The Antigen of *Bordetella pertussis* that Induces Bactericidal Antibody and its Relationship to Protection of Mice

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SUMMARY

The ability to protect mice against intracerebral infection and to elicit, after one dose, complement-mediated bactericidal antibody capable of killing a mouse-virulent phase I strain *in vitro*, was tested in seven cultures of *Bordetella pertussis*: three typical phase I strains; two strains grown in the presence of 0.5 mg nicotinic acid/ml; one phase IV strain; and the atypical strain 134. The typical strains showed both activities; the nicotinic acid-grown and phase IV strains elicited high antibody titres, but did not confer protection, whereas strain 134 protected, but did not elicit bactericidal antibody.

Pyrogenic material extracted from dried organisms by hot phenol and water (lipopolysaccharide) was not antigenic in mice; however, with all strains, except 134, multiple doses of this material coupled to stromata or *Escherichia coli* conjugated protein elicited bactericidal and precipitating antisera in mice, although the mice were not protected.

Strain 134 contained approximately one quarter of the normal amount of endotoxin, as assayed in actinomycin D-sensitized mice, but was as effective as normal strains as an adjuvant for haemolysin production.

INTRODUCTION

The injection of killed *Bordetella pertussis* organisms intraperitoneally in a mouse will protect the animal against a subsequent intracerebral challenge by a virulent strain of the same organism; and vaccines which protect mice in this way are also able to protect children against natural infection (Medical Research Council report, 1956). The measurement of whooping-cough vaccine potency by the mouse-protection test is slow and requires large numbers of animals. The potency of vaccine might be more conveniently assayed by measuring its ability to elicit an easily measured antibody with a demonstrable relationship to protection.

Following an injection of a protective dose of vaccine, circulating antibodies appear in the mouse, including a complement-mediated bactericidal antibody, the properties of which were investigated (Dolby, 1965; Dolby & Vincent, 1965). Dolby & Dolby (1969) suggested that this antibody is responsible for the protection of actively immunized mice.

Screening experiments on routine vaccines showed a good correlation between serum levels of bactericidal antibody and the degree of protection conferred on mice (A. P. Hunt, personal communication). This paper describes a more detailed examination of this correlation.

Some preliminary studies on the nature of the antigen which induces this complement-mediated bactericidal antibody, active *in vitro* against serum-sensitive phase I *Bordetella pertussis* and referred to as the 'bactericidal antigen', are also reported.

METHODS

Organisms. The strains of *Bordetella pertussis* were all obtained from the collection of freeze-dried cultures at Elstree: GL.291 – a vaccine strain originally obtained from Glaxo Laboratories; D6229, D25525, D734 – recently isolated strains used for routine vaccine production; 134 – a nutritionally less exacting but protective strain originally obtained from Lederle Laboratories and used by Pillemer to prepare his cell-free vaccine (Pillemer, 1950; Pillemer, Blum & Lepow, 1954); L84 – isolated from a case of whooping cough in Leeds in 1950; and L84 phase IV – a variant, obtained from L84, adapted by repeated subculture to grow on simple media. It grew well on nutrient agar and did not protect mice at a dose of 4×10^9 organisms per animal. These properties of phase IV organisms are not always stable (Leslie & Gardner, 1931), and we have found that when newly reconstituted from the freeze-dried state they may grow poorly on nutrient agar and show traces of mouse-protective activity; typical phase IV properties are, however, regained after a few subcultures on nutrient agar. All phase IV organisms mentioned in this paper were maintained on nutrient agar and had been subcultured on the same medium at least five times since reconstitution.

