Passive protection of mice against intracerebral infections with *Bordetella pertussis*

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SUMMARY

The passive protection of mice against an intracerebral infection with *Bordetella pertussis*, by antiserum introduced directly into the brain with the infecting organisms, was compared with the protection afforded by intraperitoneal antiserum. The antibody effective by the intracerebral route is that which is adsorbed onto the infecting organisms, although it does not affect the viability of the organisms *in vitro* in the absence of complement.

Passive protection against organisms introduced intracerebrally takes place in one of two ways, depending on the size of the challenge: (1) after 3–4 days' growth, the growth rate declines so that the number of organisms does not reach the figure lethal for the mouse; (2) the organisms do not appear to multiply, as their numbers decline from the moment of injection, so that the brain is sterile after 2 days.

Many of the mice protected against a challenge of 50,000 organisms (*ca.* 100 LD 50) by intraperitoneal or intracerebral antiserum recover by the first mechanism. The second mechanism operates after a smaller challenge of 5000 organisms (*ca.* 10 LD 50), irrespective of whether the antiserum is given intracerebrally with the challenge, or intraperitoneally within several hours of challenge. Too much antiserum given intracerebrally with a 50,000 challenge, but not with a 5000 challenge, inhibits protection.

In some mice, virulent antibody-treated organisms which have not been killed, grow very slowly over a long period, but are eventually eliminated from the brain.

INTRODUCTION

Spasojević (1962) measured the protective potency of *Bordetella pertussis* antisera for mice by means of an intracerebral passive protection test in which bacteria were incubated at 37° C. for 1 hr. with graded dilutions of antiserum, and injected intracerebrally into mice. (This will be referred to as the 'IC mix' test.) She obtained consistent and reproducible results with both rabbit sera and convalescent human sera from children; the dose–response curves were good and some sera were protective at very high dilutions. She also found (private communication) that the number of sterile brains 2 days after challenge equalled the number of survivors. Adams (1968) found that over half the mice that were to survive had sterile brains 2 days after challenge.

We were unable to reproduce the consistency and dose–response curves of

Spasojević's experiments; often the same number of mice died in each of a wide range of dilutions, giving a plateau-like or even inhibitory zone effect over lower antiserum dilutions. The preliminary investigations which were made on the course of infection in mice given serum and organisms together also indicated that the brains of recovering mice were seldom sterilized before 6 days; the curve of infection and recovery was similar to that described for intraperitoneal serum and intracerebral challenge (Dolby & Standfast, 1961).

It was felt that, apart from explaining differences between workers, a reinvestigation of the 'IC mix' test was worth while for both practical and academic reasons. Only very small amounts of serum are needed for the test and if it were measuring the same antibody as that causing protection when much larger doses are administered intraperitoneally, 3 hr. before challenge (IP/IC test), then it would be useful. If, on the other hand, there are two mechanisms for passive protection, then it is of interest to learn more about the differences.

METHODS

Strains used for challenge

Bordetella pertussis strain 18–323 was used for infections by the intracerebral route and *B. pertussis* strain Gl.353 for infections by the intranasal route.

The required suspension was made by homogenizing the growth after 20 hr. at 36°C. from Bordet-Gengou plates in 1% (w/v) Difco Casamino Acids (vitamin free) and diluting the suspension to 10 International Opacity Units (equal to $10,000 \times 10^6$ *B. pertussis* per ml.). Further dilutions were made as required in 1% Difco Casamino Acids. Routine viable counts by the method of Miles & Misra (1938) showed that about 10% of the 18–323 suspensions and 20% of the Gl.353 suspensions were viable. Doses are all cited as total, not viable, numbers of organisms. The LD50 of strain 18–323 injected intracerebrally was 500–1000 organisms; lethal doses of 5000 and 50,000 organisms were used. Strain Gl.353 was instilled intranasally in sublethal doses of 50,000 organisms (about 0·01 LD50).

Mice

White mice, 17–19 g., of Schneider-Webster, ICI, Theiler's Original, and TF1 strains were used. Mice of one strain and one sex were used in any one experiment. The first three strains were easily protected by intraperitoneal vaccine against an intracerebral challenge 14 days later. Halfway through this work, the Schneider-Webster and TF1 mice became more difficult to immunize; the implications of this are discussed below.

