Immunology 1983 **48** 351

Agglutinating and non-agglutinating antibodies in rabbits inoculated with a particulate antigen (*Salmonella typhimurium*)

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Accepted for publication 29 July 1982

Summary. Agglutinating and non-agglutinating anti-*Salmonella typhimurium* antibodies were specifically purified from the sera of immunized rabbits. Both types of antibody had the same electrophoretic mobility and were localized in the IgG fraction. It was not possible to find antigenic differences between agglutinating and non-agglutinating antibodies by immunodiffusion.

Agglutinating antibody activated the complement system, while non-agglutinating antibody lacked this capacity. Only the former increased clearance of antigen from the blood. When serum samples with different antibody titres determined by agglutination (agglutinating antibody) and Coombs test (non-agglutinating antibody) were injected in mice, clearance of antigen from the blood showed changes. These results were similar to those previously observed by us when different precipitating:co-precipitating antibody ratios were used, and indicated that competition of both antibodies for the antigen depends on their respective amounts.

When mice protection tests were set up by injection of agglutinating and non-agglutinating antibody before the inoculation of 10 LD_{50} *S. typhimurium*, non-agglutinating antibody was found to be less effective than agglutinating antibody.

Non-agglutinating antibody was detectable during the whole course of immunization. Its serum concentration was higher than that of the agglutinating antibody.

Non-agglutinating antibody behaves in a similar way to co-precipitating antibody. The initially proposed hypothesis that such antibodies could interfere with immunity to certain chronic infections was extended to include the non-agglutinating antibodies demonstrated here.

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0019-2805/83/0200-0351$02.00

INTRODUCTION

In previous papers we have demonstrated that the sera of rabbits (Margni & Binaghi, 1972; Margni, Perdigon, Abatangelo, Gentile & Binaghi, 1980), guinea-pig (Margni & Hajos, 1973), horse (Cordal & Margni, 1974), sheep (Margni, Paz & Cordal, 1976) and man (Perdigón, Margni, Gentile, Abatangelo & Dokmetjian, 1982) inoculated with soluble T-dependent antigens contain antibodies of IgG class, which are unable to precipitate with specific antigen but can nevertheless co-precipitate with the immune precipitate formed by precipitating antibodies of the same specificity.

In animals inoculated with soluble antigens, co-precipitating antibodies make their appearance during the whole course of the immune response and constitute 10%–15% of the total antibody population (Margni *et al.*, 1976). Recently (Margni, Cordal, Leoni, Hajos, Veira, Manghi & Bazzurro, 1977) we have shown that the antigen combining sites of each molecule of co-precipitating antibody apparently behave differently: one of the sites has high affinity for the antigen while the other site has low affinity. Consequently, the antibody binds the antigen but does not form insoluble Ag-Ab complexes.

Precipitating and co-precipitating antibodies differ in their biological properties: only the former fixes complement, activates phagocytosis and increases clearance of antigen from the blood (Margni *et al.*, 1980; Perdigón *et al.*, 1982).

By complement fixation and clearance of antigen from the blood tests we have demonstrated that both antibodies compete for antigen, and competition depends on their respective amounts (Margni *et al.*, 1980). Taking into account the particular behaviour of co-precipitating antibody we have proposed that it would be of interest to investigate the participation of this antibody in the induction and/or maintenance of chronicity in some microbial infections.

An increase of blocking non-agglutinating antibody has been observed in chronic brucellosis (Forget & Borduas, 1977), gonococcal infections (McCuctchan, Katzenstein, Norquist, Chikami, Wunderlich & Braude, 1978), experimental trichinosis in rat (unpublished results) and human American trypanosomiasis (Hajos, Carbonetto, Margni, Esteva & Segura, 1982). The present study was undertaken in order to examine: (i) the immune response in rabbits inoculated repeatedly with *Salmonella typhimurium* as a model of chronic infection; (ii) characteristics of the antibodies formed; and (iii) differences and/or similarities between antibodies isolated from rabbits injected with soluble (supernatant of antigen solution centrifuged at 20,000 r.p.m. for 1 hr) and particulate antigens.

