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The Antibacterial Effect of *Bordetella pertussis* Antisera

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Summary. Antisera to *Bordetella pertussis* are bactericidal for some strains of *B. pertussis* in the presence of complement and lysozyme. Phase I bacilli, virulent for mice by the intracerebral and intranasal routes, are sensitive to the bactericidal effect; most mouse avirulent strains are not.

Most of the bactericidal antisera belong to one of two types according to the concentration of antibody and complement that are optimally lethal. Type 1 antisera are bactericidal in the range 1/30 to 1/900 with guinea-pig serum as the complement source at 1/18 to 1/100. Type 2 antisera are characterized by a zone without bactericidal activity over similar antiserum and complement dilutions. Their bactericidal activity is made evident by diluting the antiserum further, by increasing the amount of complement, or by adding tissue homogenates, body fluids or crystalline lysozyme. The two types are further characterized by the differing requirements of the bactericidal system for the individual components of haemolytic complement.

The combined lethal action of antibody, complement and lysozyme is rapid. In the early stages it is partly inhibited by substances in the medium used for estimating viable counts indicating that in its initial stages the reaction is bacteriostatic rather than bactericidal.

INTRODUCTION

Mice can be protected passively against experimental *Bordetella pertussis* infections of the brain and lung. Some antisera are effective against both intracerebral and intranasal infections but it is possible, with certain fractions of the bacilli as antigens, to produce antisera active against either lung or brain infections (Dolby and Standfast, 1958). The antiserum protecting against an intranasal challenge has an immediate bactericidal effect on the organisms in the lung, causing a rapid fall in the numbers of viable organisms. This bactericidal action is demonstrable *in vitro* in the presence of fresh guinea-pig serum. Under similar conditions the antiserum protecting mice against an intracerebral challenge, although bactericidal *in vivo* (Dolby and Standfast, 1961) fails to kill the bacilli *in vitro* (Dolby, Thow and Standfast, 1961).

Increased phagocytosis of *B. pertussis* in the presence of antibody was reported by Kendrick, Gibbs and Sprick (1937). We investigated the possibility that the rapid decrease in the numbers of bacteria in the brains of protected mice was due to the action of opsonin, and discovered that peritoneal exudate without leucocytes, together with the intracerebrally protecting antibody rapidly killed the bacilli *in vitro*.

The examination of a number of pertussis antisera in the light of these findings, as related below, revealed that most of them are bactericidal *in vitro* for a serum-sensitive

strain of *B. pertussis*, but that the intracerebrally protecting sera tend to be active over a very different range of antibody dilutions from other sera. The reaction involves specific antibody, a heat-labile element of fresh guinea-pig serum referred to below as complement, and lysozyme. It is therefore a bactericidal system similar to that described previously for other Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial Strains

Most of the work was done with the well-known American mouse-virulent strain 18-323, from Dr. P. Kendrick. Other mouse virulent strains used were GL-353, originally from Dr. J. Ungar of Glaxo Research Ltd. and passaged at Elstree through mouse lungs; and 2-atox from Dr. E. Krag Andersen in Copenhagen; strains 1508, 1510, 1512, 1557, 1572, 1574, 1579, 1583 and 1622 from Belgrade children at various known stages of infection and two Belgrade vaccine strains C2-219 and 517 both mouse avirulent, were from Dr. V. Spasojevic; and L84, M6344 and G1101 were strains in the Elstree collection, all of low virulence.

The strains were grown on Bordet–Gengou solid medium and the growth was harvested after 18–20 hours and suspended in 1 per cent Casamino Acids (Vitamin free, Difco).

Bordetella bronchiseptica strains were obtained from Professor B. W. Lacey (strains 106, 116) and from the National Collection of Type Cultures (8762). *Haemophilus aegyptius* strain 181 was from Dr. M. Pittman (Bethesda). These two species were grown on nutrient agar and Fildes agar respectively and harvested into 1 per cent Casamino Acids.

Media

The Bordet–Gengou plates containing 33 per cent blood used for growing *B. pertussis* were at first also used for viable count determinations of the bactericidal mixtures. The results with successive batches of medium were very often inconsistent, hitherto lethal mixtures of complement and antiserum proving with a certain batch of medium, to be non-lethal. This effect could not be attributed solely to the blood or basic medium used; extensive investigation implicated both blood and temperature of plate-pouring as possible co-ordinated variables but no satisfactory demonstration of the responsible factors proved to be possible.

Cohen and Wheeler blood plates were made with Cohen and Wheeler's (1946) medium modified by decreasing peptone to 0·1 per cent, increasing glutamic acid to 0·5 per cent and adding 5 per cent blood. They gave reasonably consistent viable counts and were used for most of the estimations.

Antisera

These were made in rabbits by injecting whole bacterial cells or fractions intraperitoneally, intravenously or sub-cutaneously, usually over a 6–8 weeks course.

Since *B. bronchiseptica* is a natural pathogen of rabbits and shares antigens with *B. pertussis*, only rabbits proved to be free of *B. bronchiseptica* agglutinins were used for immunization. The possibilities that the rabbits may have been infected in the past so that any common antigen would stimulate a secondary response or that the rabbits were infected during immunization were covered by testing sera from several rabbits individually or as pools.

Complement

Female guinea-pigs of 250 g. weight were bled out under coal gas and ether anaesthesia. The blood was allowed to clot for 10 minutes at room temperature and held overnight at 4°. The serum was collected after centrifugation for 30 minutes at 4° and stored at −15°. Guinea-pig serum from the Venereal Diseases Reference Laboratory, London Hospital, Whitechapel and preserved by the method of Richardson (1941) was used in some experiments. Throughout, these materials are referred to as complement.

Two non-specific reactions often occurred when concentrated complement (i.e. a final concentration of 1/12 or more) was used in the bactericidal test described below. The first was a bactericidal effect in the absence of rabbit antibody and could be abolished by adding sodium citrate to a concentration of 2·4 per cent to the guinea-pig serum producing a final concentration of 0·8 per cent in the reaction mixture. This actually increased the specific bactericidal activity of the mixture of antiserum and complement above that obtained without citrate. The citrate ions presumably combined with the Mg^{2+} necessary for eliciting the 'properdin' effect of Wardlaw and Pillemer (1956). Citrate concentrations above 1 per cent decreased the bactericidal effect of antibody and complement. The second non-specific effect was occasionally observed even with citrated complement, which was still bactericidal in the absence of rabbit antibody. This activity was due to *B. bronchiseptica* antibody in the guinea-pig serum and could be absorbed out with *B. pertussis*. Such complement was not used in routine tests.

