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The effect of the antigen which elicits the bactericidal antibody and of the mouse-protective antigen on the growth of *Bordetella pertussis* in the mouse brain

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SUMMARY

The effect of antigens of *Bordetella pertussis* and their antibodies on brain infections by *B. pertussis* in mice are suppression of an infection immediately, so that the initial 90 % loss due to leakage from the brain is maintained or the numbers of bacteria are reduced even further, sometimes with complete sterilization particularly after a small lethal challenge of 10 LD 50 (mechanism 1), and a delayed antibacterial activity *in vivo* which does not begin until 3 days after challenge (mechanism 2). The first, immediate reaction is over in 2–3 days; the second is maintained from 3–4 days onwards, and results in elimination of the bacteria and protection of mice.

The parts played *in vivo* in overcoming infection in these two ways by two antigens and their respective antibodies have been investigated. These antigens are a lipopolysaccharide capable of eliciting an antibody which is bactericidal *in vitro* in the presence of complement called the 'bactericidal antigen', and the mouse protective antigen.

Considering first passive immunity, bactericidal antibody elicited by isolated antigen, and of high titre *in vitro*, is only very weakly active by mechanism (1) *in vivo*. Brains are seldom sterilized and mice not therefore protected. Antisera to whole cell vaccines whether they contain the 'bactericidal antigen' or not, or the protective antigen or not can more easily reduce infections by mechanism (1), eliminating small lethal challenges in some mice which are protected. A passive, intracerebrally protective antibody (PIPA) different from other known antibodies, has been postulated to account for this. Antisera to whole cell vaccine which is protective as defined in the potency assay, can, in additon to this, protect mice by mechanism (2) not only against 10 LD 50 but also 100 LD 50 challenge, and is the only antibody which can do this.

These antibodies have been investigated by injecting them with the challenging organisms. The antibody effects described above are given by antisera stimulated by several injections and also by the concentrated serum immunoglobulins of once vaccinated mice. The antibody, which is bactericidal *in vitro* only, is in the 7 S globulin fraction of the serum of once vaccinated mice. The protective antibody

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capable of overcoming small and large challenges is in the 19 S and 11 S globulins. The antibody, PIPA, protecting against small lethal challenges only is in the fraction A_2 containing mainly 11 S globulin.

In active immunization experiments the suppression of infection which immediately follows intracerebral vaccination, but which only lasts 2–3 days (mechanism 1), is not dependent on either 'bactericidal' or protective antigens but on a component present in all our whole cell vaccines. Vaccines which also had protective antigen eliminated the remaining infection at 4–6 days after challenge by mechanism (2).

As in passive immunity, only the protective antigen can completely overcome 100 LD 50. Suppression of a small, lethal, intracerebral infection given 14 days after *intraperitoneal* vaccination by mechanism (1) may however be correlated with protective antigen.

INTRODUCTION

The accepted method for estimating the suitability of pertussis vaccines for use in the field is an active protection test for mice (World Health Organisation, 1964). This assay consists of injecting mice once intraperitoneally with a suitable dose of vaccine and challenging them intracerebrally 14 days later with 100 LD 50 of a highly mouse-virulent strain of *Bordetella pertussis* organisms. The protection of mice by this method, and also the passive protection of mice following the intraperitoneal injection of antisera against the usual size of intracerebral challenge, have been shown to depend not on the immediate sterilization of the brain but on an *in vivo* bactericidal effect which becomes evident 3–4 days after challenge (Berenbaum, Ungar & Stevens, 1960; Dolby & Standfast, 1961).

An *in vitro* bactericidal effect of antiserum in the presence of complement has been demonstrated (Dolby, 1965). For convenience, the antigen, a lipopolysaccharide, which elicits the antibody responsible for this *in vitro* bactericidal effect, is referred to as 'bactericidal antigen'. In quantitative tests with 7 S globulin fractions of pertussis antiserum from hyperimmunized rabbits, Dolby & Dolby (1969) found that *in vitro* and *in vivo* bactericidal effects ran parallel and suggested that the two activities were the properties of one antibody. However, Ackers & Dolby (1972) showed that the 'bactericidal antigen' when injected intraperitoneally could not protect against an intracerebral challenge 14 days later. Thus the same antibody cannot be responsible for the *in vitro* and *in vivo* bactericidal action.