Antisera

Two rabbit antisera were used. Batch number 6660, a lyophilized serum used in most experiments, was a pool of sera from a dozen or so rabbits given phase I *B. pertussis*; 0·2 ml. of undiluted serum given intraperitoneally at about the same time as the intracerebral challenge usually protected all mice, whereas 0·2 ml. of a 1/10 dilution protected rather less than half. Batch number E014, a similar liquid serum, was used in a few experiments. Sera were heated at 56° C. for 30 min.

before use. The amount needed to protect half the mice (PD50) was calculated by the method of Reed & Muench (1938).

Passive protection tests with intraperitoneal serum and intracerebral challenge (IP/IC)

Mice received 0·2 ml. of the serum dilution (in saline) intraperitoneally 3 hr. before the challenge. The intracerebral challenge of 50,000 or 5000 organisms was given in 0·03 ml. of 1 % Casamino Acids. This method is referred to in brief as 'IP/IC 50,000' or 'IP/IC 5000'.

Passive protection tests with intracerebral serum and challenge ('IC mix')

Suitable dilutions of serum and challenge suspensions, both in 1 % Casamino Acids, were mixed in equal volumes so that 0·03 ml. contained either 50,000 or 5000 organisms as required. The mixtures were injected into the mice usually after incubation for 30 min. at 37° C., although incubation seemed to make very little difference to the results. There was no diminution in viable count due to *in vitro* serum treatment. This method is abbreviated to 'IC mix 50,000' or 'IC mix 5000'.

Sensitized organisms were prepared from the serum +organisms mixtures by centrifuging at 3000 rev./min. (on an MSE angle head laboratory bench model centrifuge) and replacing the supernatant with an equal volume of 1 % Casamino Acids. In one experiment, sensitized organisms were prepared by incubating serum with concentrated suspension at 10^{10} organisms per ml. and then diluting to 50,000 organisms in 0·03 ml.

Sublethal intranasal challenge

Normal mice were given 50,000 organisms of strain Gl. 353 in 0·04 ml., instilled intranasally under light anaesthesia, producing a sublethal lung infection in which the bacterial count increased up to about the 8th day and then decreased (Dolby, Thow & Standfast, 1961). Active or passive immunity suppressed the sublethal infection and counts of viable organisms in the lungs of such mice provided a sensitive method for measuring the degree of protection.

Estimation of the degree of protection and of viable organisms in brains and lungs

Dead and living animals were recorded daily in cages of treated mice. Groups of mice were taken at intervals from duplicate cages and killed with coal gas for counts. Brains or lungs were removed aseptically, each into a 2 oz. universal bottle containing 9 ml. 1 % Casamino Acids and 2 ml. 5 mm. (diam.) glass beads. The bottles were shaken on a vertical shaker ($2\frac{3}{4}$ in. throw, 325 rev./min.), 3 min. for brains and 10–15 min. for lungs. Tenfold dilutions were made of the homogenates in 1 % Casamino Acids and the dilutions counted (Miles & Misra, 1938) on solid medium (see below) which was examined after 5 days at 36° C. Volumes of 0·5 ml. of the undiluted homogenate were spread on plates for counts when low counts, between 10 and 100 organisms per brain, were expected. Brain counts of less than 10 viable organisms per 0·5 ml. were recorded as sterile.

The solid medium was made from Cohen & Wheeler (1946) liquid medium, modified by the use of 0·1 % casein hydrolysate acid (Oxoid), 0·5 % glutamic acid, 5 % blood and 1·3 % New Zeland agar.

The PD 50 dose of antiserum was calculated by the method of Reed & Muench (1938).

In vitro *measurement of circulating antibodies in mouse serum*

Bactericidal antibody was measured by incubating together for 40 min. at 37° C. 0·2 ml. antiserum dilution, 0·2 ml. *Bordetell apertussis* strain 18–323, containing 10^6 organisms, and 0·2 ml. guinea-pig serum at 1/15 dilution; all dilutions were made in Casamino Acids (Difco 1 %). After incubation, mixtures were diluted in 1 % Casamino Acids containing 7·7 % (w/v) NaCl to stop the action of complement and plated on Cohen & Wheeler blood plates. A fivefold reduction of viable count was considered significant.

