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Character of Non-Precipitating Antibodies

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Summary. A minority of rabbits immunized daily with soluble preparations of heterologous serum albumin respond with the formation of non-precipitating antibodies. Precipitating and non-precipitating antibodies, in the present study, were similar with regard to electrophoretic distribution, molecular size, and elution appearing on DEAE Sephadex. Guinea-pig antisera against purified non-precipitating antibodies did not recognize unique antigenic determinants. The present studies suggest that non-precipitating antibodies result from a limited recognition of multiple antigenic determinants on complex antigens.

INTRODUCTION

Immunization of animals with complex antigens, such as heterologous serum proteins, usually provokes the formation of antibodies that can be quantified by precipitin analysis; the quality and quantity of the immune response vary according to the route of immunization and the use of adjuvants. Antibodies formed in response to smaller, less complex antigens often require techniques other than precipitation for detection.

Prior studies of non-precipitating antibodies have utilized either selected early phase antisera or antisera partially absorbed with antigen to remove precipitating antibodies (Heidelberger and Kendall, 1935; Heidelberger, Treffers and Mayer, 1940; Pappenheimer, 1940; Burtin, 1955; Weigle and Maurer, 1957). The immunological specificity of the non-precipitating antibodies can be demonstrated by varied techniques: haemagglutination of antigen-coated cells, co-precipitation with precipitating antibodies and affinity studies using labelled antigens.

In the present studies of rabbit antisera reactive with heterologous serum albumins, the failure of antibodies to precipitate appeared to result from the limited recognition of multiple antigenic determinants on these complex antigens. Unlike the conclusions of two recent reports, there was no evidence that a physico-chemically distinct type of immunoglobulin was responsible for the non-precipitating character of antisera (Carter and Harris, 1967; Klinman and Karush, 1967).

EXPERIMENTAL METHODS

Mixed breed rabbits of either sex were immunized by daily injections (intravenous) of soluble bovine serum albumin (BSA) or human serum albumin (HSA). Crystalline BSA was obtained from Pentex, Kankakee, Illinois. The source of HSA was the American Red Cross. The antigens contained trace quantities of serum proteins other than albumin.

Isotopic labelling of antigens with ^{125}I or ^{131}I was accomplished by the iodine monochloride method (McFarlane, 1964). Labelled antigens were fractionated on Sephadex G-100 (0·15 M NaCl) to remove trace components excluded by the gel.

Blood, obtained 1–2 days after the last injection of antigen, was obtained by ear artery or cardiac puncture. Sera were separated and stored at −20°.

Quantitative precipitin reactions were incubated at 0–4° for 48–72 hours after 30 minutes at 37°. The precipitates were centrifuged and washed three times with cold buffered saline (pH 7·5, 0·01 M phosphate, 0·15 M NaCl) and dissolved in 0·1 N NaOH for protein estimations and counting of radioactivity.

Protein estimations were performed using the Folin–Ciocalteu reaction (Heidelberger and MacPherson, 1943). The nitrogen contents of standards (BSA, HSA and rabbit γ-globulin) were determined by micro-Kjeldahl analysis.

Radioactivity of ^{125}I- and ^{131}I-labelled products was measured in a Packard auto-gamma counter.

Measurements of globulin bound antigen utilized the Farr technique—precipitation of labelled antigen by 50 per cent saturated ammonium sulphate (Farr, 1958).

Density gradient centrifugation analysis employed sucrose gradients (15–45 per cent) in a Spinco SW56 rotor centrifuged at 40,000 rev/min for 15 hours. Fractions of the effluent from a pin hole in the bottom of the tube were collected.

The micro-complement fixation procedure of Wasserman and Levine (1961) was employed.

Immunoelectrophoretic analyses utilized agar Nobel (Difco) in barbital buffer (pH 8·6, 0·05 ionic strength).

Radioimmunoelectrophoretic studies were performed by adding ^{131}I-labelled antigen to the antisera troughs after precipitin lines had formed and the gels had been washed free of soluble proteins. After several hours of incubation and additional saline washes, the slides were dried and radiographic images were recorded on standard X-ray film.

DEAE Sephadex chromatography was performed on small columns (12×400 mm) utilizing a gradient from pH 8·0, 0·01 M phosphate buffer to 0·3 M NaH_2PO_4.