In passive protection tests, with antisera injected intraperitoneally or intracerebrally, Dolby (1972) has shown that two mechanisms operate: (1) an immediate clearance of organisms from the brain, (2) clearance after 4–6 days, before which time the organisms grow, apparently uninfluenced by the antisera.

Active immunization by the intracerebral route eliminates the challenge organisms by both mechanisms (Standfast & Dolby, 1972) and immunization by the intraperitoneal route by the second mechanism only as already mentioned. In the routine potency assay of vaccines where the second mechanism operates, the 'bactericidal antigen' plays no part and protection is dependent, by definition, on the protective antigen.

Table 1. *Vaccines of differing 'bactericidal' and protective antigen content used for active and passive protection experiments*

Type of vaccine	Prepared from strain	Content of antigen* 'Bactericidal'	Content of antigen* Protective	Referred to as
(*a*) Killed, whole cell	134	−	+	B − P + (134)
(*b*) Killed, whole cell	D 6229	+	+	B + P + (vac)
(*c*) Purified lipopolysaccharide†	D 6229	+	−	B + P − (LPS)
(*d*) Killed whole cell of cultures grown in nicotinic acid	D 6229	+	−	B + P − (nic)
(*e*) Lister Reference Vaccine	polyvalent	+	+	B + P + (ref)

* As defined in Methods, under vaccines.

† Combined with red-cell stromata or *Escherichia coli* conjugated protein.

Antibody to the 'bactericidal antigen' might still be responsible, however, for the immediate clearance of organisms from the brain (mechanism 1). To test this and to investigate which clearance mechanisms are or are not related to the protective antigen, active and passive protection tests were done. The vaccines previously described (Ackers & Dolby, 1972), with and without 'bactericidal antigen' or mouse-protective antigen, were used. They were injected intraperitoneally or intracerebrally into mice and their effect on a challenge given 3 hr. or 14 days later determined by brain counts. Antisera from mice given single or multiple injections of vaccine were used in passive protection tests either as separated immunoglobulins or as whole serum respectively. Antisera or immunoglobulins were mixed with the challenge organisms and injected intracerebrally.

METHODS

Vaccines

Five vaccines were used (see Table 1); the four experimental ones (*a–d*) and their production have been described (Ackers & Dolby, 1972). Several batches of the four experimental vaccines were tested and each batch was checked for its ability to stimulate bactericidal antibody production (measured as described under 'Bactericidal antibody') and protect mice (as described under 'Active immunization') after intraperitoneal injection against an intracerebral challenge of 100 LD 50 14 days later. Vaccines which were described as positive for 'bactericidal antigen' elicited bactericidal antibody to a titre of 1/120–1/480 14 days after one intraperitoneal injection, whilst negative ones had titres of less than 1/30. Vaccines which were protective contained at least 4 International Potency Units per 2×10^9 organisms, non-protective ones less than 0·2 units per 2×10^9 organisms. The whole-cell vaccines were grown in Cohen & Wheeler (1946) medium modified as previously described (Ackers & Dolby, 1972) and with the addition, where stated, of nicotinic acid to a concentration of 500 μg./ml. to suppress protective antigen

formation. Cell-free lipopolysaccharide on carrier, was as described previously. A standard reference preparation was a killed, whole cell vaccine.

Preparation of antisera

These were all prepared in mice for reasons given previously (Ackers & Dolby, 1972). Female mice of Theiler's Original strain at a starting weight of 16–18 g. were used. Vaccines were injected intraperitoneally using one of three schedules: (1) a single dose of 2 or 4×10^9 organisms in 0·5 ml.; (2) 5–7 weekly doses of 1×10^9 organisms in 0·2–0·5 ml.; (3) 4–7 weekly doses (increasing from 2–10 μg./dose) of the lipopolysaccharide antigen in 0·2–0·5 ml.

Mice were killed with coal gas 2 weeks after the last injection, or at 3 days after intracerebral challenge (17 days after vaccination). Blood was collected from the heart, and the serum was separated at 4° C. That intended for direct use was heated for 30 min. at 56° C.; the rest was stored at −25° C.