Agglutinins were measured by incubating together equal volumes of antiserum dilutions and suspensions of organisms, strain 18–323 at 5×10^9 organisms per ml., in Dreyer tubes at 37° C. for 4 hr. The tubes were held overnight at room temperature and titres of over 1/50, read the following day with a hand lens, were considered significant.

RESULTS

The effect of intraperitoneal and intracerebral antiserum on a challenge of 50,000 intracerebral organisms (100 LD 50)

Fig. 1 shows the effect of graded amounts of antiserum E 014 by different routes. It will be seen that 0·015 ml. of serum per mouse intracerebrally had the same effect as ten times that volume intraperitoneally, and 0·02 ml. serum given intraperitoneally was inactive, and the increase in viable organisms in mice given this dose was indistinguishable from that in the controls.

The course of intracerebral infection by 50,000 organisms (100 LD 50) *mixed with antiserum*

Table 1 shows the results for dilutions of 1/2 to 1/250 of the rabbit antiserum 6660. Fig. 2 shows the individual mouse counts for 1/2 serum with the intracerebral challenge of 50,000 organisms. From Fig. 2 it can be seen that at day 1 the serum-treated organisms were fewer than the control organisms, with viable counts of 100–1000 compared with 1000–10,000 in the controls. This reduction in count in the serum-treated mice was not maintained, however, and only 2/10 had sterile brains at 3 days, whereas 92 % of the mice survived. Many of the mice which would have survived were therefore fairly heavily infected at 3 and 10 days with brain counts of up to $10^{6·0}$. Recovery took place following a pattern similar to, but more extended than, what happens in mice vaccinated intraperitoneally 14 days before intracerebral challenge or given antiserum intraperitoneally near the time of challenge (Dolby & Standfast, 1961).

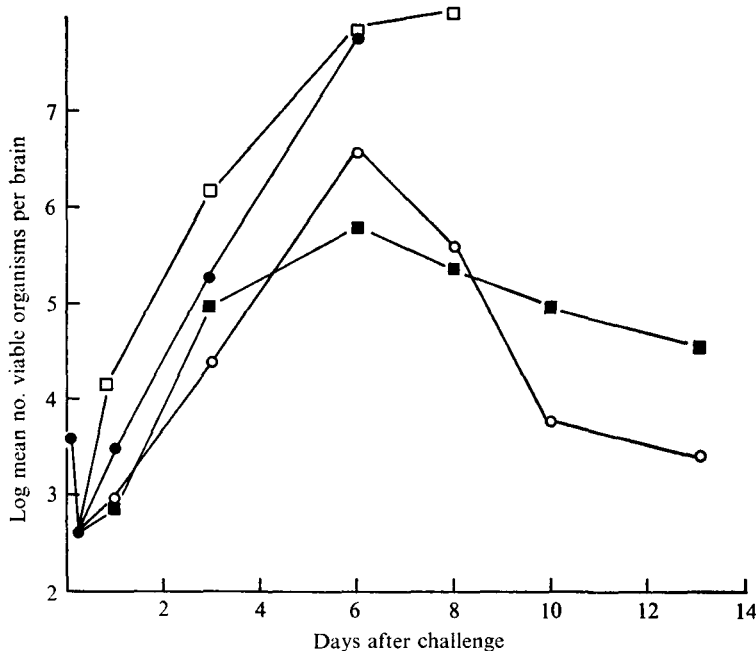To determine whether adsorbed or unadsorbed antibody was effective, a com-

Fig. 1. Growth curves of 50,000 organisms of *Bordetella pertussis*, strain 18–323, in the brains of mice treated with antiserum E014 in various ways and in untreated controls. Each point represents the average count in five mice including healthy, ill and dead individuals, selected in proportion. ●—●, Control mice untreated; 80 % dead by the 14th day. □—□, 0·02 ml. antiserum intraperitoneally 3 hr. before challenge; 74 % dead by the 14th day. ■—■, 0·2 ml. antiserum intraperitoneally 3 hr. before challenge; 10 % dead by the 14th day. ○—○, 0·015 ml. antiserum intracerebrally with challenge; 42 % dead by the 14th day.