Analysis of selected antisera by passive cutaneous anaphylaxis (PCA) employed the technique of Ovary (1952).

The procedures for tanned cell haemagglutination were as previously described (Pincus, Haberkern and Christian, 1968).

RESULTS

Previous experience with rabbits immunized daily with soluble BSA or HSA has indicated that over half of the animals respond with the formation of moderate to large amounts of precipitating antibodies (Germuth, Flanagan and Montenegro, 1957; Dixon, Feldman and Vazquez, 1961; Andres, Seegal, Hsu, Rothenberg and Chapeau, 1963). Approximately one-third are rendered immunologically tolerant and 10–20 per cent form predominantly non-precipitating antibodies in the face of trace or absent precipitins (Christian, 1969).

Table 1 summarizes the results of precipitin analyses of five sera of rabbits immunized with HSA. The supernatants were subjected to 50 per cent saturated ammonium sulfate (Farr technique), allowing detection of globulin and bound and free antigen. Two sera (17–9 and 17–18) which contained negligible precipitating antibodies were capable of

TABLE 1

PRECIPITIN ANALYSIS AND STUDY OF SOLUBLE COMPLEX FORMATION OF FIVE SERA OF RABBITS HYPERIMMUNIZED WITH SOLUBLE HSA

Serum	Character of antibody response	Distribution of [^{125}I]HSA added to 0·2 ml sera											
		5 μg			10 μg			20 μg			50 μg		
		Precipitate	Complex	Free antigen	Precipitate	Complex	Free antigen	Precipitate	Complex	Free antigen	Precipitate	Complex	Free antigen
17–4	Tolerant	0	0	5·2	0	0	9·5	0	0	22	0	0	50
17–13	Precipitating	5	0	0	10	0	0	19·9	0·1	0·1	51	0·2	0·4
17–10	Precipitating	4·8	0·2	0	9·8	0·2	0·1	20	0·3	0·1	50	0·4	0·4
17–9	Non-precipitating	1·7	3·3	0	2·5	7·5	0·1	0·3	19·4	0·2	0	45·5	7
17–18	Non-precipitating	0	4·7	0·4	0	9	1	0	17	4	0	30	22

Rabbit 17–4 was immunologically tolerant. Rabbits 17–9 and 17–18 developed non-precipitating anti-HSA antibodies.

binding 150–200 μg HSA/ml. The non-precipitating antibodies in serum 17–18 were capable of co-precipitating with serum 17–13 (Fig. 1). Triplicate sets of immune reactants were diluted in buffer, heated normal rabbit serum and heated 17–18 serum. The co-precipitating effect of serum 17–18 was evident only in the area of antigen excess. Two hundred μg of antigen resulted in the formation of 440 μg of precipitate protein where serum 17–18 was the diluent but only 30 μg of precipitate protein was formed when the reactants were diluted in normal rabbit serum.

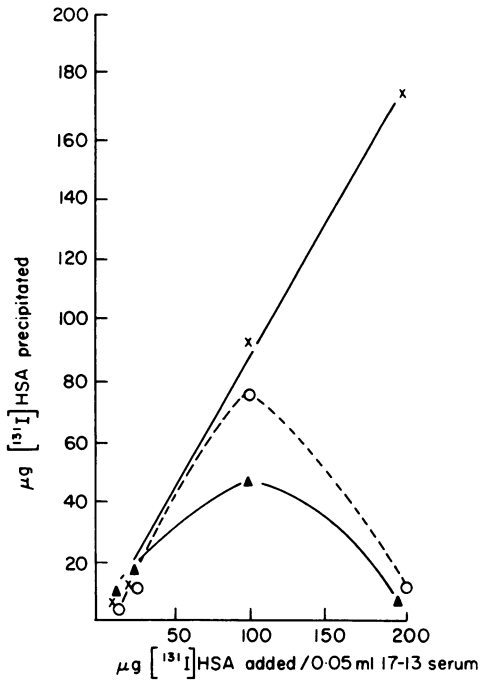

FIG. 1. Precipitin analysis with variation in diluents (buffered saline, ▲; heated normal rabbit serum, ○; and heated rabbit 17–18 serum containing non-precipitating anti-HSA antibodies ×). Each tube contained 0·05 ml of precipitating antiserum (17–13) and 0·5 ml of diluent.

