

The effects of humoral, cellular and non-specific immunity on intracerebral *Bordetella pertussis* infections in mice

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SUMMARY

When mice were injected intracerebrally with doses of *Bordetella pertussis* vaccine greater than 5 ImD 50 and challenged intracerebrally 14 days later with virulent *B. pertussis* there was an immediate reduction in the numbers of organisms. An analysis of this *in vivo* bactericidal effect has shown that large doses of an unrelated vaccine, *Salmonella typhosa*, equivalent in cell mass to about 50 ImD 50 of *B. pertussis* vaccine can achieve this effect, so for such doses the effect must be partly non-specific. This action is not maintained and so is not ultimately protective. Local immunoglobulin was also demonstrable 14 days after 300 ImD 50 of *B. pertussis* vaccine but following smaller doses of 10–20 ImD 50 it could not be found until after the mice had been infected and the blood–brain barrier impaired.

A similar immediate reduction in the numbers of infecting organisms inoculated 1 day after vaccination has been shown to follow very small, non-protective doses of vaccines unrelated to *B. pertussis* and to be achieved with lipopolysaccharide and endotoxin isolated from *B. pertussis*. Brains were not sterilized and only in mice receiving protective *B. pertussis* vaccine was the lowering of infection maintained beyond 2 days and the brains eventually sterilized.

The antibody passively protecting mice against intracerebral infection was found in the 19 S and 11 S globulin fractions of the serum of once-vaccinated mice and in the 11 S and 7 S fractions of the serum of rabbits and ascitic fluid of mice receiving repeated doses of vaccine. The IgM probably eliminated infections by immediate sterilization but had to be present locally to do so since it was unable to pass from the circulation into the brain, and was therefore inactive when injected intraperitoneally. The IgA and IgG were not so restricted and both the 11 S and 7 S globulins were capable of exerting an immediate suppressive effect on infecting organisms. The 7 S globulin was also capable of a maintained or delayed suppressive effect.

Lymphocytes from fully protected once-vaccinated mice, transferred 2–3 weeks after intraperitoneal vaccination, were able to confer some protection when injected intraperitoneally or intracerebrally into recipient mice infected 2 weeks after transfer. Homologous, non-concentrated antiserum from once-vaccinated

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mice, injected intraperitoneally 1 hr. before infection sometimes augmented the transferred immunity, whereas alone it was inactive.

INTRODUCTION

This paper records our attempts to explore some of the questions raised in trying to understand how the mouse is protected against *Bordetella pertussis* injected intracerebrally after vaccination. Although the route of infection is not natural and probably not more than six strains are known of virulence high enough to cause the death of the mouse, the mechanism of immune protection is of interest because of the correlation between the potency of vaccines protecting children against upper respiratory disease and mice against intracerebral infection (Medical Research Council, 1956, 1959; Standfast, 1958).

The type of active immunity conferred on mice by intraperitoneal vaccination can be shown by intracerebral infection 14 days later (the procedure used for assaying potency) and counting the viable organisms in the brain thereafter; after an initial fall they increase in numbers for two days and then suddenly decrease (Berenbaum, Ungar & Stevens, 1960; Dolby & Standfast, 1961). After intracerebral vaccination using doses higher than the fully protective one, however, there is an immediate suppression of intracerebral infection (Standfast & Dolby, 1972) and we have now explored the possible role of intracerebral antibody, and other effects, some of them not relating to protective antigen, in this phenomenon.

One of the non-specific effects following the injection of vaccine with or immediately preceding challenge has already been described; the number of organisms is immediately reduced but the infection is not eliminated (Andersen, 1957). Fully potent vaccines will protect mice when injected in this way (Evans & Perkins, 1954), but the protective antigen does not begin to exert its effect until 3 days or so after challenge (Andersen, 1957). Neither the antigen eliciting bactericidal antibody active *in vitro* (part of the lipopolysaccharide molecule) nor protective antigen was responsible for this initial suppressive effect *in vivo*, since vaccines in which the one is present and the other absent were equally active (Dolby, Ackers & Dolby, 1975). Iida & Tajima (1971) have also shown that endotoxin from *B. pertussis* or unrelated organisms, which does not contain protective antigen (i.e. does not protect mice actively when given intraperitoneally 14 days before an intracerebral challenge) can yet suppress growth of an infection when given intracerebrally up to 7 or 8 days before challenge, with an optimal effect at 2 days, and that this was expressed as ultimate survival. Since the effect could be mimicked by double-stranded RNA, interferon production was suggested as a contributing factor.

The results of intracerebral vaccination are therefore complex and we have here used a selection of vaccines, at both one and 14 days before challenge, to determine if any of the effects are specific to protective antigen as defined by the potency test of protection by intraperitoneal vaccination against an intracerebral challenge 14 days later.

The role of antibodies in the protection of once vaccinated mice was long doubted

because of the inability to protect recipient mice with transferred serum (Wardlaw & Jakus, 1968). Dolby *et al.* (1975) were able to demonstrate passive protection, by using concentrated serum globulins, but this left unexplained the results of Adams & Hopewell (1970), who found that the immunity of once vaccinated mice could be destroyed by X-irradiation of the body (but not of the head) 14 days after vaccination, a time when antibody should already be present. As this might be interpreted as implicating cellular immunity in the process, we also carried out experiments on transfer of immune cells.

Finally, as previous experiments with rabbit sera (Dolby & Dolby, 1969) and rabbit and mouse sera (Dolby *et al.* 1975) had given some indication of variation in the class of immunoglobulin involved in immunity to *B. pertussis* infection, we have studied the effect on intracerebral infection of serum fractions from once-vaccinated and repeatedly vaccinated animals.