Fractionation of complement into preparations containing the major components was carried out according to Kabat and Mayer (1948) for C'1, C'2, R3, R4 and RP. A sample of Zymosan Fleischman (kindly supplied by the late Professor Pillemer) was used in the preparation of RP. Heat inactivation of C'1 and C'2 was carried out in a water bath at 56° for 20 minutes.

Haemolytic Complement Titrations

Citrated sheep blood was washed three times in saline and the red cells from 0·5 ml. of whole blood suspended in 20 ml. saline. One volume of haemolysin 1/500 was mixed with one volume of red cell suspension and 0·2 ml. of the mixture added to 0·2 ml. of dilution of sample being assayed. Tubes were read for complete lysis by inspection after 1 hour at 37°.

Measurement of the Bactericidal Activity

Two tenths of a ml. of the following components in 1 per cent Casamino acids were put, in the order given, into 0·6 × 10 cm. glass tubes (final dilutions are recorded): (i) antiserum heated 56° for 30 minutes, diluted 1/30 to 1/48,000; (ii) suspension of *B. pertussis* 5×10^9 or 5×10^6 organisms per ml.; (iii) complement diluted 1/3–1/150 or in routine tests, at one dilution of between 1/30 and 1/60 depending on the strength of the guinea-pig serum complement.

Controls were included of suspension only in Casamino acids and suspension plus guinea-pig serum.

Bacterial cells first sensitized with antiserum, washed, resuspended in fresh diluent and then added to complement gave the same result but this was not done in routine tests.

The tubes were stoppered with rubber bungs (siliconed plugs gave a similar result) and incubated at 37° rotating on a gramophone turntable at an angle of 45°. After 40 minutes the contents of each tube were diluted 1/10, 1/100 and 1/1000 in 1 per cent Casamino Acids containing 7·7 per cent w./v. NaCl which was added to stop the reaction

by its anticomplementary action. Four drops of each dilution were pipetted onto the surface of Cohen and Wheeler blood plates with pipettes calibrated to deliver 0·02 ml. (Miles and Misra, 1938).

The reaction was usually complete in under 10 minutes; it did not progress with further incubation up to 5 hours.

Bactericidal Activity of Antisera

This was expressed as $\log_{10}$ ratio of mean viable counts in control tubes to mean viable count in the bactericidal mixture. Thus a log ratio of 0·0 indicates no bactericidal action; a ratio of 1·0 a ten-fold decrease in viable count, and so forth.

Collection of Brains and Lungs

In some of the experiments the bactericidal test was carried out in the presence of mouse tissue homogenates. Brains or lungs were taken from mice immediately after killing with coal gas and homogenized in Universal bottles with 2 ml. glass beads 4–5 mm. diameter for 3 minutes on a vertical shaker (throw $2\frac{3}{4}$ inch, 325 rev./min.). The resulting material was cleared of large pieces of tissue by centrifugation for about 1 minute at 2000 rev./min. at room temperature and added immediately to the other components of the bactericidal system.

In other experiments one lung or brain was homogenized in about 2 ml. of the bactericidal mixture with glass beads in universal bottles as above.

Preparation of Lysozyme-Deficient Serum and Lysozyme Assay

Lysozyme was removed from fresh guinea-pig serum and from heated rabbit antiserum by absorption for 15 minutes at 4° with 4–5 mg./ml. bentonite (Hopkin and Williams) that had been repeatedly washed in distilled water until the pH of the washings was 7·5; the bentonite was removed by centrifugation for 45 minutes at 4000 rev./min. at 0° (Wardlaw, 1962).

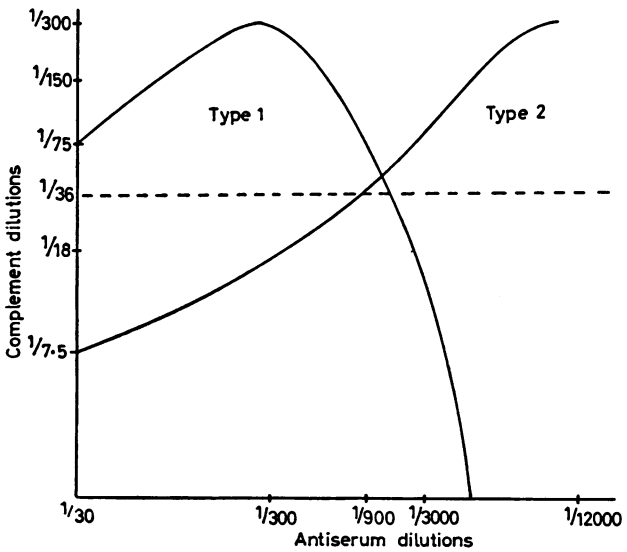

FIG. 1. The bactericidal activity of *B. pertussis* antisera produced in rabbits and tested against strain 18-323. Bactericidal activity of 0·5 or more plotted as a point on the graph.

Lysozyme was estimated by its ability to lyse completely (as read by the naked eye) a culture of *Micrococcus lysodeikticus* harvested from a beef extract, 10 per cent yeast extract, peptone and agar medium and suspended in Sorensen's buffer, pH 6·2, at an opacity equal to 20 international opacity units per ml. The test was carried out in Dreyer tubes with 0·4 ml. volumes each of suspension and suitable dilution of the sample being assayed. The mixtures were incubated in a water bath at 37° for 2 hours and read immediately. Crystalline lysozyme in a standard solution was included as the control.