Cultural conditions. Freeze-dried cultures of all strains except L84 phase IV were opened on to Bordet-Gengou medium, subcultured once at 37° and then used to inoculate Cohen & Wheeler (1946) medium (100 ml), modified to contain 0.5 % L-glutamic acid (w/v) and 0.1 % peptone. Maintenance cultures of L84 phase IV were subcultured once on nutrient agar before inoculation into 100 ml of liquid medium. The primary liquid cultures of all strains were shaken at 37° for 24 h and then used to inoculate 500 ml of the same medium in Glaxo flasks (10 ml inoculum/flask); the cultures were shaken for 40 h at 37°, killed by treatment with 0.1 % formaldehyde at 4° for 48 h, centrifuged, and resuspended in phosphate buffered saline (0.85 % NaCl + 0.006 M-phosphate buffer, pH 7.2) plus 0.01 % merthiolate. Vaccines were stored at 4°; before use they were suitably diluted in buffered saline + merthiolate and heated at 56° for 30 min to destroy heat-labile toxicity.

Bacterial concentrations in liquid cultures and vaccines were determined by visual comparison of opacity with the International Opacity Standard (10 International Units are equal to 10^{10} *Bordetella pertussis* organisms/ml).

Bacterial sonicate. The sonicate used in immunodiffusion plates was prepared by suspending bacteria in buffered saline to 1.2×10^{12} organisms/ml and exposing them to the full power of a Dawe Soniprobe (Dawe Instruments Ltd, Western Avenue, London, W.3), model 1130A, for 15 min in a water-cooled cell. The viscous product was centrifuged at 20000g for 30 min at 4° and the greenish-yellow supernatant liquid was stored at –15° until required.

Nicotinic acid. Nicotinic acid was suspended in water; sufficient 4 N-sodium hydroxide was added to dissolve the solid and the solution was diluted to 25 mg/ml. The solution was autoclaved at 121° for 15 min and 10 ml was added aseptically to 500 ml of medium in a Glaxo flask, giving a final concentration of approximately 500 µg/ml.

Tests for bactericidal antibody. Mice of Theiler's Original strain were used, and single or multiple doses of vaccine injected intraperitoneally. Whole cell vaccine was given in 0.5 ml containing 2×10^9 organisms, and extracted antigens in 0.1 to 0.5 ml containing 2 to 100 µg as described. The mice were killed with coal gas 14 days after the last injection and were bled out at once from the heart. The blood was allowed to clot and, after standing overnight at 4°, sera were collected, pooled and inactivated at 56° for 30 min.

Bactericidal antibody levels were determined by a slight modification of the method previously described (Dolby & Vincent, 1965). Serial dilutions (usually fourfold) of the

test serum were prepared in 1 % Casamino acids (Difco). Fresh guinea-pig serum (unabsorbed and stored at -15°) was used as a source of complement; it was diluted by a previously determined optimum factor (usually 1/10 to 1/25) in 1 % (w/v) bovine serum albumin (Armour, St. James', London, S.W.1) in buffered saline. The test organism was a 20 h Bordet-Gengou culture of *Bordetella pertussis* strain 18-323 (identical in all respects with the strain used for intracerebral infection of mice in the vaccine potency assay), suspended in 1 % Casamino acids and diluted to contain 5×10^6 organisms/ml. The reaction mixture consisted of 0.2 ml each of serum, complement and suspended organisms; after incubation at 37° with gentle agitation for 45 min, tenfold dilutions were made in 1 % Casamino acids containing 7 % (w/v) sodium chloride. The high salt concentration inactivated the complement and prevented further killing of bacteria. Viable counts were determined by the method of Miles & Misra (1938) on plates of modified Cohen & Wheeler medium plus 1 % (w/v) agar and 5 % (v/v) blood. Appropriate controls were included and the end-point was taken as the serum dilution killing 80 % of the bacteria.

Potency assay. Mice vaccinated intraperitoneally with graded doses of vaccine were challenged intracerebrally 14 days later with 5×10^4 *Bordetella pertussis* organisms of the mouse-virulent strain 18-323, harvested from a 20 h Bordet-Gengou plate into 1 % (w/v) Casamino acids. Deaths were counted 14 days later. The dose of vaccine protecting half the mice (ImD₅₀) was determined by the method of Reed & Muench (1938).