The heterogeneity of complexes formed by non-precipitating antibodies is indicated by density gradient centrifugation studies. The distribution of labelled antigen added to three sera (including a control non-immune serum) is shown in Fig. 2. Soluble complexes formed by most non-precipitating sera were polydisperse. In addition to the three modes illustrated in Fig. 2 (peaks in Fractions 6, 9 and 11), there were variable amounts of very large complexes that formed pellets in the bottom of the tubes.

Fig. 3 summarizes some properties of globulin preparations from two hyperimmune sera. The globulin from serum 16–31, which lacked detectable precipitins, was reactive with antigen (BSA) in haemagglutination, PCA and C fixation analyses. The non-precipitating globulin was several-fold more effective than the precipitating preparation in C fixation. In the micro-C fixation tests, it was possible to demonstrate significant fixation of C by low concentrations of non-precipitating antibodies and no fixation of C by comparable concentrations of precipitating antibodies.

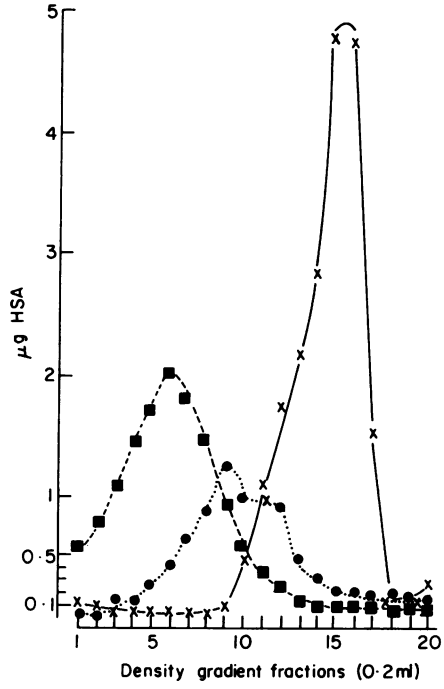

FIG. 2. Sucrose density centrifugation of soluble complexes formed with non-precipitating anti-HSA antisera and [^{131}I]HSA; serum, 0·5 ml; [^{131}I]HSA, 20 μg. Under the conditions of study, human IgM and IgG markers had peak concentrations in fractions 6 and 12, respectively. ×, Control; ■, serum 17–9; ●, serum 17–74.

Globulin preparation	Protein concentration ml(ma)	Precipitating antibody (protein/ml)	Haemagglutinin titre (reciprocal dilution)	Complement fixation titre (reciprocal dilution)	PCA titre (reciprocal dilution)
16 – 62	1·74	0·265	19,200	<40	400
16 – 31	1·46	<0·010	4,800	160	80

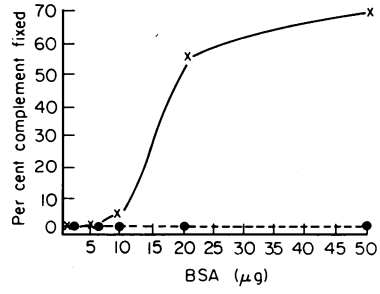

FIG. 3. Properties of globulin preparations (second peak, Sephadex G-200) of non-precipitating and precipitating anti-BSA antisera. Globulin: salt precipitation ×3, Sephadex G-200 (second peak). Microcomplement fixation (0–18 hours), globulin concentration, 0·015 mg protein/ml. ×, rabbit 16–31; ●, rabbit 16–62.

The possibility that a unique type of immunoglobulin was responsible for non-precipitating properties was examined by preparing antisera to purified soluble complexes. The distribution of [^{131}I] BSA immune complexes, formed with serum 16–31, on Sephadex G-200 filtration is illustrated in Fig. 4. The components marked A and B contained labelled antigen in the form of soluble complexes. The bulk of γ-globulin was present in peak C. Peaks A, B and C were used as immunogens in guinea-pigs. Immunoelectrophoretic analyses of antisera are illustrated in Fig. 5. The antisera reacted, in each case, with a broadly distributed γ-globulin and this reactivity was removed by absorption of the antisera with immune precipitate. In double diffusion studies of antisera against peaks A and B, it was not possible to demonstrate antigens unique to non-precipitating antibodies.