TABLE 1

THE BACTERICIDAL ACTIVITY* CHARACTERIZING TYPE 1 SERUM

Rabbit antiserum dilution	*Guinea-pig serum dilution*					
	1/7·5	1/18	1/36	1/75	1/150	1/300
1/30	2·4	1·2	1·0	0·8	0·2	0
1/300	0·6	1·4	1·2	0·9	0·8	0·5
1/900	0·4	1·0	0·8	0·5	0·3	0
1/3000	0	0·3	0·3	0	0	0
1/12000	0	0·2	0·1	0	0	0

* Activity in this table and all subsequent tables expressed as $\log_{10}$ decrease in numbers of viable bacilli in the test mixtures.

TABLE 2

THE BACTERICIDAL ACTIVITY CHARACTERIZING A TYPE 2 SERUM

Rabbit antiserum dilution	*Guinea-pig serum dilution*					
	1/7·5	1/18	1/36	1/75	1/150	1/300
1/30	1·2	0	0	0	0	0
1/300	2·3	0·5	0·2	0	0	0
1/900	1·8	1·0	0·6	0·1	0	0
1/3000	1·0	1·5	0·8	0·8	0·4	0
1/12000	0	0·7	0·6	0·6	0·8	1·4

RESULTS

BACTERICIDAL ACTIVITY PATTERNS OF *B. pertussis* ANTISERA

Over 300 *B. pertussis* antisera were tested; most of them had some bactericidal activity but the conditions of their optimal activity were by no means similar. Most of them belonged to one of two types, designated 1 and 2, characterized by the antiserum dilutions over which bactericidal activities occurred. Tables 1 and 2 exemplify bactericidal activities over a wide range of antiserum and complement dilutions which characterize types 1 and 2 sera respectively. The figures from these tables are expressed graphically in Fig. 1 by plotting the highest dilution of complement giving an activity of 0·5 or more with each dilution of antiserum.

Type 1 sera are characterized by strong bactericidal activity with strong antiserum and complement, the activity being maintained on moderate dilution falling off with high dilutions of both. Type 2 sera are active at very high serum dilutions and exhibit a zone in

which antiserum dilutions from 1/10 up to 1/1000 or so are inactive unless very strong complement is available. The weaker the complement, the greater must be the antiserum dilution before bactericidal activity results.

Not all sera were tested as fully as those exemplified in Tables 1 and 2. As a routine one level of guinea-pig serum between 1/30 and 1/60 was used. The amount was determined by titrating over this range with standard types 1 and 2 antisera so that activities similar to 1/36 in Fig. 1 were obtained. The degree of bactericidal activity over a range of antiserum dilutions with this amount of complement is plotted with typical types 1 and 2 sera (Fig. 2). The typical curve for each type of serum has been designated 'the bactericidal activity pattern'.

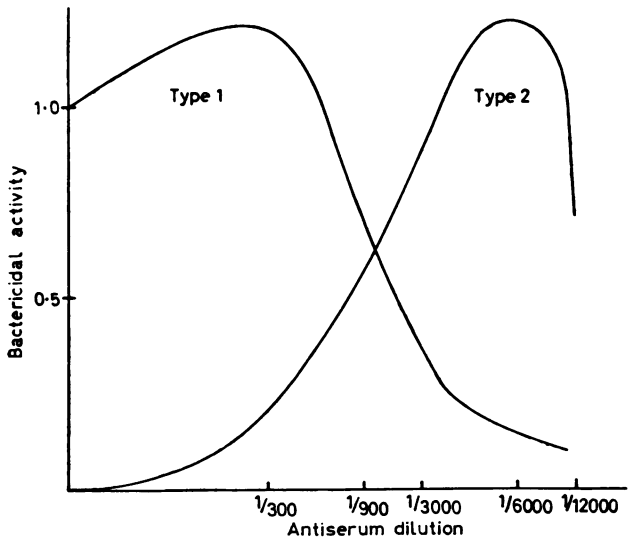

FIG. 2. The bactericidal activity patterns of the two types of *B. pertussis* rabbit antisera with complement 1/30–1/40 against strain 18-323.

The Specificity of the Activity Pattern

Normal rabbit sera were either completely inactive or active only at 1/30. The few that were bactericidal in higher dilutions always proved to have high *B. bronchiseptica* agglutination titres. Their activity against *B. pertussis* was due to antibodies to common antigens of the *B. bronchiseptica* and *B. pertussis* (Table 3).

The specificity of the bactericidal activity of various antisera towards *B. pertussis* is shown in Table 3. *Haemophilus* antisera were no more active than normal rabbit sera whereas *B. bronchiseptica* antiserum had an activity similar to that of type 1 *B. pertussis* antisera.

The Inhibition Zone of Type 2 *Sera*

With 1/36 complement, only type 2 antisera were non-bactericidal in dilutions up to 1/1000 (Fig. 1). In these dilutions the antisera also inhibited the bactericidal activity of type 1 sera when the two were mixed. Thus a type 1 antiserum diluted 1/100 was inactive in the presence of 1/10 or 1/100 type 2 antiserum. Most type 2 sera did not block type 1 activity in dilutions greater than 1/100.

The inhibition of bactericidal activity could not be shown to be due to anticomplementary substances. It is possible that since complement is fixed during antigen–antibody reactions, sera with a high content of complement fixing antibodies may reduce the complement to less than the required amount for the bactericidal action; however, type 2 sera did not always contain more (at least demonstrable) antibodies than type 1 antisera. Agglutinins could be absorbed almost completely from strongly agglutinating type 2 serum without affecting either the activity pattern or the degree of bactericidal activity.