Table 1. *Data for mice injected intracerebrally with* 0·015 ml. *antiserum* 6660 *mixed with* B. pertussis 18–323, 50,000 *organisms per mouse*

(The mice were kept for 35 days. Fig. 2 shows the viable count of organisms in mice receiving 1 in 2 dilution of antiserum.)

Initial serum dilution	Survivors/ total at 35 days	Percentage survivors	Average time to death (days)
1/2	23/25	92	15·7
1/10	4/14	29	16·4
1/50	5/15	33	12·9
1/250	1/15	7	9·1
No serum	0/10	0	6·6

31/32 survivors from first three cages had sterile brains 35 days after infection. One mouse given 1/2 serum was infected with 80 organisms at the 35th day.

parison was made of the protective effect of antiserum and bacteria, and bacteria alone, after sensitization *in vitro* (Table 2); there was little difference in protection, indicating that the free antiserum in the mixture, which was presumably rapidly eliminated from the brain, took no part in protection. Such small amounts given intraperitoneally were not effective (Fig. 1). The degree of infection by sensitized
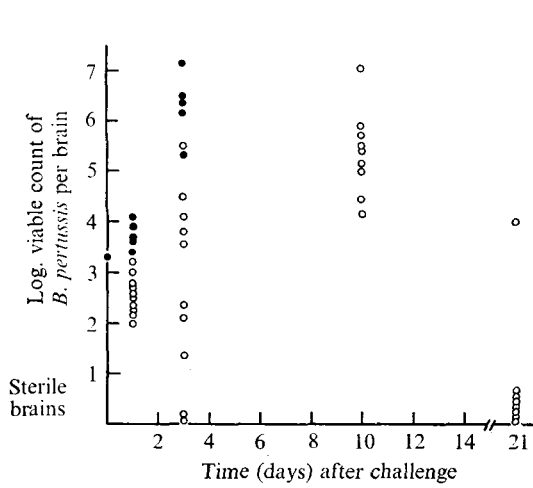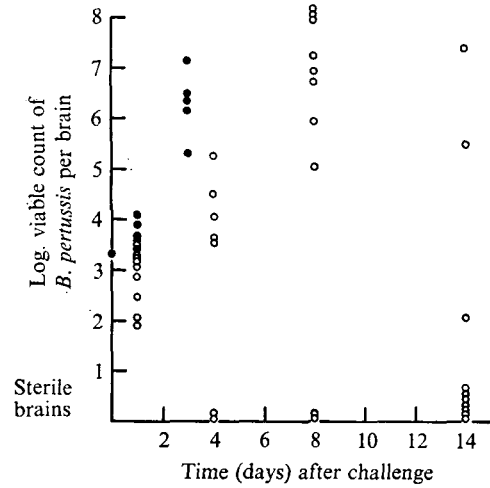
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Fig. 2. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given anti-serum 6660 dilution 1/2 (final) and 50,000 organisms intracerebrally. ○, Infection with a mixture of antiserum and organisms; 92 % survived the 21st day. ●, Control mice given infecting organisms only; none survived beyond the 6th day.

Fig. 3. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given, intracerebrally, 50,000 organisms which had been sensitized with antiserum 6660 at a dilution of 1/2 (final), centrifuged and resuspended in Casamino Acids. ○, Infection with sensitized organisms; 50 % survived the 14th day. ●, Control mice given unsensitized organisms only; none survived beyond the 6th day.

Table 2. *Comparison of 'IC mix' passive protection tests using antiserum–organisms mixture or sensitized organisms resuspended in Casamino acids*

(Each challenge volume of 0·03 ml. contained 50,000 organisms of strain 18–323.)

Initial serum dilution	State of organisms on injection	No. of viable organisms in inoculum	Survivors/ total at 21 days	Degree of infection in brains of survivors at 21 days		
				No. investi-gated	No. sterile	No. of organisms in infected brain
1/2	Mixed with antiserum	1270	5/12	5	4	3200
1/2	Mixed with antiserum, washed and resuspended	1110	6/12	6	6	—
1/100	Mixed with antiserum	1170	9/12	5	5	—
1/100	Mixed with antiserum, washed and resuspended	1160	7/12	5	4	40

organisms is shown in Fig. 3. In this group, mortality was 50 %, yet all mice were infected at days 1 and 8.

