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The Antibody Activities of 19S and 7S Fractions from Rabbit Antisera to *Bordetella pertussis*

JEAN M. DOLBY AND D. E. DOLBY

Lister Institute of Preventive Medicine, Elstree, Hertfordshire

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Summary. *Bordetella pertussis* antisera were fractionated on Sephadex G-200 to give 19S and 7S globulins, the latter refractionated on DEAE-cellulose, and the antibody properties of the two purified materials compared.

The minimal weights of 19S and 7S globulins required to neutralize haemagglutinin and to protect mice against an intranasal challenge causing lung infection were similar. In agglutination 7S globulin was 1·5–100 times more effective than 19S, the ratio depending on strain and serum, but there was no apparent correlation with specific agglutinogens.

7S globulin was at least 100 times more effective than 19S both in the complement-dependent bactericidal reaction *in vitro* and in the protection of mice against an intracerebral challenge. It is suggested that the two reactions measure the same antibody.

INTRODUCTION

Recent studies on the immunological activities of the various classes of immunoglobulin have shown that, although antibodies of one class can take part in a number of reactions, they may do so less efficiently than antibodies of another class and in some cases may fail to react at all (see Pike, 1967). Thus in opsonization and bactericidal reactions IgM antibody appears to be more effective than IgG (Robbins, Kenny and Suter, 1965; Landy, Sanderson and Jackson, 1965), and most workers have found the same to hold for agglutination. On the other hand precipitation reactions and complement fixation with bacterial antigens are usually stronger with IgG antibodies. The degree of correlation of these *in vitro* activities with the ability to protect the animal against bacterial infection has not, however, been established to any significant extent.

In the present study 19S and 7S immunoglobulins from rabbit sera to *Bordetella pertussis*, prepared by different methods, were separated and their properties examined by various *in vitro* tests in an attempt to discover which were most closely correlated with mouse protective activity. Three serum pools were used, one produced by intravenous injection of six to eight doses of whole bacterial cells, the second a hyperimmune serum prepared by the intravenous injection of whole and fractionated cells, and the third produced as a result of intraperitoneal injection of cells with adjuvant. All three methods gave similar results. Because there is no evidence as to the avidity of these antibodies and, because there is evidence that not all antibodies are firmly adsorbed to bacteria (Herzberg, Kenny and Robbins, 1966) but that non-antibody IgG *is* adsorbed (Frommel, Grob, Masouredis and Isliker, 1967), no attempt was made to isolate specific antibodies by adsorption to

and elution from bacterial cells. In consequence, quantitative comparisons of antibody concentrations were not possible. Comparison of the ratios of activities in different sera demonstrated the close similarity between the ratios of the minimal effective amounts of 19S and 7S globulins required either for the *in vitro* bactericidal reaction or for protecting mice against intracerebral infection, suggesting that the same antibody is responsible for both effects.

METHODS

Preparation of antisera

Serum I was made by injecting a number of rabbits with heat killed *B. pertussis*, strains A 21-523, A 5373 (from Dr G. Eldering, Michigan) or 360E (from Dr N. W. Preston, Manchester). Equal volumes of the antisera to each strain were pooled. Suspensions of 10×10^9 organisms/ml were given intravenously in six to eight doses of 0·2–1·0 ml over a period of 3–4 weeks, and the animals bled 10–14 days after the last injection.

Serum II was a pool of sera from rabbits hyperimmunized with whole cells of *B. pertussis*, killed and living; a total of about twenty doses was given intravenously in three or four courses over a period of 6–9 months, followed by ten to fifteen intravenous doses of various fractions of the organism. The pool was lyophilized and reconstituted for use as a standard, and designated 6660.

Serum III was raised in rabbits by giving intraperitoneally ten to sixteen injections of a soluble fraction of *B. pertussis* in 100-μg doses with aluminium hydroxide as adjuvant. The rabbits were bled 2 weeks after the last injection.

All antisera and fractions thereof were heated for 30 minutes at 56° before use.

Fractionation of antisera

This was carried out at 4° and the protein content of fractions measured by the adsorption of ultraviolet light at 280 mμ. Primary fractionation was performed on Sephadex G-200 (Pharmacia, Uppsala). Five millilitres of antiserum were run on a column of Sephadex G-200, 2·5 × 100 cm, equilibrated with 0·1 M Tris–HCl buffer containing 1·0 M NaCl; the apparent pH of the undiluted buffer was 8·0. Of the three peaks eluted (Fig. 1a) the one eluted last (peak C) contained mainly albumin and was not usually treated further. The macroglobulins in the first peak (peak A) were re-run on Sephadex G-200 under the same conditions (Fig. 1b).

