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Characterization of the Antibodies Responsible for the 'Bactericidal Activity Patterns' of Antisera to *Bordetella pertussis*

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Summary. Antisera to fractions of *Bordetella pertussis* have been investigated to determine the nature of the antibodies responsible for bactericidal activity and the cause of prozoning observed with some (type 2) sera. Bactericidal activity in both type 1 and type 2 sera tends to be correlated with the titre of agglutinins, anti-haemagglutinin and intracerebral protective antibody; it is not constantly associated with any other function. It is almost certainly not a property of anti-toxin, antibody to histamine sensitizing factor or intranasal protective antibody.

The inhibition zone of type 2 antisera appears to be due to an antibody present in the crude β-globulin fraction of antiserum. The antigen stimulating this antibody is probably a protein but is not the normal agglutinin, haemagglutinin or the histamine sensitizing factor.

INTRODUCTION

B. pertussis antisera are specifically bactericidal in the presence of complement and lysozyme. The antisera belong to one of two types each characterized by the range of complement and antiserum dilutions over which it is active (Dolby, 1965).

During chemical fractionation of *B. pertussis* cells, antisera against the fractions were routinely prepared. Nearly all were bactericidal and were typed according to the bactericidal activity patterns obtained at one concentration of complement. It is the purpose of this paper to determine whether these patterns bear any relationship to the known antibodies, and whether they are affected by the chemical nature of the antigen and the length of the course of immunization.

It has been supposed for some time that not all homologous antibodies, even when reacting with antigens at the surface of the cell are bactericidal (Schlemmer, 1920 quoted by Adler, 1953; Felix and Olitski, 1926; Dingle, Fothergill and Chandler, 1938; Adler, 1953). The lethal effect at the cell wall may be primarily due to the reaction of antibody with one antigen only in any given system of bacterial cells and antiserum, although it may be complicated by other antigen–antibody reactions (Wardlaw, 1963). The inhibition prozone characteristic of what we have called type 2 sera may be caused by another antigen–antibody reaction different from, but blocking, the bactericidal antigen (Thjøtta, 1920).

From the outset it was hoped that the antibody or antibodies capable of killing *B. pertussis in vitro* were the same as the antibody capable of protecting mice either against an intranasal or intracerebral challenge (Dolby and Standfast, 1958). The antibody protective against intracerebral challenge is presumably the one that protects children since

the child-protective antigen is almost certainly the one that protects mice against an intracerebral challenge (Standfast, 1958). If a correlation exists between a bactericidal effect *in vitro* and the antibody to the child-protective antigen a less cumbersome method of assay of pertussis vaccines than the mouse protective test of Kendrick, Eldering, Dixon and Misner (see U.S., N.I.H. (1948) Minimum Requirements) would be available.

METHODS

Bacterial Strains

Bordetella pertussis. The classical Kendrick strain 18-323 was used for intracerebral challenge of mice, for the bactericidal test and for routine agglutination tests. Strains 3865 and 3851 were two of Dr. Krag Andersen's phase 1 strains typed by her with respect to their agglutinogens. Strain GL-353 obtained from Dr. J. Ungar was passaged through mouse lungs at Elstree. It is rich in haemagglutinin and was used for assay of anti-haemagglutinin, and for intranasal challenge of mice. Strain 134, used for some antigen preparations was sent to us by Dr. H. Piersma; most of the antigen preparations were made from strains in the Elstree collection used for routine vaccine production.

B. bronchiseptica. Strains 95, 106 and 116 used for agglutination tests in the screening of rabbits for current bronchiseptica infections were obtained from Professor B. W. Lacey.