Endotoxin assay. Mice sensitized with actinomycin D (Cosmogen Lyovac; Merck, Sharp & Dohme, Hoddesdon, Hertfordshire) are killed with very small amounts of endotoxin (Pieroni & Levine, 1970). This method was used to assay the endotoxin content of *Bordetella pertussis* whole cell vaccines. The actinomycin D was injected intraperitoneally (10 μ g in 0.25 ml of buffered saline), into 15 to 17 g mice of the T.F. 1 strain. Graded doses of vaccine, also in 0.25 ml, were injected intraperitoneally 30 min later into groups of 10 mice for each dose level of vaccine. Deaths were counted at seven days and the results were expressed as the dose of whole organisms required to kill half of the sensitized mice (the LD₅₀) calculated by the method of Reed & Muench (1938). No mice died when given either the highest dose of vaccine alone or actinomycin D alone.

Assay for adjuvant activity. The method was based on that of Finger and his co-workers (Finger, Beneke & Fresenius, 1970; Finger, Emmerling & Brüss, 1970). Groups of five mice (Theiler's Original strain) were used; the adjuvant to be tested (0.25 ml) was given intraperitoneally, followed by the antigen, 0.25 ml of 1/2.5 dilution of human group A whole blood in buffered saline. Seven weeks later the mice were bled out and the sera were prepared as already described; haemolysin titrations were done on plastic tiles by mixing 0.02 ml volumes of test serum, 1/5 guinea-pig serum, and a 1 % (v/v) suspension of washed human group A red cells. All dilutions were made in buffered saline. The test was incubated for 90 min at 37° and the tile read from above, by eye, using a 50 % haemolysis end-point.

Immunodiffusion-precipitin tests. Ouchterlony plates were made by placing 10 ml of 1.5 (w/v) New Zealand agar (containing 0.01 % merthiolate) in an 8 cm plastic Petri dish. Plates were incubated at room temperature and 100 % relative humidity for 2 to 4 days.

Preparation of lipopolysaccharides. These were prepared at the Lister Institute from acetone-dried *Bordetella pertussis* organisms of strain GL291 by phenol + water extraction (MacLennan, 1960) and from the other strains by Dr L. Szabo of the University of Paris, who used a similar method. These materials were pyrogenic in rabbits (L. Szabo, personal communication) and were therefore presumed to be typical Gram-negative bacterial lipopolysaccharides.

Coupling of lipopolysaccharide to red-cell stromata. Red-cell stromata were prepared

either from sheep blood or from human blood of groups O or A by the method of Staub (1964). Lipopolysaccharide (LPS) was coupled to stromata by mixing 1 mg LPS (which had been uniformly suspended in 5 ml of buffered saline by 30 s treatment with a Dawe Soniprobe) with 1 ml of packed stromata, and incubating the mixture at 37° with gentle agitation for 30 min. The coated stromata were centrifuged and washed three times with 10 ml volumes of buffered saline, resuspended in 10 ml of buffered saline containing 0.01 % merthiolate, and heated at 56° for 30 min.

To enhance the efficacy of coupling, LPS (10 mg) was suspended in 20 ml of 0.02 N-sodium hydroxide and kept at 37° with gentle agitation for 18 h. At the end of the treatment all the material had dissolved; it was approximately neutralized by adding the theoretical amount of 0.2 N-hydrochloric acid, and dialysed against several changes of buffered saline containing 0.01 % merthiolate at 4°. The alkali-treated material was subsequently coupled to stromata in the same way as the untreated LPS.

Preparation of Bordetella pertussis endotoxin. A Boivin-type endotoxin was prepared from strains D6229 and D734 of *Bordetella pertussis* by trichloroacetic acid extraction as described by Staub (1967), except that the ethanol precipitation was done at 4°.

Preparation of Escherichia coli conjugated protein. *Escherichia coli* strain NCTC86 was grown for 18 h on nutrient agar at 37°, harvested and washed in saline; endotoxin was prepared as from *Bordetella pertussis*. Acetic acid was added to the endotoxin solution to a final concentration of 1 % (v/v) and the acidified material was kept at 100° for 4 h. After standing overnight at 4°, the small precipitate was centrifuged and purified by twice dissolving it in 0.05 N-sodium hydroxide and reprecipitating by adjusting the pH to 4.0 with acetic acid. The final product was suspended in water and coupled to *B. pertussis* LPS by the method of Davies (1956) after estimating the protein content colorimetrically.