TABLE 3

SPECIFICITY OF THE BACTERICIDAL REACTION OF ANTISERA TO RELATED AND UNRELATED ORGANISMS AGAINST *B. pertussis* STRAIN 18-323 USING GUINEA-PIG SERUM AT 1/36

Rabbit antiserum prepared against	*Activity*					
	1/30	1/300	1/600	1/900	1/3000	1/12000
Nil (*B. bronchiseptica* titre 1/50)	0·3	0·1	0·1	0·1	0	0
Nil (*B. bronchiseptica* titre 1/6400)	0·6	1·3	1·2	1·1	0·3	0·1
B. bronchiseptica (*B. bronchiseptica* titre 25,600)	2·3	1·1	0·3	0·4	0	0
H. influenzae	0·1	0·2	0	0	0	0
H. aegyptius	0·9	0·3	0·2	0·1	0·1	0·1
S. typhi	0·6	0·2	0	0	0	0
B. pertussis (two injections)	1·1	1·4	1·1	1·0	0·5	0·2
B. pertussis (six injections)	0·2	0·8	1·3	1·5	1·5	1·0

TABLE 4

THE EFFECT OF MOUSE TISSUE HOMOGENATES OR ORGANS ON THE BACTERICIDAL ACTIVITY OF A TYPE 2 RABBIT ANTISERUM AGAINST STRAIN 18-323 WITH GUINEA-PIG SERUM 1/36

Procedure	*Activity*						
	1/30	1/300	1/900	1/3000	1/12000	1/27000	1/∞
Usual	0	0	0·7	1·3	0·9	0·9	0
Complement diluted in brain homogenate	0	0	0·7	1·3	0·9	0·8	0
Antiserum diluted in brain homogenate	0·1	0·4	0·8	1·4	1·1	0·5	0
One brain shaken with 1 ml. 1/10 antiserum; further dilution as usual	0·1	0·3	1·0		1·5	0·5	0
One brain added to each mixture of antiserum dilution + organisms + complement and shaken before incubation	0·2	0·5	0·8	1·3	1·0		0

The addition of more of the guinea-pig serum used as a source of complement abolished the inhibition zone (Table 2). It either removed an inhibitor of the bactericidal reaction or increased a factor that was in sub-threshold concentration with strong antibody. The addition of mouse brain or lung homogenates or of lysozyme decreased or abolished the inhibition zone (Tables 4, 9 and 10); these did not act by potentiating the complement present, at least to any degree, since there was no detectable increase in the haemolylic effect in their presence when mixtures were tested against sensitized red cells. The mixtures tested did however contain the antigen and the complement that was estimated was excess only.

In view of the lysozyme effect, the lysozyme content of several types 1 and 2 sera were assayed. The bactericidal activity patterns were in no way connected with the varying lysozyme content of different antisera.

In an attempt to characterize the inhibitor in serum, a strongly prozoning serum was fractionated into crude γ- and β-globulins and crude albumin by the method of Levy and Sober (1960) modified and carried out by Dr. D. E. Dolby (Dolby, 1964). The protective antibodies and agglutinins were present in the β- and γ-globulins, the β being the stronger. The γ- and β-globulins both had type 2 activity but the inhibition zone of β-globulin was the stronger. The albumin preparation had type 1 activity presumably due to small amounts of contaminating globulins. The inhibition zone of the β-globulin was almost identical with that of non-fractionated serum and of the three fractions combined (Table 5). A sample of human globulin from an immunized adult (Cutter Laboratories) was strongly bactericidal at 1/30 and bactericidal to 1/6000 with no inhibition zone. This and the very much smaller inhibition zone of our γ-globulin compared with the β-globulin and whole serum suggest that the inhibition zone may be characteristic of either β-globulin antibodies or of antibodies separating out with our crude β-globulin fraction. If β-globulin antibodies are responsible for the inhibition zone, then all sera with antibody in the β-globulin fraction (that is all except early bleeding, very short course sera) may be expected to have an inhibition prozone. This is generally so, though not all sera with inhibition zones are long-course sera and not all long-course sera are inhibitory as exemplified in the next paper (Dolby and Vincent, 1965).

TABLE 5

THE BACTERICIDAL ACTIVITY OF FRACTIONS OF TYPE 2 ANTISERUM AGAINST STRAIN 18-323 USING GUINEA-PIG SERUM 1/36

Rabbit antiserum fraction	*Activity*					
	1/30	1/300	1/600	1/3000	1/6000	1/12000
Non-fractionated serum	0	0	0	1·2	1·2	1·2
Mainly γ-globulin	0	0·2	1·0	1·2	1·2	1·0
Mainly β-globulin	0	0	0	1·0	0·8	1·0
Impure albumin	0·7	1·0	0·5	0	0	0
γ-globulin + β-globulin + albumin	0	0	0	1·2	1·2	1·2

THE NATURE OF THE GUINEA-PIG SERUM COMPONENT

Guinea-pig serum stored for 2–3 months at −15° retained its complementary activity for the bactericidal test, but lost it on standing at room temperature for about 5 days. During storage, bactericidal complement and haemolytic complement titres fell at about the same rate. Serum with haemolytic complement titres of over 1/100 were satisfactory for use in the bactericidal test but 1/80 or less was too weak for the conditions of the test. There were enough exceptions to the general finding however to suggest that haemolytic and bactericidal activities are not identical (Table 6). In mixtures of active guinea-pig sera and aged sera there was no correlation between bactericidal and haemolytic complement.

Citrate at a concentration of 0·8 per cent in the final reaction mixture with strong guinea-pig serum did not decrease the bactericidal complement but with more dilute sera of 1/30 0·8 per cent citrate reduced or abolished the bactericidal activity. Under these

TABLE 6

BACTERICIDAL ACTIVITY OF DILUTIONS OF GUINEA-PIG SERA WITH SIMILAR HAEMOLYTIC COMPLEMENT TITRES AND TYPE 2 RABBIT ANTISERUM AT 1/12,000 AGAINST STRAIN 18-323

Guinea-pig serum sample	*Haemolytic complement titre*	*Activity*		
		1/21	1/27	1/33
251062	1/120	0·2	0	0
15162	1/120	1·4	1·3	1·2
12262	1/120	0·8	0·7	0·8

last conditions, the citrate may remove for instance the Mg^{2+} and Ca^{2+} necessary for the complement system (Levine and Mayer, 1954; Levine, Mayer and Rapp, 1954), and the Ca^{2+} involved in the lysozyme system (Myrvik and Weiser, 1955).

The heating to 56° for 30 minutes that destroys haemolytic complement also renders guinea-pig serum inactive for the bactericidal test.

The Action of Various Preparations of C′ Components

The preparations C′1, C′2, R3 and R4 separately and in combination were tested in the bactericidal and haemolytic systems. The preparations containing C′1, the most unstable component, had to be used in higher concentration than the others, to produce haemolysis.

