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A Comparison of Passive Protection Tests against Intranasal and Intracerebral Challenges with *Bordetella pertussis*

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Summary. A survey of a number of rabbit antisera to *Bordetella pertussis* revealed the existence of two distinct antibodies, one passively protecting mice against lethal infection by the intracerebral route, the other passively protecting mice against lethal infection by the intranasal route. Neither is the antitoxin. Antisera against most, if not all, S forms of *B. pertussis* contain both types of protective antibody, and so to a lesser extent do *B. parapertussis* and *B. bronchisepticus*. Neither of the protective antigens is an agglutinogen, or the haemagglutinin. The two antigens can also be distinguished by active protection tests. Extensive investigations, however, had not led to an *in vitro* test for either of the protective antigens or their antibodies that would replace the mouse test.

The practical importance of the two distinct antigens and their antibodies is shown by the fact that assay by the intranasal and intracerebral routes of challenge will not arrange vaccines in the same order of potency either in active or in passive protection tests.

INTRODUCTION

THERE are many references in the literature to passive protection of mice against *Bordetella* (*Haemophilus*) *pertussis* by antiserum given intramuscularly, intraperitoneally or intravenously from 4 hr. before to 4 hr. after the challenging dose of organisms. In attempting to evaluate these experiments it is important to consider the route of administration and the type of antiserum and the route of the challenge. Most authors distinguished between antibacterial serum and antitoxin, and some realized that the intraperitoneal route for the challenge organisms only measures the toxin content of the challenge dose, since *B. pertussis* does not grow intraperitoneally.

Protection against a small intraperitoneal challenge was demonstrated by Mishulow, Klein, Liss and Leifer (1939), after Powell and Jamieson (1937) had found some evidence of protection. North, Keogh, Anderson and Williams (1939) and Scherp, Bradford and Wold (1939) observed protection of mice against an intranasal challenge, and Ospeck and Roberts (1944), protection against an intracerebral challenge.

Sera were known to contain 'antitoxin' because of their ability to neutralize the ability of live cultures or toxin to produce necrosis in the skin. Some sera were also considered to contain 'antibacterial' antibody, but this was invariably measured in terms of agglutinin content; these sera were protective for mice.

Attempts were made by several workers to correlate antitoxin content and protective potency of antisera. Antisera to *B. pertussis* can be obtained from rabbits injected with whole cell suspensions either living or killed by heat; such sera contain agglutinins but will not neutralize toxin (Evans and Maitland, 1937; Wood, 1940). Sera containing *B. pertussis* antitoxin and antibacterial antibodies have been obtained by injecting formalin

treated water extracts of cells disintegrated at low temperatures (Evans, 1940) or by sonic disintegration (Flosdorf *et al.*, 1941). Antibacterial antibodies can be absorbed from such sera with cells heated to 56° for 1 hr. leaving behind the antitoxin (Evans, 1940).

Anderson and North (1943), Ospeck and Roberts (1944) and Proom (1947) demonstrated that antitoxin was protective against a large intraperitoneal challenge, whereas antibacterial sera were ineffective. With an intranasal challenge, Anderson and North (1943) found that the limited protection afforded by pertussis antiserum was not related to its antitoxin content but was due to the antibacterial antibody present in the serum. Evans (1944) confirmed these findings using small and large infecting doses and showed that the absorption of the antibacterial antibodies leaving the pure antitoxin removed the protective ability of the sera when given parenterally (as Anderson and North). He showed, however, that such absorbed sera were able to protect mice if administered intranasally with the infecting organisms. To be effective, antitoxin must be present in an adequate dose at the site of infection to neutralize the toxin of the infecting dose. Antitoxin therefore is important for protection only where a toxic effect is being exerted, as when a very large challenging dose is used or when a particularly susceptible site is involved, and only then, if it is present at the site in adequate amount.

The reports of the activity of antibacterial antibodies are rather more contradictory, partly due perhaps to the fact that most workers had regarded antibacterial sera in terms of agglutinin titre despite the demonstration by Mishulow *et al.* (1939) and North *et al.* (1939) of a lack of correlation between protective action and agglutinin titre in both human and rabbit sera. In 1952 Masry showed that the protective antibodies are distinct from haemagglutinin neutralizing antibodies; and in 1953 Horton and Standfast published a preliminary report on the separation of the protective and agglutinating antibodies in rabbit antisera.

The results reported in this paper were collected during the investigation into the differences between the intranasal and intracerebral route of infection of mice with *B. pertussis*. An inspection of the results of many passive protection tests showed that whereas sera prepared from most strains of *B. pertussis* were active against challenges by both routes, some sera behaved differently and were active against the intranasal challenge or the intracerebral challenge, but not both.