The globulins of the middle peak (peak B) were sometimes re-run on Sephadex G-200 and always fractionated on DEAE-cellulose (Whatman, DE 52). Middle fraction, 250–300 mg for a column 2·5 × 45 cm, or 750–1000 mg for a column 4 × 50 cm, was equilibrated against 0·0175 M sodium phosphate buffer, pH 6·3, put on the column and eluted first with this buffer, then successively with 0·03 M sodium dihydrogen phosphate, 0·05 M sodium dihydrogen phosphate containing 0·02 M NaCl and 0·1 M sodium dihydrogen phosphate containing 0·5 M NaCl (Fig. 2).

Electrophoresis and ultracentrifugation

Immunoelectrophoresis was carried out on microscope slides by the method of Hirschfeld (1959), using goat anti-rabbit serum. Ultracentrifugation was done with the Spinco Model E analytical ultracentrifuge at 25° and 50,740 rev/min.

In vitro *tests*

Bactericidal, agglutination and anti-haemagglutination tests were those described by Dolby and Vincent (1965).

The index of bactericidal activity (Table 4) was derived by subtracting the log of the number of viable organisms in 0·02 ml (one drop) of the tube containing the serum under test from the log of the number of viable organisms in the same volume of a control preparation lacking one component of the system. The log titre in the control was usually 3·0–3·5, which is therefore the highest figure the bactericidal activity index can attain.

Passive protection tests

White mice of Teiler's original strain, weighing 17–20 g, were used.

Protective activity against a lung challenge was measured by determining the fall in viable count of a sublethal dose of organisms injected into the lung. One volume of serum fraction was mixed with 1 volume of a suspension of *B. pertussis*, strain G1.353, harvested from 20-hour Bordet–Gengou plates into 1 per cent Casamino Acids (Difco), so that the final mixture contained a sublethal dose of 100,000 organisms in 0·04 ml. The mixture was incubated for 30 minutes at 37°, during which time there was no fall in the viable count, which was about one-fifth of the total count. The mixture was instilled into the noses of mice under ether anaesthesia and the course of infection followed by determining the numbers of organisms per lung at various intervals, as described hereafter.

Protective activity against an intracerebral challenge was measured: (a) by injecting serum intraperitoneally in 0·2-ml volumes and challenging intracerebrally between 2 hours and 3 days later, and (b) by injecting a mixture of serum and challenge dose after incubation, as in the lung protection tests (the 'IC mix' challenge).

The challenge dose for intracerebral tests was made from 20-hour Bordet–Gengou plates of strain 18-323 harvested into 1 per cent Casamino Acids and contained a lethal challenge of 50,000 organisms, about one-tenth of which were viable, in the 0·03 ml given.

Protection against intracerebral challenge was expressed as the PD_{50} dose (Reed and Muench, 1938) of serum from death rates at 14 days or estimated in terms of modification of the course of infection in brains.

For estimating the degree of infection animals were killed with coal-gas and their organs removed aseptically into Universal bottles containing 9 ml each of 1 per cent Casamino Acids and glass beads 4–5 mm in diameter. Bottles were shaken on a vertical shaker, throw 2¾ in., 325 rev/min for 15 minutes for the lungs and for 3 minutes for the brains and 0·02-ml volumes of ten-fold dilutions of the homogenate of each organ dropped on to the plates. Counts were made after 4–5 days at 37°.

The plates for viable counts were modified Cohen and Wheeler liquid medium with 5 per cent blood and 1 per cent agar (Dolby, 1965).