MATERIALS AND METHODS

Antigens

Smooth *S. typhimurium* was grown in a nutrient solid medium for 24 hr at 37° and (i) killed by formolinization, washed with phosphate-buffered saline (PBS) and suspended (1×10^9 bacteria/ml); or (ii) killed by heating for 1 hr at 60°, washed three times with PBS and disrupted with a Megason ultrasonic disintegrator. After centrifugation the dry weight of the supernatant was determined and adjusted to 2 mg/ml.

Labelled S. typhimurium *(^{131}I-S. typhimurium)* Bacteria killed as indicated above were labelled with ^{131}I (National Atomic Center Ezeiza, Argentina) by McFarlane's method (McFarlane, 1958).

Animals and immunization

Ten adult white rabbits of both sexes weighing 2–2·5 kg each were injected subcutaneously with 1 ml of formolinized *S. typhimurium*. Further injections were given weekly, over 42 weeks, and the animals were bled before each injection.

Determination of agglutinating antibody titre

Purified antibody (0·2 ml) or serum dilutions was mixed with 0·2 ml of *S. typhimurium* (5×10^9 bacteria/ml killed by formolinization as indicated above). Readings were taken after incubation for 1 hr at 37°.

Determination of agglutinating antibody titre

In the tubes of the above series in which agglutination was not observed, non-agglutinating antibodies were titrated by Coombs test (Coombs, Mourant & Race, 1945). As agglutinating reagent rat anti-rabbit IgG serum diluted up to its optimal activity was used.

Isolation and purification of agglutinating antibodies

The isolation method was similar to that used for precipitating antibodies (Margni & Binaghi, 1972). Antibody was isolated from a mixture of serum samples with minimal differences in antibody titre determined by agglutination and Coombs test. Sera with high agglutinating activity and low non-agglutinating activity were chosen. Successively 1/20 of the bacteria required for adsorption of the total antibodies were added until no agglutination reaction was obtained. After each addition the mixtures were incubated 1 hr at 37° and 24 hr at 4°.

The agglutinates from agglutinations one to five were collected, pooled, washed with saline phosphate and suspended in glycine-HCl buffer 0·1 M, pH 3 at 37° for 30 min. After centrifugation the supernatant was immediately brought to pH 6 with $NaHCO_3$, dialysed against phosphate buffer 0·01 M, pH 7·5 and passed through a DEAE-cellulose column equilibrated with the same buffer. Before use the eluate containing antibody was centrifuged for 1 hr at 10,000 r.p.m., at 4°.

The purified antibodies were analysed by immunodiffusion (Ouchterlony, 1958), immunoelectrophoresis (Scheidegger, 1955) and acrylamide gel electrophoresis (Orstein & Davis, 1962).

Isolation and purification of non-agglutinating antibodies
Non-agglutinating antibodies were isolated from serum samples with low agglutinating activity and high antibody titre determined by Coombs test. They were recovered from the supernatant remaining after adsorption of agglutinating antibodies by adding an excess of bacteria and subsequently eluted with glycine-HCl buffer 0·1 M pH 3.

Purification was done as indicated for agglutinating antibodies.

Rat anti-rabbit serum
Rat anti-rabbit serum was prepared as previously indicated (Margni & Binaghi, 1972).

Rat anti-rabbit IgG serum
Rats were injected subcutaneously with 0·5 ml of purified rabbit Fc (γ) (500 μg/ml) emulsified with an equal volume of Freund's complete adjuvant (Difco). Inoculations were done on days 1, 7, 21 and 28 and the animals were bled a week after the last injection.

The rabbit Fc (γ) used for inoculations was obtained by Porter's method (Porter, 1959) from IgG purified from normal rabbit serum by chromatography on DEAE-cellulose. Column stabilization and IgG elution were done with phosphate buffer 0·01 M, pH 7·5.