METHODS

Preparation of immunoglobulins from vaccinated animals

Rabbit antiserum I, prepared by repeated injections of a bacterial suspension, was fractionated into 19 S and 7 S globulins on Sephadex G-200 and DEAE cellulose (Dolby & Dolby, 1969). Fractions 2-5 of the 7 S globulin, re-run on DEAE cellulose, were compared with fraction 1 in experiments attempting to separate the different activities of 7 S immunoglobulin.

Immunoglobulins from the serum of once vaccinated mice were as used by Dolby *et al.* (1975). Ascitic fluid was stimulated in large mice as described by Anacker & Munoz (1961) and cited by Chase (1967).

The antigen was a mixture of a suspension of killed *Bordetella pertussis* and Freund's complete adjuvant (FCA) containing 5 international potency units of *B. pertussis* and 5 mg. of mycobacteria per ml. Volumes of 0.2 ml. were injected into mice intraperitoneally, the fluid collected at intervals, and stored at -15°C . Before processing, the clot was removed from bulked, thawed material by centrifugation or filtration through Whatman No. 1 filter paper and discarded. The immunoglobulins were fractionated as described for mouse serum.

All globulin fractions were stored at -25°C . as 25 mg./ml. of protein stock solutions and heated at 56°C . for 30 min. before use.

Immuno-electrophoresis of mouse brain extracts

Brains were dissected from vaccinated, challenged and control mice which had been killed and immediately bled out from the heart. Each brain was rinsed in 9 ml. 1% Casamino acids and then extracted into 1% Casamino acids (1-2 ml. for each) by shaking in groups of 3-10 with glass beads in bottles on a vertical shaker (2½ in. throw, 325 rev./min.) for 3 min. at room temperature, and then centrifuging for 30 min. at 3000 rev./min. at 4°C . The supernatant extracts were stored at -15°C . until ready for use, concentrated about ten times by ultra-filtration and subjected to immuno-electrophoresis on microscope slides (Hirschfeld, 1959) using rabbit anti-mouse serum.

Mice

Unless specified, female Theiler's Original strain have been used throughout, at about 16 g. in weight in vaccination experiments started 14 days before infection, at 20 g. for most other experiments, and at 30 g. for the production of ascitic fluid. Syngeneic mice were used for some of the cell transfer experiments and details are given later.

Intraperitoneal injections were given in 0.2–0.5 ml. and intracerebral inoculations in 0.03 ml. under light ether anaesthesia.

Vaccines

Bordetella pertussis strains 134 and D6229 were used as whole cell vaccines for the preparation of antisera from once vaccinated mice. These, and other phase I strains, routinely employed in the preparation of vaccines, were used as intracerebral vaccines, grown on either the usual liquid or solid media, and heat or formalin killed. Routine, Phase I *B. pertussis* vaccine or 'triple vaccine' plain or adsorbed was used to immunize donor mice in cell transfer experiments. *B. pertussis* phase IV vaccine was prepared from a nutrient-agar-grown, heat-killed derivative of a phase I strain L84, obtained by repeated subculturing. *Salmonella typhi* vaccine was prepared from overnight cultures, grown on nutrient agar, of mouse virulent strains T5501 and T4904 and heat-killed. All experimental bacterial vaccines were adjusted in opacity by eye to match the International Opacity Standard of 10 international units per ml. which was equivalent to 10^{10} *B. pertussis* and 10^9 *S. typhi* per ml. and made up in phosphate buffered saline.

Lipopolysaccharide and endotoxin isolated from various *Bordetella pertussis* strains were as used by Ackers & Dolby (1972).

The ImD50 (amount of vaccine required to protect 50 % of mice) was calculated by the usual statistical methods.

Intracerebral challenge strain

Bordetella pertussis strain 18-323 was harvested from 20 hr. cultures on Bordet-Gengou medium, suspended in 1 % Casamino acids, adjusted by eye to the International Opacity Standard, and diluted to contain 50,000 organisms in 0.03 ml. of which 10 % were viable.

Suspensions of *Salmonella typhi* strain T 4904 were harvested from 6 hr. nutrient agar plates and prepared as described by Standfast (1960) to contain 10,000 organisms in 0.03 ml.

Estimation of viable organisms in mouse brains

Brains were removed from animals killed at the appropriate time in groups of 5–10 and suspended individually in 9 ml. of 1 % Casamino acid, homogenized, diluted and pipetted onto solid medium as described by Dolby (1972).

Table 1. *The effect on intracerebral B. pertussis of 19 S and 7 S antibody from rabbit multi-dose antisera*

Day	Antibody fraction	Percentage of mice infected with numbers of viable organisms/brain					Survivors (%)
		< 20	20-1000	10^3-10^5	10^5-10^7	> 10^7	
1	19 S	0	60	40	0	0	0
	7 S	30	60	10	0	0	75
	Control	0	26	74	0	0	0
3	19 S	0	0	0	95	5	0
	7 S	45	5	10	40	0	75
	Control	0	0	0	74	26	0

The antibody-containing fraction at a concentration of 12.5 mg. protein/ml. (375 μ g./mouse) was injected intracerebrally with a challenge of 50,000 organisms of strain 18-323. The degree of infection is expressed in numbers of viable organisms/brain, and the percentage of mice infected at each level is shown.

The preparation of cell suspensions and serum for transfer of immunity

Mice were killed with coal gas and bled out from the heart to provide leucocytes and serum. For leucocytes the blood was collected into plastic syringes or siliconed glass pipettes rinsed out in 100 u./ml. heparin, and placed in siliconed tubes containing 2 u. heparin per ml. of blood to which was added 3% dextran (T 2000, Pharmacia, Uppsala, Sweden) to sediment the red cells and allow collection of the leucocytes (Rabin & Rose, 1970). For serum it was allowed to clot for 4 min. at room temperature in glass bottles, refrigerated for 4 hr. and serum collected by centrifugation at 4° C. The serum was either used immediately or stored at -15° C. until use, as described in the text.