Preparation of mouse immunoglobulins

Unheated sera from groups of 60–80 mice were treated to remove lipoproteins by the addition of 1/50th vol. of 10% dextran sulphate (Pharmacia) followed by 1/10 vol. of 1 M-$CaCl_2$ added drop by drop with stirring (Burstein & Samaille, 1958). The lipoprotein precipitate was centrifuged and discarded. In a few cases, the lipoprotein-free serum was also freed of albumin by precipitating the globulins with slow addition of 1/2 vol. of 21% polyethylene glycol (M.W. 6000; Hopkin & Williams, Chadwell Heath, Essex), standing overnight at 4° C., and centrifuging (Chesebro & Svehag, 1968). The supernatant was discarded and the globulins in the precipitate dissolved in 0·1 M tris-HCl buffer containing 1 M-NaCl, pH 8·0, and ultrafiltered to concentrate them and remove polyethyleneglycol.

In all cases the sera were run in 0·1 M tris-HCl containing 1 M-NaCl, pH 8·0, on columns of Sephadex G. 200 (Pharmacia), either $2{\cdot}5 \times 100$ cm. with downward elution, or $2{\cdot}2 \times 90$ cm. with upward elution. Fractions were 5 ml. In early experiments the first and second peaks (called '19 S globulins' or fraction A and '7 S globulins' or fraction B respectively) were separated and re-run on the same column, only the middle part of the peak on the second run being used. In later experiments the first peak was further divided into leading and trailing portions which were separated again ('19 S globulins' or fraction A 1 and '19 S + 11 S globulins' or fraction A 2 respectively). Nomenclature of the globulin fractions was determined by identity checks against Nordic anti-mouse IgM, IgA and IgG. The fractions were ultra-filtered and dialysed against phosphate saline, pH 7·6, to give a concentration of 25 mg. protein/ml. and stored at −25° C. Yields of protein per ml. serum were about 1·5 mg. of A 1, 1·5 mg. of A 2 and 8–10 mg. of B. Before use, fractions were sterilized by treating at 56° C. for 30 min.; this had no appreciable effect on their activity.

Active immunization

Female mice of Theiler's Original strain were used. They were given either one dose of vaccine intraperitoneally in 0·5 ml. and challenged 14 days later or

vaccinated intracerebrally in 0·03 ml. and challenged 3 hr. or 14 days later. Graded fivefold dilutions of vaccine were used, to determine the ImD 50 (the dose of vaccine protecting half of the mice) based on mice surviving at 14 days after challenge, calculated as previously (Ackers & Dolby, 1972). Challenges were of 5000 or 50,000 total organisms approximately 10 or 100 LD 50 of infecting organisms of strain 18-323 in 1 % Casamino acids (Difco).

Passive protection

Female mice of Theiler's Original strain were given antiserum either intraperitoneally in a dose of 0·2 ml. (dilutions in saline) followed by intracerebral challenge 3 hr. later, or intracerebrally (dilutions in 1 % Casamino acids), mixed with the infecting organisms of 10 or 100 LD 50, incubated 30 min. at 37° C. and injected in a total volume of 0·03 ml. The viable count of challenges alone or mixed with serum were estimated from tenfold serial dilutions pipetted on Bordet-Gengou plates. Survivors were recorded 14 days later.

Bactericidal antibody

The titres of antisera were based on an 80 % *in vitro* bactericidal effect and determined as described by Ackers & Dolby (1972) on mixtures of antibody, guinea pig complement and organisms of strain 18-323.

Degree of infection of brains

Mice were killed in groups of 5–10, at intervals after infection, the brains removed into 1 % Casamino acids, homogenized (by shaking with glass beads), and serial dilutions were plated as described previously (Dolby, 1972). One group of 10–15 mice was kept for determining survivors at 14 days.

RESULTS

The passive protection of mice by antisera with high and low bactericidal antibody titres

Most antisera produced in response to several doses of *B. pertussis* whole cell vaccines have high titres of both *in vitro* bactericidal antibody and protective antibody. The sera are protective against all challenge doses when given either intraperitoneally or intracerebrally, but the effect may be either immediate or delayed. Use of the special vaccines described under Methods has allowed differentiation of these two activities.

Sera from mice injected repeatedly with these special vaccines were given intracerebrally mixed with either 5000 organisms (about 5–10 LD 50) or with 50,000 organisms (50–100 LD 50). Their effect in protecting mice for 14 days and on the numbers of organisms present 2 days after challenge is shown in Table 2.