Rat anti-rabbit IgG agglutinating antibody serum and rat anti-rabbit IgG non-agglutinating antibody serum
These were prepared as indicated for rat anti-rabbit IgG serum. Purified rabbit IgG agglutinating antibody and IgG non-agglutinating antibody were used as antigen, respectively, instead of Fc (γ).

Blocking reaction by non-agglutinating antibodies
One part *S. typhimurium* (5×10^9 bacteria/ml) was mixed with two parts of a solution of non-agglutinating antibody (2 mg/ml in PBS) and incubated for 1 hr at 37°. After centrifugation, the original volume of the bacterial suspension was restored. This suspension (0·2 ml) was added to 0·2 ml of agglutinating antibody (2 mg/ml) dilutions. The readings were taken after incubation for 1 hr at 37°.

As control a similar reaction was performed in which non-agglutinating antibody solution was replaced by PBS or normal rabbit IgG. The inhibitory capacity was established by the difference in the agglutinating titres obtained in both series.

Co-precipitating activity of non-agglutinating antibody
This was estimated by quantitative precipitin analyses (Margni & Binaghi, 1972) using agglutinating antibody alone and agglutinating antibody mixed with non-agglutinating antibody. The supernatant of disrupted *S. typhimurium* obtained as above indicated was used as antigen. After incubation for 1 hr at 37° and 24 hr at 4°, the precipitates were dissolved in 0·1 N NaOH and analysed by reading the absorbance at 280 nm in a Zeiss PMQII spectrophotometer. The $E^{1\%}_{280}$ employed for antibody estimation was 15·8. The amount of non-agglutinating antibody incorporated into the precipitate (co-precipitation) was calculated from the difference between the precipitate in the two series.

Complement fixation
The complement fixing activity of the antibodies was investigated by determination of 100% haemolysis after incubation at 37° for 1 hr.

One-tenth millilitres of antigen (5×10^9 *S. typhimurium*) and 5–50 μg antibody in 0·1 ml were used, and the CH_{100} dose was adjusted to 0·3 ml. As sensitized cells, 0·25 ml of 2×10^8 sheep red cells/ml mixed with an equal volume of haemolytic rabbit antibody (2 AbH_{100}/ml) were employed. The haemoglobin liberated was measured spectrophotometrically by OD at 413 nm.

Passive cutaneous anaphylaxis (PCA)
The PCA reaction were assayed in guinea-pig skin, according to Ovary (1952). Challenge with antigen (the same used in immunodiffusion; 1 mg/ml) was made after a 3 hr latent period. The antibody and antigen dose injected was 0·1 ml and 1 ml, respectively.

Opsonic activity
It was measured in mice (BALB/c) as previously indicated (Margni et al., 1980). The dose of ^{131}I-*S. typhimurium* was 1×10^8 bacteria/20 g mouse body weight. Antibodies were injected intravenously at different doses 5 min after antigen injection and the clearance of the radioactivity was followed by analysing blood samples taken from the retro-orbital plexus at various times. The phagocytic index K (Biozzi, Stiffel, Halpern, Le Minor & Mouton, 1961) was calculated according to the equation: $K = \log C_1 - \log C_2/t_2 - t_1$ where C_1 and C_2 represent the blood radioactivity at the times t_1 and t_2, respectively.