Subcutaneous axillary, subclavian and inguinal lymph nodes from three mice were collected in 1.5 ml. Hanks' balanced salt solution containing 4 u./ml. heparin, in siliconed glass tubes. Cell suspensions were prepared by sucking into and expelling from a 1 ml. plastic syringe; the homogenate was then filtered through a double layer of 15 denier nylon. Spleen cell suspensions were prepared similarly from three mice.

Cell suspensions at 10^8-10^9 cells/ml. were injected intraperitoneally in volumes of 1 ml. or intracerebrally in 0.03 ml. within 4 hr. of collection when over 90% were still viable.

RESULTS

The antibacterial properties of antibody globulins from vaccinated animals

Rabbit antiserum collected after repeated doses of vaccine

Antiserum from rabbits given repeated doses of *Bordetella pertussis* vaccine were able to protect mice when injected intracerebrally together with the challenge organisms and, as shown by Dolby & Dolby (1969), the protective activity was in the 7 S globulin fraction. The 19 S globulin was completely inactive whereas both 19 S and 7 S were equally effective in protecting against an intranasal infection.

We have now re-examined the results of these previous experiments for the effect of these globulins in individual mice after injection intracerebrally with a challenge. The results for individual mice have been grouped together, according to the degree of infection, in Table 1. The pooled results of three experiments in which serum I fractions were injected are given. These represent a total of 15–20 mice injected with 375 μg . (expressed as protein) of each globulin, or uninoculated for the control group, all killed for brain count determinations at 1 day after challenge; the same number were killed for brain count determinations 3 days after challenge and 30–40 mice in each group were kept for 14 days for the estimation of the percentage protected by the antiserum fraction.

Table 1 emphasizes the almost complete ineffectiveness of the 19 S globulin. There was a transient effect one day after challenge because 60% of the mice had counts below 1000 organisms per brain as compared to 26% of the control mice, but by day 3 this had disappeared and all mice had high brain counts; none of the group kept for 14 days survived. The 7 S globulin on the other hand protected 75% of the mice at 14 days, but by two methods. Only 30% of brains were effectively sterilized 1 day after challenge. Although 60% had a fairly low count of 20–1000 organisms per brain at this time, by 3 days nearly half had high brain counts. As 75% of mice survived for 14 days, some of these must have been heavily infected at 3 days after challenge and their brains became sterile later, as often happens when whole antiserum from rabbits vaccinated several times is injected (Dolby, 1972).

Attempts at separating the 7 S globulin fraction into antibody which sterilized immediately and that which was effective at later than 3 days after challenge were not very successful. Globulin run on DEAE cellulose could be split into 5 fractions and although some separation of the effects, which appeared to be independent of concentration, could be achieved, these results were not consistent. Part of the difficulty lay in the instability of separated 'late sterilizing antibody'.

The 7 S antibody was also protective against an intracerebral challenge when injected intraperitoneally (Dolby & Dolby, 1969).*

Mouse ascitic fluid collected after repeated doses of vaccine

Ascitic fluid was fractionated and the protective potency of the 19 S, 11 S (containing a little 19 S) and 7 S fractions was measured, by injecting intracerebrally with the challenge. All three fractions had some activity, unlike fractions from multidose rabbit antisera, but the 7 S was the most active. For example the pooled figures of experiments with three preparations injected in 375 μg . doses gave percentage survivals of 19 S, 25%; 11 S, 30%; 7 S, 45%. These were not so potent as we had hoped, but at least the large quantities available enabled us to explore further the way in which protective antibody in different globulin fractions was effective. As in the rabbit antiserum experiments, globulins were injected intracerebrally into the brain in doses of 375 μg ., but this time immediate sterilization was assessed by the degree of infection of brains 1 day after globulin had been injected with the infecting organisms; and the ability to suppress infection at a

Table 2. *The effect on intracerebral B. pertussis of 19 S, 11 S and 7 S antibody from mouse anti-pertussis ascitic fluid*

	Antibody fraction	Percentage of mice infected with numbers of viable organisms/brain				Survivors (%)
		< 20	20-1000	10 ³ -10 ⁵	10 ⁵ -10 ⁷	
1 day after	11 S (+ 19 S)	50	30	20	0	50
after globulin	7 S	50	10	40	0	50
+ challenge	Control	0	20	80	0	0
4 hr. after	11 S (+ 19 S)	0	0	10	90	50
globulin to	7 S	0	0	50	50	50
3-day infected mice	Control	0	0	0	100	0

The antibody-containing fraction at a concentration of 12.5 mg. protein/ml. (375 µg./mouse) was injected intracerebrally with a challenge of 50,000 organisms of strain 18-323, or intracerebrally 3 days after infection. The degree of infection is expressed in numbers of viable organisms/brain, and the percentage of mice infected at each level is shown.

later time tested by injecting globulin intracerebrally 3 days after infection and determining the effect on the brain infection 4 hr. later.

The results given in Table 2 are those of two experiments on groups of five mice each time, injected with the mainly 11 S and 7 S fractions, which protected 40 and 50 % of mice respectively. They suggest that the 7 S globulin lowered the bacterial count within 24 hr. of administration with the challenge and also on injection 3 days after infection which confirms the dual effect with the 7 S globulin from rabbit antiserum. This 7 S fraction was shown not to be contaminated with IgA (by the use of anti-mouse IgA, Nordic) whereas the 19 S fraction used in the experiments shown in Table 1 contained a little. The 19 S + 11 S fraction, on the other hand, behaved more like the 19 S globulin in Table 1 and was only effective if injected with the challenge.