The results are pooled ones of two or three experiments on two or three sera in each group; the findings with these sera were consistent. They show a difference between the antisera with high bactericidal titres (B+) and those with high protective titres (P+). Serum prepared against cell-free lipopolysaccharide

Table 2. *The passive protection of mice by sera, with high and low bactericidal antibody titres, given intracerebrally with infecting organisms of strain* 18-323

Serum from mice vaccinated with*	Bactericidal antibody† (inverse titre)	Effect of anti-serum against 10 LD 50 Protection‡ (%)	10 LD 50 Sterile brains§ (%)	100 LD 50 Protection‡ (%)	100 LD 50 Sterile brains§ (%)
(*a*) B−P+(134)	<30	75	80	40	0
(*c*) B+P−(LPS)	>7680	0	20	12	20
(*d*) B+P−(nic)	1920	30	40	0	0
No vaccine	<30	0	2	0	0

* See code in Table 1.

† Measured *in vitro* against the serum-sensitive strain 18-323 in the presence of complement.

‡ Protection expressed as percentage of survivors/total mice at 14 days.

§ Sterile brains at 2 days after challenge = % of mice with less than 20 organisms in the brain.

(B+P−), although it had a high bactericidal titre (1/7680) showed no real protection against a challenge of 10 or 100 LD 50, for although 20 % of mouse brains had less than 20 organisms 2 days after challenge, many of these mice eventually died. Sterilization of the challenge was not therefore complete and the few remaining organisms multiplied and killed the mice, after the suppressive initial effect of the serum had been exerted.

Sera against whole cell vaccine B+P−(nic) with a bactericidal titre of 1/1920, showed moderate protection against 10 LD 50 but no protection against 100 LD 50. These sera were effective because of their ability to suppress the challenge initially and brains which had few organisms at 2 days really had been sterilized. They were not, however, able to achieve this result against 100 LD 50.

Sera against whole cell vaccine B−P+(134), with no demonstrable bactericidal activity *in vitro* were able to protect against 10 LD 50 *in vivo* by immediate sterilization and were also moderately protective against 100 LD 50 but by a later more prolonged action since no brains were sterile at 2 days and effective action took place later than this, unlike that of bactericidal sera. Normal sera had no effect.

Experiments with intraperitoneal serum given 4 hr. before an intracerebral challenge of 10 LD 50 gave the expected results. Antiserum to vaccine (*a*), B−P+ (134), protected most of the mice by the delayed effect.

The passive protection of mice with immunoglobulin from the sera of mice once vaccinated with vaccines containing different amount of 'bactericidal' and protective antigens

Table 3 shows the degree of protection, measured as percentage survivors 14 days after challenge, of groups of mice given various immunoglobulin fractions at 12·5 mg. of total protein per ml., injected intracerebrally with 10 or 100 LD 50. As fractions prepared from normal mouse serum showed slight protection against

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Table 3. *The passive protection of mice by the purified immunoglobulins of the sera of mice vaccinated once with vaccines of different 'bactericidal' and protective antigen content*

Serum from mice vaccinated once intraperitoneally with:*	Fraction	Percentage protection†		Relative *in vitro* bactericidal activity‡
		10 LD 50	100 LD 50	
Vaccine (*a*) B − P + (134)	A 1 (19 S)	**70**	**40**	–
	A 2 (11 S + 19 S)	**70**	**40**	–
	B (7 S)	**50**	0	–
Vaccine (*b*) B + P + (vac)	A 1 (19 S)	40	**30**	1
	A 2 (11 S + 19 S)	**60**	**30**	1
	B (7 S)	**50**	0	3·3
Vaccine (*d*) B + P − (nic)	A 1 (19 S)	40	20	–
	A 2 (11 S + 19 S)	**70**	0	–
	B (7 S)	20	0	20
Unvaccinated; normal mouse serum control	A 1 (19 S)	40	0	–
	A 2 (11 S + 19 S)	30	0	–
	B (7 S)	30	0	–

* See code in Table 1.

† Serum fractions at a concentration of 12·5 mg./ml. total protein were injected intracerebrally with 10 or 100 LD 50 of strain 18–323.

‡ $\frac{1000}{\text{Minimum } \mu\text{g. protein/ml. showing an 80 \% bactericidal effect}}$. Negative samples were those inactive at 1000 μg./ml., i.e. < 1.

10 LD 50, although none against 100 LD 50 and no *in vitro* bactericidal activity, only the results in bold type are considered to show significant protection.

The *in vitro* bactericidal activity, where present was, almost entirely in the 7 S globulin, fraction B. Protective power against 100 LD 50 was in the A 1 and A 2 fractions representing 19 S and 11 S globulins, although against 10 LD 50 the 7 S antibody showed some protective activity as well. The results with antiserum to vaccine (*d*) (see Table 1), B + P − (nic), show 70 % protection against 10 LD 50 in the A 2 fraction which had no bactericidal activity *in vitro*; this result suggests that it may not be the bactericidal antibody in antisera to whole cell, non-protective vaccine which is responsible in all the *in vivo* protective effect.