RESULTS

SERUM FRACTIONATION

A typical first fractionation of 5 ml of Serum I on Sephadex G-200 is shown in Fig. 1(a). The material excluded from the gel (peak A) contained roughly 60 per cent of protein with a sedimentation coefficient of 19S, together with 5–10 per cent of aggregated material,

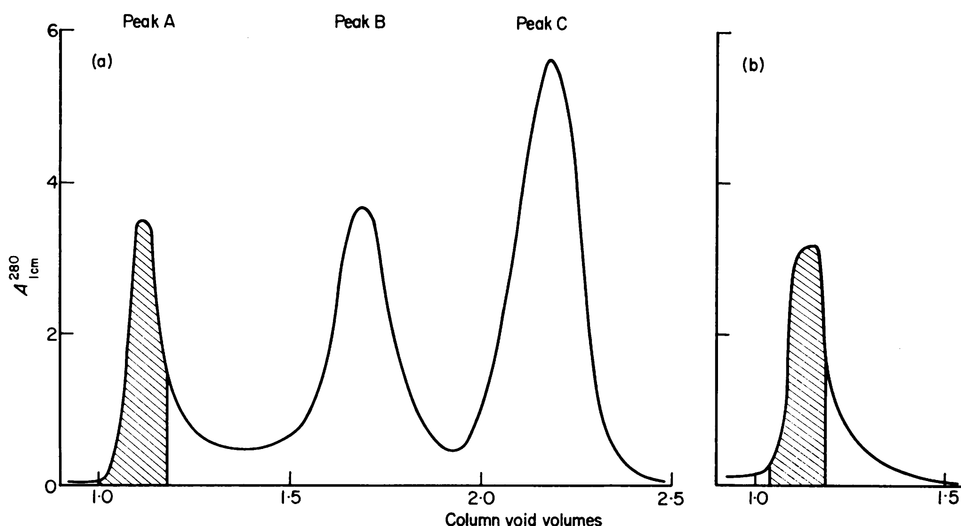

FIG. 1. (a) Chromatography of 5 ml rabbit antiserum on a column of Sephadex G-200, 2·5 × 100 cm in 0·1 M Tris–HCl buffer, pH 8·0, containing 1 M NaCl. Five millilitres effluent/tube; void volume 145 ml. Peak A contained the 19S globulins. (b) Material represented by the cross-hatched area of Peak A was concentrated and re-run on the same column. The cross-hatched portion from this peak was again concentrated for test.

almost certainly 19S, the remainder was composed of roughly equal amounts of proteins with sedimentation coefficients of 12S and 7S. Concentration and rechromatography of peak A on Sephadex G-200 under the same conditions (Fig. 1b) decreased the 12S and 7S components to negligible amounts, but increased the aggregated material with a sedimentation coefficient greater than 19S to 20 per cent. The remaining 80 per cent was 19S. The principal immunological properties of the fractions from Sephadex G-200 are shown in Table 1.

The protein from peak C consisted mainly of albumin, and was not further investigated. The 7S globulins of peak B from several runs of a single serum on Sephadex G-200 were combined, concentrated by ultrafiltration at 4°, and fractionated on DEAE-cellulose. The results of a typical fractionation on DEAE-cellulose of material from peak B of Serum I are shown in Fig. 2. Material from tubes under each peak was combined as shown and concentrated by ultrafiltration. On immunoelectrophoresis the first three fractions gave

TABLE 1

RELATIVE STRENGTHS OF ANTIBODIES IN THE THREE SEPHADEX FRACTIONS A, B AND C OF THE THREE SERA INVESTIGATED

	Antibody activity of fraction		
	A	B	C
Bacterial agglutinin* as measured by tube agglutination of whole cells at 5000×10^6 organisms/ml	±	++	+
Anti-haemagglutinin* against 2 m.h.d. of *B. pertussis* strain G1.353	±	++	−
No. of precipitin bands in gel diffusion plates	0	2–5	0
Bactericidins* as measured by complement dependent bactericidal activity *in vitro*	± or −	++	+

* − indicates no measurable antibody, ±, + and ++ increasing amounts.