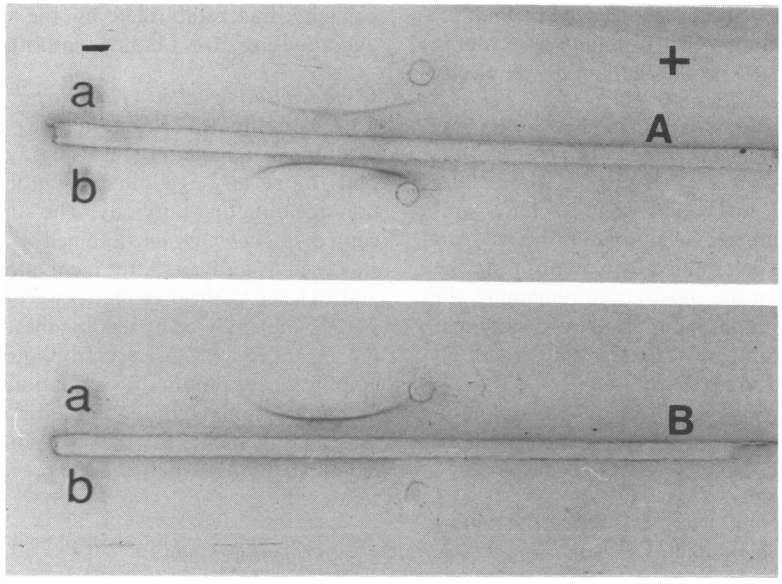

Figure 1. Immunoelectrophoretic analysis of rabbit anti-*S. typhimurium* agglutinating (a) and non-agglutinating (b) antibodies. A, rat anti-rabbit serum; B, supernatant of disrupted *S. typhimurium*.

Groups of five animals were used to establish each point.

Determination of the ED_{50} of agglutinating and non-agglutinating antibodies

Five groups of six female mice each were injected intraperitoneally with 400, 200, 100, 50 and 25 μg of agglutinating and non-agglutinating antibodies, respectively. Immediately afterwards they were challenged intravenously with 10 LD_{50} of *S typhimurium*. Deaths were recorded 72 hr later.

The LD_{50} and ED_{50} for *S. typhimurium* and antibodies, respectively, were calculated by the Reed and Muench method (Reed & Muench, 1938).

RESULTS

The above mentioned methods enabled agglutinating and non-agglutinating antibodies to be isolated from the rabbit sera obtained at different bleedings.

Agglutinates from agglutinations one to five of rabbit anti-*S. typhimurium* serum mixture, with high agglutinating antibody titre and low non-agglutinating activity, were used for agglutinating antibody isolation. The purified agglutinating antibody contained very little non-agglutinating antibody. On the other hand, non-agglutinating antibody was easily purified from an immune serum mixture with low agglutinating activity and high Coombs test activity.

Immunoelectrophoretic analysis was unable to show a difference between the precipitation bands of agglutinating and non-agglutinating antibodies when rat anti-rabbit serum and rat anti-rabbit IgG serum were used. Both antibodies were localized in the IgG fraction and had the same electrophoretic mobility. Precipitin lines of IgM and IgA were not detected (Fig. 1).

When diffused against rat anti-rabbit serum and rat anti-rabbit IgG serum, IgG agglutinating antibody gave a band of precipitation which showed complete identity with the band given by IgG non-agglutinating antibody. Similar results were obtained when rat anti-rabbit IgG agglutinating antibody serum and rat anti-rabbit IgG non-agglutinating antibody serum were used for precipitation.

When supernatant of disrupted *S. typhimurium* was used as antigen, only agglutinating antibody (3 mg/ml) gave a precipitin line by immunodiffusion and immunoelectrophoresis (Fig. 1). Different antigen doses were used and the best Ab/Ag ratio was 8 (w/w). Under the same conditions non-agglutinating antibody did not form insoluble Ab-Ag complexes.