Analytical methods. Protein was measured by the Lowry, Rosebrough, Farr & Randall (1951) modifications of the Folin-Ciocalteu method, using crystalline bovine serum albumin as standard. When appropriate, results were corrected for the interference produced by merthiolate. Dry weights were determined after drying samples at 105° for 18 h.

Preparation of anti-LPS sera. All preparations were injected intraperitoneally into mice of Theiler's Original strain. Lipopolysaccharide from *Bordetella pertussis* strain GL.291, either alone, mixed with aluminium hydroxide (100 µg/dose), coupled to *Escherichia coli* protein, or coupled, after treatment with alkali, to stromata, was given in one dose (of 0.2, 1.0, 5.0, 10 or 100 µg), or in four doses (2, 5, 5, 5 µg) at weekly intervals. Lipopolysaccharide from all strains, including GL.291, which had been coupled to stromata without prior treatment with alkali was given in seven weekly doses of 2, 2, 4, 4, 10, 10, 10 µg. All mice were bled out 2 weeks after their last injection and sera were prepared as already described.

Preparation of anti-endotoxin sera. These were prepared in the same way as the anti-LPS sera by injecting single doses of 3 to 70 µg (dry wt).

RESULTS

The ability of killed whole-cell *Bordetella pertussis* vaccines to protect mice and to elicit bactericidal antibody is shown in Table 1, which demonstrates that organisms grown with nicotinic acid have lost the mouse-protective antigen, and that strain 134 is deficient in the antigen which elicits the complement-mediated bactericidal antibody (the 'bactericidal antigen'). Multiple doses of vaccines prepared from strain D6229 grown with nicotinic acid were completely devoid of protective antigen, but multiple doses of strain 134 organisms eventually gave bactericidal antibody titres at least as high as those induced by a single dose

Table 1. *Protective potencies and bactericidal antibody titres induced in mice after single injections of whole-cell vaccine*

Potency was assayed with doses of 2000, 400 and 80×10^6 organisms. Five mice were removed from the highest-dose group just before challenge and the serum from these mice used for titrating the bactericidal antibody.

Strain	ImD 50×10^{-6} (organisms/mouse)	Bactericidal antibody titre*
GL.29I	500	120
D25525	640	480
D25525/NA†	≥ 2000	480
D6229	860	120
D6229/NA†	≥ 2000	480
L84 phase I	260	480
L84 phase IV	≥ 2000	480
I34	630	≤ 30

* Reciprocal of highest dilution killing 80% of bacteria.

† Organisms grown with 500 μ g of nicotinic acid/ml.

Table 2. *Protection of and bactericidal antibody titres in mice after multiple doses of whole-cell vaccine*

Strain	No. of organisms/ dose	No. of doses	No. of survivors/ number challenged	Bactericidal antibody titre*
D6229/NA†	2×10^9	6	0/10	30,000
I34	2×10^9	7	N.D.	1,920

N.D. = Not done.

* Reciprocal of highest dilution killing 80% of bacteria.

† Organisms grown with 500 μ g of nicotinic acid/ml.

Table 3. *Protection of and bactericidal antibody titres in mice after multiple doses of Bordetella pertussis LPS on carriers*

Strain from which LPS was prepared	Carrier	No. of doses	No. of survivors/ number challenged	Bactericidal antibody titre*
GL.29I	<i>Escherichia coli</i> protein	4	0/10	480
GL.29I	Alkali stromata†	4	3/10	1920
GL.29I	Stromata	7	1/8	7680
D6229/NA‡	Stromata	7	N.D.	7680
I34	Stromata	7	N.D.	< 30
—	<i>E. coli</i> protein alone	4	N.D.	< 30
—	Human stromata alone	4	N.D.	< 30
—	Sheep stromata	4	N.D.	480

N.D. = Not done.

* Reciprocal of highest dilution killing 80% of bacteria.

† LPS treated with alkali before coupling to stromata.

