

## The influence of the route of immunization on the protection of mice infected intracerebrally with *Bordetella pertussis*

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### SUMMARY

The development of immunity in mice to *Bordetella pertussis* induced by intracerebral, intravenous or intraperitoneal vaccination was analysed in terms of the viable bacteria in the brain after intracerebral challenge, the serum antibodies, and protection against the sublethal infection of the lung that follows intranasal inoculation.

A vaccine introduced intracerebrally was five to ten times more effective than that given intraperitoneally or intravenously, as measured for each route by the amount of vaccine required to protect half the mice against an intracerebral challenge 14 days later (ImD50). Intracerebral vaccination induced higher antibody titres than vaccination by the other two routes. The survival of infected mice given 1-3 ImD50 doses of vaccine intracerebrally 14 days before, followed a pattern similar to that after intraperitoneal or intravenous vaccination with up to 10 ImD50 of vaccine: the numbers of organisms increased for 3 days and then declined. Injection of about four ImD50 of vaccine intracerebrally produced a local immunity, resulting in an immediate kill of challenge organisms given 14 days later. Such an effect following intraperitoneal vaccination was achieved only against challenges with an avirulent strain. It is suggested that better stimulation of circulating antibody and local immunity in the brain together account for the better protection induced by intracerebral vaccine.

Immunity to an intracerebral infection appears therefore to have at least three components, each specific for pertussis. The first, like that induced by intraperitoneal and intravenous vaccination, reaches a maximum in 2 or 3 weeks and is probably an expression of a general response by the animal operating not earlier than 3 days after infection. The second is a local immunity, appearing after the same interval. The third is a short-lived local immunity which has been described by previous workers; it immediately follows the injection intracerebrally of ten times less vaccine than that needed to protect against a challenge 14 days later and lasts only 2-3 days. The second and third types result in immediate sterilization of the infection.

Mice recovering from sublethal brain infection with avirulent organisms were immune to a second infection with a virulent organism, but this was achieved not by the ability to kill the re-infecting organisms immediately on injection into the brain, but only after the 3-4 days lag such as follows intraperitoneal vaccination.

## INTRODUCTION

Throughout this paper, vaccination and challenge routes are referred to by the abbreviations IP (intraperitoneal(ly)); IV (intravenous(ly)); and IC (intracerebral(ly)). The vaccination route is given first and the challenge route second: IP/IC indicates IP vaccination followed by IC challenge.

The protective property of a *Bordetella pertussis* vaccine is assayed in mice, 14 days after a single IP injection of vaccine, by an IC challenge with infecting organisms (Kendrick, Eldering, Dixon & Misner, 1947). Experience has shown that the results are reasonably correlated with those in children in the field (Medical Research Council, 1956). The test, however, is artificial in the sense that *B. pertussis* is not a natural pathogen of mice, and protection against an IC infection has little obvious connexion with the disease in children.

The course of infection in the brains of IC infected mice, unvaccinated and IP vaccinated, was described by Dolby & Standfast (1961); even in mice completely protected by the vaccine, the number of living organisms in the brain increases for 3–4 days before falling. From a comparison of active and passive protection, it seemed to us that the IP vaccine stimulated the production of antibody that reached the brain tissue 3–4 days after infection, when the blood–brain barrier became ‘leaky’ (Holt, Spasojević, Dolby & Standfast, 1961).

Doubt has been cast on the role of antibody in this mouse protection test by several workers. Evans & Perkins (1954*a*, 1955) and Wardlaw & Jakus (1968) could find no protective circulating antibody from 5 hr. to 14 days after one strongly protective IP dose of vaccine.

Evans & Perkins (1954*b*) and Andersen (1957) used very small IC doses of vaccine to protect mice against IC challenge given immediately or up to 3 days later, suggesting some form of local immunity which may not be mediated by antibody. Evans & Perkins suggested that two types of immunity were operative in the mouse potency assay of vaccine. Blake & Wardlaw (1969), however, quote an experiment of Dr Fischel’s in which this short-lived immunity was abolished after body irradiation and have shown that the immunity present one day and 14 days after vaccination IP is inhibited by the immuno-suppressive drug, cyclophosphamide.

The experiments described below were done to compare the course of infection in the mouse brain after IC, IP and IV vaccination. An antigen introduced into the brain tissue leaks into the circulation (Cairns, 1950; Mims, 1960) and so stimulates a general immune response as well as having a local effect.

The general immune response to vaccination was investigated by measuring (i) serum antibodies *in vitro* by assay or by passive protection against sublethal lung infections and (ii) the active protection against a sublethal lung infection, a state that may be independent of circulating antibody.

*Mice*

METHODS

Four strains of mice (Theiler's Original, Schneider-Webster, ICI and a National Institutes of Health strain, NIH-BS, derived from Webster-Swiss mice) were used, but there was no difference in the results obtained attributable to mouse strain. Male and female mice were used, but only one sex in any experiment.

*Vaccination*

Mice were vaccinated at 16-17 g. in weight. The ImD 50 values were estimated by the method of Reed & Muench (1938).

The Lister Institute Reference pertussis vaccine, K 278, formalin-killed and resuspended in thiomersal saline at  $2 \times 10^{10}$  organisms/ml., was used in all experiments. This vaccine was about ten times more potent when given IC than IP or IV (Table 2). As a non-specific control, a batch of Lister Institute alcohol-killed TABC vaccine was used.