The figures reported in Table 3 are an average of repeated tests in a total of 30–50 mice for each fraction on duplicate pools, each collected from groups of 60–80 mice. Some of these groups were bled 14 days after vaccination, and some 17 days after vaccination and 3 days after challenge to include any stimulating effects of the challenge on the globulin levels. No differences in the sera collected by the two different procedures was detected. Experiments, to determine how the protection of the fractions from antisera to vaccines (*a*) and (*b*) (Table 1) was achieved, will be published elsewhere (Dolby, Dolby & Bronne-Shanbury, 1975).

Active protection by intraperitoneal injection of vaccines containing different amounts of 'bactericidal' and protective antigens against 10 LD 50 intracerebrally 14 days later

The numbers of viable *B. pertussis* organisms in the brains of mice vaccinated intraperitoneally with a standard vaccine such as (*b*) or (*e*), both B + P + (see

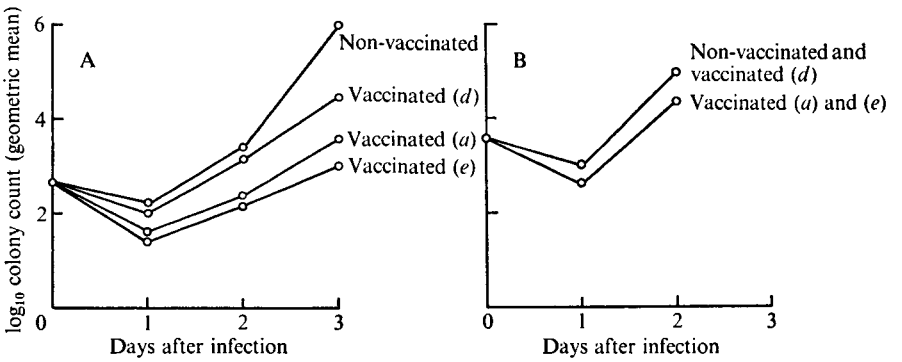

Fig. 1. The colony counts of *B. pertussis* strain 18-323 in the brains of mice infected with 10 or 100 LD 50 14 days after IP vaccination.

(*A*) Ten LD 50 of challenge after vaccination with (i) 10 ImD 50 of vaccine (*a*), B−P+, or (*e*), B+P+, each protecting 14/15 mice; (ii) an equivalent number of organisms of vaccine (*d*), B+P−, protecting 4/15 mice. Challenge given to non-vaccinated mice killed all.

(*B*) One hundred LD 50 of challenge after vaccination with (i) 10 ImD 50 of vaccines (*a*) and (*e*). The curves were identical and all mice were protected. (ii) An equivalent number of organisms of vaccine (*d*). The curve was identical to that in non-vaccinated mice and none survived in either group.

Table 1), and challenging 14 days later with 100 LD 50 are very similar to those of non-vaccinated mice 24–72 hr. after challenge. In one experiment, for instance, an inoculum of $10^{3\cdot6}$ became $10^{3\cdot0}$ and $10^{2\cdot6}$ in non-vaccinated and vaccinated mice respectively at 24 hr. Increasing the dose of vaccine has little effect unless a virulent strain other than 18-323 is used as a challenge (Standfast & Dolby, 1972). If the challenge by strain 18-323 is reduced to one of 10 LD 50, however, there is a difference between vaccinated and non-vaccinated similar to that achieved by using another strain; an inoculum of $10^{2\cdot5}$ became $10^{2\cdot3}$ in non-vaccinated but $10^{1\cdot3}$ in vaccinated mice. Since the ImD 50 of the vaccine was the same whether 10 or 100 LD 50 were used as challenge, this early drop in bacterial count in vaccinated mice could not have been of importance in protection from death.