The Bactericidal Test

The test was carried out in $0{\cdot}6 \times 10$ cm. glass tubes plugged with rubber bungs with 0·2 ml. of each reagent. Antiserum dilutions in 1 per cent Casamino Acids (vitamin-free) ranging from 1/10 to 1/12,000 were added to a suspension of *B. pertussis* strain 18-323 made by harvesting growth from an 18 hour Bordet–Gengou plate at 37° in 1 per cent Casamino Acids and diluting to contain 5×10^6 organisms per ml. by opacity. A suitable dilution, usually 1/12–1/16 of guinea-pig serum, referred to as complement (collected and stored as in the previous paper) was added to each tube as the complement source and the contents rotated for 40 minutes at 37°. Estimation of viable count and expression of bactericidal activity are as described previously (Dolby, 1965).

Leucocyte Suspensions for Use in Bactericidal Test

Mouse peritoneal exudate was stimulated by injecting 1 ml. of a 5 per cent casein solution intraperitoneally. Four hours after injection the peritoneum was washed out with a tissue culture medium made by adding 3 g./l. of 'Panmede' (Paines & Byrne Ltd.) to Earle's saline. The white cells of the exudate were centrifuged down gently in siliconed tubes, washed twice and resuspended in tissue culture medium to a concentration two to five times that of the exudate. The preparation usually contained twice as many polymorphs as macrophages.

The bactericidal test involving leucocytes was carried out in siliconed tubes or Bijou bottles in which were mixed 0·2 ml. volumes each of antiserum dilution, organisms, complement and exudate suspension. Each sample was then centrifuged slowly to sediment the white cells. Viable pertussis cells were estimated in the supernatant fluid.

Preparation of Antisera

Rabbits were used. Relatively undegraded fractions were injected by the intravenous or intraperitoneal routes; purer fractions were given by the subcutaneous route. The importance of using *B. bronchiseptica* free rabbits has already been stressed (Dolby, 1965).

Definition of Serum Types

With complement of about 1/40 final dilution, each serum diluted over a wide range produced characteristic bactericidal activity:

Type 1—sera with bactericidal activity at dilutions of up to 1/300 or 1/900 but with maximum activity at 1/300, and no inhibition zone.

Type 2—sera with bactericidal activity at 1/1000 or more and with an inhibition zone at higher concentrations (Dolby, 1965).

Antitoxin Determinations

These were carried out as described by Dolby and Standfast (1958).

Agglutinations

Tests were carried out in Dreyer tubes for 4 hours at 37° and read after standing at room temperature overnight. Suspensions were made by harvesting from overnight Bordet–Gengou plates, into saline for *B. pertussis* strains or from nutrient agar plates into 0·4 per cent formol saline for *B. bronchiseptica* strains and adjusting to 5000×10^6 organisms per ml. by opacity.

Antihaemagglutination Test

Strain GL-353 was harvested from 18 hour Bordet–Gengou plates incubated at 37° to give a suspension of 10×10^9 organisms per ml. in saline. One minimal haemagglutinating dose of suspension was estimated by titrating in doubling dilutions in saline with equal volumes of washed sheep red cell suspension in saline (at a concentration of cells from 0·5 ml. blood resuspended in a final volume of 20 ml. saline). Tubes were incubated at 37° for 1½ hours and read. One haemagglutinating dose was usually about 2×10^9 cells per ml. The bulk of bacterial suspension was then adjusted to slightly more than one haemagglutinating dose, i.e. usually 3×10^9 bacterial cells per ml.

To assay the antihaemagglutinin content of the antisera one volume of this bacterial suspension was added to one volume of serial three-fold dilution of antisera. The tubes were shaken and left for 10 minutes at room temperature. One volume of red cell suspension was then added and the tubes were incubated at 37° for 1½ hours and read immediately.

Antihistamine Sensitizing Factor Assay

This was carried out by determining if antisera could prevent mice from becoming sensitized to histamine resulting from an injection of *B. pertussis* suspension (Dolby, 1958).

The LD50 of histamine in treated and untreated mice was obtained by the method of Reed and Muench (1938). The LD50 of histamine in mice sensitized with vaccine was usually less than 1·0 mg. histamine; serum-protected mice often survived 16 mg.