METHODS

PREPARATION OF HIGH POTENCY ANTISERA IN RABBITS

Bordetella pertussis, smooth and rough, *B. bronchisepticus* and *B. parapertussis* antisera were prepared by injecting rabbits intravenously once or twice weekly for 3–4 weeks with 0·1–0·5 ml. formalin or heat-killed organisms suspended in saline to an opacity of 2500–5000 $\times 10^6$ organisms/ml., followed by weekly injections of 1 ml. of a suspension of killed or living organisms at the same opacity for a further 3–4 weeks. Two weeks later the animals were bled, rested and further courses of 3–4 weekly injections given before the next bleeding.

The antigen used to stimulate antitoxin formation was prepared by making a water or phosphate buffer extract of bacterial cells crushed in the frozen state in a Hughes (1951) press. Formalin was added to 0·08 per cent concentration. Rabbits were given 0·1–0·5 ml. intravenously in increasing doses at 3-day intervals followed by four 1 ml. injections at weekly intervals. They were bled after 2 weeks, rested and re-injected before another bleeding.

Antisera to S.P.A., the 'protective antigen' of Pillemer *et al.* (1954), were prepared in rabbits by 1–6 subcutaneous injections at weekly intervals. Antisera to other fractions were prepared by injecting intravenously increasing doses, up to 1 mg. Six to eight injections were given before the last bleeding.

Abbreviations

Throughout this paper the following abbreviations are used. *IC* for intracerebral; *IN* for intranasal; *IP* for intraperitoneal. *IP/IC* assay means the test in which antiserum is given intraperitoneally 4 hr. before an intracerebral challenge. *IN Mix* assay means the assay in which the serum and challenge are mixed together and given intranasally. Hence *IN Mix* serum means an antiserum active by the *IN Mix* or intranasal assay, etc.

Passive Protection Tests

In the early stages of this work various methods of determining the potency of sera in brain and lung infections were investigated. The following routes were used: Intraperitoneal serum 4 hr. before intracerebral challenge (*IP/IC*), intraperitoneal serum 4 hr. before intranasal challenge (*IP/IN*), serum and challenge mixed and given intracerebrally (*IC Mix*), serum and challenge mixed and given intranasally (*IN Mix*).

With good phase I antisera protection was obtained in the *IP/IC* and *IN Mix* routes and the results with different sera were consistent and reproducible, but were less consistent and reproducible by the *IP/IN* and *IC Mix* routes.

The dose of serum which would protect 50 per cent of mice (PD50) was calculated by the method of Reed and Muench (1938). The average PD50 of sera was calculated from results obtained in 2 or 4 titrations and as sera were grouped together and tested at the same time, their values are comparable. A Reference serum was included in each test.

The PD50 by *IP/IN* is invariably much larger than that by *IN Mix*, presumably because only a proportion of the injected antibody reaches the alveoli and comes in contact with the infecting organisms, whereas most of the antibody mixed with the challenge (*IN Mix*) should be effective.

The failure of the *IC Mix* test to produce results as consistent as the *IP/IC* is more difficult to explain. The quantity of serum may be insufficient, 0·03 ml. being the maximum volume of liquid that can be injected, of which only half is serum; the agglutinins in the serum may clump the organisms in the challenge mixture despite all shaking and mixing; or some toxic substance may be liberated (see p. 151). Many attempts were made to perfect the *IC Mix* test, particularly for reasons of economy of serum. A titration on 40 mice at 4 dose levels with 10 mice at each dose may need 15 ml. of serum *IP/IC* or 0·03 ml. *IC Mix*; and where the titration of human sera or sera from vaccinated children is concerned 0·03 ml. is practical, 15 ml. is not, without pooling the sera, which may defeat the object of the test. As the main difficulty seemed to arise from mixing, a trial was made giving both serum and challenge intracerebrally but separately. All these attempts failed (Dr. V. Spasojevic, unpublished results, 1956) and it was necessary to use the *IP/IC* test and the *IN Mix* test, although the latter entails variation both of challenge and administration route of serum.

Tests were carried out in 18–22 g. white mice as follows:

(i) *Intraperitoneal/Intracerebral Test (IP/IC)*

Serum was injected intraperitoneally usually in a volume of 0·2 ml. containing 0·2, 0·1,

0·05 and 0·025 ml. serum. Occasionally with low-potency sera it was necessary to give 0·4 ml. or 0·8 ml. undiluted serum. Four hr. later a challenge of 50,000 organisms strain 18–323 in 0·03 ml. Casamino acids was given intracerebrally. Mice were kept for 14 days.