Undiluted serum, mixed with organisms at 10,000 times the challenge strength, was unable to sensitize them enough to protect the mice.

It was surprising how long strain 18–323 could persist in the brain after 'IC mix 50,000' experiments. The organisms isolated after 35 days from two brains of
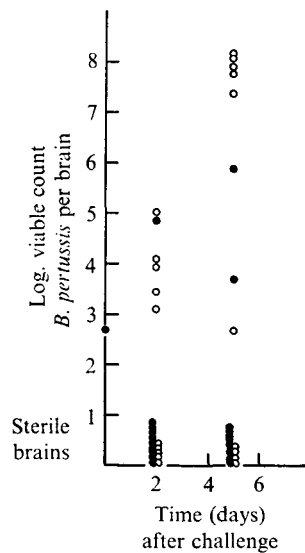
Fig. 4. *Bordetella pertussis*, strain 18–323, in the brains of individual mice given 5000 organisms intracerebrally with: O, 6660 at a dilution of 1/250 (final), 50 % survived; ●, 6660 at a dilution of 1/5 (final), 90 % survived.

mice given 1/10 antiserum were typical smooth *Bordetella pertussis*, but another isolate from a mouse given 1/2 antiserum was less easily agglutinated by the antiserum (to almost the same titre, but very weakly) and was 3–4 times less virulent.

Since some mice with these prolonged infections survived, one might expect them to possess some demonstrable active immunity. This was tested on the survivors of 'IC mix 50,000' experiments, after treatment with various amounts of antiserum, by reinfecting with 50,000 organisms intracerebrally; none survived and the course of infection was identical with that in untreated control mice. Neither serum bactericidal antibody nor agglutinins nor active response of the mice to a sublethal lung infection was positive in mice surviving 'IC mix 50,000' experiments with 1/20 or 1/200 antiserum, but serum antibodies and active immunity to small lung infection were detected in mice surviving 'IC mix 50,000' experiments with 1/2 antiserum.

These positive results were due to an active response of the mice and not to persistent antibody from the top dose of antiserum injected. When mice were given 1/2 antiserum intracerebrally and tested 4 weeks later, they had no immunity to a sublethal intranasal infection, nor were antibodies demonstrable *in vitro*.

The course of intracerebral infection by 5000 *organisms*
(10 *LD* 50) *mixed with antiserum*

With the smaller, but still lethal, challenge the result was different (Fig. 4). In 'IC mix 5000' experiments with 1/5 and 1/250 antiserum, the viable count dropped from day 0 and the proportion of mice free of organisms on the second day corresponded to the proportion of ultimate survivors. These results are similar to those of Spasojević (1962) and quite different from those in Figs. 2 and 3.

Table 3. *Passive protection experiments with* 6660 *antiserum in Theiler's Original mice against an intracerebral challenge of* 18–323.

(Paired results from two similar experiments: 20 mice on each dose; brain counts at two days; LD 50 of 18–323 was 1300; PD 50 IP/IC against 50,000 challenge was 0·06 ml., against 5000 challenge was 0·006 ml.; 0·2 international units of vaccine protected 50% of these mice.)

| | | Brains at 2 days | | | | Brains at 2 days | | | |
| | | Infected | | | | Infected | | | |
	Dilutions of antiserum	No. infected	Log. geom. mean of organisms	No. sterile brains	S/T*	No. infected	Log. geom. mean of organisms	No. sterile brains	S/T*
IP/IC	1/1	4	2·0	16	10/10	14	4·0	6	16/20
serum	1/5	6	2·9	14	18/20	16	4·6	4	7/19
in	1/25	14	3·5	6	10/19	16	4·6	4	2/10
0·2ml.	1/125	16	3·5	4	8/19				
	0/	18	3·6	2	3/19	18	5·0	2	1/20
'IC	1/2	0	—	20	16/20	14	3·6	6	10/20
mix'	1/10	4	4·6	16	18/20	18	3·6	2	6/19
in	1/50	3	3·0	16	17/21	11	3·6	9	11/20
0·03ml.	1/250	3	3·7	17	19/22	13	3·8	6	14/22
total	1/250	8	2·3	12	·9/20	18	4·4	2	3/20
	0	17	3·9	3	4/21	18	4·8	2	3/20
		5000 challenge				50,000 challenge			

* S/T Survivers over total in groups of mice kept for 14 days.