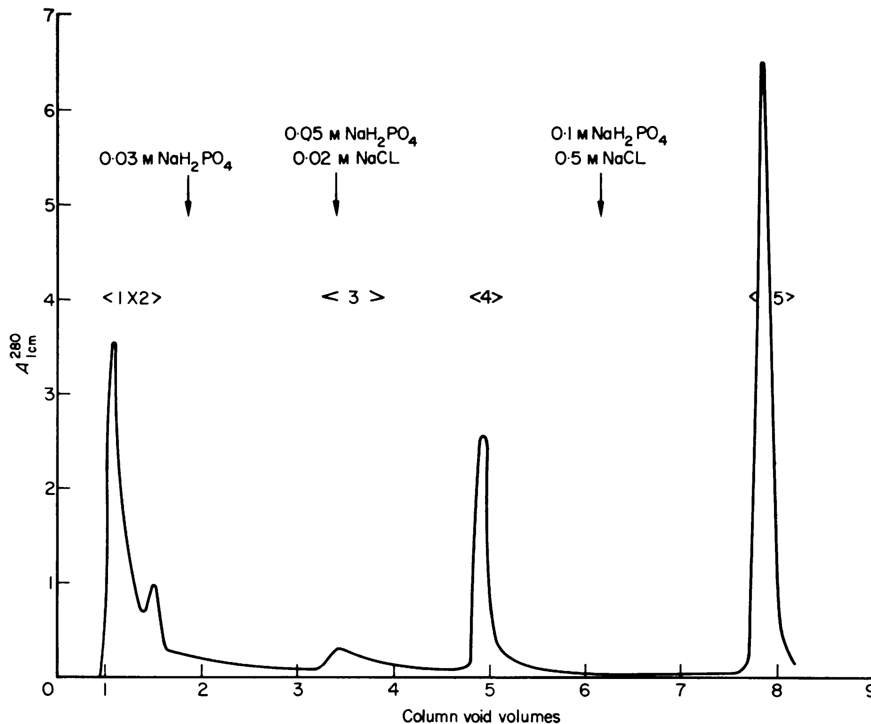

FIG. 2. Chromatography of 290 mg of 7S globulins from Sephadex G-200 fractionation on a column of DEAE-cellulose, 2·5 × 45 cm. The starting buffer was 0·0175 M sodium phosphate, pH 6·3 and was changed stepwise. Five millitres effluent/tube; void volume 124 ml. The eluates were pooled into fractions as indicated by the arrows.

a single line in the γ-globulin region, the mobility increasing slightly from fraction 1 to fraction 3; fractions 4 and 5 contained the remaining 7S globulins and some albumin. There seemed little difference in the biological properties of fractions 1–3, so attention was concentrated on a comparison of the properties of fraction 1 with those of the 19S globulins. Ten millilitres of serum yielded about 40 mg of 19S globulins and 20–30 mg of IgG in fraction 1.

THE ANTIBODY PROPERTIES OF PURIFIED 19S AND 7S GLOBULIN FRACTIONS

Bacterial agglutinins and anti-haemagglutinins

Table 2 shows the minimal agglutinating amount of 19S and 7S globulins for *B. pertussis* 18-323, which contains agglutinogens 1, 2, 3, 4, 5 and 6 (Andersen, 1953; Eldering, Hornbeck and Baker, 1957) and is readily agglutinable. 7S globulins are more potent than 19S. This is so whether the parent antiserum was produced by a course of six injections (Serum I), thirty injections (Serum II); or by the intraperitoneal (Serum III) or the intravenous route (Serum I).

In an attempt to differentiate between agglutinogens, fractions from Serum I were tested not only against the suspensions of *B. pertussis* against which the serum was raised (homologous strains) but also against three others (Table 3). The ratios of the minimal weights of 19S and 7S globulins required to agglutinate suspensions ranged from 1·5 to

125. A similar experiment using fractions from the hyperimmune Serum II to agglutinate strains 3865, G1.353 and B 16 gave ratios of about 60 in each case.

The anti-haemagglutinating activities of the 19S and 7S fractions from these sera were of the same order (Table 2).

Table 2

Agglutinin and anti-haemagglutin content of 19S and 7S globulin preparations

Serum	Minimal amount agglutinating strain 18-323 (μg protein/ml)		Minimal amount inhibiting haemagglutination by 2 m.h.d. of strain G1.353 (μg protein/ml)	
	19S	7S	19S	7S
I	15	0·5	100	50
II	60	0·4	250	125
III	112	7		

Table 3

Agglutinin content of fractions from Serum I (minimal agglutinating amounts in μg protein/ml)

Fraction	Strain and agglutinogen type					
	Homologous			Heterologous		
	3865* 1, 2, 4	21-523 1, (2), 3, (5)	5373 1, (2), 3, (6)	18-323 1–6	G1.353 1	B16 1,3
19S	125	500	10	30	15	100
7S	2	4	2	1	10	12·5
Ratio 19S/7S	62·5	125	5	30	1·5	8

* Strain 3865, a routine typing strain, has an agglutinogen make-up similar to that of strain 360E, used in raising the antiserum, and has been substituted for it in these agglutination tests.