(ii) *Intranasal Mix Test (IN Mix)*

Suitable dilutions of sera were made up (usually a dilution factor of 5) and one volume of serum mixed with one volume of double strength challenge (strain G353) and 0·04 ml. of the mixture instilled intranasally by the method of Burnet and Timmins (1937). The mice were kept for 28 days.

Antitoxin Tests

Toxin consisted of a watery extract of *ca.* 2×10^{12} organisms crushed in the Hughes press with 1 ml. water, from which debris was removed by centrifugation. Such extracts contain 200–800 minimal lethal doses (m.l.d.) per ml. assayed by intraperitoneal injection in 18 g. mice. The extracts were kept at $-15°$; at 4° they gradually lost toxicity. One volume of antitoxin dilution was mixed with 1 volume of extract diluted to give a final concentration of 2–4 m.l.d. per mouse dose (0·05 ml.). The mixtures were kept at room temperature for 1 hr. and injected intraperitoneally into mice, which were then kept for one week.

Serum Absorptions

(i) Agglutinogen. Antiserum was absorbed repeatedly at 37° with freeze dried samples of agglutinogen prepared by the method of Smolens and Mudd (1943) until the agglutinin titre could not be reduced further. (ii) Haemagglutinin was prepared by Masry's method (1952). Freeze dried samples were added repeatedly to serum at 37° until the haemagglutinin titre was negative. Excess haemagglutinin was neutralized by the addition of very small quantities of unabsorbed serum. (iii) Bacterial suspensions. Antisera absorbed repeatedly with whole bacterial suspensions had all the antibodies removed; those absorbed with agglutinogen-free suspensions lost the protective and other antibodies but not the agglutinins.

In vitro Tests

(i) Agglutinins. One volume of suspension of *B. pertussis* in saline at 5000×10^6 organisms/ml. was added to one volume of serum dilution. Tubes were incubated 4 hr. 37°, left overnight on the bench and read the following morning. The end point was determined with a ×4 lens. (ii) Antihaemagglutinins were measured by the method of Keogh and North (1948) using sheep red cells. (iii) Complement lysis. The sensitized red cells (sheep) were prepared as described by Fisher (1950) with heated culture supernatant. One drop of sensitized red cell suspension was added to 4 m.h.u. guinea pig complement and 0·5 ml. serum dilution absorbed with sheep red cells. The tubes were incubated 1 hr. at 37° and read.

RESULTS

THE DISTINCTION BETWEEN ANTISERA ACTIVE AGAINST THE INTRACEREBRAL ROUTE AND THE INTRANASAL ROUTE

In a preliminary survey by the passive protection test various pertussis antisera fell into three groups: (1) sera of approximately equal potency by *IP/IC* and *IN Mix* assays;

(2) sera more potent by *IP/IC* than *IN Mix* assays; and (3) sera more potent by *IN Mix* than by *IP/IC* assays (Table 1). Thus a group 2 serum might protect 9 per cent of mice in the *IN Mix* test and 53 per cent in the *IP/IC* test. Group 3 sera, on the other hand, protected 52 per cent mice in an *IN Mix* test *c.* 8 per cent in an *IP/IC* test. This strongly suggests that two antibodies are involved.

TABLE 1

PAIRED PASSIVE PROTECTION TESTS WITH *Bordetella pertussis* ANTISERA

Protection measured as survivors over total numbers (S/T) and percentages of mice treated with undiluted serum.

Serum	*Intracerebral challenge (IP/IC)*		*Intranasal challenge (IN Mix)*	
Group 1: 26 sera ..	314/720	44%	235/596	40%
Group 2: 10 sera ..	133/253	53%	16/188	9%
Group 3: 11 sera ..	15/193	8%	100/193	52%
Control mice no serum	12/380	3%	28/401	7%

Group 1: sera of approximately equal potency against intracerebral and intranasal challenge (*IC=IN*).
Group 2: sera more potent against intracerebral challenge than intranasal (*IC>IN*).
Group 3: sera more potent against intranasal challenge than intracerebral (*IN>IC*).

All sera produced against smooth phase I strains fell in group 1, in which both antibodies are present. Rough *B. pertussis* and *B. bronchisepticus* antisera also fell into group 1, although the sera were not nearly so potent as the phase I sera (Table 2).