Non-agglutinating antibody did not form aggregates

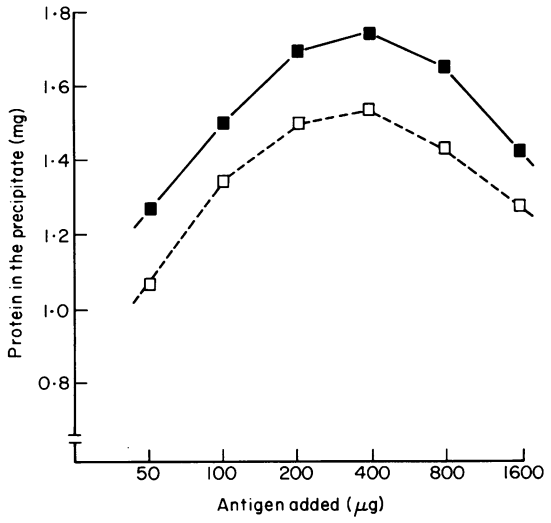

Figure 2. Co-precipitation assay. (□ - - - □) Precipitin curve obtained with agglutinating antibody alone (1·6 mg/ml). (■—■) Precipitin curve obtained with a mixture of agglutinating (1·6 mg/ml) and non-agglutinating (0·3 mg/ml) antibody. As antigen supernatant of disrupted *S. typhimurium* was used.

with soluble antigen, though it was incorporated into the immune precipitates formed by agglutinating antibody (Fig. 2).

The antibody activity of the purified preparations of agglutinating and non-agglutinating antibodies was determined by agglutination and Coombs test, respectively. The minimum concentration of agglutinating antibody required to obtain a positive reaction in agglutination was 6 μg/ml, whereas 3 μ g/ml of non-agglutinating antibody was required for a positive Coombs test.

Inhibition of agglutination was observed when purified non-agglutinating antibody in free solution was mixed with the antigen before adding the agglutinating antibody over a wide range of antibody concentrations (Table 1). Similar results were obtained when dilutions of different serum samples with high antibody titre determined by Coombs test and low agglutinating activity were used (Table 2).

Anti-*S. typhimurium* antibodies were quantified by agglutination and Coombs test in sera obtained at different bleedings. Figure 3 shows the average of the results obtained in the rabbits studied (± SD).

The complement fixing capacity of rabbit agglutinating and non-agglutinating anti-*S. typhimurium* antibodies was investigated. Only agglutinating antibody activates the complement system and a minimum of 10 μg of antibody was required to produce 100% complement consumption. Non-agglutinating antibody was ineffective at all the doses tested (Table 3).

Both agglutinating and non-agglutinating antibody gave passive (PCA). The activity of the non-agglutinating antibody was six times less. The minimum concentration required to obtain a positive reaction in PCA was 1·25 μg/ml and 7·5 μg/ml for agglutinating and non-agglutinating antibody, respectively.

The blood clearance of ^{131}I-*S. typhimurium* (K) for 1·5 μg of purified agglutinating and non-agglutinating antibodies was determined (Fig. 4a). This antibody dose was used because for rabbit IgG precipitating antibody it was found to be the most effective (Margni *et al.*, 1980). The phagocytic index (K) of agglutinating and non-agglutinating antibody was 0·25 and 0·015,

Table 1. The agglutinating activity of agglutinating antibody and its binding inhibition by non-agglutinating antibody

Inhibitor (2 mg/ml)	Agglutination of *S. typhimurium* by IgG agglutinating anti-*S. typhimurium* antibody (μg/ml)							
	400	200	100	50	25	12	6	3
IgG non-agglutinating anti-*S. typhimurium* antibody	+	±	−	−	−	−	−	−
Non-specific rabbit IgG*	+	+	+	+	+	+	±	−
PBS	+	+	+	+	+	+	+	−

* Non-specific rabbit IgG was purified from the sera of non-immunized rabbits by DEAE-cellulose chromatography.

Table 2. Agglutination of *S. typhimurium* by serum sample 17 and its inhibition by serum sample 55

Inhibitor (0·5 ml)	Agglutination of *S. typhimurium* by serum sample 17 dilutions							
	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
Serum sample 55* (dilution 1/1024)	+	+	−	−	−	−	−	−
Serum of non-immunized rabbit (dilution 1/10000)	+	+	+	+	+	+	±	−
PBS	+	+	+	+	+	+	+	−

* Antibody activity determined by agglutination and Coombs test: 1/512 and 1/8192, respectively.