TABLE 7

BACTERICIDAL ACTIVITIES OF FRACTIONATED GUINEA-PIG COMPLEMENT COMPONENTS WITH TYPE 1 RABBIT ANTISERUM AT 1/300 AND TYPE 2 RABBIT ANTISERUM AT 1/3000 AGAINST *B. pertussis* STRAIN 18-323

Fractions (h.t. = 56° 20′)	*Factors present**	*Fractions = 1/8 guinea-pig serum*			*Fractions = 1/18 guinea-pig serum*		
		Haem. C′ (units/tube)	*Activity*		*Haem. C′ (units/tube)*	*Activity*	
			Type 1	*Type 2*		*Type 1*	*Type 2*
R3, RP, R3+RP	1, 2, 4	n.t.	n.t.	n.t.	<1	0·3	0·2
R3+C′2	1, 2, (3), 4	n.t.	n.t.	n.t.	2·7	0·3	0·2
R4	1, 2, 3	n.t.	n.t.	n.t.	<1	0	0
R4+C′1	1, 2, 3, (4)	1·3	0·6	0·1	<1	0·2	0·2
C′1, C′1+ht	1, 3, (4) or 4	1·0	0·2	0	<1	0	0
C′2	2, (3), 4	n.t.	n.t.	n.t.	<1	0·3	0·9
C′2+ht	2, 3, 4	n.t.	n.t.	n.t.	1·0	0·4	0·4
ht	3, 4	n.t.	n.t.	n.t.	<1·0	0	0

* Numbers in parentheses indicate a trace. n.t. = not tested.

It can be seen from Tables 7 and 8 that the correlation between the number of haemolytic units of complement and the bactericidal activity is not perfect and also that the two types of antisera appear to need components in different ratios. Only combinations containing C′2, C′3 and C′4 are active; C′1 may not be essential for either type but C′4 may be the limiting factor for type 2.

Substitution of the Guinea-pig Serum Component

Guinea-pig serum can be substituted in the bactericidal reaction by mouse plasma (heparinized or citrated), mouse serum, mouse peritoneal exudate, rabbit serum and human

plasma. During tests of the effect of mouse lung and brain homogenates on the bactericidal activity of type 2 antiserum with complement, it was observed that tissue homogenate increased the bactericidal activity and narrowed the inhibition zone (Table 4). With high dilutions of antiserum it was also found that the tissue homogenate could replace guinea-pig serum. In these tests the antisera and organisms were mixed first, the brain removed from the mouse and immediately added to the mixture, the whole being shaken for 3 minutes and incubated as usual (Table 9).

TABLE 8

BACTERICIDAL ACTIVITY OF MIXTURES OF GUINEA-PIG SERUM COMPLEMENT COMPONENTS EQUIVALENT TO COMPLETE HAEMOLYTIC COMPLEMENT WITH TYPE 1 RABBIT ANTISERUM AT 1/300 AND TYPE 2 RABBIT ANTISERUM AT 1/3000 AGAINST *B. pertussis* STRAIN 18-323

Fractions (*h.t.* = 56° 20′)	*C*′1 = 1/6; *other fractions* ≡ 1/18 *guinea-pig serum*			*All fractions* ≡ 1/18 *guinea-pig serum*		
	Haem. C′ (*units/tube*)	*Activity*		*Haem. C*′ (*units/tube*)	*Activity*	
		Type 1	*Type* 2		*Type* 1	*Type* 2
R3+R4	n.t.	n.t.	n.t.	1	0·2	0
R3+ht	n.t.	n.t.	n.t.	1·3	0	0
R3+C′1	1·3	1·3	2·2	<1	0·4	0
RP+R4	n.t.	n.t.	n.t.	1	0·5	0·4
RP+ht	n.t.	n.t.	n.t.	n.t.	0·7	0·1
RP+C′1	2	1·7	2·5	<1	0·5	0·1
R4+ht	n.t.	n.t.	n.t.	2	1·2	0·4
R4+C′2	n.t.	n.t.	n.t.	2·7	0·9	1·5
C′1+C′2	2·7	0·8	1·5	<1	0·2	0·3

TABLE 9

REPLACEMENT OF GUINEA-PIG SERUM BY WHOLE MOUSE BRAIN (OR LUNG) WITH TYPE 2 RABBIT ANTI-SERUM AGAINST STRAIN 18-323

	Activity					
	1/300	1/900	1/3000	1/9000	1/27000	1/∞
Guinea-pig serum	0	1·0	2·3	2·6	2·0	0
Prehomogenized brain (or lung)	0	0	0	0	0	0
Brain (or lung) homogenized in the reaction mixture	0·7	0·6	0·2	0·3	0·3	0

THE ROLE OF LYSOZYME

Amano, Inai, Seki, Kashiba, Fujikawa and Nishimura (1954), Muschel, Carey and Baron (1959), Michael and Braun (1959) and Wardlaw (1962) all showed that lysozyme was implicated in at least part of the bactericidal effect of specific antibody on certain Gram-negative bacteria. Absorption of the antisera and of guinea-pig serum with bentonite sufficient to remove lysozyme but not agglutinins and haemolytic complement decreased the bactericidal activity of both type 1 and 2 antisera. The activity could be restored by adding crystalline lysozyme (Light & Co.) (Table 10).

Two interesting effects occurred with crystalline lysozyme in a concentration greater than that commonly found in serum and tissues. The inhibition zone of type 2 serum was narrowed, as it was by strong complement and tissue homogenate, and the activity of type 1 serum was increased (Table 10).

TABLE 10

THE EFFECT OF DIFFERENT LYSOZYME CONCENTRATIONS ON THE BACTERICIDAL ACTIVITY PATTERNS OF TYPES 1 AND 2 RABBIT ANTISERA AGAINST STRAIN 18-323

Lysozyme concentration per tube	*Activity*											
	Type 1						*Type* 2					
	1/30	1/300	1/600	1/900	1/3000	1/12000	1/30	1/300	1/600	1/900	1/3000	1/12000
From 0·45 to 0·04 µg. with dilution of component	0·6	0·6	0·8	0·6	0	0	0·1	0	0	0·6	1·0	0·5
0 (absorbed sera)	0·5	0·2	0	0·1	0	0	0·1	0·2	0·1	0·1	0·2	0·1
0·8 µg. as crystalline lysozyme	0·2	0·2	0·3	0·7	0·3	0·1	0	0	0	0·1	0·4	0·9
10 µg. as crystalline lysozyme	0·6	1·0	1·3	1·6	0·3	0·2	0	0	0·3	0·5	0·6	0·4

SENSITIVITY OF STRAINS OF *B. pertussis* AND OTHER ORGANISMS TO THE BACTERICIDAL ACTION OF ANTISERA

Both types 1 and 2 bactericidal antisera are active only against some strains of *B. pertussis*, phase I. About twenty strains were tested for serum sensitivity; of these, three were highly virulent for mice by both the intranasal and intracerebral routes and were also very sensitive to the bactericidal antisera. The rest were either avirulent or of low virulence for mice and of these strains only one, M6344, was serum sensitive (Table 11).