Passive protection experiments, comparing the effect of the route of administration of antiserum and size of challenge on type and degree of protection

Serum was given intraperitoneally followed by an intracerebral challenge ('IP/IC 50,000' or 'IP/IC 5000') or mixed with the challenge ('IC mix 50,000' or 'IC mix 5000'). Two days after infection, half the mice were killed for brain counts; the other half were observed for 14 days (Table 3).

In the left-hand side of Table 3, giving the 5000 challenge results, the proportion of sterile brains at 2 days for all these mice over a wide range of serum doses injected either intraperitoneally or intracerebrally mixed with the challenge, was fairly close to the proportion of survivors at 14 days, though less close in mice receiving intraperitoneal serum. Survival after a 5000 challenge was therefore associated with the ability of the animal to overcome the infection very quickly.

The top right-hand side of Table 3, showing the results of serum given intra-peritoneally against a 50,000 challenge, indicates (as did Fig. 1) that for 2 days the organisms increased in numbers only slightly more slowly than in control mice, but that many of the infected mice did not die; a killing mechanism was effective at later than 2 days.

The 'IC mix 50,000' results (lower right-hand part of Table 3), though typical

Table 4. *Comparison of passive protection (pooled results) by serum 6660 against an intracerebral challenge of 18–323 in Theiler's Original (TO) and TF1 mice.*

Route and challenge	Serum dilution	Percentage survival in	
		TO	TF1
'IC mix' 5000	1/2	70	60–70
	1/20	80	75
	1/200	70	60–70
'IC mix' 50,000	1/2	45	10–20
	1/20	40	10–20
	1/200	70	10–20
IP/IC 50,000	1/1	96	25
	1/5	33	17
	1/25	20	4

of most of these experiments, are more difficult to interpret. Although the proportion of survivors at 14 days was greater than the proportion of sterile brains at 2 days with 1/2, 1/10 and 1/250 antiserum, as with intraperitoneal serum and a 50,000 challenge, there was no consistent association of serum dose and its effect. At the 1/50 and 1/250 serum dilutions, the proportion of sterile brains was greater than at other dilutions, and at these dilutions the corresponding proportion of survivors was greater.

The PD50 for the antiserum given intraperitoneally was 10 times less against the smaller challenge than the larger; such a comparison was impossible in the 'IC mix' test because of the lack of dose response, but 1/250 at both challenge levels produced the maximum protection with a decrease in protection at the next dilution used.

The influence of mouse strain on the degree of protection conferred

During the course of this work, it became increasingly more difficult to protect the Schneider–Webster strain of mice against an intracerebral challenge with intraperitoneal antiserum. At the same time, the ImD50 of pertussis vaccine increased by more than 10 times. The strain TF1 was also difficult both to immunize and to protect passively IP/IC. The amount of vaccine needed to protect the TF1 mice to the same extent as Theiler's Original (TO) mice was 6–10 times; but the virulence of 18–323 for the two strains of mice was the same. The Schneider–Webster mice, on the other hand, were very susceptible to 18–323 and for this reason less suitable for comparison with the TO mice.

Table 4 shows the passive protection in TO and TF1 mice. There is little difference in the ability of antiserum to protect both strains of mice when the antiserum was mixed with a 5000 challenge, where the mechanism of protection appears to be an immediate sterilization. There was only a little protection of TF1 mice against a 50,000 challenge, with antiserum either intraperitoneally or intracerebrally.

Blake & Wardlaw (1969) showed that the immunosuppressive reagent, cyclophosphamide, inhibited both active and passive protection of mice by vaccine or

intraperitoneal serum against a 50,000 challenge. In one experiment by my colleague, Dr J. P. Ackers, cyclophosphamide-treated TO mice given intraperitoneal antiserum were protected only to the same degree as TF1 mice.

Serum from TF1 mice 14 days after vaccination had, however, as high an agglutinin titre and content of complement-mediated bactericidal antibody *in vitro* as TO mice similarly vaccinated, whereas antibodies were not found in cyclophosphamide-treated TO mice. The TF1 mice were not, therefore, behaving as immunosuppressed animals, even though the superficial results in passive protection experiments were similar.