A small amount of 19 S globulin was given to four mice intracerebrally with the challenge organisms. This particular preparation was not protective, but even so, it lowered the brain count of three mice; two brains had fewer than 20 organisms, one brain 20 and one over 1000 as in the control group. All of these mice would have died if kept for 14 days; sterilization was complete in none, and once the initial effect had been exerted and failed, the infection regained its hold. This effect was similar to but stronger than that seen with the non-protective 19 S globulin from the antiserum of rabbits vaccinated several times.

A 19 S globulin preparation which was able to protect 30 % of mice when injected intracerebrally was used to try to establish whether it would have any effect if given intraperitoneally in two or three doses of 5 mg. each time, over the 2-3 days following challenge, to ensure a constant supply over the critical period. One dose was given within a few hours of challenge, the second 24 hr. later and the third on the 2nd day. There were no survivors out of 5 mice so treated, compared with 2 or 3 survivors out of groups of 5 injected similarly with the 19 S + 11 S or 7 S globulins. By the intracerebral route the 11 S and 7 S fractions protected

Table 3. *The presence of γ -globulin in brains of mice vaccinated with B. pertussis*

Route of vaccine	Amount of vaccine in units*	ImD 50 of vaccine	Challenge	γ -globulin
IP	0.1	2	—	—
	0.1	2	+	—
	0.6	30	—	—
	0.6	30	+	—
IC	0.1	20	—	—
	0.1	20	+	+
	0.6	300	—	+

Brain extracts were collected 17 days after vaccination. Challenge, where given, was at 14 days after vaccination with 50,000 organisms.

* As defined by ability to protect mice after intraperitoneal injection followed at 14 days by intracerebral challenge; 0.1 unit = 4×10^8 organisms.

40 and 50 % of mice respectively as against 30 % by the 19 S fraction. The complete absence of any protective effect, even prolonging time to death, with the 19 S globulin suggests that it is completely ineffective by the intraperitoneal route.

Mouse antiserum collected after one dose of vaccine

In a previous paper (Dolby *et al.* 1975), it was shown that when serum was collected from mice 14 days after a single vaccination or 3 days later after challenge, and fractionated, the 7 S globulin fraction unlike the same fraction from multiply vaccinated animals was not protective when injected intracerebrally; fractions 19 S and 11 S both protected 50–80 % of mice when injected in 375 μ g. doses intracerebrally with the challenge.

Brain counts were made in only a few experiments. The 7 S globulin was completely inactive. The 11 S fraction was protective but the means by which this was achieved varied; in one experiment, early sterilization of the brain accounted for all the survivors but for only half in another experiment. Comparable experiments with the 19 S fraction against the usual challenge of 50,000 organisms were not done, but a challenge of 5000 organisms mixed with 19 S globulin and injected intracerebrally was completely sterilized by 2 days. When 19 S globulin was injected intraperitoneally into mice in three doses of 5 mg. at the time of infection, 24 and 48 hr. later, there was no protection, whereas half of the mice were protected by non-fractionated antisera from repeatedly vaccinated animals.

The demonstration of intracerebral immunoglobulin in intracerebrally vaccinated mice

Having established that some antibody globulin from vaccinated animals could sterilize an infection immediately, when introduced intracerebrally with the challenge, we wondered if local antibody acting in this way could be the explanation of immediate suppression of a challenge given to mice vaccinated intracerebrally with more than 3 ImD 50 of vaccine 14 days earlier (Standfast & Dolby, 1972).

Mice were vaccinated intracerebrally or intraperitoneally; half of each group

Table 4. *The effect on intracerebral B. pertussis of vaccination intracerebrally 1 or 14 days before infection*

Vaccine	Percentage of mice infected with viable organisms at 2 days				Survivors (%)
	< 20	20-100	10 ³ -10 ⁵	10 ⁵ -10 ⁷	
Challenged 1 day post-vacc.					
Typhoid	0	20	60	20	n.d.
Pertussis phase I	30	40	30	0	60-70
Pertussis phase IV	0	40	60	0	40
Parapertussis	25	0	50	25	20
Pertussis LPS	10	10	70	10	20
Pertussis endotoxin	0	20	80	0	0
Non-vaccinated	0	10	20	70	0
Challenged 14 days post-vacc.					
Typhoid	10	45	45	0	0
Pertussis phase I	85	0	15	0	100
Pertussis phase IV	0	0	0	100	0
Pertussis LPS	0	0	25	75	0
Pertussis endotoxin	0	20	5	75	0
Non-vaccinated	10	0	30	60	0

The whole cell vaccines were all given in doses of 10⁹ organisms. The results for vaccination with endotoxin from a typical phase I strain are pooled from experiments using 6-35 μ g. Lipopolysaccharide (LPS) from a variety of strains, was injected in 6 μ g.

n.d. = not done.

were challenged intracerebrally 14 days later. Brains were collected 3 days after this from all groups of mice, extracted, concentrated and examined by immunoelectrophoresis.