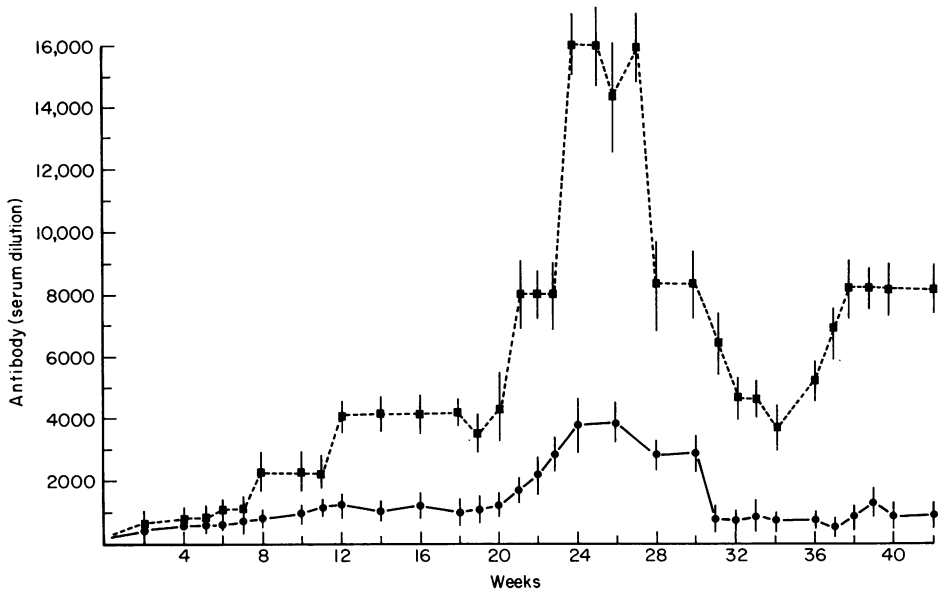

Figure 3. Rabbit anti-*S. typhimurium* antibodies quantified by agglutination and Coombs test. Average of the ten inoculated animals (± SD). (●—●) Antibody detected by agglutination; (■) antibody detected by Coombs test.

Table 3. Complement-fixing activity*

	Antibody (μg/0·1 ml)											
	Agglutinating						Non-agglutinating					
	5	10	20	30	40	50	5	10	20	30	40	50
Complement consumption (%)	33	100	100	100	100	100	0	0	0	0	0	0

*Antigen used: *S. typhimurium* 5×10^8 bacteria/0·1 ml.