Vaccine was given IP either neat or diluted in sterile buffered saline in a volume of 0.2 ml.; the large dose of  $10^{10}$  organisms was given in 0.5 ml.

For IC use, 0.03 ml. volumes were injected. The inoculum was concentrated by centrifugation from the thiomersal saline suspension and resuspended in buffered saline. This material was diluted suitably for other routes of injection in experiments where direct comparison was being made.

For IV vaccination, 0.2 ml. volumes were given into the tail vein.

*Challenge infections*

All mice were infected under ether anaesthesia. For lethal brain infections, strain 18-323, strain 2 atox. from Dr E. K. Andersen (derived from Albany 40103), and strain B 5533 from Dr M. Haire (passed through mice to make it virulent) were used. For intranasal, sublethal infections, a strain, originally from Glaxo laboratories, mouse-passaged at this Institute and referred to as Gl. 353, was used. Strain L 84, from the Lister Institute collection, was used for sublethal brain infections. Growth was harvested from 20 hr. Bordet-Gengou plates, suspended in 1% Casamino Acids (Difco), and the suspension first adjusted until equal to an opacity of 10 international units, approximately equivalent to  $10^{10}$  total organisms/ml., of which 10-20% were viable. Further dilutions were made in 1% Casamino Acids as required.

Mice were challenged with strain 18-323 in 0.03 ml. volumes containing 50,000 organisms, representing a challenge of about 100 LD<sub>50</sub>. Strain L 84 grew in mice, but did not kill them in IC doses up to  $10^6$  organisms in 0.03 ml., and was used to determine the immunity induced by the cerebral growth of an avirulent strain and the effect of vaccination immunity on a non-virulent strain.

Sublethal lung infections, used to determine the general immune response in contrast to local brain immunity, were established by instilling strain Gl. 353 in 0.04 ml. volumes containing 10,000-50,000 organisms.

*Brain and lung counts*

Animals were killed with coal gas, and brains or lungs removed aseptically into 2 oz. universal bottles containing 9 ml. Casamino Acids and 2 ml. of 5 mm. diameter glass beads. The bottles were shaken - 3 min. for brains, 10-15 min. for lungs - on a vertical shaker, throw  $2\frac{3}{4}$  in., 325 rev./min. The homogenates were diluted tenfold in 1% Casamino Acids and viable counts were estimated by the method of Miles & Misra (1938) on Cohen & Wheeler blood plates (see below); when the count was expected to be low, 0.5 ml. of brain homogenate was spread over one plate.

The Cohen & Wheeler blood plates were made from medium as described by Cohen & Wheeler (1946), but with 0.1% acid casein hydrolysate (Oxoid), 0.5% glutamic acid, 5% blood and 1.3% New Zealand agar.

*Passive protection tests; protective index for lung infection*

Equal volumes of antiserum and double-strength challenge suspension of strain Gl. 353 were mixed, and 0.04 ml. instilled into the noses of 40 mice. A similar number of control mice received the same challenge dose, without antiserum, at the same time. Ten mice from each group were killed after 2 hr. or 1, 4 or 6 days, and viable counts made of the organisms in the lungs of each mouse. The protective index of the serum is calculated by subtracting the mean viable count of the 40 serum-treated mice from the mean viable count of the 40 control mice; thus the higher the figure, the greater the protection.

*In vitro tests for antibodies*

*Agglutinin titrations.* Dilutions of serum in saline and living organisms of strain 18-323, freshly harvested from Bordet-Gengou plates into saline at a concentration of  $5 \times 10^9$  organisms/ml., were incubated in Dreyer tubes at 37° C. for 4 hr.; the results were read after a further 20 hr. at room temperature (*ca.* 18° C.). Eight doubling dilutions of serum were put up, the final dilution in the first tube being 1/40. The degree of agglutination in each tube was given a numerical value: 4, 3, 2, 1 where 4 = complete agglutination and 1 = smallest trace distinguishable from no agglutination. The figures were added together and called the agglutinin rating. It was felt that this rating gave a better evaluation of two sera which gave agglutinations: 4, 4, 4, 3, 0, 0, 0, 0 and 2, 1, 1, 1, 1, 1, 0, 0. The first serum had a rating of 15; the second of 7. The 'titre' of the first serum would usually be regarded as 1/320 and of the second as 1/1280.

*Antihaemagglutinin and bactericidal antibody.* These were measured as described by Dolby (1965) and Dolby & Vincent (1965).

## RESULTS

*Comparison of intraperitoneal and intracerebral vaccination*

Figures 1-5 illustrate the courses of infection in the brain resulting from an IC challenge 14 days after IC or IP vaccination. Ninety per cent of the organisms, both vaccine and challenge, injected IC are lost very quickly from the brain by

draining away through the cerebrospinal fluid (Cairns, 1950; Mims, 1960). The vaccine 'lost' to the brain is, however, available for antigenic stimulation in the rest of the body. Nevertheless, there is a pronounced difference between the two results.

Titration showed that, IC/IC, one ImD50 of vaccine was  $1 \times 10^8$  organisms with the infection curve shown in Fig. 2; this dose of vaccine had no effect IP/IC and the mice behaved as the uninfected controls in Fig. 1. One ImD50 of vaccine IP/IC was  $1 \times 10^9$ , or ten times as great, with a similar infection curve (Fig. 4). In these three curves, Figs. 1, 2 and 4, the viable counts rose to the 4th or 5th day; the counts in mice destined to die continued to rise as in the controls, but the counts in recovering mice fell and by the 9th or 10th day surviving mice had sterile brains (Figs. 2, 4).