‡ Organisms grown with 500 μ g of nicotinic acid/ml.

Table 4. *Protection of and bactericidal antibody titres in mice after single doses of Bordetella pertussis Boivin endotoxin*

Parent strain	Material	Dose	No. of survivors/ number challenged	Bactericidal antibody titre*
D734	Whole organisms	2×10^9	11/13	480
D734	Endotoxin	70 μ g	2/14	480
D6229	Endotoxin	3 μ g	2/14	30
D6229	Endotoxin	14 μ g	4/15	480
D6229	Endotoxin	70 μ g	1/15	120

* Reciprocal of highest dilution killing 80% of bacteria.

of a typical strain (Table 2). Changing the route of injection and administering a single dose of 2×10^9 strain 134 organisms intravenously did not induce detectable bactericidal antibody; nor did an equivalent dose that had been sonically disrupted and mixed with human red-cell stromata.

Despite its atypical nutritional requirements, strain 134 is in most other respects a typical phase I *Bordetella pertussis* strain, possessing, for example, histamine-sensitizing factor, haemagglutinin, agglutinogens, and protective antigen. Nevertheless, extraction of acetone-dried organisms of this strain by the phenol+water procedure yielded only about one-quarter of the normal amount of LPS (W. A. Vincent, personal communication), whereas bacteria grown with nicotinic acid yielded at least as much LPS as normal organisms.

These two sets of results suggested that the 'bactericidal antigen' might in fact be the *Bordetella pertussis* LPS, and attempts were therefore made to raise bactericidal antisera in mice with extracted LPS. However, this material was poorly antigenic, and single or divided doses of 0.2 to 100 μ g given either alone or with aluminium hydroxide as adjuvant failed to elicit any response. To improve its antigenicity, the LPS was coupled to red-cell stromata (with or without prior alkali treatment) or to the conjugated protein (Morgan, 1943) extracted from *Escherichia coli* strain NCTC86.

Table 3 gives the results of antibody production and mouse protection experiments with these materials. Sheep stromata alone induced moderate titres of bactericidal antibody; such antisera also gave a precipitin line with isolated pertussis LPS (see below). Human stromata prepared from either group O or group A blood gave no cross-reaction and were used for all coupling experiments.

A single dose of LPS+carrier never induced bactericidal antibody, even after treatment of LPS with alkali, a procedure which improves its ability to bind to stromata. (Since alkali treatment causes some loss of antigenic determinants from *Salmonella typhimurium* LPS (Čižnár & Shands, 1970), it was not used routinely.) Five or seven doses of LPS from all strains examined, except strain 134, were sufficient to produce a serum with a bactericidal antibody titre at least as high as that given by a single dose of a normal vaccine strain. However, similar multiple doses of LPS+carrier did not protect mice (Table 3). Seven injections of either *Escherichia coli* protein or human stromata alone gave no detectable bactericidal antibody. A similar dosage of the material extracted by the Westphal procedure from strain 134 did not induce any bactericidal activity, a finding that accorded with the lack of effect of a single dose of whole-cell vaccine.

Extraction of Gram-negative organisms with hot phenol+water yields a highly degraded product containing very little protein; however, the much gentler cold trichloroacetic acid

Fig. 1. Identity of LPS from phase I *Bordetella pertussis* and precipitating antigens in supernatant liquids of sonicates (S/N) from other strains in phases I and IV. Centre well: 6660, hyperimmune rabbit anti-pertussis serum. Outer wells: 1, GL.291 LPS in saline; 2, D6229 S/N; 3, D6229/NA* S/N; 4, 134 S/N; 5, L84 phase I S/N; 6, L84 phase IV S/N.

Fig. 2. Identity of LPS from strains of *Bordetella pertussis*. Centre well: 6660, hyperimmune rabbit anti-pertussis serum. Outer wells: 1, GL.291 LPS; 2, L84 phase IV LPS; 3, D6229/NA* LPS; 4, GL.291 LPS; 5 and 6, 134 LPS (two different preparations).