Precipitins

Fractions from Serum I (25 mg/ml) were tested by the Ouchterlony method. The antigen was the supernatant from a suspension of *B. pertussis* disintegrated ultrasonically and centrifuged at 20,000 ***g***. Antibody was dispensed either at the same time as the antigen, or up to 8 hours later, to allow the antigen time to diffuse. No arcs of precipitation ever appeared between antigen and 19S antibody; two or three appeared between antigen and 7S antibody.

Bactericidal tests

Fractions from Sera I, II and III were all very similar in their ability to kill *B. pertussis*, strain 18-323 *in vitro*, in the presence of complement. A typical result with serum I is shown in Table 4. In these experiments the amounts of 19S globulin used were very much higher than in the experiment illustrated in Table 1. An inhibition zone can be demonstrated with 300 μg/ml of protein and bactericidal activity at 0·3 μg/ml. It is evident that the 7S globulin is at least 100 times as active as the 19S, whether measured at optimum kill or at minimum inhibiting concentration.

TABLE 4

THE BACTERICIDAL ACTIVITY *in vitro* OF 19S AND 7S GLOBULINS OF SERUM I

	Concentration of protein (μg/ml)						
	300	30	3	0·3	0·03	0·003	0·0003
Bactericidal activity index*							
19S	0	0·6	1·4	2·4	0·2	0	0
7S	0	0	0	0·3	1·8	2·0	0·2

* This is expressed as the difference: $\log_{10}$ mean viable count in control tube minus $\log_{10}$ mean viable count in a bactericidal mixture (see 'Methods'). 0 indicates no bactericidal activity, 1·0 a ten-fold decrease in viable count and so on.

Mouse protective antibodies

Intraperitoneally administered 7S globulins from Serum II protected mice against an intracerebral challenge with *B. pertussis* when given 3 hours before infection. The protective dose (PD_{50}) was 1·5 mg protein. In the same circumstances 5 mg 19S globulin were completely ineffective, and even 7 mg, given in two doses on the 2nd day after challenge, when injected antibody begins to leak from the circulation into the brain tissue (Holt, Spasojevic, Dolby and Standfast, 1961), was ineffective.

The viable count of *B. pertussis* in the brains of mice given mixtures of antibody and organisms intracerebrally is a very sensitive indicator of protective antibody effects.

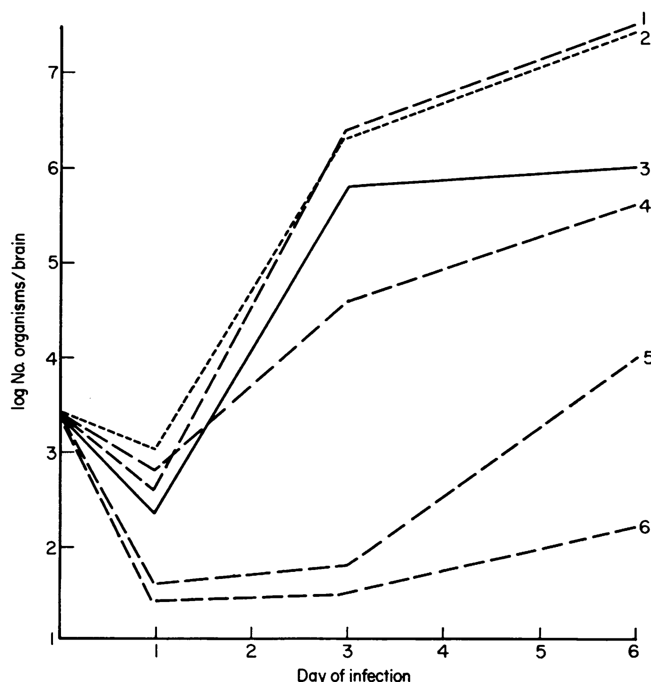

FIG. 3. The effect of 19S and 7S globulins (Serum I) on a lethal brain infection ('IC mix' test). Average viable count of organisms in brain. Each point represents the average count for five mice. 1, 7S: 0·025 mg/ml; 2, organisms only; 3, 19S: 50 mg/ml; 4, 7S: 0·25 mg/ml; 5, 7S: 25 mg/ml; 6, 7S: 2·5 mg/ml.