Passive Protection Tests

The antibody that protects mice against an intranasal challenge of *B. pertussis* was assayed by mixing three-fold dilutions of serum with an equal volume of a suspension of strain GL-353 organisms harvested from overnight, 37° plates into 1 per cent Casamino Acids, containing 200×10^6 organisms per 0·04 ml. Two drops of the mixture from a 50 dropping pipette were instilled into the noses of anaesthetized mice. The percentage survival with the serum at the dilution giving the most protection was calculated 28 days after infection. The antibody that protects mice against an intracerebral challenge of *B.*

pertussis was measured by injecting 0·2 ml. of two-fold dilutions of antiserum intraperitoneally into mice and challenging intracerebrally 3–4 hours later with 50,000 organisms of strain 18-323 in 1 per cent Casamino Acids. The percentage surviving at the most protective level was calculated 14 days after infection. Both challenges contained about 100 LD50.

RESULTS

EFFECT OF ANTIGEN AND NUMBER OF INJECTIONS ON THE BACTERICIDAL ACTIVITY PATTERNS OF SERA

Effect of Length of Immunization Course

Fig. 1 shows the effect of the number of injections on the activity patterns of rabbit sera when a complex mixture of pertussis antigens exemplified by whole cells was injected intravenously. After one injection a type 1 serum was produced (Fig. 1a), after five injections a serum that had an inhibition zone up to 1/300 (Fig. 1b), and after twelve injections a serum with a very strong inhibition prozone typical of type 2 (Fig. 1c). A partially purified fraction of *B. pertussis* (but still a complex mixture of antigens), the F 68 of Cruickshank and Freeman (1937) similarly induced sera varying from type 1 after one injection to a type 2 after nine to fifteen injections (Fig. 3a). Many antigens behaved similarly and repeated immunization nearly always produced type 2 sera.

Effect of the Qualitative and Quantitative Differences in Antibody

Experiments have already been described (Dolby, 1965) which showed that (i) the β-globulin fraction, with which antibody is associated after repeated immunization, had a stronger inhibition zone than γ-globulin antibody which is produced early; (ii) a sample of Cutter γ-globulin had bactericidal activity up to 1/12000 dilution without the inhibition zone. The inhibition zone, the bactericidal activity at very high serum dilutions, or both could therefore depend upon the distribution of antibodies in different serum proteins.

Another possibility is that the bactericidal activity pattern is influenced by the *amount* of antibody present. Some antigen preparations, e.g. F 68, certainly produced type 2 antisera much more quickly than other preparations; but we had no means of knowing whether, in comparing development of such activity in antisera to two different antigens, we were witnessing a response to a similar fraction in different states of antigenicity due to chemical treatment, or to different amounts of antibody *per se*.

