 93 per cent for γ_1 and γ_2 non-precipitating antibodies.

IMMUNOCHEMICAL ANALYSES

By immunoelectrophoretic analyses it was not possible to demonstrate any difference between the precipitation bands of precipitating and non-precipitating antibodies of the same class when rabbit anti-guinea-pig sera was used for precipitation. When DNP-BSA

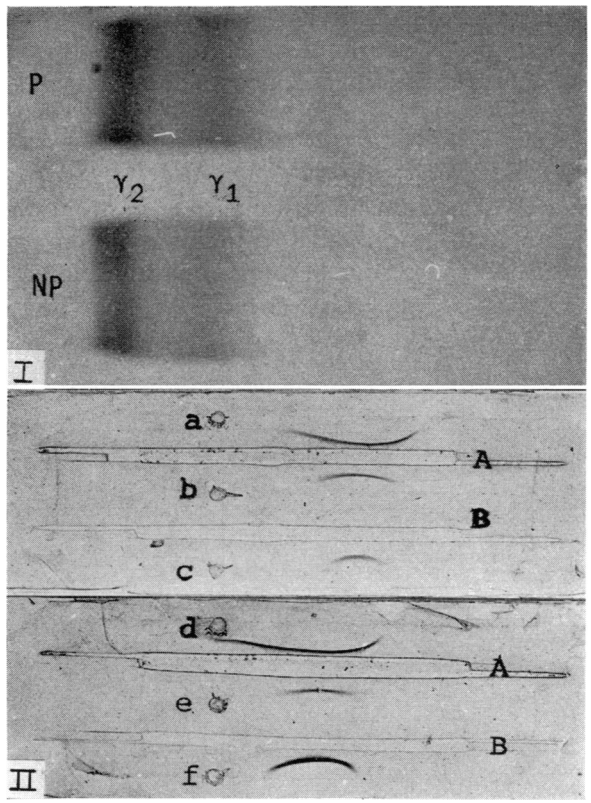

FIG. 1. (I) Cellulose acetate electrophoresis. P = precipitating antibodies (10 mg/ml). NP = non-precipitating antibodies (7 mg/ml). γ_1, γ_2 = antibodies localized in the γ_1 and γ_2 immunoglobulin fractions. (II) Immunoelectrophoresis. a and c = γ_2 precipitating antibody (4 mg/ml). b = γ_2 non-precipitating antibody (2 mg/ml). d and f = γ_1 precipitating antibody (5 mg/ml). e = γ_1 non-precipitating antibody (3 mg/ml). A = Rabbit anti-guinea-pig serum. B = DNP-BSA (300 μg/ml).

was used, only precipitating antibodies gave precipitation arcs (Fig. 1). The same was observed by Ouchterlony's analyses. For these assays different concentrations (1–10 mg/ml) of non-precipitating antibodies were used.

Sedimentation coefficients for γ_2 and γ_1 precipitating and non-precipitating antibodies were approximately 7S.

The simultaneous gel filtration on Sephadex G-200 showed that precipitating and non-precipitating γ_1 and precipitating and non-precipitating γ_2 antibodies eluted together.

BINDING PROPERTIES

The results obtained by equilibrium dialysis studies are expressed in Fig. 2. It shows that the valency of precipitating and non-precipitating antibodies of both immunoglobulin classes approaches two. The K_0 of non-precipitating antibodies is less than that of precipitating antibodies of the same class. The differences of these values are $10{\cdot}6\ M^{-1}$ for γ_1 antibodies and $5{\cdot}3\ M^{-1}$ for γ_2 antibodies. The index of heterogeneity (a) is

approximately the same for all the antibodies. The results obtained are the following. γ_1 precipitating antibody: 0·62; γ_1 non-precipitating antibody: 0·66; γ_2 precipitating antibody: 0·59; and γ_2 non-precipitating antibody: 0·61.

COPRECIPITATION

By addition 20 per cent of non-precipitating antibodies to a known concentration of precipitating ones, the amount of antibodies in the precipitate increases 15–18 per cent when antibody concentration is determined by the quantitative precipitin technique.