TABLE 11

THE SERUM SENSITIVITY OF *Bordetella* AND *Haemophilus* SPECIES TO *B. pertussis* RABBIT ANTISERA TYPES 1 AND 2

Organism	*Strain No.*	*Virulence for mice (intracerebral route) (LD_{50} in total orgs.)*	*Activity*					
			Type 1 *serum*			*Type* 2 *serum*		
			1/300	1/2000	1/6000	1/300	1/2000	1/6000
B. pertussis	18-323	300	0·4	0·2	0	0·4	1·0	1·0
B. pertussis	2 atox	250	1·4	1·2		0·5	0·9	
B. pertussis	G1 353	300	0·5	0·3	0	0	0·6	1·1
B. pertussis	C2 219	>50,000	0	0	0	0·2	0	0
B. pertussis	517	300,000	0	0	0	0	0	0
B. pertussis	L84	2×10^6	0	0	0	0	0	0
B. pertussis	G 1101	5×10^6	0	0	0	0	0	0
B. pertussis	M6344	5×10^6	0·8	0·3	0	0·6	0·9	0·8
B. bronchiseptica	8762	90,000	0	0	0	0·2	0·1	0
H. aegyptius	181	>500,000	0	0	0	0	0	0

Strains from Dr. V. Spasojevic's collection were serum insensitive on the whole but with occasional strains in some tests showing sensitivity. The stage of the disease when the organisms were isolated from children was known but it was not in any way correlated with serum sensitivity.

The serum insensitive strains of *B. pertussis* stimulated the production of antisera which were bactericidal against the serum sensitive strains. *B. bronchiseptica* was not serum sensitive but produced bactericidal antisera, whereas *H. aegyptius* was insensitive and a very poor antigen for bactericidal antiserum production (Tables 3 and 11).

DISCUSSION

The bactericidal action of antisera against *B. pertussis* fits into the general pattern observed for other Gram-negative organisms such as *Escherichia coli* and *Salmonella typhi*. This pattern is now becoming clarified; complement, lysozyme and antibody are required —the last often only in minute traces, so that 'normal' serum may be active (Amano *et al.*, 1954; Muschel *et al.*, 1959; Carey, Muschel and Baron, 1960; Wardlaw, 1962; Michael, Whitby and Landy, 1962). Two interesting observations emerge from the work with *B. pertussis*, the one connected with the two types of antiserum and the implications of the inhibition zone and the other with the sensitivity of organisms.

The two types of antisera are characterized by the range of dilutions over which they are bactericidal, by their differing quantitative and qualitative complement requirements for the bactericidal reaction and by the absence or presence of the inhibition prozone. At one concentration of complement, sera fall readily into one or other types—there is no graduation of activity patterns; changing the complement level alters the shape of the activity patterns but the distinction remains. For example, even when very strong complement is used with type 2 sera there is still a zone of low bactericidal activity covering the inhibition zone with weak complement while stronger bactericidal activity persists at very high dilutions. Type 1 sera bactericidal activity is either high in strong serum and falls off with dilution or it increases steadily to a maximum before falling. Type 2 sera always have the pronounced plateau of the inhibition zone.

The incomplete parallelism between haemolytic complement and bactericidal complement was mentioned in 1938 by Dingle, Fothergill and Chandler in their work with *H. influenzae*; the findings reported in this paper support this work. Dozois, Seifter and Ecker (1943) on the other hand had results compatible with complete identity of the haemolytic and bactericidal complement.

Mouse tissues, tissue fluids and sera can supply all the factors required for the *B. pertussis* bactericidal system other than antibody. This has a practical importance in attempting to translate any *in vitro* effects into protection against *B. pertussis* in the mouse, the animal commonly used for *in vivo* work. It also supports the work of McGhee (1952), Muschel and Muto (1956) and Borsos and Cooper (1961) who have all shown, using sensitive methods, that haemolytic complement is in fact present in the mouse in spite of earlier reports to the contrary; C'2 and C'3 are relatively deficient in comparison with other animals. Carey *et al.* (1960) showed that immune mouse blood used immediately on withdrawal induced spheroplast formation of *S. typhi in vitro*.

The inhibition prozone like that characterizing type 2 sera, has been noted by many workers since Neisser and Wechsberg first described it in bactericidal systems in 1901 and showed that the addition of more complement obliterated the prozone. Subsequent work suggests that the prozone effect is due to competition between antigen–antibody systems for a limiting component and that, as in so many immune reactions, there is a ratio of complement to antibody at which the bactericidal effect is greatest (Thjotta and Waaler, 1932; Nagington, 1956a; Muschel and Treffers, 1956). As we have shown, complement,

mouse tissues and fluids and lysozyme can all reduce the inhibition zone of the type 2 *B. pertussis* bactericidal system. They may all supply limiting factors other than those in haemolytic complement.

In the bactericidal antigen–antibody reaction taking place presumably at the surface of the bacterial cell an inhibition zone could also be due to blocking of one antigen–antibody system by another by reason of size or physico-chemical forces (Thjotta, 1920; Adler, 1953b). Muir and Browning (1931) discussed the possible causes of the Neisser–Wechsberg phenomenon. Attention was then focussed on the antigen and there seems little to add to this as a possibility.

With increasing knowledge of antibody structure however, it is equally possible that the antibody may be responsible. The preliminary experiments on antibody fractionation reported here suggests this. Kourilsky, Bloch, Benacerraf and Ovary (1963) showed that in passive immune lysis of red cells, guinea-pig $7S\gamma_1$ antibody can block $7S\gamma_2$, producing an inhibition zone. The $7S\gamma_1$ antibody does not fix complement so the block cannot be due to competition for complement.