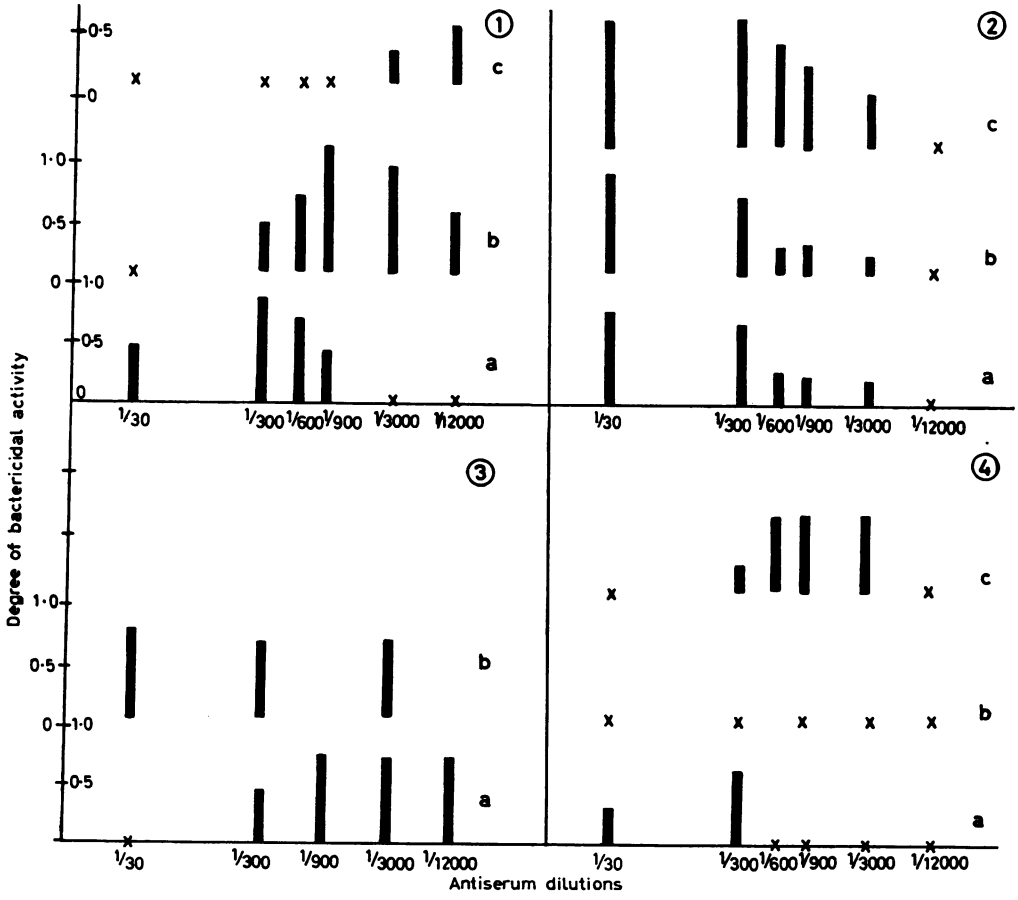
Antiserum to one antigen preparation, however, the 'Boivin antigen', did not produce a type 2 serum even after twenty-one injections. The serum remained strong type 1 (Fig. 2). That this antigen was a poor one and the antibody response therefore weak seemed unlikely since this antiserum could overcome the inhibitory zone of a typical type 2 serum when the two were mixed.

The 'Inhibition Zone Antigen'

The chemical nature of the antigen may influence the development of the inhibitory zone. From the results so far obtained it seems probable that exhibition of this zone by an antiserum is dependent on immunization with a protein-like antigen. The fraction F 68 prepared by tryptic digestion produced an inhibitory zone after five injections (Fig. 3a). Treatment of the antigen with trichloracetic acid to precipitate proteins reduced the ability of F 68 to produce antisera with inhibition zones (Fig. 3b).

Another protein-like antigen 'V' prepared from a methanol precipitate of a water extract of broken *B. pertussis* cells and then fractionated on a DEAE cellulose column, produced a weak type 2 serum after two injections (Fig. 4a), but after eleven injections the serum was so inhibitory that no bactericidal activity was recorded (Fig. 4b). Treatment of the antigen with trypsin rendered it far less capable of producing such strong inhibitory zones (Fig. 4c).

It can be seen from Table 1 that the inhibition zone cannot be associated with the presence or absence of antihistamine sensitizing factor, agglutinins, antihaemagglutinins or protective antibodies.



FIGS. 1–4. The influence on the bactericidal activity pattern of *B. pertussis* rabbit antisera of the length of immunization course and the nature of the antigen injected intravenously or intraperitoneally.

FIG. 1. Whole cells: (a) one injection, (b) five injections, (c) twenty-one injections (two rabbits).

FIG. 2. Boivin antigen: (a) six injections, (b) ten injections, (c) twenty-one injections (one rabbit).

FIG. 3. Five injections of F68: (a) untreated (two rabbits), (b) treated with trichloracetic acid (one rabbit).

FIG. 4. Fraction V: (a) two injections, (b) eleven injections, (c) eleven injections of trypsinized V (one rabbit each).

The bactericidal activity is expressed as $\log_{10}$ decrease in numbers of viable bacilli in the test mixtures. × represents no activity of sera tested at the corresponding dilution.