TABLE 2

PASSIVE PROTECTION—COMPARISON OF *B. pertussis* SMOOTH AND ROUGH, *B. parapertussis* AND *B. Bronchisepticus* ANTISERA

	PD50 in ml. serum		*Ratio of PD50 IC : IN*
	IP/IC test	*IN Mix test*	
B. pertussis—S	0·031	0·0013	23·9
B. pertussis—R	0·32	0·014	22·9
B. parapertussis	0·42	0·015	28
B. bronchisepticus ..	0·22	0·012	18·3

Further evidence of two separate antibodies (Table 3) was obtained during the immunization of a rabbit (308), which received eight courses of an S.P.A. (Pillemer *et al.*, 1954) preparation; one antibody appeared much more rapidly than the other.

All attempts to distinguish the two antibodies by precipitin test, complement fixation, red-cell agglutination or by the plate diffusion technique of Ouchterlony have failed. Though eight or more lines were obtained consistently when testing with an antibacterial and/or an antitoxic serum it was not possible to correlate any of these lines with the *IP/IC* or *IN Mix* antibody. So far the two antibodies can be distinguished only *in vivo*, and are not associated in any way with agglutinins, haemagglutinin neutralizing antibodies or antitoxin.

TABLE 3

PASSIVE PROTECTION TESTS WITH UNDILUTED SERA FROM RABBIT NO. 308 AFTER 2, 6 AND 8 COURSES OF VACCINE, AGAINST INTRACEREBRAL AND INTRANASAL CHALLENGE

Survivors/total number of mice and % survivors

	Serum after					
	2nd course		*6th course*		*8th course*	
Intracerebral challenge ..	2/20	10%	9/33	27%	17/20	85%
Intranasal challenge	7/19	37%	16/27	60%	16/19	85%

THE DISTINCTION BETWEEN THE PROTECTIVE (*IP/IC*) AND AGGLUTINATING ANTIBODIES

In 1953 Horton and Standfast reported briefly on the distinction between the protective antibodies as measured by the *IP/IC* assay, from the agglutinating antibodies.

Although North *et al.* (1939), using an intranasal challenge, reported a lack of correlation between the protective action of sera and their agglutinin titre, it has been widely assumed that the protective potency of *B. pertussis* antisera was due to the contained agglutinins. In fact, it has been suggested that an agglutinin absorption test might replace the mouse protection test (Smolens *et al.*, 1947); and an agglutinin production test (Evans and Perkins, 1953, 1954, and M.R.C. Report, 1956) gave good correlation with protection in the field for a number of vaccines. However, the experiments recorded in Tables 4 and 5 show that the agglutinins and *IP/IC* antibody are not the same and hence that the agglutinogens and *IC* antigen cannot be the same. Sera prepared in rabbits given a long course of injections of living *B. pertussis* contain both agglutinins and protective antibodies. Absorption with smooth cells removes both the protective antibodies and the agglutinins.

Certain suspensions of *B. pertussis* stored at 2°–4° in thiomersalate saline lose their surface agglutinogen into the suspending saline and after washing and resuspending in fresh saline are not agglutinable and will not absorb agglutinins, though (Horton and Standfast,

TABLE 4

TYPICAL EXAMPLES OF RABBIT ANTISERA PREPARED AGAINST LIVING SMOOTH *Bordetella pertussis* ABSORBED WITH INAGGLUTINABLE CELLS CONTAINING PROTECTIVE ANTIGEN BUT NO (OR PRACTICALLY NO) AGGLUTINOGEN AND CELL-FREE AGGLUTINOGEN WHICH CONTAINS LITTLE OR NO PROTECTIVE ANTIGEN

Serum	*Absorbed by*	*Passive protection tests IP/IC*								*Agglutination tests*	
		Dose in ml.						*PD50 ml.*	*% Reduction*	*Titre (reciprocal)*	*% Reduction*
		0·8	*0·4*	*0·2*	*0·1*	*0·05*	*0·025*				
P.7	—	—	—	14/15	11/15	7/15	1/15	0·06	—	25,000	—
P.7	Smooth cells	—	—	0/15	0/15	1/15	1/15	∞	100	80	99·7
P.13	—	—	—	—	18/20	11/20	6/20	0·042	—	17,000	—
P.13	Inagglutinable cells	0/20	1/20	0/20	—	—	—	∞	100	14,000	18
P.7	—	—	—	—	35/55	—	—	—	—	16,000	—
P.7	Cell-free agglutinogen	—	—	—	31/55	—	—	—	11	800	95

1953) they retain potency as protective vaccines. Such a suspension absorbs (Table 4, serum P.13) the protective antibodies but reduces the agglutinin titre from 1/17,000 only to 1/14,000. Decreases in agglutinin titre were tested for at 10 per cent steps in place of the more usual two-fold dilutions. Absorption with cell-free agglutinogen prepared by the method of Smolens and Mudd (1943) had no significant effect on the protective power of the serum but reduced the agglutinin titre by 95 per cent, from 1/16,000 to 1/800. Other sera gave similar results.