* Organisms grown with 500 µg of nicotinic acid/ml.

method of Boivin gives a protein-containing product which is a much better antigen. A single dose of material extracted by this method from *Bordetella pertussis* strains D6229 and D734 induced bactericidal antibody, but did not confer significant protection (Table 4).

Further evidence of the connexion between LPS and the 'bactericidal antigen' was provided by immunodiffusion plates. When the supernatant liquid of a sonicate centrifuged at 20,000g was tested against a rabbit hyperimmune antipertussis serum (denoted 6660, Dolby & Dolby, 1969), a strong precipitin line was given by material from strains D6229, D25525 (grown with or without nicotinic acid), and L84 phase I or phase IV, but not by two separate lots prepared from organisms of strain 134 (Fig. 1). Moreover, this line was identical with the single line obtained by using GL.291 LPS as antigen; sonicates from three other phase I strains gave the same strong precipitin line and single doses of them induced bactericidal antibody in mice.

The identity between the LPS present in all strains examined except 134 is also shown in Fig. 2; LPS from strains GL.291, D6229 grown with nicotinic acid, and L84 phase IV, all gave a single identical line against serum 6660. Two lots of extract from strain 134 (two separate extractions) gave no line.

Similarly, when the mouse sera prepared by repeated doses of LPS coupled to stromata or to *Escherichia coli* protein were examined, all, with the exception of anti-134 LPS, gave a single precipitin line against GL.291 LPS. Identical patterns were obtained when the centre well contained LPS extracted from D6229 (grown with or without nicotinic acid), L84 phase IV, or two other normal vaccine strains; with LPS from strain 134, no lines appeared.

The striking immunological difference between the LPS prepared from strain 134

Table 5. *Ability of Bordetella pertussis whole organisms and LPS to act as an adjuvant to haemolysin production in mice*

Antigen, given to all mice, was 0.25 ml of a 1/2.5 dilution of human group A blood.

Strain	Adjuvant		Haemolysin titre*
	Material	Dose	
D6229	Organisms	3×10^9	80
D6229/NA†	Organisms	3×10^9	80
184 phase I	Organisms	3×10^9	160
184 phase IV	Organisms	3×10^9	80
134	Organisms	3×10^9	160
GL.291	LPS	100 µg	80
—	Saline	—	10

* Reciprocal of highest dilution lysing 50 % of the red cells.

† Grown with 500 µg of nicotinic acid/ml.

organisms and that prepared from all other strains examined is emphasized by the fact that even serum from mice given as many as seven doses of 134 whole-cell vaccine, although bactericidal (Table 2), did not give a precipitin line with any *Bordetella pertussis* LPS, including that extracted from strain 134 itself.

Sheep stromata could not be used as carriers for LPS because injected alone into mice they induced bactericidal antibody. It is therefore interesting that with 6660 serum these stromata gave a precipitin line identical with that given by GL.291 LPS; moreover, the bactericidal serum produced by five injections of stromata alone gave a precipitin line against GL.291 LPS.

The Boivin-type endotoxin prepared from strains D734 and D6229 gave single strong lines against 6660 serum identical with that formed by GL.291 LPS.

In view of the close association between LPS and the 'bactericidal antigen', the endotoxin content of the whole-cell vaccines was assayed by measuring their toxicity in mice sensitized with 10 µg of actinomycin D. The LD₅₀ of strain 134 was about four times that of D6229 (5.3×10^8 organisms compared with 1.4×10^8 for D6229). Strain D6229 grown with nicotinic acid had an LD₅₀ similar to that of normally grown D6229, whilst 184 phases I and IV were both slightly more toxic (LD₅₀ 1.0×10^8 organisms).

The effectiveness of various strains as adjuvants to the production of haemolysin by mice was also investigated, since the adjuvant activity of Gram-negative organisms is believed to be related to their content of endotoxin. The results (Table 5) show that whole bacteria of strain 134 are as good an adjuvant as D6229, and that approximately the same effect is produced by 100 µg of GL.291 LPS.

DISCUSSION

Since the report of the Medical Research Council trial (1956) there has been much interest in the mechanism by which the mouse is protected in the potency assay for *Bordetella pertussis* vaccines, and in the reason why the results of the test correlate with the ability of vaccines to protect children.