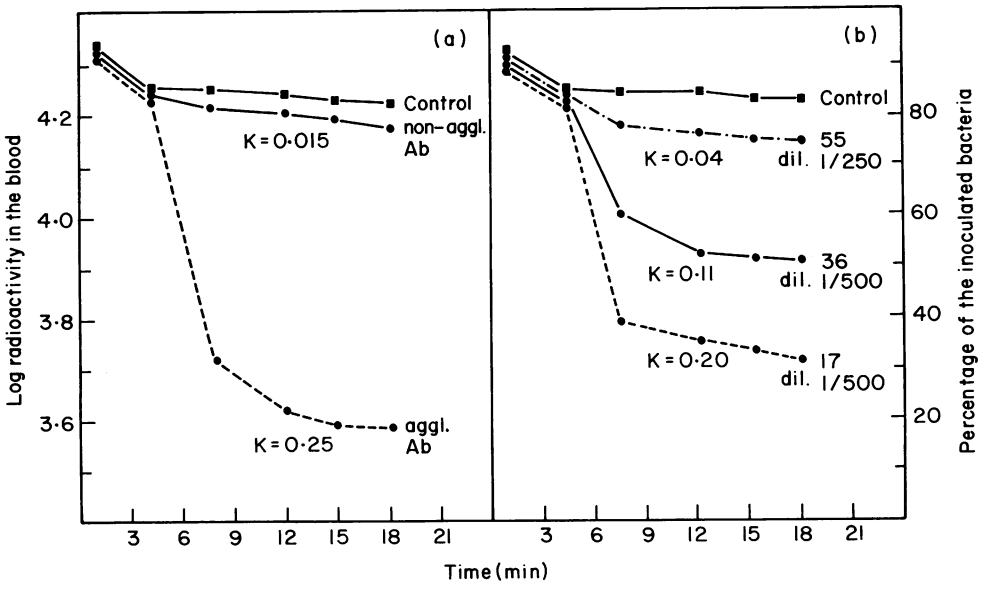

Figure 4. Blood clearance of ^{131}I-*S. typhimurium*. (a) After injection of rabbit anti-*S. typhimurium* agglutinating or non-agglutinating antibody. (b) After injection of serum samples obtained from rabbits inoculated with *S. typhimurium*. (17) Agglutinating activity 1/1024, Coombs test positive up to 1/2048; (36) agglutinating activity 1/1024, Coombs test positive up to 1/4096; (55) agglutinating activity 1/512, Coombs test positive up to 1/8192. As control serum of non-immunized rabbit diluted 1/250 was used.

Table 4. Agglutinating and non-agglutinating antibody ED_{50}

Antibody (μg)	Survived	Died	Total Survived	Total Died	Deaths (%)
Agglutinating					
400	5	1	18	1	5
200	4	2	13	3	19
100	4	2	9	5	36
50	3	3	5	8	62
25	1	5	2	13	87
12·5	1	5	1	18	95
ED_{50} (agglutinating Ab) = 67 μg					
Non-agglutinating					
400	2	4	6	4	40
200	2	4	4	8	67
100	1	5	2	13	87
50	1	5	1	18	95
25	0	6	0	24	100
12·5	0	6	0	30	100
ED_{50} (non-agglutinating Ab) = 310 μg.					
ED_{50} (non-agglutinating Ab)/ED_{50} (agglutinating Ab) = 4·5.					

respectively. Similar determinations were done with serum samples with different antibody titre determined by agglutination and Coombs test (Fig. 4b). For serum 17 (dilution 1/500), with agglutinating activity 1/1024 and Coombs test positive up to 1/2048, K was 0·20, while for serum 55 (dilution 1/250), with agglutinating activity 1/512 and Coombs test positive up to 1/8192, K was 0·04.

Mouse protection by injection of agglutinating and non-agglutinating antibodies before inoculation of 10 LD_{50} of *S. typhimurium* was investigated. For agglutinating antibody the ED_{50} was 67 μg, while for the non-agglutinating antibody the ED_{50} was 310 μg (Table 4). This difference was statistically analysed by the X^2 test. From X^2 tables $P(X^2 < 7{\cdot}88;\ 1\ \text{d.f.}) = 0{\cdot}995$ so that the result of $X^2 = 9{\cdot}0$ observed is statistically significant ($P \leqslant 0{\cdot}005$).

DISCUSSION

Rabbits inoculated with a particulate antigen (*S. typhimrium*) formed agglutinating and non-

agglutinating antibodies. The latter did not agglutinate the bacteria but firmly attached to them and gave positive Coombs test. When agglutinating and non-agglutinating antibody were reacted with a supernatant of disrupted *S. typhimurium*, only the agglutinating antibody gave a precipitin line. The non-agglutinating antibody did not form Ab-Ag insoluble complexes, though it was incorporated into the precipitates formed by the agglutinating antibody as shown by co-precipitation analyses. These results indicated that non-agglutinating antibody isolated from rabbit had similar immunochemical characteristics to the non-precipitating antibody previously described (Margni & Binaghi, 1972; Margni *et al.*, 1980).

Rabbit non-agglutinating antibody was considered to be blocking antibody because it did not agglutinate the antigen and inhibited agglutination when mixed with the antigen before the addition of the agglutinating antibody. McLeod Griffiss & Beltram (1977) have shown that IgA will block the bactericidal activity of human IgM directed against meningococci. IgA did not appear to be involved in the case of anti-*S. typhimurium* agglutinating and non-agglutinating antibodies. The antibodies were localized in the IgG fraction and contamination with IgM and IgA was not detected by immunoelectrophoresis when rat anti-rabbit serum was used as precipitating reagent. It was not possible to find antigenic differences between the antibodies by immunodiffusion.