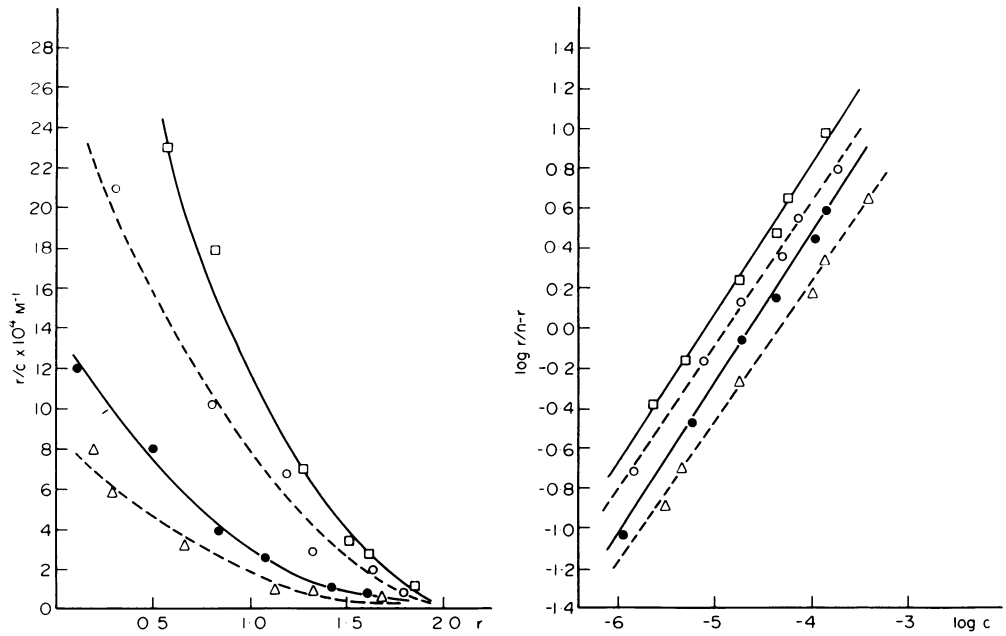

FIG. 2. Binding capability of precipitating and non-precipitating antibodies. (a) As we can see in the figure all the *n* values (valence) are 2. (b) The Ko for the different antibodies are the following: (□), γ_1 precipitating antibody $1{\cdot}51 \times 10^{-5}$ M^{-1}; (○), γ_2 precipitating antibody 7·69 M^{-1}; (●), γ_1 non-precipitating antibody $4{\cdot}50 \times 10^{4}$ M^{-1}; (△), γ_2 non-precipitating antibody $2{\cdot}39 \times 10^{4}$ M^{-1}.

Coprecipitation was possible between γ_1 precipitating and γ_2 non-precipitating antibodies or vice versa. It was also possible to obtain coprecipitation between guinea-pig non-precipitating antibodies and precipitating antibodies of the same specificity from other mammalian species, e.g. sheep, rabbit, horse, goat and donkey.

In the control series the amount of antibodies in the precipitates were the same.

BIOLOGICAL PROPERTIES

Precipitating and non-precipitating anti DNP guinea-pig γ_1 and γ_2 antibodies were able to agglutinate dinitrophenylated sheep red cells.

The ability to mediate PCA in the guinea-pig was confined to precipitating and non-precipitating γ_1 antibodies and none of them were able to fix complement.

γ_2 Precipitating and non-precipitating antibodies bound complement and can transfer anaphylactic sensitivity to the mouse but not to the guinea-pig.

Reverse Arthus reaction was positive for the precipitating antibodies but negative for both γ_1 and γ_2 non-precipitating ones, in the conditions tested.

The results of Table 1 show that the activity of purified non-precipitating antibodies are less than the precipitating ones.

TABLE 1

Biological properties		Antibodies															
		Precipitating								Non-precipitating							
		γ_1				γ_2				γ_1				γ_2			
Precipitation	mg/ml	0·5	1	5	10	0·5	1	5	10	1	5	10	20	1	5	10	20
	Results	+	+	++	++	+	+	++	++	−	−	−	−	−	−	−	−
Complement fixation	μg/ml	3	6	12	24	3	6	12	24	3	6	12	24	3	6	12	24
	Results	−	−	−	−	±	+	++	++	−	−	−	−	−	−	+	++
PCA (guinea-pig)*†	μg/ml	0·5	1	5	10	0·5	1	5	10	0·5	1	5	10	0·5	1	5	10
	Results	—	+	++	+++	−	−	−	−	−	−	+	++	−	−	−	−
PCA (mouse)*‡	μg/ml	0·5	1	5	10	0·5	1	5	10	0·5	1	5	10	0·5	1	5	10
	Results	−	−	−	−	−	−	+	++	−	−	−	−	−	−	±	++
Passive	μg/ml	3	6	12	24	3	6	12	24	3	6	12	24	3	6	12	24
haemagglutination	Results	—	+	++	++	−	+	++	++	−	−	+	++	−	−	+	++
Arthus reverse	μg/ml			500				500				500				500	
	Results			+				+				−				−	

* Time of sensitization 3 hours.
† 1 mg of DNP-BSA was used as challenge dose.
‡ 0·5 mg of DNP-BSA was used as challenge dose.