Lines in the albumin and β -globulin region were shown by the electrophoresis of extracts of brains of normal and vaccinated mice (Plate 1, fig. 1). The presence of blood in the extract could not account for these two components since other serum proteins were not present. They were either present locally or had diffused selectively into the brain from the blood or CSF. γ -Globulin was detected in certain extracts only as shown in Table 3 and Plate 1, fig. 2. It was not detectable in mice given 20 ImD 50 vaccine intracerebrally, until after the mice had been challenged. Mice vaccinated with 30 ImD 50 of vaccine intraperitoneally (representing six times more total antigen) gave no evidence of γ -globulin in the brain even after challenge, so that γ -globulin stimulated by intracerebral vaccination must have been formed locally, and not produced by diffusion from the serum. Use of enough vaccine intracerebrally made it possible to detect γ -globulin produced locally in uninfected brains 17 days after vaccination (last line, Table 3), without a secondary stimulus or augmentation by serum protein.

The suppression of the growth of Bordetella pertussis in the brains of mice vaccinated intracerebrally 1 or 14 days before infection

Earlier experiments had shown that previous infections and small doses of intracerebral vaccine 2-3 weeks before subsequent infection, stimulated conditions in

the brain such that the infection was suppressed by the mechanism operating after a delay of 3 days. Large doses of intracerebral vaccine caused immediate suppression of an infection given two weeks later (Standfast & Dolby, 1972). From the work of Iida & Tajima (1971) it seemed that the large doses were acting non-specifically. This has been re-investigated by vaccinating intracerebrally with homologous and heterologous vaccines and determining the immediate effect on a challenge 1 or 14 days later, by brain counts at 2 days and the delayed effect as measured by percentage survival (Table 4).

Large doses of vaccine given intracerebrally in mice cause immediate suppression of the challenge organisms (Standfast & Dolby, 1972) in contrast to the 3-day growth of the challenge before its elimination when small doses are used. In view of the demonstration by Iida & Tajima (1971) of the non-specific protective effect of endotoxin and double-stranded RNA it seemed possible that large doses of pertussis vaccine were also acting non-specifically. In our previous experiments the only non-specific control was a small dose of typhoid (TAB) vaccine with a cell mass equivalent to that of 3 ImD 50 of *B. pertussis* vaccine, which had no effect on the bacterial count.

To test large doses of heterologous vaccine, heat-killed *Salmonella typhi* in doses of $0.5-2.0 \times 10^9$ organisms was injected intracerebrally and the mice challenged with *B. pertussis* 14 days later. Bacterial counts on the brains after two days (Table 4, lower half) showed less than 1000 organisms in 55% of the brains vaccinated with *S. typhi* as compared with 10% of those not vaccinated. The converse was also true: *B. pertussis* vaccine even in relatively small doses (equivalent to 2-3 ImD 50 against a *B. pertussis* infection) suppressed an *S. typhi* challenge to about 10^4-10^5 organisms per brain compared with 10^8 in non-vaccinated controls.

Table 4 also shows the effect of a variety of vaccines made from *B. pertussis* and related but not protective organisms, and of *B. pertussis* lipopolysaccharide and endotoxin. All these vaccines had some effect on the brain counts, but only when the brains were infected at one day but not fourteen days after vaccination, with some slight subsequent protective effect.

Only vaccination with *B. pertussis* vaccine was able to maintain the lowering sufficiently to exert any appreciable protective effect in our experiments, however. Nevertheless, the typhoid vaccine effect was clearly different from that of other vaccines and was not antibody mediated, since anti-typhoid serum was completely ineffective when mixed with *B. pertussis* organisms and injected intracerebrally.

The cellular transfer of protection from immune to normal mice

Intraperitoneal transfer

In preliminary experiments, cells from the lymph nodes, thymus, bone-marrow, spleen and peritoneum of mice immunized 1-21 days previously were transferred to normal mice and the recipients challenged intracerebrally 1-21 days later. The greatest effect of this treatment - for example, the survival of 1 or 2 out of 7 infected mice with 1 or 2 prolonged times to death as compared with times for control mice - was found in transfer of donor cells about 3 weeks after vaccination

Table 5. *The intraperitoneal transfer of protection against intracerebral B. pertussis infection to recipient mice given lymph node (L) or spleen (Sp) cells from immunized donor mice*

Mice	Vaccine to donors	Survivors/total and percentage protection of mice receiving			
		L	L + S	Sp	Sp + S
TO/1	4-15 ImD 50, 100 % protection	0/14	15/41 (38 %)	.	.
TO/1	1-2 ImD 50, less than 100 % protection	0/13	0/11	.	.
TO/2	6-30 ImD 50, 100 % protection	4/24 (17 %)	0/15	1/12	3/10 (30 %)

Cells were transferred at about 20 days after immunization, to mice infected 14-19 days later. Serum (S) was given intraperitoneally 1 hr. before challenge.

and the challenge of recipients 2-3 weeks after transfer. Shortening either of these time intervals abolished the slight protective effect.

These first experiments were done with the syngeneic mouse strain CBA/J to provide optimal compatibility, but unfortunately these, and other syngeneic strains we tested, were relatively insensitive to immunization, so that very large doses of vaccine were needed to obtain even 50 % protection of donors. It is not surprising that protection of recipients was limited.

These inbred mice were replaced by those from a closed colony of Theiler's Original mice, referred to as TO/1. These mice provided more satisfactory results than those of Theiler's Original from another breeder, referred to as TO/2, in spite of the ease with which the second could be immunized; the average ImD 50 was ten times lower for TO/2 than TO/1. In addition to this strain variation for ImD 50, variations of 5 times were also experienced from batch to batch of one strain of mice. We were able to set up our experiments in TO mice in parallel with routine vaccine potency testing so that the number of immunizing doses received by the donor mice and the amount of vaccine needed to immunize the recipient mice were eventually known.