Much less antibody is protective given in this way than when given intraperitoneally (J. M. Dolby, to be published). The 19S and 7S fractions from Serum I were examined by this 'IC mix' test (Fig. 3). At 0·025 mg/ml 7S globulin did not protect and at 0·25 mg/ml it was partially protective; 19S globulin at 50 mg/ml was also partially protective, but less protective than 0·25 mg/ml 7S. The PD_{50} for 19S globulin was greater than 50 mg/ml whilst the PD_{50} for 7S globulin was 0·8 mg/ml. The 7S globulin is thus over sixty times as effective as 19S globulin. The individual mouse brain counts conformed closely to the averages. The addition of guinea-pig complement to the mixture before injection did not affect the result. The results in Fig. 3 also suggest that there is inhibition by excess antibody *in vivo*, because 25 mg/ml of 7S globulin is less protective than 2·5 mg/ml.

In protective activity against sub-lethal lung infection, however, the 19S and 7S globulins differed little (Fig. 4), the 19S being only slightly less active than the 7S.

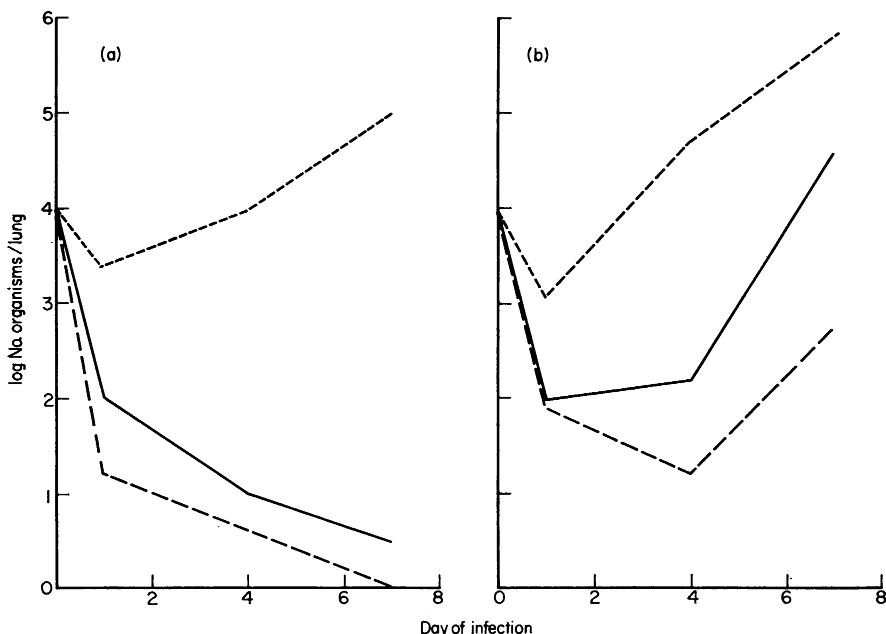

Fig. 4. The effect of 19S and 7S globulins (Serum I) on a sublethal lung infection. Average viable count of organisms in lung. Each point represents the average count for five mice. (a) 5 mg/ml, (b) 0·25 mg/ml. ——, 19S+organisms; – – –, 7S+organisms; - - - -, organisms only.

DISCUSSION

It is tempting to attribute *in vivo* protection by antisera to the antibody responsible for complement-mediated killing of the bacteria *in vitro*. The idea has been entertained since the early days of bacteriology, but so far no concrete evidence exists, because no one has produced a serum monospecific for the 'protective antigens' alone, with which bactericidal tests could be made.

Dolby and Vincent (1965) approached this problem by comparing the mouse-protective activity of antisera with other activities, using antisera raised against fractions of *B. pertussis* prepared in many different ways. The correlation of mouse-protective activity

with activity in the *in vitro* bactericidal test was good, but not so much better than its correlation with other antibody activities as to provide convincing proof of the identity of the protective and bactericidal antibodies.

This paper reports a further attempt to test this identity, by comparing 7S and 19S fractions of the antisera. The sera used were not monospecific, and no attempt was made to make them so by absorption and elution of the antibodies. Nevertheless, the results indicate an association of bactericidal activity *in vitro* with protective activity against intracerebral, but not intranasal, challenge.

Both 7S and 19S globulins are active at low concentrations in the bactericidal test and both inhibit this effect at high concentrations, but 7S globulin is over 100 times as effective as 19S. On the other hand, the anti-haemagglutinating activities of the two fractions are of the same order whereas the ratio of agglutinins varies with strain.