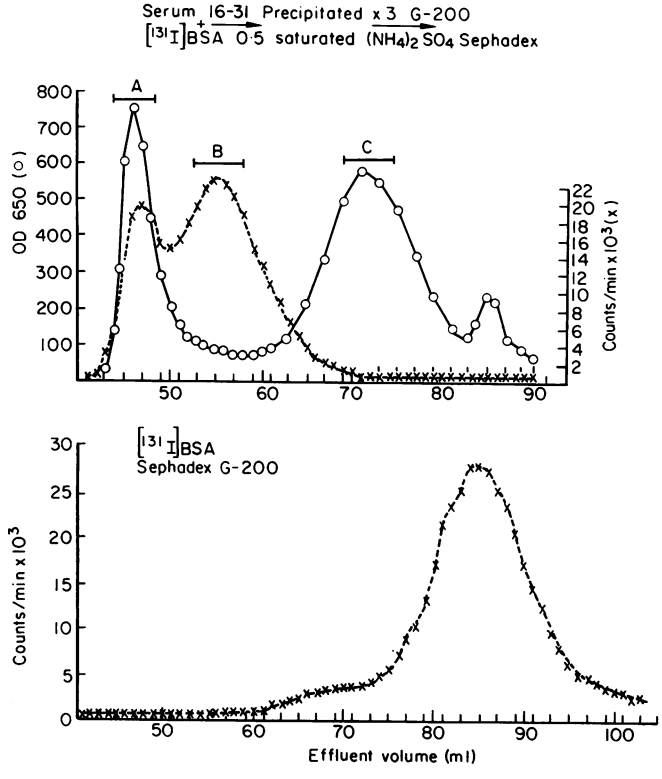

FIG. 4. Distribution of soluble complexes formed with anti-BSA antiserum (16–31) on Sephadex G-200 filtration. Fractions A, B and C were used as immunogens in guinea-pigs (see Fig. 5).

Antisera with either precipitating or non-precipitating properties were subjected to density gradient centrifugation and DEAE Sephadex chromatography and the fractions derived tested for antigen binding by the Farr globulin precipitation technique. With both types of antisera, antibody activity was associated with the low molecular weight (7S) fraction and the elution patterns from DEAE Sephadex were identical. Radioimmunoelectrophoretic studies, using ^{131}I-labelled antigen, also demonstrated similar electrophoretic properties of precipitating and non-precipitating antibodies.

Fig. 6 illustrates the variable recognition of heterologous serum albumins by a potent precipitating rabbit anti-HSA antiserum. With most antisera there was decreasing reac-

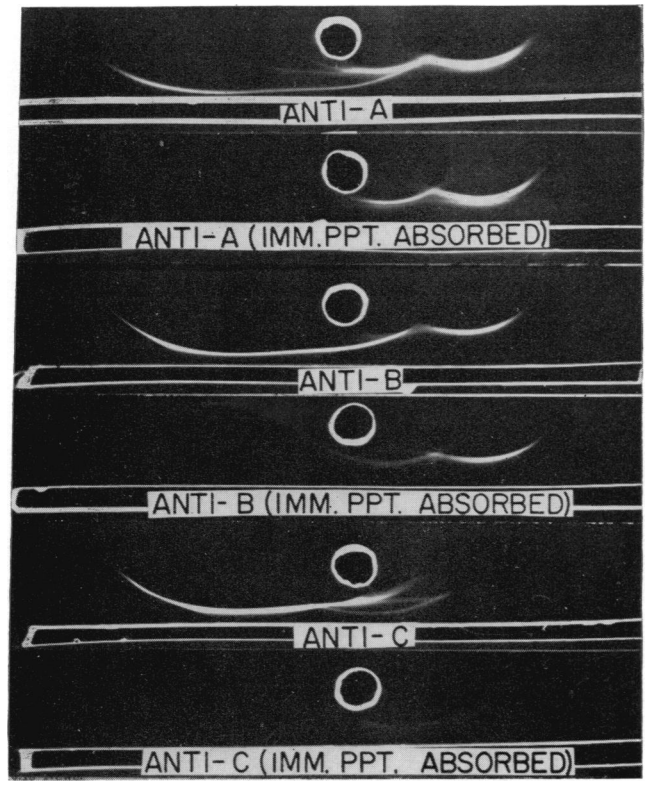

FIG. 5. Immunoelectrophoretic analysis of guinea-pig antisera to fractions A, B and C (Fig. 4). The antigen in each study (16–31 serum) diffused against a single trough of antiserum. Antisera were absorbed with washed immune precipitate formed by antiserum 16–62.