Groups of 20 mice of TO/1 or TO/2 strains were immunized with vaccine, and used as donors for 5 or 6 recipient mice. Serum, separated from the pooled blood of donor mice and stored at -15°C . until 1 hr. before challenge, was thawed and injected intraperitoneally in volumes of 1.5-2.0 ml. The results, shown in Table 5, are grouped together on the basis of the amount of vaccine given to the donors. The ease with which TO/2 mice could be immunized meant that an acceptable amount of vaccine contained up to 30 ImD 50 yet these mice still gave poor transfer protection. We found that it was irrelevant to the transfer of protection whether the dose of vaccine given to the donor mice contained 4 or 30 ImD 50 and the ease with which the recipient mice could be immunized also seemed relatively unimportant.

Table 5 shows that about 35 % of mice could be protected by the transfer of

Table 6. *The intracerebral transfer of protection against intracerebral B. pertussis infection to recipient mice given lymph node cells from immunized (LV) or normal (LN) donor mice*

Lymph node cells	Challenge (no. of organisms)	Survivors/total mice in experiment no.				
		1	2	3	4	5
LV/S	50,000	.	1/3	.	2/5	.
LV	50,000/S	3/5	1/13	0/5	.	.
LV	50,000	0/6	.	1/5	.	0/6
LN	50,000/S	1/5	.	0/5	.	.
.	50,000/S	1/2	1/5	0/5	.	.
.	50,000	0/3	1/5	.	1/5	0/5
LV (IPS)	50,000	2/5

The mixing of either challenge organisms of lymphocytes with serum from once-vaccinated mice to give a final serum dilution of 1/2 is denoted by S. All intracerebral injections were in 0.03 ml. In Expt. 5 serum alone was given intraperitoneally daily in 0.5 ml. on days 0-4 (IPS).

lymph node or spleen cells and that serum injected just before challenge improved the chances (TO/1 for lymph node cells and TO/2 for spleen cells). None of the untreated mice survived nor those given serum alone, nor those receiving less than an immunizing dose of vaccine (Table 4).

Intracerebral transfer

In these experiments TO/2 mice were used throughout. Suspensions of lymph node cells were prepared as before from mice vaccinated 2 weeks earlier, but the challenge was given only on the day of the transfer. The lymphocyte suspension was inoculated intracerebrally in 0.03 ml. volumes, at a concentration varying from 1.6×10^6 to 9×10^6 cells in five experiments, alone or with an equal volume of fresh, homologous mouse serum from the once-vaccinated mice. The challenge was given 4 hr. later alone or made up in 1/2 fresh homologous mouse serum also from once-vaccinated mice, incubated for 30 min. at 37° C. before injection. This procedure had no effect on the viable count of the challenge. Results are given in Table 6, Expts. 1-4.

In one experiment (1, Table 6), in which lymphocytes from donors vaccinated with 8 ImD 50 were injected 4 hr. before a challenge sensitized with antiserum, 3/5 mice survived whereas most of the control mice, including those given lymphocytes and unsensitized challenge, died; attempts to repeat this result have failed. In another experiment (4), 2/5 mice survived challenge with unsensitized organisms after receiving lymphocytes with homologous antiserum, both from mice vaccinated with 6 ImD 50. Expt. 5 (Table 6) was carried out in an attempt to imitate conditions in a vaccinated mouse. Lymphocytes from vaccinated mice were injected intracerebrally and challenged 4 hr. later. Immediately before challenge, fresh, homologous antiserum from the same batch of vaccinated mice was injected in 0.5 ml. volume intraperitoneally. The serum injection was repeated, using antiserum stored since collection at -15° C. on days 1 and 2. On day 3, fresh serum given within 4 hr. of collection from once vaccinated mice kept alive and challenged the

previous day was used. Two mice out of five were protected in the group given lymph node cells and repeated serum, none in the group receiving only cells. The serum-only control was not done in this experiment but on previous occasions there had been no survivors from repeated serum injections.

As with intraperitoneal transfer, the results are again disappointing, although there is a suggestion that antiserum sometimes given with the challenge, sometimes with the cells, increases the chances of protection to 25% compared with 6% for transfer of lymphocytes without serum and 13% for controls.

During the course of these experiments, the virulence of the challenge organisms in antiserum from once vaccinated mice was compared with that of organisms in fresh serum from normal mice, for controls. In all experiments the usual challenge of 50,000 total organisms killed all the mice and serum treatment did not alter the viability of the inoculum. The virulence of the organisms mixed with antiserum from once vaccinated mice was, however, consistently lower by 2 or 3 times than that of organisms in normal serum. This difference would not be sufficient to affect the outcome of the experiment unless other factors were operating, but demonstrates a slight difference between the antiserum and normal serum, which we had previously only been able to do by using concentrated antibody.

DISCUSSION

In response to repeated doses of vaccine, rabbits and mice produce 11 S and 7 S antibody capable of protecting normal recipient mice against an intracerebral challenge (Dolby & Dolby, 1969). These globulins appear able to cross the blood-brain barrier and so be effective when injected either intraperitoneally or intracerebrally. This *in vivo* antibacterial action has nothing to do with the bactericidal antibody, which can be demonstrated *in vitro* in the presence of guinea-pig serum although it is found in the same antiserum fractions as protective antibody (Dolby *et al.* 1975), nor is vaccine potency correlated with the antigen which elicits this antibody (Ackers & Dolby, 1972).