To test whether 'bactericidal antigen' was able to bring about this initial drop in count, groups of mice were immunized intraperitoneally with the same amount of vaccines (*a*) B−P+ (134), (*d*) B+P−(nic) and (*e*) B+P+(ref) (see Table 1) and challenged with 10 LD 50 14 days later. Five mice from each group were killed at 1, 2 and 3 days after challenge and 10–15 mice observed for 14 days for deaths. The ImD 50 was calculated from survivors given three fivefold doses of each vaccine. Results are shown in Fig. 1 A and these illustrate very similar counts in mice receiving 10 ImD 50 of protective vaccines (*a*) and (*e*), whether 'bactericidal antigen' was present (*e*) or absent (*a*). On the other hand, counts in mice given vaccine (*d*) B+P−(nic) were more like those of non-vaccinated mice, thus suggesting that 'bactericidal antigen' was not responsible for this early suppressive effect on the challenge. For comparison, the results following vaccination with (*a*) only and challenge with 100 LD 50 are shown in Fig. 1 B. Here, the difference in counts at 1 day between vaccinated and non-vaccinated mice is very small.

Table 4. *The effect of two protective vaccines with and without 'bactericidal antigen' against* 100 *LD*50 *of strain* 18-323 14 *days after intracerebral vaccination*

Vaccine*	B−P+ (*a*)	B+P+ (*e*)	B−P+ (*a*)	B+P+ (*e*)	B−P+ (*a*)	B+P+ (*e*)
Dose in ImD 50	1·5		6		12	
No. sterile brains	1	0	2	3	2	2
No. infected brains	4	5	3	2	3	3
Log colony count† (infected brains)	4·9	4·4	4·4	4·4	2·1	2·6
Log colony count (all brains)	4·4	4·4	2·8	1·8	1·3	1·7
Survivors/15 mice at 14th day	12	12	13	15	15	15

* See Table 1. † Geometric mean.

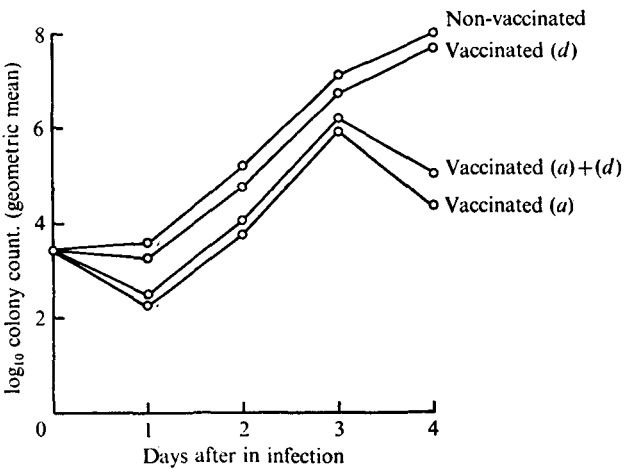

Fig. 2. The colony counts of *B. pertussis* strain 18-323 in the brains of mice infected with 100 LD50 13 days after IC vaccination with (i) 2·5 ImD50 of vaccine (*a*), B−P+, giving 12/14 survivors, (ii) the same amount of vaccine (*d*), B+P−, 0/10 survivors, (iii) vaccines used in (i) and (ii) together, 7/13 survivors. Non-vaccinated mice were not protected.

Active protection by intracerebral injection of vaccines containing different amounts of 'bactericidal' and protective antigens against intracerebral challenge

Effect against 100 *LD*50 14 *days later*

Standfast & Dolby (1972) showed that 3 ImD50 of whole-cell vaccine given by the intracerebral route protected mice in the same way as intraperitoneal vaccination, that is the inoculum increased in numbers for 3–4 days and not until then was suppressed. A dose greater than 3 ImD50 intracerebrally caused a suppression of the bacterial growth by 2 days, however, whereas increasing the dose of intraperitoneal vaccine had no such effect. To determine whether 'bactericidal antigen' played any part in this early suppression by larger doses of intracerebral vaccine, the two vaccines (*a*) B−P+ (134) and (*e*) B+P+(ref) (see

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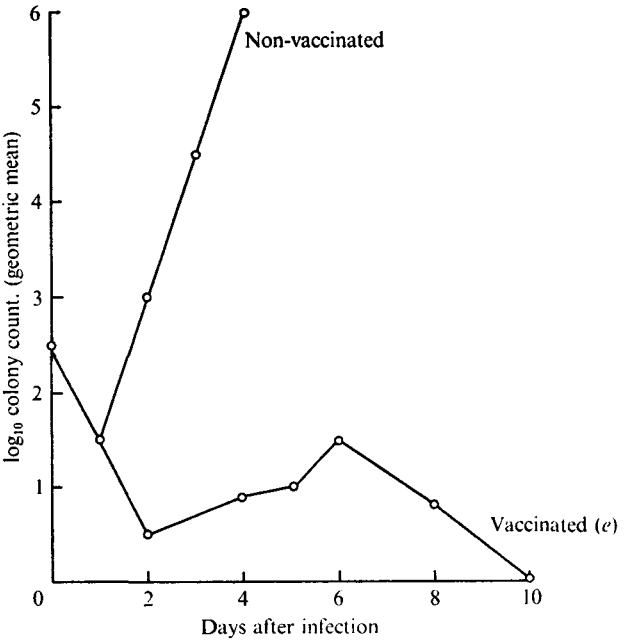