Turning now to the sensitivity of the organisms, our findings fit less well into the general picture of bactericidal reactions in Gram-negative organisms. With other bacteria, the mouse avirulent strains were most serum sensitive (Thjotta and Waaler, 1932; Muschel, Chamberlin and Osawa, 1958; Wardlaw, 1963). With *B. pertussis* the mouse virulent strains are usually sensitive whereas the avirulent ones tend not to be; but factors other than virulence appear to be involved in making the cell wall susceptible to attack. The 'bactericidal antigen' must be present in all strains since antisera to them are bactericidal, irrespective of the serum sensitivity of the inducing strain but presumably in avirulent strains the antigen is partly masked by what perhaps makes the organism avirulent. Variations in serum sensitivity of similar strains have been noted (Adler, 1953a, b; Michael and Landy, 1961; Landy, Michael and Whitby, 1962). In *S. typhi* O, R and Vi antigen can all act as 'bactericidal antigens' with the appropriate antisera and each can block the others (Nagington, 1956b; Muschel *et al.*, 1958).

Wardlaw (1963) analysed the cell walls of *E. coli* R. (serum sensitive) and *E. coli* (insensitive) and found differences in the composition of the lipopolysaccharide and in the amount. The serum insensitive strain had nine times more lipopolysaccharide than the other, and he suggests that it is this component that blocked the bactericidal antigen–antibody reaction.

The lysozyme requirements of the *B. pertussis* bactericidal system are similar to those reported for other Gram-negative bacteria in spite of the cell walls of *B. pertussis* being more difficult to lyse than those of other organisms. Gram-negative organisms are not by themselves sensitive to lysis by lysozyme but can be made so by pretreatment with ethylenediamine tetra acetic acid (EDTA) (Repaske, 1956, 1958). Noller and Hartsell (1961) have listed other 'co-lytic' physical treatments to make them lysozyme susceptible. It has been suggested that the combination of the antibody, responsible for the bactericidal action, with its antigen is also co-lytic, rendering the mucopeptide of the cell wall susceptible to lysozyme. The spheroplasts formed can be kept alive by increasing the osmotic pressure of the medium thus decreasing the bactericidal effect (Amano, Inoue, Tanigawa, Morioka and Utsumi, 1957; Muschel *et al.*, 1959; Michael and Braun, 1959; Wardlaw, 1963; Freeman, Musteikis and Burrows, 1963).

B. pertussis however is not lysed by mixtures of EDTA and lysozyme, as other Gram-negative bacteria are; isolated cell walls although adsorbing lysozyme are not dissolved

even in the presence of EDTA (Dr. W. A. Vincent, personal communication). Whether the bactericidal reaction causes spheroplast formation in *B. pertussis* is not known. Our attempts to maintain the viability of antibody-treated cells and to reduce the bactericidal activity by adding sucrose have given variable and non-conclusive results. Wittler (1952) found that *B. pertussis* injected into the lungs and peritoneal cavities of mice changed into L-forms with even greater loss of the cell wall than spheroplast formation.

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REFERENCES

ADLER, F. L. (1953a). 'Studies on the bactericidal reaction. I. Bactericidal action of normal sera against a strain of *S. typhosa*.' *J. Immunol.*, **70**, 69.

ADLER, F. L. (1953b). 'Studies on the bactericidal reaction. II. Inhibition by antibody and the antibody requirements of the reaction.' *J. Immunol.*, **70**, 79.

AMANO, T., INAI, S., SEKI, Y., KASHIBA, S., FUJIKAWA, K. and NISHIMURA, S. (1954). 'Studies on the immune bacteriolysis. I. Accelerating effect on the immune bacteriolysis by lysozyme-like substance of leucocytes and egg-white lysozyme.' *Med. J. Osaka Univ.*, **4**, 401.

AMANO, T., INOUE, K., TANIGAWA, Y., MORIOKA, T. and UTSUMI, S. (1957). 'Studies on the immune bacteriolysis. XII. Properties of coccoid-form bacteria produced by immune bacteriolysis.' *Med. J. Osaka Univ.*, **7**, 819.

BORSOS, T. and COOPER, M. (1961). 'On the haemolytic activity of mouse complement.' *Proc. Soc. exp. Biol. (N.Y.)*, **107**, 227.

CAREY, W. F., MUSCHEL, L. H. and BARON, L. S. (1960). 'The formation of bacterial protoplasts *in vivo*.' *J. Immunol.*, **84**, 183.

COHEN, S. M. and WHEELER, M. W. (1946). 'Pertussis vaccine prepared with phase 1 cultures grown in fluid medium.' *Amer. J. publ. Hlth*, **36**, 371.

DINGLE, J. H., FOTHERGILL, L. D. and CHANDLER, C. A. (1938). 'Studies on *Haemophilus influenzae*. III. The failure of complement of some animal species notably guinea-pig to activate the bactericidal function of sera of certain other species.' *J. Immunol.*, **34**, 357.

DOLBY, D. E. (1964). 'The action of pepsin on protein fractions from horse antisera to diphtheria toxin.' *Biochem. J.*, **92**, 112.

DOLBY, J. M. and STANDFAST, A. F. B. (1958). 'A comparison of passive protection tests against intranasal and intracerebral challenges with *Bordetella pertussis*.' *Immunology*, **1**, 144.

DOLBY, J. M. and STANDFAST, A. F. B. (1961). 'The intracerebral infection of mice with *Bordetella pertussis*.' *J. Hyg. (Camb.)*, **59**, 205.

DOLBY, J. M., THOW, D. C. W. and STANDFAST, A. F. B. (1961). 'The intranasal infection of mice with *B. pertussis*.' *J. Hyg. (Camb.)*, **59**, 191.

DOLBY, J. M. and VINCENT, W. A. (1965). 'Characterization of the antibodies responsible for the "bactericidal activity patterns" of antisera to *Bordetella pertussis*.' *Immunology*, **8**, 499.