The antibodies produced in animals by repeated immunizing doses can protect either by immediate sterilization of the infecting organisms or by a bactericidal reaction which does not manifest itself against the challenge until 3 days after infection. In the first instance the percentage of survivors is equal to the percentage of mice with sterile brains when examined 2 days after challenge: to be protective this sterilization must be complete as any surviving organisms will multiply and eventually kill. In the second instance, more mice survive than have sterile brains two days after infection. The 11 S fraction of antiserum from repeatedly immunized animals operated by the first mechanism as did some 7 S preparations, particularly those re-run on DEAE Sephadex columns and divided into sub-fractions. Many 7 S preparations, shown to consist only of IgG, caused a slightly reduced infection initially which was maintained at a lower level than in control mice, and finally suppressed 3-5 days after infection. The heterogeneity of not only whole antisera but also antibodies of the IgG class explains the range of recovery patterns seen when following brain counts; for an antiserum from a

repeatedly vaccinated animal capable of protecting 80–90% of mice the percentage of sterile brains at 2 days may vary from 0–60% (Spasojevic & Holt, 1962; Adams, 1968; Dolby, 1972). It seems possible that the delayed mechanism for delayed protection by 7 S immunoglobulin involves another factor which has to pass into, or accumulate in, the brain and Holt (1972) has raised again the suggestion that an opsonin is implicated.

The distribution of protective immunoglobulins from the serum of once vaccinated mice was quite different; when the serum was taken at either 14 days after intraperitoneal vaccination or 17 days after vaccination and 3 days after challenge, at a time when the infection was beginning to be suppressed (Dolby & Standfast, 1961) the protective antibody was in the 19 S and 11 S fractions of the sera. Our few experiments on brain counts following intracerebral infection with organisms mixed with 19 S globulin suggested that sterilization was immediate, as was that for 11 S globulin, but whereas infection was overcome by intraperitoneal injection of 11 S globulin, 19 S globulin was inactive intraperitoneally. Most of our 19 S fraction was used up in repeated intraperitoneal injection after challenge to ensure that, in spite of the short half-life of IgM *in vivo*, some was available at the optimum time for effectiveness, but none of the mice were protected.

We have already pointed out (Dolby *et al.* 1975) that when serum was taken from once-vaccinated mice, fractionated, concentrated and injected into the brain, the quantity of immunoglobulin injected was no greater than might circulate naturally through the brain, but we were thus able to inject an effective dose in 0.03 ml. whereas this volume of unconcentrated serum did not overcome a challenge of 100 LD₅₀ of organisms. The slight reduction in virulence of organisms, achieved by mixing with unconcentrated antiserum as in our cell transfer experiments suggests the presence of antibody in the serum of once-vaccinated mice but made more easily demonstrable by concentration. After repeated immunization, larger amounts of antibody present would make concentration unnecessary to demonstrate it on intracerebral injection. Antiserum from repeatedly immunized animals would also be expected to be effective when given intraperitoneally since the effective antibody is in the 11 S and 7 S globulin fractions, both of which can pass the blood–brain barrier. That mice which have been injected intraperitoneally with 2–3 ml. of serum from once-vaccinated mice are not protected (Wardlaw & Jakus, 1968; Dolby & Bronne-Shanbury, unpublished), could thus be explained qualitatively as well as quantitatively if the most effective antibody is in the 19 S fraction which could not enter the brain, and not in the 11 S or 7 S which could penetrate the brain. Antibody was found within the brain after a moderate dose (20 ImD₅₀) of intracerebral vaccine only after challenge. Without challenge it was present only after a large dose (300 ImD₅₀) of intracerebral vaccine. No antibody was detected in the brain after intraperitoneal vaccination, with large or small doses or with or without challenge. Some serum proteins do cross into the brain 3 days after infection (Holt, Spasojevic, Dolby & Standfast, 1961), but since repeated dosage of concentrated 19 S globulin intraperitoneally fails to protect the mice, 19 S globulin is not among them. Is protection in a once-vaccinated mouse therefore dependent on antibody?

Our experiments on the transfer of immunity by intraperitoneal injection of cell suspensions suggested that effective sterilization of the brain may require both cells present in lymph node preparations, and an antiserum component. These results were disappointing and the best protection given by lymphocytes of 18–21 day vaccinated mice protected only 20–30% of mice when the interval between transfer and challenge was 14 days or more. Professor A. C. Wardlaw and his colleagues in Toronto had already done similar investigations to ours (personal communication). They had little success with intravenous or intraperitoneal transfer 4–5 days after vaccination when challenging 10 days later or with transfer 14 days after vaccination and challenging immediately. Although they probably used fewer cells than we did, their sources were similar.

More rewarding were the Toronto experiments in which peritoneal exudate cells were transferred 4 days after vaccination directly into the brains of mice, mixed with the challenge. Under optimum conditions 65–70% of the mice were protected which is much better than in any of our experiments. These experiments were incomplete and there were other possible but uninvestigated reasons why the exudate cells protected recipient mice, such as bactericidal activity of the exudate for the organisms mixed with it (Dolby, 1965) or transfer of antigen along with macrophages which might have produced an effect similar to that produced by injecting antigen and challenge together (Evans & Perkins, 1954).

One explanation of the lack of immediate transfer of protection by intraperitoneal injection of cells may be that the immunocompetent ones must have time to penetrate to the site of infection, i.e. lymphocytes must pass into the brains, which they can do. Non-compatible cells may however have greater difficulty in achieving this, as they do in migration and 'homing' (Zatz, Gingrich & Lance, 1972). For this reason, intracerebral transfer of immune lymphocytes was next carried out but protection was not demonstrated with any greater success than intraperitoneal transfer, the protection being about 25–30% in the presence of antiserum. The role of antiserum is still not clear since, at least in once-immunized mice, both cells from immunized animals *and* antiserum seem necessary, unlike the system described for cholera by Actor & Pitkin (1973) in which either immune cells or normal cells plus antiserum were protective.

Two other possible reasons for our failure in the cell transfer of immunity may have been that we did not use irradiated animals and so reduced the chance of acceptance of donor cells (Mäkelä & Mitchison, 1965) and also that our animals may have been beyond the optimum age (Celada, 1966).