Fig. 3. The colony counts of *B. pertussis* strain 18·323 in the brains of mice infected with 10 LD 50 (i) 3 hr. after IC vaccination with 10 ImD 50 of vaccine (*e*), B + P +, which protected all mice, and (ii) in the brains of non-vaccinated mice.

Table 1) were again used. These were of the same protective potency so that the same amount of vaccine could be given. They were injected intracerebrally into mice in three different doses of 1·5, 6 and 12 ImD 50 and groups of five mice were killed 2 days after challenge for the estimation of viable bacteria in the brains. Groups of 15 mice were observed for 14 days for deaths and potency was determined from three fivefold doses of vaccine as previously. The results in Table 4 show that both vaccines (*a*) without 'bactericidal antigen' and (*e*) with the antigen behaved similarly, reducing the bacterial count by 2 days; this action is thus independent of 'bactericidal antigen'.

To make sure that results with vaccine (*a*), B − P + (134), would have remained unchanged had 'bactericidal antigen' been present, an experiment was carried out, injecting this vaccine (*a*) alone intracerebrally in a dose of 2·5 ImD 50 or together with vaccine (*d*) B + P − (nic); a dose of 2·5 ImD 50 of (*a*) and an equal amount of non-protective vaccine (*d*) were combined. The growth of pertussis in the mouse brains of these vaccinated, challenged mice was followed and the results are shown in Fig. 2. Vaccine (*d*) was unable to alter the effect of vaccine (*a*) on the numbers of organisms *in vivo*. The decrease in protective activity of vaccine (*a*) injected with (*d*) over that of (*a*) alone could not be consistently obtained, and is not regarded as significant.

Table 5. *The effect at 2 days of vaccines with and without 'bactericidal antigen' on 10 LD 50 3 hr. after intracerebral vaccination*

Vaccine	B+P+(vac) (*b*)	B−P+(134) (*a*)	B+P−(nic) (*d*)	Control
Dose in ImD 50	50	50	1	0
No. sterile brains*	2	1	3	0
No. infected brains*	8	9	7	10
Log GM† colony count of infected brains	2·0	1·9	2·1	2·6
Log GM† colony count of all brains	1·6	1·7	1·5	2·6

* Out of 10. † Geometric mean.

Effect against 10 LD 50 3 hr. later

Evans & Perkins (1954) described an interference phenomenon which occurred when they challenged mice with 10 LD 50 3 hr. after mice had been vaccinated intracerebrally. This they suggested was due to the vaccine interfering with the establishment of the infecting organisms. In previous investigations (Standfast & Dolby, 1972) it was found that small doses of vaccine of the type (*b*) or (*e*) equivalent to one tenth of a protective dose could suppress challenge injected 3 hr. later but that this ability disappeared if the interval between vaccination and challenge extended to 3–4 days or more, unless the dose of vaccine were increased to a protective dose. A typical infection curve for about 10 ImD 50 of vaccine (*e*), B+P+(ref), is shown in Fig. 3. The bacterial inoculum was reduced from $10^{2\cdot5}$ to less than 10 for up to 3–4 days. As this effect decreased the bacterial count began to rise slowly until about the 6th day when an antibacterial mechanism capable of sterilizing the brains of all infected mice came into effect. To determine the activity of a non-protective vaccine containing 'bactericidal antigen' compared with a protective vaccine, 50 ImD 50 of the latter and the same number of cells of the non-protective vaccine were injected intracerebrally into mouse brains 3 hr. before 10 LD 50 of challenge. Vaccines (*d*) B+P−(nic), (*a*) B−P+ (134) and (*b*) B+P+ (vac) were used. The results, shown in Table 5, suggest that all vaccines, with and without 'bactericidal antigen' and irrespective of their ultimate ability to protect mice, had a similar initial effect on the challenge, but thereafter only vaccines containing protective antigen could sustain the low bacterial counts and eventually eliminate the infection. This effect has been investigated further (Dolby *et al.* 1975).