DISCUSSION

In 'IC mix' experiments, we are dealing with the fate of sensitized organisms as antiserum given with infecting organisms intracerebrally is effective only when adsorbed on to the organisms. In spite of this, the protective effects of antiserum against 50,000 and 5000 organisms are completely different, and the difference is independent of the route of administration of antiserum. All effective doses of antiserum given either IP or IC with 50,000 organisms intracerebrally sterilize the brains after an initial rise in the bacterial content of the brain; all effective doses of antiserum given IP or IC with 5000 organisms sterilize the brain immediately. The difference does not seem to be an expression of the ratio of organisms to serum. A 1/250 dilution by the 'IC mix' route protects nearly all the mice by immediate sterilization of a 5000 challenge; over 100 times more antiserum by the same route protects half the mice against a ten-times bigger challenge, but not by immediate sterilization.

There is an apparent inhibition of the protective effect with strong antiserum, mixed with a 50,000 challenge, for more dilute serum gives the better protection. The sharing out during bacterial division of not-immediately-lethal antibody that has been adsorbed to bacteria was beautifully demonstrated for *Salmonella typhi* by Cole (1964). The inhibition zone of poor protection (i.e. non-killing of the bacteria *in vivo*) is so like the non-killing zone in the complement-mediated bactericidal action of antibacterial sera *in vitro*, the Neisser–Wechsberg phenomenon (Dolby, 1965), that it was tempting to consider that both *in vivo* and *in vitro* bactericidal inhibition were caused by the same antibody. Attempts were made to correlate the *in vivo* and *in vitro* bactericidal inhibition zones, but were not very successful, a result perhaps only to be expected in dealing with a system of more than one effective antibody. Moreover, if such a correlation existed, then an inhibition zone would be expected against a 5000 as well as a 50,000 challenge (Table 3, bottom left-hand side); it is not evident here. All of this suggests that the bactericidal antibody which acts *in vitro* may have little to do with the *in vivo* killing of bacteria. This question is investigated more fully elsewhere (Ackers & Dolby, 1972; J. M. Dolby & S. Stephens, to be published).

Although we were able to isolate 18–323 consistently from the brains of surviving mice in passive protection tests after 14 days, in active immunization experiments 18–323 was isolated only once from 50 survivors after the 14th day (C. J.

Shanbury, personal communication). Dolby & Standfast (1961) showed that a single organism of 18–323 which lodged in the brain of a 'normal' mouse would cause death in 12–14 days and that it was rare for deaths to occur later. In these passive protection 'IC mix' experiments, however, deaths occurred up to 28 days and 18–323 was isolated as late as 35 days after challenge. There must have been organisms which escaped being killed at 6–8 days but whose growth was slowed down so that either death resulted eventually or a host–parasite balance was achieved. Small amounts of circulating antibody were detected in long-surviving, infected mice. Organisms isolated 3 weeks after challenge from passively protected mice were fully virulent (C. J. Shanbury, personal communication), but two out of three strains isolated 5 weeks after had decreased virulence. Avirulent infections which are too low to stimulate a general response in the host can cause local immunity which eventually overcomes the infection (Standfast & Dolby, 1972), but no local immunity to a re-infection could be detected in mice with long drawn-out infections with 18–323.

The experiments reported here only seem to add to the complexity of relating active and passive immunity. They also emphasize the importance of the size of the challenge dose in the 'IC mix' passive protection test. It is the immediately acting antibody which is effective only against the smaller challenge and organisms not killed in this way grow up to be victims of the second antibody at 4 days. Antisera such as those we used contained both antibodies. If the two antibodies were separable, then it might be possible to use a serum having only immediate or long term activities, but not both.

As judged by the course of infection in passively protected mice, the 'IP/IC 50,000' and 'IC mix 50,000' tests resemble the results after active immunization IP. The role of antibody in the active protection test is, however, controversial and the possibilities have been set out by Blake & Wardlaw (1969). The inability to protect passively mice that do not immunize supports their experiments, suggesting that protection of mice by pertussis antiserum may not be such a passive phenomenon as is generally thought.

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