TABLE 1

LACK OF CORRELATION OF INHIBITION ZONE OF TYPE 2 RABBIT SERA WITH OTHER ANTIBODIES

Antigen	*Serum No.*	*Inhibitory zone*	*Protective antibodies*		*Antihaem-agglutinin*	*Agglutinin*	*Anti-HSF*
			Intracerebral	*Intranasal*			
V	G 435	+	+	+	Trace	±	+
Mickle disintegrate SN	G 602	++	±	+	−	+	Trace
Stroma adsorbed antigen	B 821	+	−	±	N.D.	−	N.D.
V trypsinized	G 610	−	+	+	±	+	+
Arcton methanol ppt.	G 516	−	+	+	+	+	+
Sodium lauryl sulphate lysate methanol SN	G 569	±	+	+	Trace	+	−

COMPARISON OF BACTERICIDAL ACTIVITY WITH OTHER ANTIBODY FUNCTIONS

Antitoxin

Most of the sera used in these experiments were produced by injecting fractions of bacterial cells, and were free of antitoxin. Five sera were checked for antitoxin and all found to be negative, i.e. no mice survived when given five toxic doses mixed with undiluted serum. Of these five sera two were of type 1 and three of type 2. Conversely, a serum with a high antitoxin titre had bactericidal activity, similar to that of an antiserum without antitoxin. Antitoxin therefore is not obviously connected with the bactericidal property of sera.

Agglutinins, Antihaemagglutinins, Antihistamine Sensitizing Factor and Protective Antibodies

The 150 or so sera used for this analysis were prepared by injecting fractions collected during procedures designed to separate the known antigens from each other. Titres to the known antibodies in this heterogeneous collection of antisera were recorded on punch cards together with the bactericidal activity patterns. The cards were sorted to determine whether any correlation existed between agglutinating, antihaemagglutinating, antihistamine sensitizing or protective antibodies and the bactericidal activity.

TABLE 2

AGGLUTININS AND BACTERICIDAL ACTIVITY OF *B. pertussis* RABBIT ANTISERA

Bactericidal activity strongest at antiserum dilution	*No. of sera with agglutinin titres to strain* 18-323 *of:*		
	<1/100	1/100–1/2000	>1/4000
1/30	6	16	0
1/300	3	14	3
1/300–1/3000	1	18	7
1/3000 or more	1	6	34

The sera were both short and long course but a late bleeding serum was recorded only when it was different from an early bleeding from the same animal. The high titres of type 2 sera are of course likely to be correlated with high titres of any of the various antibodies since the greater the number of vaccine injections given, the higher will be antibody titres to all the contained antigens including impurities. For this reason, only lack of correlation

between properties is therefore of any definite value. Positive correlations can only indicate tendencies. The results of the punch card analysis are recorded in Tables 2–6.

There is a positive correlation between high agglutinin titres and a type 2 serum pattern (Table 2); thirty-four out of forty-one type 2 sera were moderate or strongly agglutinating whereas only three out of forty-two type 1 sera had agglutinin titres of over 1/4000. Routine agglutinations were carried out with strain 18-323 which has main agglutinogen 1 (common to all *B. pertussis*) exposed. Some of the anomalous sera were checked with either typed or homologous strains to determine if the results could be due to the degree of correlation of only one agglutinogen. The grouping, however, remained unchanged.

TABLE 3

ANTIHAEMAGGLUTININS AND BACTERICIDAL ACTIVITY OF *B. pertussis* RABBIT ANTISERA

Bactericidal activity strongest at antiserum dilution	*No. of sera with antihaemagglutinin titres of:*		
	<1/3	1/3–1/9	>1/9
1/30	14	2	0
1/300	15	0	1
1/300–1/3000	6	9	4
1/3000 or more	9	5	12

There is only a tendency to a positive correlation between antihaemagglutinins and type 2 sera (Table 3). Of twenty-six type 2 sera, only twelve had moderate or good antibody titres and nine type 2 sera were negative. Correlation at the other end of the scale was better; only three out of thirty-two type 1 sera were positive. Correlation of a similar degree between the intracerebral protective antibody and type 2 bactericidal activity patterns is seen in Table 4.