The course of infection following 10 ImD50 was quite different with the two routes. In the IC/IC series there was an immediate fall and by the 3rd day most of the brains were sterile (Fig. 3). In the IP/IC series, the curve (Fig. 5) resembled the one ImD50 dose curves (Figs. 2, 4) and, though all the mice survived, there were no sterile brains before the 6th day.

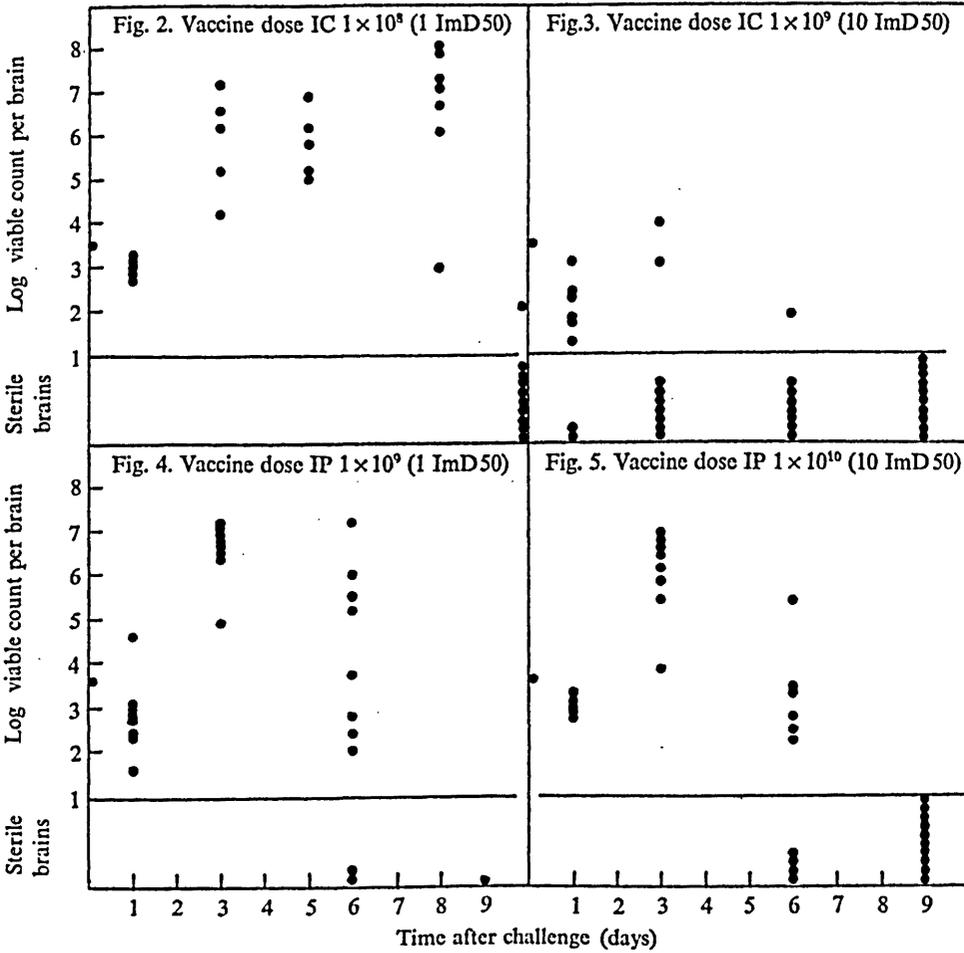
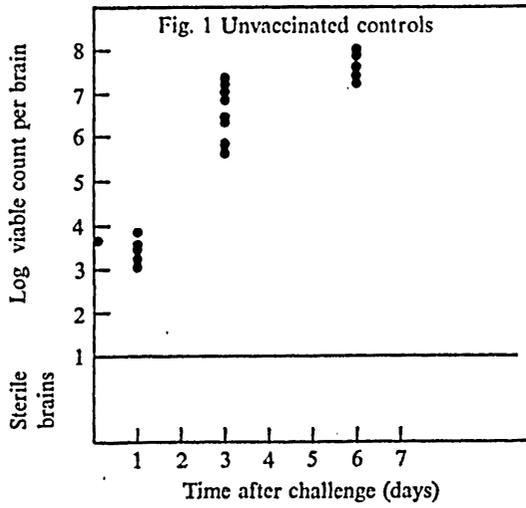
Bacterial growth curves following infection 14 days after IV vaccination were similar to those of IP vaccinated mice. The ImD50 was a little lower,  $0.8 \times 10^9$  instead of  $1 \times 10^9$ .

The possibility of a local non-specific effect of vaccine given IC was tested by estimating protection in terms of lowered brain counts following a small dose of TABC vaccine given IC 14 days before the *Bordetella pertussis* challenge. The counts obtained were indistinguishable from the unvaccinated controls in Fig. 1. A second experiment, in which TABC vaccine IC was combined with one ImD50 pertussis vaccine IP, gave a result similar to Fig. 4. From these experiments we conclude that a measurable local non-specific immunity was not elicited by IC vaccination, or, if it were, it had disappeared by the 14th day when the IC challenge took place.