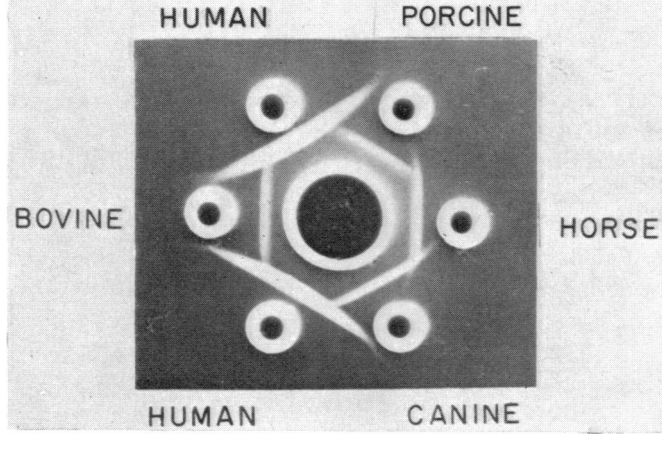

FIG. 6. Double gel diffusion analysis of a precipitating anti-HSA antiserum (17–13) using heterologous serum albumins. The concentration of antigens was 0·05 per cent.

tivity, as indicated by precipitin spurs, in the order of human, dog, horse and pig. Pig serum albumin, which was most deficient, lacked at least three determinants that were present on HSA. Table 2 summarizes the results of haemagglutination studies which employed tanned sheep cells coated with HSA, BSA and pig serum albumin. Non-precipitating anti-HSA sera (17–9 and 17–18) which were equivalent to precipitating antisera in reacting with HSA coated cells did not demonstrate significant agglutination of cells coated with BSA and pig serum albumin.

TABLE 2

HAEMMAGLUTINATION PATTERNS OF ANTI-HSA ANTISERA FOR TANNED CELLS COATED WITH HSA, BSA AND PIG SERUM ALBUMIN

Rabbit No.	Tanned cells coated with:	Haemagglutination patterns, tube Nos. (serial dilutions)											
		1	2	3	4	5	6	7	8	9	10	11	12
17–9	HSA	+	+	+	+	+	+	+	+	+	+	±	±
	Pig SA	0	0	0	0	0	0	0	0	0	0	0	0
	BSA	0	0	0	0	0	0	0	0	0	0	0	0
17–18	HSA	+	+	+	+	+	+	+	+	+	+	+	±
	Pig SA	0	0	0	0	0	0	0	0	0	0	0	0
	BSA	±	±	0	0	0	0	0	0	0	0	0	0
17–3	HSA	+	+	+	+	+	+	+	+	+	+	+	±
	Pig SA	+	+	+	±	0	0	0	0	0	0	0	0
	BSA	+	+	+	±	0	0	0	0	0	0	0	0
17–13	HSA	+	+	+	+	+	+	+	+	+	+	±	0
	Pig SA	+	+	+	+	+	+	±	±	0	0	0	0
	BSA	+	+	±	0	0	0	0	0	0	0	0	0

Antisera were diluted so that serial two-fold dilution titres were equivalent for HSA coated cells.

DISCUSSION

The failure of some antibodies to form insoluble complexes with antigen was recognized in early studies by Heidelberger and associates (1935, 1940). Variables influencing the development of this type of immune response have included: the species immunized, the route of immunization, and the phase (early *versus* late) of immunization. Although past interpretations of the failure of antibody to precipitate with antigen relied largely on theories of antibody univalence, Heidelberger and Kendall in 1935 offered an explanation that is consistent with the current studies and conclusions. In discussion of the observation that the early immune response, characterized by non-precipitating antibodies, was followed by formation of precipitating antibodies, they postulated that successive courses of immunization led to the production of 'more and more antibody capable of reacting with a larger number of chemical groupings in the antigen molecule' (Heidelberger and Kendall, 1935).

In comparisons of precipitating and non-precipitating anti-HSA and anti-BSA antibodies, no evidence could be derived for mediation of these properties by the physicochemical characteristics of the antibodies themselves. Both types of antibody were similar with regard to their properties in electrophoretic, zone centrifugation and DEAE Sephadex chromatographic studies; and guinea-pig antisera to purified non-precipitating rabbit antibodies did not recognize antigenic determinants unique to non-precipitating antibodies.