TABLE 4

INTRACEREBRAL PROTECTIVE ANTIBODY AND BACTERICIDAL ACTIVITY OF *B. pertussis* RABBIT ANTISERA

Bactericidal activity strongest at antisera dilution	*No. of sera protecting against* 200 LD_{50} *by:*		
	<10 *per cent*	10–30 *per cent*	>40 *per cent*
1/30	22	0	3
1/300	18	1	3
1/300–1/3000	9	4	14
1/3000 or more	7	10	26

Complete lack of correlation between any bactericidal activity and intranasal protective antibody and antihistamine sensitizing factor is shown in Tables 5 and 6 respectively. As negative correlations these results are therefore valid.

The three groups of sera analysed in Tables 2–4 were then further examined to determine whether information could be obtained on the absolute correlation or not of agglutinins, antihaemagglutinins, protective intracerebral antibody and bactericidal patterns by considering the individual sera, which were exceptions to this and trying to determine

if there were any circumstance which could be responsible for the exception other than that the antibody and bactericidal activity were unrelated. Leaving out all the intermediate lack of correlations and borderline cases the chief exceptions were four not correlating on agglutinin titres, ten on antihaemagglutinin titres and twelve on intracerebral protective antibody.

TABLE 5

INTRANASAL PROTECTIVE ANTIBODY AND BACTERICIDAL ACTIVITY OF *B. pertussis* RABBIT ANTISERA

Bactericidal activity strongest at antiserum dilution	*No. of sera protecting against 200 LD_{50} by:*		
	<10 per cent	*10–30 per cent*	*>40 per cent*
1/30	6	2	11
1/300	8	2	10
1/300–1/3000	6	4	6
1/3000 or more	5	3	23

TABLE 6

ANTIHISTAMINE SENSITIZING FACTOR AND BACTERICIDAL ACTIVITY OF *B. pertussis* RABBIT ANTISERA

Bactericidal activity strongest at antiserum dilution	*No. of sera (0·5 ml. dose) protecting sensitized mice against LD_{50} histamine of:*		
	<1 mg. histamine	*1–4 mg. histamine*	*4–16 mg. histamine*
1/30	5	3	1
1/300	8	2	1
1/300–1/3000	8	0	1
1/3000 or more	11	3	4

With respect to agglutinins, two short course antisera had agglutinin titres of less than 200 but with type 2 bactericidal patterns. These two antigenic preparations were very weak in agglutinogen, as repeated injections showed. Both these sera occurred again in the twelve exceptions with respect to intracerebral antibody but continued injections produced protective sera so that in this instance the lack of correlation was due to insufficient immunization for the less sensitive test to register as positive. A type 2 serum with an agglutinating titre of 1/10,000 was absorbed with cell free agglutinogen (Smolens and Mudd, 1943) to produce the third exception in this group. The absorbed serum had an agglutinin titre of 1/160 yet the bactericidal pattern was identical with that of unabsorbed serum. The fourth serum had a high agglutinating titre but its lack of bactericidal activity was due to a very strong inhibition zone (serum anti-V, Fig. 4b). This group therefore presents only one antiserum which does not fit the assumption that agglutinins and bactericidal activity are related—namely the absorbed serum.

Ten sera had type 2 bactericidal activity but were without antihaemagglutinins. The sera were both short and long course and produced in response to various antigenic fractions obtained by a variety of methods. There were no sera with high antihaemagglutinin activity but not type 2.

Ten sera also had type 2 bactericidal activity without being protective—again a heterogeneous batch of antigens were used and the sera were short and long course. At the other end of the scale two sera were protective whilst having little or no bactericidal activity. One of these was the antiserum to V with inhibition zone; the other was a serum to a similar protein fraction but no inhibition zone was demonstrable. It had very weak bactericidal activity at 1/30 serum dilution and protected 60 per cent of mice.