The pronounced difference between 10 ImD50 of vaccine IC and IP on the ensuing IC challenge was not therefore non-specific. Although the ImD50 values differed tenfold, one ImD50 for each route had the same effect on infection. Figure 6 illustrates that, at about 3 ImD50, the difference between IC and IP vaccination becomes apparent. The one-day lowering of count in mice given 2.5 ImD50 by the IC route was extended to 2 days. Increasing the dose of vaccine IC increased the initial bactericidal effect until a curve like Fig. 3 was reached.

#### *The mechanisms of the greater efficacy of intracerebral over intraperitoneal or intravenous vaccination*

*General immunity.* The vaccine introduced into the brain and leaking into the circulation makes an IC vaccination equivalent to an IV injection of perhaps 80% of the IC dose. To determine the general immune response 14 days after IC vaccine, lung counts were made at intervals after a sublethal (intranasal) infection. The animals were also bled out and the serum tested for antibodies. These tests were chosen as indicators of a host response and no assumption is made as to lung protection or positive antibody response being correlated with the protection of mice in the routine assay of potency.



For figure legends 1-5 see opposite page.

The effect of IC vaccine on lung infection is shown in Figs. 7–10. Doses of  $10^5$  and  $10^6$  were inactive and the mice behaved like the controls (Fig. 7);  $10^7$  had a very slight effect (Fig. 8) and  $10^8$  organisms had a pronounced effect (Fig. 9);  $10^9$  organisms almost sterilized the lung (Fig. 10). A dose of  $10^8$  was equivalent to about one ImD50 IC/IC and affects a brain infection as in Fig. 2. Thus, doses of IC vaccine effective against IC challenges were similarly effective against lung infections.

The effects of a  $2 \times 10^8$  dose of vaccine by the three routes IP, IC and IV on a sublethal, intranasal infection 14 days later were next compared. In the IP vaccinated mice, the lung counts were only slightly lower than the controls; mice on this dose were not protected against an IC challenge. After IC and IV vaccination the lung infection was suppressed to about the same extent and lung counts were similar to those shown in Fig. 9. Increasing the dose of vaccine to  $1 \times 10^9$  increased the protection against lung infection so that the IP route was now effective, but the relative efficacies by the three routes were the same as for the smaller dose. In spite of the similarity of lung counts in IV and IC inoculated mice, IV vaccination protected mice against an IC challenge less well than IC vaccination; this is reflected in the ImD50 values (Table 1). Thus, although IC and IV vaccination were both far more efficient than IP, the IV vaccination route occupied a more intermediate position in protecting mice against an IC challenge.

The antibody response in mice vaccinated by the three routes was measured in groups of five or ten mice 14 days after  $10^9$  organisms. The sera were tested for antihæmagglutinin, total agglutinins, complement-mediated bactericidal antibody, and potency in protecting mice passively against a sublethal lung infection (Table 1). Vaccine given IC was the most efficient as an antibody stimulus and IP vaccine the worst; IV vaccine was intermediate in two of the antibody tests, but similar to the IP vaccine in the other two. The ImD50 values for each route of vaccination against an IC challenge fall in the same order as the average figures for antibody assays, with IV vaccine in an intermediate position.

There is no doubt that vaccine inoculated IC stimulates a better response in the

Figs. 1–5. Viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with large or small doses of pertussis vaccine either IP or IC 14 days before they were challenged with 50,000 organisms (ca. 5000 viable). Each point represents one mouse.

Fig. 1. Unvaccinated controls.