DISCUSSION AND CONCLUSIONS

Isolated 'bactericidal antigen' in the form of lipopolysaccharide injected repeatedly into mice, stimulated antisera which were highly bactericidal *in vitro* but when injected intracerebrally with the challenge organisms, such sera had at most only a very slight effect in reducing the numbers of organisms *in vivo*; brains were not sterilized and no protection ensued even with a small challenge of 10 LD 50. Dolby & Stephens (1973) had previously found a rather poor correlation between the ability of child sera to suppress infection in mice and their bactericidal antibody titres.

Antiserum to several injections of any whole cells vaccine, whether containing 'bactericidal', or protective antigen reduced a 10 LD 50 challenge of organisms with which it was injected into the brain, and in some mice eliminated the infection entirely in 1–2 days. This effect was over in 3 days after challenge and any organisms remaining multiplied and killed the mouse unless protective antibody was also present. We suggest calling the antibody the 'passive intracerebrally protective antibody' or PIPA. It is exemplified by antibody to vaccine (*d*) Table 2, and is separate from bactericidal or mouse-protective antibodies. Protective antibody was the only one with late and sustained activity capable of eliminating 100 LD 50.

When one dose of any whole cell vaccine, with or without 'bactericidal antigen' or protective antigen, was injected intracerebrally with, or shortly before 10 LD 50 of challenge, there was an immediate reduction in the numbers of organisms in the brain. This effect must therefore be independent of both antigens. It was described by Evans & Perkins (1954), who showed that protection was only achieved by vaccines protecting when given intraperitoneally. Andersen (1957) demonstrated, however, that an immediate reduction in brain-count was non-specific and followed *E. coli* vaccination. The non-specificity of this effect is explored in more detail elsewhere (Dolby *et al.* 1975).

The non-specific bacterial cell component capable of suppressing an infection after intracerebral vaccination has been suggested as being endotoxin. Iida & Tajima (1971) produced an inhibition of the growth of *B. pertussis* intracerebrally after endotoxin injection and could reproduce the effect with double-stranded-RNA. Their effect differs from ours however because their suppression did not begin until 3–4 days after challenge, was maximal at 7 days and was correlated with ultimate survival which means that the brains must have been completely sterilized. Whether the antigen we have demonstrated is that eliciting PIPA is not known.

The three antibodies were demonstrated in the concentrated immunoglobulins of mice once-vaccinated intraperitoneally. The bactericidal antibody is a 7 S globulin and has no protective activity. The antibody PIPA, capable of protecting mice against 10 LD 50 of challenge and stimulated by vaccines independently of their 'bactericidal' and mouse-protective antigen content is in the A 2 (mainly 11 S) fraction. The mouse-protective antibody elicited by potent vaccines and capable of eliminating challenge of 100 LD 50 by an antibacterial action extending from 3 days after challenge is in both the 19 S and in the 19 S + 11 S fractions. Previous attempts to protect mice passively with the sera from once-vaccinated animals have been made (Wardlaw & Jakus, 1968) but none of these has been with concentrated immunoglobulins, which we used to ensure that enough antibody could be introduced in the restricted volume. In doing so, we showed that an amount of immunoglobulin not more than would be present in each mouse could, when introduced into the brain, protect against infection. Use of these globulins by the intraperitoneal route is described elsewhere (Dolby *et al.* 1975).

Protective antigen cannot therefore be measured specifically by estimating antibody capable of protecting against 10 LD 50 of challenge, which measure PIPA, but only against 100 LD 50; it cannot be measured by ability of vaccine to suppress

a challenge injected intracerebrally with it or up to two days later because of the non-specific clearance effects which may protect.

Protective vaccine injected *intraperitoneally* followed by a small challenge of 10 LD 50 intracerebrally 14 days later, however, can depress the initial bacterial growth before the dramatic elimination of organisms from the brain begins at 3 days after challenge. This is not dependent on the presence of 'bactericidal antigen' and is only exerted by protective vaccine. Standfast & Dolby (1972) described the suppression of brain infections in vaccinated mice, with the virulent strain Gl 353 and the avirulent strain L 84. This was present over the first 3 days after challenge and to a much greater extent than we ever found using 18-323 for challenge, even 10 LD 50, but might well be due to the same phenomenon.

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