Our experiments concerning intracerebral vaccination were designed to determine the possible role of local antibody and also to dissociate the non-specific effects from that induced by protective antigen. Some of the complexities have been mentioned in the introduction. Suppression of intracerebral infection was achieved by vaccines not containing protective antigen when the interval between vaccination and challenge was only 1 day, but this was not manifested as ultimate protection. Extending the interval from vaccination to challenge to 14 days removed all this initial non-specific suppression except for that of *S. typhi* vaccine. Lipopolysaccharide both from 'bactericidal antigen' positive and negative strains

was able to suppress infection in mice vaccinated only 1 day earlier but the more purified samples had less effect. This could well be an interferon effect as suggested by Iida & Tajima (1971) and might explain the inhibition by virus of the growth of *B. pertussis* in mouse brains (Cohen, 1953). On the other hand, only *B. pertussis* vaccines containing protective antigen can prevent ultimate death of infected mice, even when an interferon-like inhibition of the challenge is present initially. Interferon production is, however, complex and can be a one- or two-step process (Nagano & Maehara, 1972; Salvin, Younger & Chederer, 1973).

The suppression of intracerebral infections of *B. pertussis* by lipopolysaccharide, as described here together with earlier observations of Dolby & Vincent (unpublished) on the decreased virulence of lipopolysaccharide-treated organisms might well provide a clue to the intracerebral virulence of some strains for mice. Sensitivity *in vitro* to the bactericidal antibody (antilipopolysaccharide) of an antiserum, and intracerebral virulence for mice tend to be properties of the same few strains of *B. pertussis* (Dolby, 1965). Coating the organisms of such strains with lipopolysaccharide made them insensitive to the action of bactericidal antibody, as might be expected, and also 5–100 times less virulent for mice. Treated organisms usually established themselves in the brain but they either remained constant in number for 3–4 days before being completely suppressed or the numbers increased and then fell again at about 3 days. This can now be regarded as non-specific suppression of the organisms by the adsorbed lipopolysaccharide which would allow time for local immunity to the challenge to develop before the numbers became lethal.

The properties and amounts of lipopolysaccharide isolated from virulent, serum-sensitive strains were similar to those isolated from serum-insensitive, avirulent strains and both were equally able to interfere with the bactericidal reaction and virulence when adsorbed on to test bacteria (W. A. Vincent, unpublished). Virulent serum-sensitive strains might thus be ones in which the lipopolysaccharide is below the surface and unable to exert its effect. Virulence in this context refers of course only to performance in mice. Very few strains isolated from children are mouse-virulent.

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EXPLANATION OF PLATE

Figs. 1 and 2. The immuno-electrophoretic patterns of 5 μ l. of concentrated mouse brain extract (upper) and 2.5 μ l. of a 1/6 dilution of rabbit anti-mouse serum (lower). The brain extract shown in Fig. 1 was from normal mice, that in Fig. 2 from mice vaccinated intracerebrally with 0.1 unit of *B. pertussis* vaccine (20 ImD 50) and infected intracerebrally 14 days later, 3 days before the brains were collected.

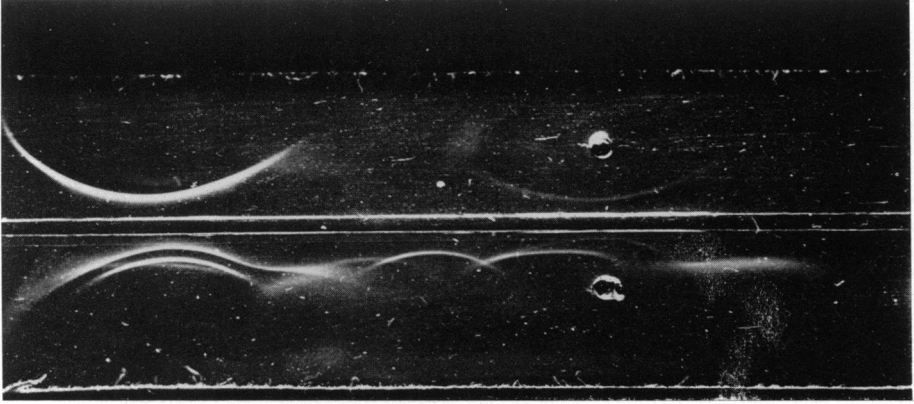


Fig. 1

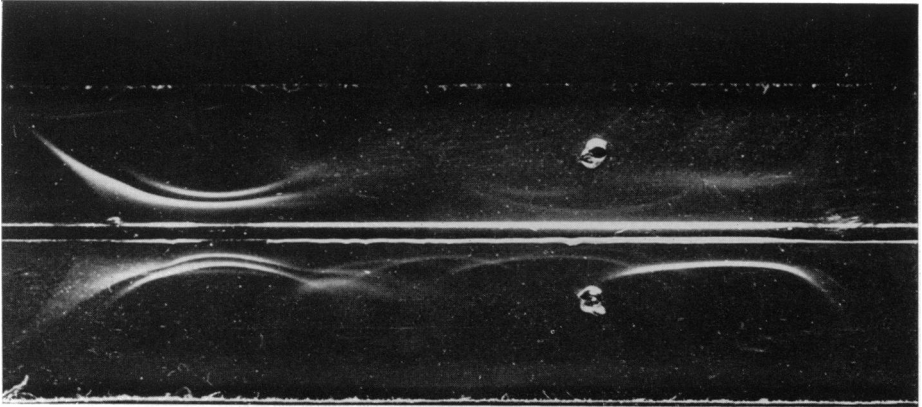


Fig. 2