In zone centrifugation studies, the distribution of complexes formed by non-precipitating antisera was heterogeneous (Fig. 2). This heterogeneity was consistent with the variable

recognition of multiple antigenic determinants on serum albumin antigens. Comparable variation in complex size has been observed in hapten immune systems where purified anti-hapten antibody formed complexes with hapten–protein conjugates with a range of hapten–protein ratios (Lightfoot, Drusin and Christian, 1969).

Haemagglutination studies, utilizing serum albumins of several species, offered additional evidence that non-precipitating antisera recognized, relative to precipitating antisera, a smaller number of antigenic determinants on the complex antigens (Table 2).

Non-precipitating antisera, in the present studies, did not significantly inhibit the formation of insoluble complexes by precipitating antisera. In the zone of antigen excess, non-precipitating antibodies were capable of co-precipitation, rendering otherwise soluble complexes insoluble (Fig. 1). It is suggested that the soluble complexes formed in antigen excess are precipitated by the additional cross-linking function of non-precipitating antibodies, even though the latter are unable to form a high degree of lattice-work by themselves.

Antisera that lacked precipitating properties demonstrated antibody activity by the techniques of complement fixation, haemagglutination, passive cutaneous anaphylaxis and Farr globulin precipitation. As illustrated in Fig. 2, the soluble complexes formed by most non-precipitating antisera were heterogeneous in size. In studies of complexes composed of hapten immune reactants, it was observed that complexes less dense than an IgM marker were relatively inefficient in fixation of C, while the very large complexes which sedimented as a soluble pellet in zone centrifugation studies had marked anti-complementary effect (Lightfoot *et al.*, 1969). These large soluble complexes, under the conditions of the present study, were more efficient than insoluble complexes in C inactivation.

Why do a minority of rabbits immunized with heterologous albumins respond with the formation of non-precipitating antibodies? The explanation suggested is that such animals are partially tolerant to the determinants of a multivalent antigen: they recognize a limited number of antigenic sites, a number too few to permit complex lattice formation.

In two previous studies, evidence was offered that non-precipitating anti-hapten antibodies were physico-chemically distinct from precipitating antibodies. (Carter and Harris, 1967; Klinman and Karush, 1967). Although the present effort failed to demonstrate comparable uniqueness of non-precipitating anti-HSA and anti-BSA antibodies, the possibility that a subtle variation in antibody structure determines this property remains.

Klinman and Karush (1967) concluded that a non-precipitating horse anti-hapten antibody was incapable of reacting with determinants on two different molecules of antigen. The high density of some of the soluble complexes formed with non-precipitating anti-HSA antibodies (serum 17–9 in Fig. 2) and the effect of these sera on complexes solublized in antigen excess (Fig. 1) suggest that these non-precipitating antibodies can react with more than one molecule of antigen. A group of rabbits with three patterns of immune response (precipitating and non-precipitating antibodies and immune tolerance) to HSA were immunized with soluble hen egg albumin and keyhole limpet haemacyonin. There was no correspondence between the magnitude of the immune response to the latter two antigens and the pattern of response to HSA (Christian, unpublished observations). It was also observed, in immunoelectrophoretic studies, that non-precipitating anti-HSA antisera were equivalent to precipitating antisera in the recognition of serum protein antigens other than serum albumin which were trace contaminants in the preparations of serum albumin. It would appear from such studies, that animals which are tolerant to

HSA and those that form predominantly non-precipitating anti-HSA antibodies are not inherently hyporesponsive to all antigens. There is the possibility that allotypic variations in rabbit serum albumins influence the immune response to heterologous serum albumin—one explanation for the observed inheritance of immunological unresponsiveness of rabbits to BSA (Sobey, Magrath and Reisner, 1966; Thorbecke and Benacerraf, 1967).

To what extent the non-precipitating quality of the immune response may influence immunopathological events is not known, although there is evidence that the soluble complexes circulate for periods of hours and that rabbits manifesting this type of immune response represent the minority that develop chronic glomerulitis (Pincus *et al.*, 1968; Christian, 1969). As a class of antigens, autologous substances, with limited antigenicity for the host, would be the best candidates for the induction of non-precipitating antibodies. The failure of potent anti-globulin antibodies (rheumatoid factors) to precipitate with their corresponding antigen (IgG) may be an expression of this phenomenon.

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