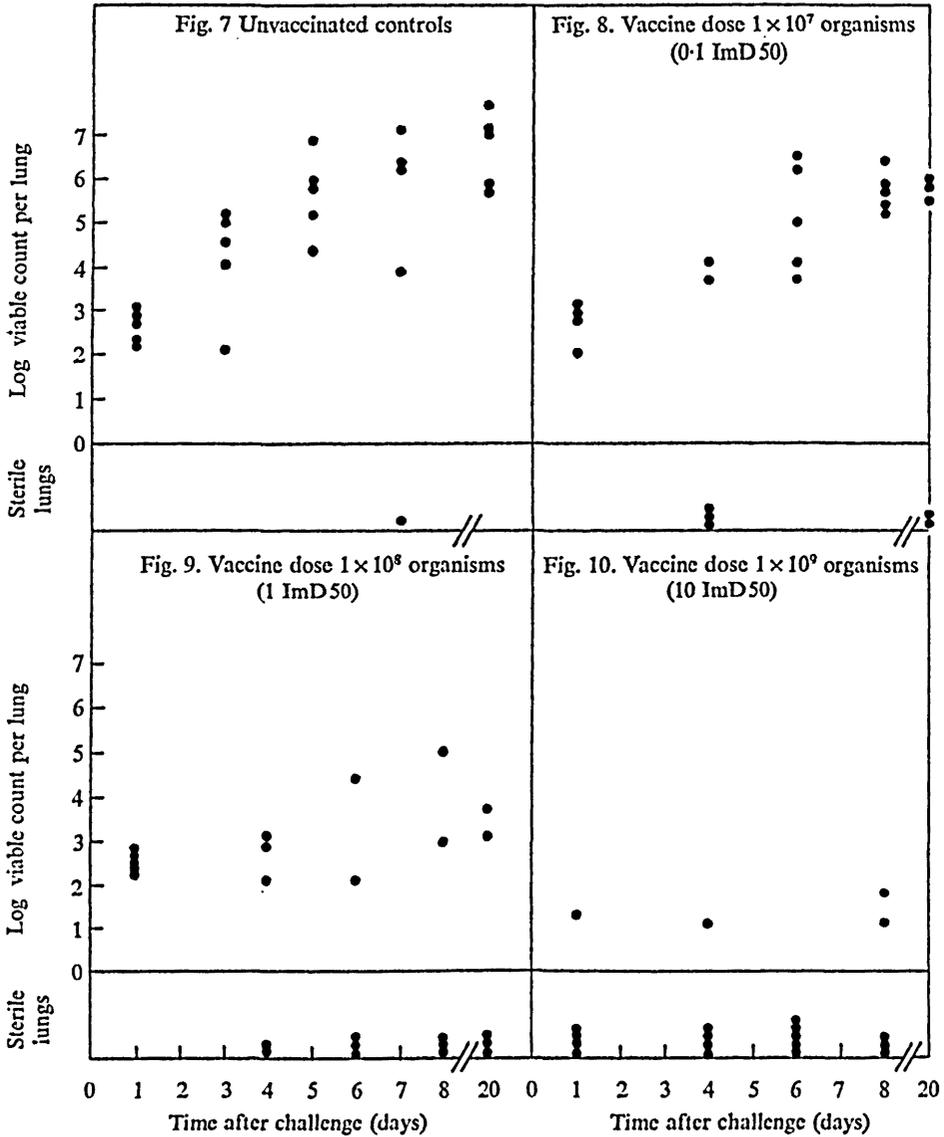
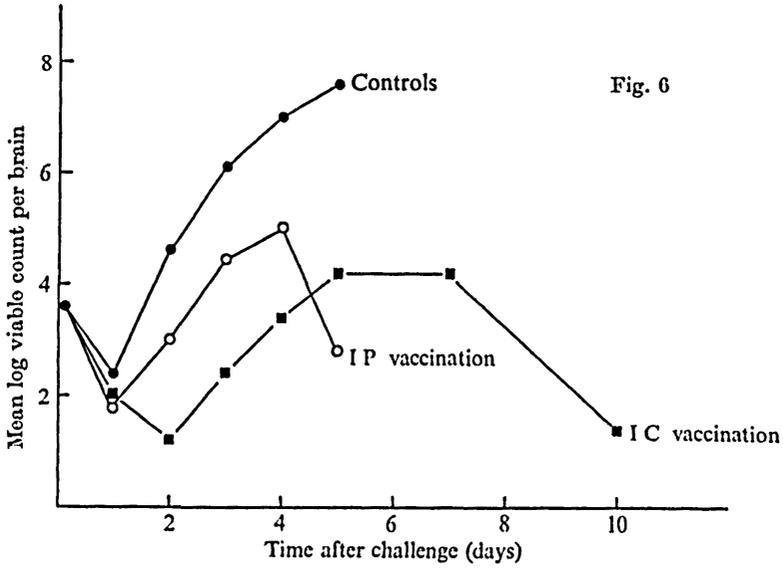
Fig. 2. Vaccine dose  $1 \times 10^8$  organisms (= 1 ImD50) IC.

Fig. 3. Vaccine dose  $1 \times 10^9$  organisms (= 10 ImD50) IC.

Fig. 4. Vaccine dose  $1 \times 10^9$  organisms (= 1 ImD50) IP.

Fig. 5. Vaccine dose  $1 \times 10^{10}$  organisms (= 10 ImD50) IP.

Similar groups of 10 mice were kept for 14 days after challenge to determine the number of mice dying on each vaccine. These were: unvaccinated controls, 10/10 (number dead/total number at risk); vaccine dose 1 ImD50 IC, 6/10; 10 ImD50 IC, 1/10; 1 ImD50 IP, 5/10; 10 ImD50 IP, 0/10.



For figure legends 6-10 see opposite page

body as a whole than vaccine given IP. The question to be considered is whether this alone accounts for the dramatic effect on IC infection.

*Local immunity in the brain.* We have shown above that one ImD 50 of vaccine IC/IC ( $10^8$  organisms) (Fig. 2) had the same effect on brain infection as one ImD 50 IP/IC ( $10^9$  organisms) (Fig. 4). All the mice were infected, the organisms first grew and were then killed. With larger doses of vaccine, the course of infection diverged in IC and IP vaccinated mice (Fig. 6); with an IC vaccination of 10 ImD 50, which protected all the mice, the brains were sterilized within 3 days or so (Fig. 3), whereas the organisms grew in IP vaccinated mice given 10 ImD 50 for 3–4 days before being killed (Fig. 5).

It is to be expected that the ability of IC vaccine to stimulate a better immune response than IP vaccine would be reflected in differences in ImD 50 in the IC/IC and IP/IC tests; and indeed the characteristic courses of infection after 10 ImD 50 doses of vaccine IC and IP (an immediate decline in numbers IC/IC (Fig. 3) and a decline only after 3 days IP/IC (Fig. 5)) suggest different mechanisms of overcoming the brain infection. Figure 3 suggests an immediate specific local action.

Evans & Perkins (1954*b*), Blyth (1955) and Andersen (1957) demonstrated a type of immunity in the brain, the so-called interference effect of vaccine given 3 hr. to 3 days before challenge. We have confirmed their results. This effect is, however, quite separate from that illustrated in Fig. 3. Doses of IC vaccine between  $10^7$  and  $8 \times 10^7$ , too small to produce a response in any of the tests we have used or to protect against IC challenge at 14 days, protected mice against a challenge 3 hr. later. This ability had disappeared in 3 days, even with bigger doses of vaccine, to be replaced, provided the dose was above  $10^8$  organisms, by the usual specific immunity to the challenge as described above.