In mice challenged with 50,000 organisms of strain 18-323 the bacteria multiply on the ciliated epithelium of the brain for 3 days whether the mouse is immune or not (Berenbaum, Ungar & Stevens, 1960; Dolby & Standfast, 1961). In non-protected mice the bacteria continue to multiply until the viable count reaches 10^7 to 10^8 organisms/brain, when the

animal dies; in mice which have been protected by active immunization, however, the viable count starts to decline at about 4 days after challenge, and by 7 to 10 days after challenge the brain is sterile. At about the same time as the brain count in protected mice is beginning to decline the blood-brain barrier becomes permeable to bovine serum albumin (Berenbaum *et al.* 1960), to the dye Pontamine Sky Blue, and to human serum (Holt, Spasojevic, Dolby & Standfast, 1961), and to horse gamma globulin (Iida, Kusano, Yamamoto & Konosu, 1966). The blood-brain barrier is normally impermeable to all these substances and, at least in the case of Pontamine Sky Blue, becomes so again in surviving mice at about the same time as the brain becomes sterile.

These results have naturally led to the suggestion that protection in vaccinated mice is due to a circulating antibody which cannot, however, gain access to the infecting organisms until the blood-brain barrier has been breached. Among the many antibodies induced by a whole-cell *Bordetella pertussis* vaccine, the obvious candidate for a protective antibody in such a mechanism is the complement-mediated bactericidal one; and a considerable amount of evidence indirectly supporting its involvement has been collected (Dolby & Dolby, 1969).

However, the results presented here seem to show that such a correlation must be fortuitous. Pusztai & Joó (1967) found that *Bordetella pertussis* grown with 500 µg of nicotinic acid/ml suffers a phenotypic loss of mouse-protective antigen; we have confirmed this observation, and have also demonstrated that as many as six spaced doses of such organisms were unable to protect mice. Nevertheless, one dose of bacteria grown with nicotinic acid induced bactericidal titres at least as high as those produced by normal protective strains. In addition, phase IV variants of normal vaccine strains, adapted to grow on simple media by repeated subculture (Leslie & Gardner, 1931), still induced normal bactericidal antibody titres, although their ability to protect mice was almost, if not entirely, lost.

Conversely, one dose of 2×10^9 organisms of strain 134 will protect mice well, without producing detectable bactericidal antibody. This antibody thus appears to be neither necessary nor sufficient for the protection of mice; whether protection is conferred by a different circulating antibody or by a cell-mediated process is not known.

Little is known of the chemical nature of the protective antigen, although it has been suggested to be a lipoprotein (Wardlaw, 1966; Ayme, Mynard, Donikian & Triau, 1970; Sato, 1970). However, Pusztai, Csizér & Joó (1971) produced evidence that the 'bactericidal antigen' may be the lipopolysaccharide. This is known to be the case with *Neisseria gonorrhoeae* (Glynn & Ward, 1970) and the results in this paper support the suggestion that the same is true of *Bordetella pertussis*. The LPS prepared by the Westphal procedure is a poor antigen. In order to produce a bactericidal serum in rabbits, Pusztai, Csizér & Joó administered repeated large doses (a total of 35 to 40 mg/animal), the first three with Freund's complete adjuvant. Working with mice, we found that LPS coupled to either *Escherichia coli* conjugated protein or human red-cell stromata induced a high bactericidal titre after 4 to 7 spaced doses totalling 30 µg/mouse. Only with a Boivin-type endotoxin, prepared by extraction with cold trichloroacetic acid, could we induce bactericidal antibody after a single injection. Neither LPS on carrier nor Boivin-type endotoxin conferred any active protection against intracerebral challenge.

Pusztai and her colleagues were also unable to protect mice actively with LPS, although their hyperimmune rabbit serum would confer passive protection. However, passive protection does not necessarily involve the same mechanism as active protection: some types at least of passive protection are not correlated with active protection. These results will be reported elsewhere.