Opsonins

Cells of *B. pertussis* incubated with a leucocyte suspension are phagocytosed readily whether living or heat killed and whether normal or anti-whole cell serum is used in the mixture.

Whereas the major role of antibody, *in vitro* at least, appears to be a direct killing of bacteria which may then be phagocytosed and disposed of, it is possible that dilutions of antisera in the bactericidal or non-bactericidal range are capable of acting as opsonins. Experiments similar to those of Maaløe (1946) were carried out. Our findings with type 1 antisera were similar to his for antisera to *Salmonella typhi*. Over a wide range of dilutions of antiserum with complement the numbers of viable bacteria were the same in the presence and absence of leucocytes, making allowance for the very small decrease due to phagocytosis without antibody.

With a type 2 serum, however, the presence of leucocyte suspension reduced the inhibitory zone so that the serum was active at 1/300 serum dilution. At the usual bactericidal serum dilutions the numbers of organisms killed was much greater in the presence of peritoneal leucocytes. These results bore a striking similarity to those achieved by adding more guinea-pig serum or other tissue homogenates (Dolby, 1965). The effect could also be produced by increasing the concentration of lysozyme. A comparison of these is made in Table 7.

Further experiments with type 2 serum and leucocytes smashed by shaking with glass beads showed an even greater reduction in inhibition zone and increase of bactericidal activity than with live ones. The action is thus independent of phagocytosis.

TABLE 7

COMPARISON OF THE EFFECT OF LYSOZYME, LEUCOCYTE SUSPENSION AND HOMOGENIZED MOUSE TISSUE ON THE BACTERICIDAL PATTERN OF TYPE 2 RABBIT ANTISERA AGAINST 18-323

Deviations from usual procedure by adding or omitting constituents	*Activity*						
	1/30	1/300	1/600	1/900	1/3000	1/9000	1/27000
None	0	0	0	0	0·6	1·0	0·4
Lysozyme removed from antiserum and complement by bentonite	0·1	0·2	0·1	0·1	0·2	0·1	0
0·8 μg./tube of lysozyme added	0	0	0	0·1	0·4	0·9	0
5 μg./tube of lysozyme added	0	0	0·3	0·3	0·8	0·4	0·4
50 μg./tube of lysozyme added	0	0	0·3	0·5	0·6	0·4	0·4
'Pan' tissue culture medium added	0	0		0·3	0·6	0·8	0
Leucocyte suspension in 'pan' medium added	0	1·3		2·6	2·6	1·1	0
Brain homogenate added	0·2	0·5		0·8	1·3	0·9	

DISCUSSION

Agglutinins, antihaemagglutinin and the intracerebral protective antibody all showed some correlation with type 2 bactericidal pattern.

It could be shown from individual sera however, that for all these antibodies there was at least one example of clear-cut absence of correlation. The tendency to correlation of two properties within a large group may, of course be due to close association of the antigens, say the agglutinogens and haemagglutinin with the protective antigen. It might be mentioned that Dingle, Fothergill and Chandler (1938) working with *H. influenzae* and Landy, Michael and Whitby (1962) using a variety of Gram-negative organisms, were not able to correlate bactericidal antibody with agglutinins or precipitins.

A complete correlation of intracerebral protective mouse antibody and bactericidal serum type 2 would be a satisfying one. In the passive or active protection tests, an antibody that is active at 1/3000 dilution or more is likely to be active after intraperitoneal injection, dilution in the circulation of a mouse, and leakage through the blood brain barrier 3 days after infection (Dolby and Standfast, 1961).

Antitoxin and antihistamine sensitizing factors could not be correlated in any way with the bactericidal activity patterns. The intranasally protecting antibody also appeared not to have any direct correlation with activity, which is surprising in view of the immediate drop in viable count of organisms instilled into an immune lung (Dolby, Thow and Standfast, 1961).