The effect of a live IC vaccine was next investigated using L 84, an avirulent strain of *Bordetella pertussis* known to establish itself in the mouse brain, multiply, and then die out. With an inoculum of  $10^6$  organisms of L 84, the brain counts increased tenfold by the 3rd–4th day after infection and then declined; by 20 days the brains were sterile. We re-infected these mice with 18-323 3–4 weeks after the primary L 84 infection. The course of the 18-323 infection in the twice-infected and

Fig. 6. Mean viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with  $2.5 \times 10^8$  organisms (2.5 ImD 50) IC or  $5 \times 10^9$  organisms (5 ImD 50) IP 14 days before they were challenged with 50,000 organisms (3980 viable). Each point represents the geometric mean brain count of five mice. ●—●, Unvaccinated controls; ■—■, IC vaccinated mice (2.5 ImD 50); ○—○, IP vaccinated mice (5 ImD 50).

Figs. 7–10. Viable counts of *Bordetella pertussis* strain Gl. 353 in the lungs of mice vaccinated with various doses of pertussis vaccine IC 14 days before they were challenged with 10,000 organisms intranasally. Each point represents one mouse.

Fig. 7. Unvaccinated controls.

Fig. 8. Vaccine dose  $1 \times 10^7$  organisms (0.1 ImD 50) IC.

Fig. 9. Vaccine dose  $1 \times 10^8$  organisms (1 ImD 50) IC.

Fig. 10. Vaccine dose  $1 \times 10^9$  organisms (10 ImD 50) IC.

Vaccine doses of  $1 \times 10^5$  and  $1 \times 10^6$  were similar to the unvaccinated controls.

Table 1. *Effect of vaccination route on serum antibody and resistance to IC challenge 14 days after Bordetella pertussis vaccine*

Route of vaccination	Pooled serum of 10 mice 14 days after vaccination with $10^9$ organisms				ImD 50 against an IC challenge of 50,000 organisms at 14 days
	Protective index* lung infection	Antihaemagglutinin titre	Agglutinin rating†	Bacteriocidal index‡ at 1/30 dilution	
IC	1.6	27	22	1.6	$200 \times 10^6$
IV	1.1	0	8	0.6	$800 \times 10^6$
IP	0.6	0	7	0.3	$1000 \times 10^6$

\* See p. 490.

† See p. 490.

‡ Difference between log number of organisms in control tube (no killing) and log number of organisms with antiserum and complement.

control mice is shown in Fig. 11. The brains were not immediately sterilized. The immunity to 18-323 was well over one ImD 50 since nearly all the mice survived. Yet the course of infection was similar to that after one ImD 50 of dead vaccine IC (Fig. 2) and not like a bigger protective dose (Fig. 3). The counts increased for 3 days and then decreased, suggesting the operation of a general immunity stimulated by antigen leaked-out from the brain, rather than a local brain immunity. If such were the case, it might be expected that the mice would also be immune to a sublethal lung infection, as after one ImD 50 of dead vaccine IC (Fig. 9). However, the L84-infected mice were not immune to sublethal doses of Gl. 353 intranasally.

Immunization with lower doses of live L 84 were less effective: 50,000 organisms protected 26% and 500,000 protected 60% of mice against 18-323.

#### *Intraperitoneal vaccination and infection with other strains*

As already described, an IC vaccination of more than 5 ImD 50 produced an immediate effect on virulent *Bordetella pertussis* 18-323 introduced into the brain 14 days later, whereas 10 ImD 50 of vaccine IP did not do so; at most, there was a slightly lower infection curve for the vaccinated mice than for the controls, but always with a rise and fall (Fig. 6). Challenge with another virulent challenge strain, 2 atox., produced similar curves.

The course of infection in IP vaccinated mice after challenge with three other strains was different, however, from that after 18-323 and 2 atox. infection. Counts were held down at the challenge level or actually reduced from it as early as one day after infection. For example, when mice were vaccinated IP with about one ImD 50 of vaccine as measured IP/IC against an 18-323 challenge, and challenged with the avirulent but establishing strain L84, there was no dramatic immediate sterilization (Fig. 12), but the bacterial count fell to 100 and was held at this level for 4 days until the usual IP/IC effect operated and clearance was achieved faster than in non-vaccinated mice. The ImD 50 could not, of course, be measured for the L84 infection because non-vaccinated mice recovered eventually without vaccination.