The Ouchterlony immunodiffusion technique gave a strong precipitin line, identical to

that produced by isolated GL.291 LPS, from sonicates of all organisms that induced bactericidal antibody in mice, including L84 phase IV. Moreover, the LPS isolated from these strains appeared to be homogeneous and identical by immunodiffusion; by contrast, the LPS isolated from strain 134 was clearly different and did not give a precipitin line, even with an homologous antiserum. In addition, the Boivin-type antigens cross-reacted strongly with the isolated LPS as expected. Thus all strains of *Bordetella pertussis* examined, with the significant exception of strain 134, seem to contain a common antigen, identical, in terms of the immunodiffusion test, with isolated LPS. All sera prepared against LPS combined with stromata, except that raised against 134 LPS, gave a single anti-LPS precipitin line. Although there is thus a strong correlation between the presence of precipitating and bactericidal antibodies, the two are not identical, for the serum of mice given seven doses of strain 134 organisms, although now strongly bactericidal, did not give any precipitin lines against GL.291 LPS. MacLennan (1960) found that LPS extracted from phase IV *B. pertussis* did not cross-react with phase I LPS; however, the supposedly phase IV strain G154E (supplied to Dr MacLennan from the Elstree culture collection) is not in fact *B. pertussis* but a *Brucella* species.

The finding that mice given seven doses of strain 134 organisms developed appreciable bactericidal antibody titres indicates that this strain must possess some 'bactericidal antigen'.

We have suggested that the antigenic group which induces bactericidal antibody forms part of the bacterial endotoxin, and two possible explanations for the low 'bactericidal antigen' activity of strain 134 are that it contains (i) only a very small amount of the whole *Bordetella pertussis* endotoxin, or (ii) a normal amount of an endotoxin in which the bactericidal antibody-inducing ability has been diminished. The latter would not necessarily affect the pharmacological properties of the bacterial endotoxin.

The endotoxin toxicity of strain 134, as measured in actinomycin D-sensitized mice (Pieroni & Levine, 1970), was four times less than that of strain D6229, suggesting that 134 had a lower than normal content of endotoxin. Gram-negative organisms have a pronounced stimulatory effect on antibody production, and this adjuvant effect appears to be due to their endotoxin content (for a review, see Neter, 1969). Specifically, isolated *Bordetella pertussis* LPS has been shown to act as an adjuvant by Farthing & Holt (1962) and by Nakase, Tateisi, Sekiya & Kasuga (1970). The ability of strain 134 to increase the primary response of mice to human group A erythrocytes was therefore examined; this strain was found to be as good an adjuvant as any of the strains tested.

The evidence obtained so far therefore suggests that strain 134 possesses appreciable quantities of an endotoxin having at least some of the properties expected of such a material, and that its inability to induce bactericidal antibody is due to a loss of relevant immunological determinant group(s). An attempt by Dr L. Szabo to detect this loss by examination of the composition of isolated strain 134 LPS is now in progress.

It is now clear that bactericidal antibody is not involved in the protection of mice. Vaccine made from strain 134 protected both mice (Pillemer, 1950) and children (Medical Research Council report, 1956). Vaccine made from strain 134 did not induce bactericidal antibody in mice unless given in a prolonged series of injections, but it did do so in rabbits much more readily; children may well be more like rabbits than mice in their ability to respond. Since strain 134 is not now in general use as a vaccine strain, the bactericidal antibody response of children cannot be tested directly.

There is at present no evidence to suggest that the mouse-protection test is not measuring the same antigen as that which protects children, and so it seems reasonably likely that

bactericidal antibody production, if any, by vaccinated children would be of no value in protecting against subsequent infection. Certainly Dolby & Stephens (unpublished findings) were unable to show any correlation in vaccinated children between the bactericidal antibody titres and their ability to avoid a subsequent attack of whooping cough.

A consequence of the lack of identity between the 'bactericidal' and protective antigens of *Bordetella pertussis* is that it is now not possible to contemplate replacing the expensive and time-consuming mouse-protection assay for vaccine potency by an assay for bactericidal antibody *in vitro*.

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