PRECIPITATING ACTIVITY OF REDUCED AND ALKYLATED ANTIBODIES AND F(AB')$_2$ FRAGMENTS

By Ouchterlony's analyses the F(ab')$_2$ fragment obtained from γ_1 and γ_2 precipitating antibodies gave a precipitin line with the antigen. The F(ab')$_2$ fragments of γ_1 and γ_2 non-precipitating antibodies and the reduced and alkylated non-precipitating antibodies did not precipitate with the antigen.

DISCUSSION

The results confirm the presence in the guinea-pig of non-precipitating antibodies localized in γ_1 and γ_2 immunoglobulins, with similar physicochemical properties to the γ_1 and γ_2 precipitating antibodies.

The non-precipitating antibodies are not capable of giving a precipitin line when reacted directly with the antigen, but coprecipitate with precipitating antibodies from the guinea-pig and other mammalian species. These results indicate that coprecipitation is a general phenomenom and not a singular property of non-precipitating antibodies of a particular vertebrate.

Gel filtration and sedimentation coefficient determinations show that the molecular weight of guinea-pig precipitating and non-precipitating antibodies are approximately the same.

The biological properties of the same class of both antibodies are similar, excepting the

reverse Arthus reaction which is positive only with the precipitating antibodies. The activity of γ_1 antibodies in reverse Arthus reaction has been reported previously (Oliveira, Osler, Siragamian and Sandberg, 1970) and appears to be due to the ability of this class to activate later components of the complement sequence (Sandberg, Osler, Shin and Oliveira, 1970).

In order to examine the possibility that the lack of precipitation was a consequence of the high content of hydrophilic groups in the molecule, specially in the Fc fragment, we have prepared $F(ab')_2$ fragments of precipitating and non-precipitating antibodies. The immuno precipitation reaction between the $F(ab')_2$ of the non-precipitating antibodies and the specific antigen did not show any precipitation, while the $F(ab')_2$ of the precipitating antibodies reacted in the same way as the whole molecules.

When reduced and alkylated non-precipitating antibodies were tested against DNP-BSA, no precipitin bands were observed. That minimizes the possibility of an extra S-S bridge that restrains the binding capability of the two Fab of the whole molecule. From these results and those obtained with the $F(ab')_2$ fragments, it seems unlikely that the guinea-pig non-precipitating antibodies have to be confined to a particular class of immunoglobulin.

At present the agglutination of passively coated erythrocytes by non-precipitating antibodies is under study in our laboratories (Margni *et al.*, unpublished results). The contribution of the Fc fragment in the formation of complexes could be possible.

In a previous communication (Margni and Binaghi, 1972) we have studied the non-precipitating rabbit antibody. In that opportunity we have speculated with the facts that should participate in the behaviour of this antibody, principally the binding capability, antibody valence, molecular size, number of antigenic determinants per mole of antigen, class of immunoglobulin involved, electric charge of the immunoglobulin and others.

All these speculations could be repeated for the non-precipitating guinea-pig antibodies.

The equilibrium dialysis studies showed that precipitating and non-precipitating antibodies of both immunoglobulin classes had valency two and approximately the same index of heterogeneity (a). The precipitating antibodies Ko are less than the non-precipitating ones. These values (not less than $2{\cdot}3 \times 10^4$ M^{-1}) and the Ko difference between precipitating and non-precipitating antibodies, suggest that the inability of the non-precipitating antibodies to form insoluble complexes with antigen must be due to reasons other than low affinity.

We think, according to these results and with the biological and physicochemical properties of these bivalent non-precipitating antibodies, that the non-precipitation with the antigen could be a consequence of some steric impediment that makes difficult or impossible the formation of insoluble antigen–antibody complexes.

We have not found non-precipitating antibodies localized in the IgM immunoglobulin fraction. It is possible that we could not detect them because of their lower concentration in the supernatant from which we isolated the non-precipitating antibodies.

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