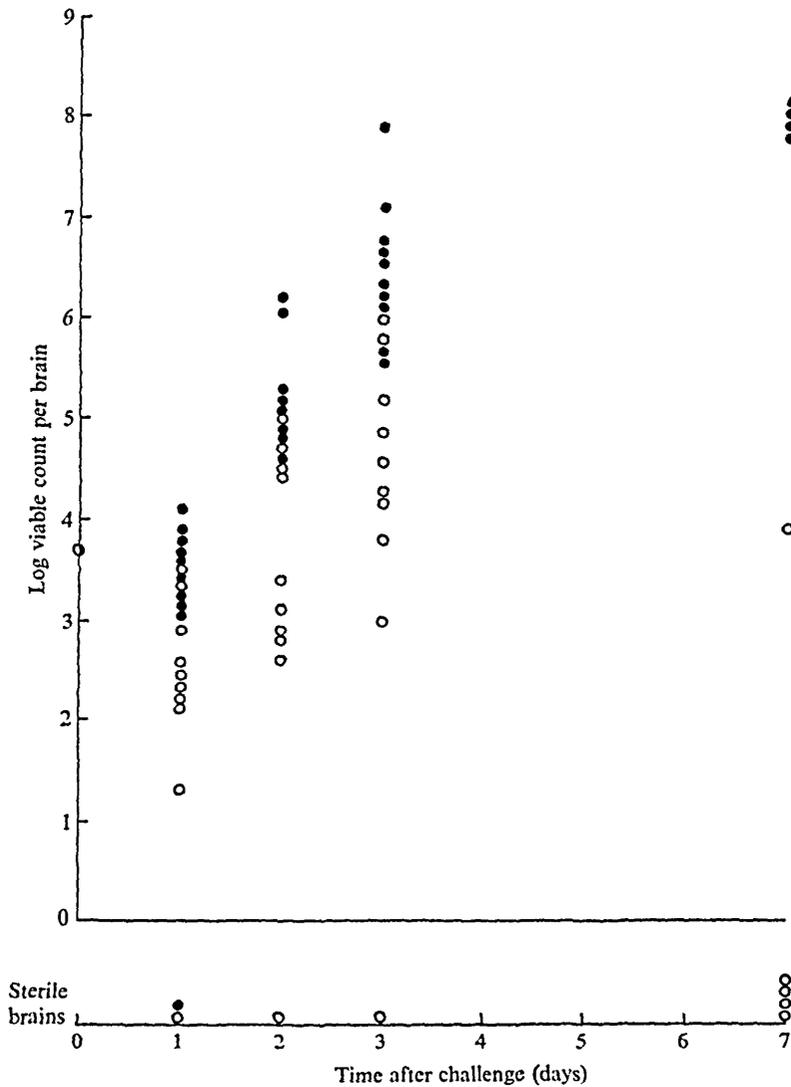


Fig. 11. Viable counts of *Bordetella pertussis* strain 18-323 in the brains of mice vaccinated with a living vaccine IC 3½ weeks before they were challenged with 50,000 organisms (5000 viable). Vaccinated mice were injected with  $5 \times 10^8$  total organisms of the avirulent strain of *B. pertussis*, L 84; a group of mice from the 90 % or more survivors from this treatment had sterile brains 10 days after vaccination. Each point represents one mouse. ●, Unvaccinated controls; ○, Vaccinated mice. In groups of mice kept 14 days after challenge to determine the number of mice dying, 0/10 survived in the control group and 19/21 in the vaccinated mice.

Two other strains, this time virulent, were next used for challenge, following IP vaccination, and the course of infection measured. These were Gl. 353 and a strain made virulent by mouse passage, B5533 P5. The LD50 for both of these in non-immune mice was about the same as that of 18-323; and the organisms grow similarly to 18-323 in non-immunized mice; Adams (1970) illustrated the growth curve for Gl. 353. The ImD50 of vaccine against these strains was, however, very

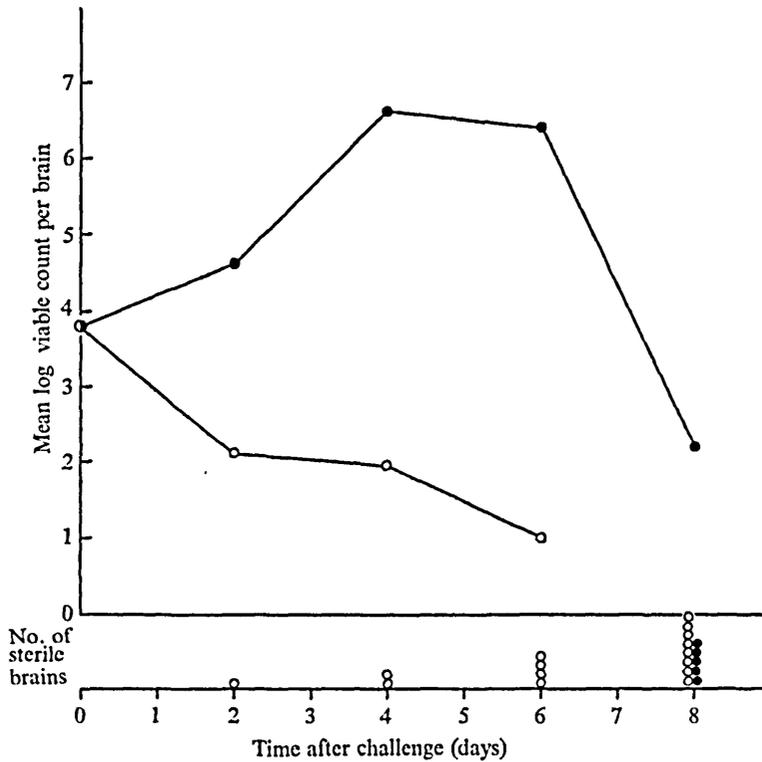


Fig. 12. Mean viable counts of *Bordetella pertussis* strain L84 in the brains of mice vaccinated with  $1 \times 10^9$  organisms IP 14 days before challenge with 100,000 organisms (6300 viable). Each point represents the geometric mean brain count of infected mice in each group of eight (sterile brains shown separately). ●-●, Unvaccinated controls; ○-○, Vaccinated mice.