Titres of agglutinating and non-agglutinating antibodies were measured during the whole of the immunization period. They showed an early and persistent response.

Animals inoculated with soluble antigens synthesize co-precipitating antibodies which constitute 10%–15% of the total antibody population during the whole course of the immune response (Margni *et al.*, 1976). On the other hand, in rabbits inoculated with a micro-organism the amount of non-agglutinating antibody made during the same period of time represents 25%–75% of the total antibody activity.

When complement fixing capacity and opsonic activity of rabbit IgG agglutinating and non-agglutinating antibodies were analysed, the results obtained showed that those antibodies behaved in a similar fashion to rabbit precipitating and co-precipitating antibodies (Margni *et al.*, 1980). Agglutinating antibody activated the complement system whereas non-agglutinating antibody lacked this capacity. The results in Fig. 4a show that different opsonic capacity is possessed by rabbit agglutinating and non-agglutinating antibodies. Clearance of antigen from the blood was greatly increased by the injection of an optimal dose of agglutinating antibody. Non-agglutinating antibody was ineffective at all the doses tested.

When serum samples with different antibody titres as determined by agglutination (agglutinating antibody) and Coombs test (non-agglutinating antibody) were injected into mice, clearance of antigen from the blood showed changes. The results obtained (Fig. 4b) are similar to those when different precipitating:co-precipitating antibody ratios were employed (Margni *et al.*, 1980), and indicate that agglutinating and non-agglutinating antibodies compete for the antigen, and competition depends on their respective amounts. These results also indicate that non-agglutinating antibody is not a laboratory artifact.

Agglutinating and non-agglutinating antibody were able to elicit anaphylaxis phenomena (PCA) in the guinea-pig when disrupted *S. typhimurium* were used as challenge. It is evident that whatever the mechanism of sensitization, the non-agglutinating antibody possesses the necessary structures and can be activated as well as the agglutinating type, although perhaps with somewhat less efficiency.

In mouse protection tests non-agglutinating antibody was four and half times less effective than agglutinating antibody. This difference was statistically significant.

Non-agglutinating antibody appeared early in the course of the immune response and its serum concentration, was in general, higher than that of the agglutinating antibody (Fig. 2). Animals inoculated with T-dependent soluble antigens elaborate co-precipitating antibodies, but the serum concentration never represents more than 10%–15% of the total antibody population (Margni *et al.*, 1976). These results suggest that antigen characteristics (particulate or soluble) probably would play an important role in immune response modulation.

Non-agglutinating antibody can also function as a blocking antibody and has immunochemical characteristics and biological properties similar to co-precipitating antibody. Co-precipitating and non-agglutinating antibodies are not able to trigger immune mechanisms inducing antigen damage.

An increase of blocking antibodies has been observed in chronic bacterial and parasitic infections (Forget & Borduas, 1977; McCuctchan *et al.*, 1978; Hajos *et al.*, 1982). We have shown that non-agglutinating antibodies can compete with agglutinating

antibody for antigen; that they occur in appreciable amounts during the whole course of the immunization schedule, especially when antigen is repeatedly injected; and that they possess certain biological properties similar to co-precipitating antibodies. We originally proposed that co-precipitating antibody could affect the course of certain chronic, bacterial and viral infections, and also tumour growth, among other chronic processes (Margni *et al.*, 1980). We wish to extend this hypothesis to include non-agglutinating antibodies.

ACKNOWLEDGMENTS

This work was supported in part by grant 8072b/80 from the National Research Council of Argentina, and grants 3122/77 and 7603/79 from Scientific Research Commission, Pcia., Buenos Aires.

R. A. Margni and Gabriela Perdigón are members of the Researcher Career, National Research Council of Argentina.

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