DOZOIS, T. F., SEIFTER, S. and ECKER, E. E. (1943). 'Immuno-chemical studies on human serum. IV. The role of human complement in bactericidal phenomena.' *J. Immunol.*, **47**, 215.

FREEMAN, B. A., MUSTEIKIS, G. M. and BURROWS, W. (1963). 'Protoplast formation as the mechanism for immune lysis of *Vibrio cholerae*.' *Proc. Soc. exp. Biol. (N.Y.)*, **113**, 675.

KABAT, E. A. and MAYER, M. M. (1948). *Experimental Immunochemistry*, p. 121. Thomas, Springfield.

KENDRICK, P., GIBBS, J. and SPRICK, M. (1937). 'The opsonocytophagic test in the study of pertussis.' *J. infect. Dis.*, **60**, 302.

KOURILSKY, F. M., BLOCH, K. J., BENACERRAF, B. and OVARY, Z. (1963). 'Properties of guinea-pig 7S antibodies. V. Inhibition by guinea pig γ_1 antibodies of passive immune lysis provoked by γ_2 antibodies.' *J. exp. Med.*, **118**, 699.

LANDY, M., MICHAEL, J. G. and WHITBY, J. L. (1962). 'Bactericidal method for the measurement in normal serum of antibody to Gram-negative bacteria.' *J. Bact.*, **83**, 631.

LEVINE, L. and MAYER, M. M. (1954). 'Kinetic studies on immune haemolysis. V. Formation of the complex EAC_x' and its reaction with $C'_y{}^{1,2}$.' *J. Immunol.*, **73**, 426.

LEVINE, L., MAYER, M. M. and RAPP, H. J. (1954). 'Kinetic studies on immune haemolysis. VI. Resolution of the C'_y reaction step into two successive processes involving C'2 and C'3.' *J. Immunol.*, **73**, 435.

LEVY, H. B. and SOBER, H. A. (1960). 'A simple chromatographic method for the preparation of γ-globulin.' *Proc. Soc. exp. Biol. (N.Y.)*, **103**, 250.

McGHEE, R. B. (1952). 'The presence of complement in the serum of the mouse.' *Proc. Soc. exp. Biol. (N.Y.)*, **80**, 419.

MICHAEL, J. G. and BRAUN, W. (1959). 'Serum spheroplasts of *Sh. dysenteriae*.' *Proc. Soc. exp. Biol. (N.Y.)*, **100**, 422.

MICHAEL, J. G. and LANDY, M. (1961). 'Endotoxic properties of Gram-negative bacteria and their susceptibility to the lethal effect of normal serum.' *J. infect. Dis.*, **108**, 90.

MICHAEL, J. G., WHITBY, J. L. and LANDY, M. (1962). 'Studies on natural antibodies to Gram-negative bacteria.' *J. exp. Med.*, **115**, 131.

MILES, A. A. and MISRA, S. S. (1938). 'The estimation of the bactericidal power of blood.' *J. Hyg. (Camb.)*, **38**, 732.

MUIR, R. and BROWNING, C. H. (1931). *A System of Bacteriology*, vol. 6, p. 274. Medical Research Council.

MUSCHEL, L. H., CAREY, W. F. and BARON, L. S. (1959). 'The formation of bacterial protoplasts by serum components.' *J. Immunol.*, **82**, 38.

MUSCHEL, L. H., CHAMBERLIN, R. H. and OSAWA, E. (1958). 'Bactericidal activity of normal serum against bacterial cultures. I. Activity against *Salmonella typhi* strains.' *Proc. Soc. exp. Biol. (N.Y.)*, **97**, 376.

MUSCHEL, L. H. and MUTO, T. (1956). 'Bactericidal reaction of mouse serum.' *Science*, **123**, 62.

MUSCHEL, L. H. and TREFFERS, H. P. (1956). 'Quantitative studies on the bactericidal actions of serum and complement. II. Some implications for the mechanism of the bactericidal reaction.' *J. Immunol.*, **76**, 11.

MYRVIK, Q. M. and WEISER, R. S. (1955). 'Studies on the anti-bacterial factors in mammalian tissues and fluids. I. A serum bactericidin for *B. subtilis*.' *J. Immunol.*, **74**, 9.

NAGINGTON, J. (1956a). 'The bactericidal action of typhoid antibody.' *Brit. J. exp. Path.*, **37**, 385.

NAGINGTON, J. (1956b). 'The sensitivity of *Salmonella typhi* to the bactericidal action of antibody.' *Brit. J. exp. Path.*, **37**, 397.

NOLLER, E. C. and HARTSELL, S. E. (1961). 'Bacteriolysis of Enterobacteriaceae. II. Pre- and co-lytic treatments potentiating the action of lysozyme.' *J. Bact.*, **81**, 492.

REPASKE, R. (1956). 'Lysis of Gram-negative organisms by lysozyme.' *Biochim. biophys. Acta*, **22**, 189.

REPASKE, R. (1958). 'Lysis of Gram-negative organisms and the role of versene.' *Biochim. biophys. Acta*, **30**, 225.

RICHARDSON, G. M. (1941). 'Preservation of liquid complement serum.' *Lancet*, **ii**, 696.

THJØTTA, TH. (1920). 'On the so-called Neisser-Wechsberg inhibiting phenomenon in bactericidal immune sera.' *J. Immunol.*, **5**, 1.

THJØTTA, TH. and WAALER, E. (1932). 'Dissociation and sensitiveness to normal serum in dysentery bacilli of type III.' *J. Bact.*, **24**, 301.

WARDLAW, A. C. (1962). 'The complement-dependent bacteriolytic activity of normal human serum. I. The effect of pH and ionic strength and the role of lysozyme.' *J. exp. Med.*, **115**, 1231.

WARDLAW, A. C. (1963). 'The complement dependent bacteriolytic activity of normal human serum. II. Cell wall composition of sensitive and resistant strains.' *Canad. J. Microbiol.*, **9**, 41.

WARDLAW, A. C. and PILLEMER, L. (1956). 'The properdin system and immunity. V. The bactericidal activity of the properdin system.' *J. exp. Med.*, **103**, 553.

WITTLER, R. G. (1952). 'The L-form of *H. pertussis* in the mouse.' *J. gen. Microbiol.*, **6**, 311.