Table 2. Course of infection in mice vaccinated IP with one level of vaccine and challenged IC, various strains

Challenge strain $5 \times 10^4$ total (= log 3.5/brain)	LD50	ImD50 of vaccine IP	Day after infection	Viable count of organisms per brain	
				Log count (geometric mean, 5 mice)	
				Vaccinated	Non-vaccinated
Gl. 353	320	20	1	3.8	3.4
			3	4.2	6.1
			6	1.4	7.8
B5533 P5	1050	100	1	2.9	nd
			3	2.9	5.2
			4	2.5	nd
			5	2.2	nd
18-323	1500	6	1	2.8	2.9
			3	4.8	5.2
			6	1.8	7.3

nd = not done.

low and a dose equivalent to 6–10 ImD50 against 18-323 was equal to one of 20–100 ImD50 against these strains.

Viable counts in vaccinated mice are shown in Table 2 and are similar to those after challenge with the avirulent strain. Brains were not sterilized immediately but the counts were held down until clearance began at about 3 days. Bactericidal antibody levels in the serum of the three groups of mice could not be correlated with the events in the brain.

#### DISCUSSION AND CONCLUSIONS

In spite of objections based on the absence of direct evidence (Wardlaw & Jakus, 1968), protection by IP vaccination against brain infection 14 days later with 18-323 *could* be due to circulating antibody leaking into the brain when the blood-brain barrier becomes permeable 4 days after infection (Holt *et al.* 1961). A curve of intracerebral growth similar to that for mice adequately protected in the IP/IC active immunization test and illustrated in Fig. 5 was obtained in mice passively protected against IC infection by IP antiserum (Dolby & Standfast, 1961, Figs. 7 and 8). The experiments of Blake & Wardlaw (1969) raise the question whether anything other than antibody is involved.

The order of efficacy of the three vaccination routes for producing (i) protection against IC challenges at 2 and 14 days, (ii) an immediate reduction in bacterial count of an IC challenge at 14 days, and (iii) suppression of sublethal lung infections, is shown in Table 3. This order was the same for protection against IC challenge as for induction of serum antibodies (Table 1). Vaccination IC was the most effective way of inducing high titre antisera, with IV vaccination intermediate and IP vaccination the least effective. Circulating antibody entering the brain could therefore account for the drop in the brain count 3–4 days after a challenge given 14 days after vaccination by any of the three routes, and the better stimulus of IC injected vaccine (although for what reason?) could explain its efficacy against IC infection reflected in ImD50 values. There is also, however, the *shape* of the infection curves in vaccinated mice to be considered. For an 18-323 challenge these are different in IC vaccinated mice from those given equivalent ImD50 of vaccine IP or IV in all doses greater than 2 ImD50. Beginning with 2–3 ImD50, it is clear that the drop in bacterial numbers in the IC vaccinated mice is greater during the first two days after challenge than in the IP vaccinated mice, as shown in Fig. 6. This becomes more exaggerated with higher doses until, with 10 ImD50, the local effect in the IC vaccinated mice is so strong that sterilization begins at once and is complete at a time when the brain count has not yet started to fall in IP and IV vaccinated mice.

We have not investigated whether the same protective dose of vaccine IC and IP produces the same amount or the same kind of circulating antibodies, bearing in mind that ten times less vaccine is needed to immunize mice by the IC route. The Table 1 data were collected on sera of mice immunized with one given number of organisms. The greater efficacy of the IC route of vaccination might reflect the induction of different classes or kinds of antibody globulin (Dolby & Dolby, 1969) or the production of a local immune response.

Table 3. *A comparison of IC, IV and IP vaccinations*

Route of vaccination	Minimum number of organisms in killed vaccine inducing			
	Local interference phenomenon 2 days later	50% protection against IC challenge with strain 18-323	Suppression of sublethal lung infection	Immediate bactericidal effect against IC challenge with strain 18-323
IC	$2 \times 10^7$	$1 \times 10^8$	$1 \times 10^8$	$4 \times 10^8$
IV	—	$8 \times 10^8$	$3 \times 10^8$	$> 2 \times 10^9$
IP	—	$1 \times 10^9$	$1 \times 10^9$	$> 1 \times 10^{10}$

In contrast to IC vaccination, the infection curve produced by 18-323 in the brain two weeks after IP vaccination suggested that no recovery began until 3 days after challenge. Use of an avirulent challenge, L84, or two other virulent strains, exposed an earlier antibacterial effect; the mechanism was there, but unable to operate against 18-323 (or 2 atox.) until 3 days after challenge.

The shape of the infection curve after IC challenge with 18-323 3 weeks after primary IC infection with an avirulent strain was surprising: we had expected an immediate reduction in count in the protected mice similar to that shown after IC vaccination, instead of the rise and fall typical of IP vaccination and subsequent IC challenge. Adams (1970) had demonstrated the slowing down of the growth of 18-323 introduced into the brain 2 days after an avirulent infection, but had found no effect  $4\frac{1}{2}$  days after such an infection, whereas re-infection with an avirulent strain at 2 and 4 days after the primary infection had been suppressed with an immediate drop in count. No inhibitory effect on the re-infecting strain was observable at 1 hr. after the primary infection. This suppression resembled the Evans & Perkins interference phenomenon except in its absence at 1 hr.

The Medical Research Council trials (1956) showed that the IP/IC mouse test correlates well with protective potency in the child. Clearly therefore any test that might replace it must correlate in the same way. Table 3 suggests that more factors are operating to overcome infection in the IC/IC vaccination test than in the IP/IC test, although one of the factors may be common to both. It seems to us that the analysis of the mechanism promoting recovery only 3 days after infection is still of great importance.

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