The nature of the inhibition zone of type 2 sera is still in doubt. Chemical treatment of the antigen used for producing the antiserum, and length of course of immunization influenced the activity pattern with respect to this zone, indicating both that a particular antigen and localization of antibody in the one antibody fraction may be involved (Dolby, 1965).

Analysis of all our antiserum bactericidal patterns supports the conclusions of Thjøtta (1920) and Adler (1953) that the inhibition zone is due to another antigen–antibody reaction, which is not bactericidal but blocks the bactericidal activity. Either this 'inhibitory' antigen is closely associated with the type 2 bactericidal antigen, or it is an impurity in all preparations which produces circulating antibody only after more than four or five injections.

Purer antigen preparations and fractionation of antibodies at intervals during immunization are needed to determine which factors are responsible for the bactericidal activities and inhibition zone. Difficulties have been experienced in this because of the absolute requirement of rabbits free from *B. bronchiseptica* infection for such quantitative and qualitative work.

Results with the antiserum to Boivin antigen not only in not having an inhibition zone but in overcoming the inhibition zone, complicate matters further. If it is postulated that the inhibition prozone is due to the presence of an antibody different from the bactericidal antibody and preventing union of the bactericidal antibody with its antigen spatially or because of deviation of complement, etc., then it seems necessary to postulate the presence in the Boivin antiserum of another antibody which, by combining with its antigen, can remove this blocking affect on bactericidal antigen–antibody reaction. These reactions at the surface of the bacterial cell are obviously complex and we hope to investigate these further with purer antigen preparations and their antisera.

Antibody in the 19S macroglobulin fraction of antisera has been implicated in the bactericidal reaction (Rosen, 1962). In passive immune lysis of red blood cells

$7S\gamma_1$ antibody has been shown to block the lytic activity of $7S\gamma_2$ (Kourilsky, Bloch, Benacerraf and Ovary, 1963). A similar phenomenon may occur in antisera reacting on the surface of a bacterial cell, a small molecular weight antibody blocking a larger molecular weight bactericidal one. The results of our crude antisera fractionations are not unopposed to these conclusions; further purification of rabbit antibodies is being carried out. The observation that endotoxin inhibits the formation of $7S\gamma$ antibodies (LoSpalluto, Miller, Dorward and Fink, 1962) is interesting in view of our rabbit antiserum to Boivin antigen being without an inhibition zone (Fig. 2) and supports the hypothesis that both nature of antigen and type of antibody are important in defining bactericidal activity patterns.

Since the inhibition zone of type 2 serum can be overcome by increasing complement or lysozyme or adding mouse tissue homogenates, or mouse leucocyte suspension or homogenate, the blocking effect on the bactericidal antigen and antibody may only be effective under conditions in which certain factors are limiting.

Contrary to the results of Kendrick, Gibbs and Sprick (1937) and subsequent workers, our evidence for an opsonizing action of either type of serum is negligible. The effect of leucocyte suspensions in narrowing the inhibition zone of type 2 sera could be attributable to limiting factor(s) such as complement or lysozyme contained in the phagocytes (Cohn and Hirsch, 1960).

Although there is abundant lysozyme in alveolar macrophages, Mryvik, Leake and Fariss (1961) reported that, in rabbits at least, peritoneal macrophages were poor in lysozyme. Phagocytin (Hirsch, 1960a) and perhaps other non-specific antibacterial factors (Hirsch, 1960b) may also be implicated.

The bactericidal reaction is a sensitive test of antigen–antibody reaction but with *B. pertussis* the presence of the inhibition zone complicates its use in titration of antisera (Muschel and Treffers, 1956; Nagington, 1956; Osawa and Muschel, 1960; Landy *et al.*, 1962). The stipulation of these other workers that the titrations must be carried out under conditions in which the limiting factor is the antiserum only may be more difficult to fulfil with *B. pertussis* than with *S. typhi*. Increased information about the inhibition zone might help to solve this difficulty.

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