These results give no indication whether the inhibition of the bactericidal reaction is due to antibodies specific for antigens other than the antigen concerned in the bactericidal effect or to different forms of the bactericidal antibody. Similar inhibition by an excess of 7S antibody, but not by the corresponding 19S antibody, occurs in precipitin reactions with endotoxin (Pike, Schulze and Chandler, 1966) and with blood group A substance (Tada and Ishizaka, 1965) whereas Daguillard and Edsall (1968) found as we did that both 19S and 7S globulins inhibit the bactericidal reaction at high concentration. In agglutinin reactions no inhibition was found with five hundred bacterial agglutinating doses of 19S and 7S globulins; or in anti-haemagglutinin reactions with ten effective doses. There was, however, inhibition with high concentrations of 7S globulin in protection tests against an intracerebral challenge.

Most other workers have found that fewer molecules of 19S than of 7S antibody are needed for agglutination and lytic reactions, whether haemolytic or bactericidal [see the review by Pike (1967) and Daguillard and Edsall (1968)]. With *B. pertussis* antibodies, agglutination required rather less 7S than 19S globulin, whereas the bactericidal reaction required considerably less 7S than 19S. The previous workers dealt with antibodies to *Salmonella typhosa*, *Escherichia coli*, blood group antigens and endotoxin, and their work has thus been mainly concerned with antibodies to polysaccharide and lipopolysaccharide antigens. However, according to Heremans, Vaerman and Vaerman (1963), with antibodies to *Brucella suis* and to the protein antigen, diphtheria toxin, 7S antibodies are *more* active than the 19S as agglutinins. Possibly antibodies to antigens of different chemical classes give different results.

Heremans and his colleagues also found that the complement-fixing activity of the 7S fraction from patients with brucella infections was greater than that of the 19S. To the extent that the bactericidal activity is dependent on complement-fixation, this accords with our *B. pertussis* results. On the other hand, Borsos and Rapp (1965) suggested that the greater haemolytic activity of 19S globulin in their experiments with red cells resulted from the ability of a single molecule of 19S antibody to combine with its antigen at the cell surface and to fix a molecule of C′1a, whereas at least two molecules of 7S antibody in the correct orientation were needed. In consequence the binding of complement and lysis of the red cell require very much more than double the molar concentration of 7S antibody. Humphrey (1967) found, however, that in the presence of anti-IgG (7S) antibody complement is fixed and 7S antibody is as efficient as 19S at lysing red cells.

Borsos and Rapp (1965) also found that fixation of C′1a to *S. typhosa* and *E. coli* required one molecule of 19S antibody but at least two of 7S. Lesions very similar to those on the

membranes of red cells lysed by 19S antibody were found by Bladen, Evans and Mergenhagen (1966) in the cell membranes of *E. coli* treated with complement and an unfractionated hyperimmunized rabbit antiserum at a dilution of 1 : 10,000. In our experiments only 7S antibody was active at dilutions as high as this.

The lability of 19S antibody might explain its comparatively low protective activity against intracerebral infection in mice if it were inactivated during the 3-day period after injection, before it penetrates from the circulation to the brain tissues (Holt *et al.*, 1961). This explanation is unlikely since 19S globulin given at about the time when antibody leaks into the brain is still ineffective; nor are mice protected when the infecting organisms are treated with 19S globulin in concentrations at which 7S is effective.

Antibody of one specificity appears to be measured by the two passive protection tests in which infection is by the intracerebral route (J. M. Dolby, to be published): the 'IC mix' test, where antibody is also given intracerebrally, and that in which serum is given intraperitoneally and organisms intracerebrally [if mice vaccinated intraperitoneally are in fact protected against intracerebral challenge by circulating antibody (Wardlaw and Jakus, 1968)]. This same antibody is presumably measured in the potency assay of vaccines in actively immunized mice and is thus comparable with the antibody that protects children (M.R.C. Report, 1956). There is strong evidence from our results that the same antibody is measured by the *in vitro* bactericidal test.

The potency of vaccines assayed in intranasally infected mice, on the other hand, is not correlated with their protective power in children (M.R.C. Report, 1956), and our demonstration that 19S and 7S globulins are equipotent in overcoming lung infections indicates that a different mechanism is at work. Possibly an opsonin is involved, though some preliminary experiments show that approximately the same number of organisms are taken up by mouse peritoneal macrophages in the presence either of 19S or 7S antibody.

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