TABLE 5

PRODUCTION OF AGGLUTININS AND 'INTRACEREBRAL' PROTECTIVE ANTIBODIES BY TYPICAL RABBITS FROM A SERIES OF 54 RABBITS GIVEN 2–5 COURSES OF VARYING INTENSITIES.

Protective antibodies are shown as the average % protection in all tests in 0·4 ml. undiluted serum was given.

Immunizing antigen	*Rabbit No.*	*After 1st course*		*After 2nd course*		*After 3rd course*		*After 4th course*		*After 5th course*	
		Titre	*% protection*	*Titre*	*% protection*	*Titre*	*% protection*	*Titre*	*% protection*	*Titre*	*% protection*
Living pertussis cells	A451	400	0	1,600	0	800	10	1,600	50	1,600	50
	A436	12,800	70	32,000	90						
Killed pertussis vaccine	A467	400	20	800	20	800	20	1,600	50	1,600	80
	A460	8,000	40	32,000	80						
Cell-free agglutinogen	B5	200	0	1,600	0	3,200	0	6,400	0	25,600	25
	B731	640	0	12,800	0	12,800	10	25,600	10		
	A331	8,000	0	32,000	0	64,000	0				
	B478	16,000	0	32,000	0						

Table 5 shows typical examples of the different rates of production of agglutinins and protective antibody in rabbits. There is no correlation between agglutinin titre and protective antibody production, and it is not until after four or five courses of immunization that any protective antibody is produced by cell-free agglutinogen. Most batches of agglutinogen gave rise to antisera with some protective power, indicating that they were not antigenically pure or as pure as batches used for A331 and B478 which gave no protective power though high agglutinin titre after two or three courses.

THE DISTINCTION BETWEEN THE PROTECTIVE (*IN MIX*) AND AGGLUTINATING ANTIBODIES

The lack of correlation in pertussis antisera between agglutinin titre and ability to protect mice against intranasal challenge was noted by North *et al.* (1939). Table 6 shows the failure of agglutinin absorption to remove protective antibodies in both *IN Mix* and *IP/IC* tests. Serum 20954 was absorbed with cell-free agglutinogen, losing agglutinins for all five of Andersen's (1953) *B. pertussis* antigens and for the challenge strain G353. Although the agglutinin titre fell from 1/12,800 to 1/50 against the challenge strain, the PD50 only rose from 0·001 ml. to 0·0027 ml. (At the same time the PD50 (*IP/IC*) had fallen from 0·13 ml. to 0·061 ml; these differences in PD50s are within the experimental error and the protective power of this serum is obviously unchanged.) Other experiments showed that neither the intranasal nor intracerebral protection was associated with the S, L or O agglutinogens of the system of Kasuga *et al.* (1953).

TABLE 6

ANDERSEN* AGGLUTININS AND 'INTRANASAL' PROTECTION OF SERUM NO. 20954 BEFORE AND AFTER ABSORPTION WITH CELL-FREE AGGLUTINOGEN

Serum 20954 absorbed by	*Passive protection tests PD50 ml.*	*Agglutination tests*			
		Strain No.	*3851*	*3747*	*3865*	*G353†*
		**Antigen No.*	*1,3*	*1,2,5*	*1,2,4*	
			Titre (reciprocal)			
—	0·001		12,800	>25,600	12,800	12,800
Cell-free agglutinogen	0·0027		640	160	800	50

* See Andersen (1953).
† *B. pertussis* strain used for challenge in passive protection tests of 20954.

PROTECTIVE POTENCY OF ANTISERA OF HIGH AND LOW ANTITOXIN CONTENT

The lack of correlation between antitoxin and either of the protective antibodies was shown by titrating antisera to whole cell vaccine (low antitoxin content) and Hughes press extracts of cells (high antitoxin content). (Table 7.)

TABLE 7

LACK OF CORRELATION BETWEEN ANTITOXIC TITRES AND ANTIBODY CONTENT (PD50 IN ML. SERUM) OF SERA AGAINST INTRACEREBRAL (*IC/IP*) AND INTRANASAL (*IN Mix*) CHALLENGES

Serum No.	*Antitoxin content and PD50*	*Antibody content against IC challenge and PD50*	*Antibody content against IN challenge and PD50*
17	High 0·0006 ml.	Low 0·12 ml.	High 0·001 ml.
23	High 0·0003 ml.	High 0·035 ml.	High 0·0013 ml.
24	Low >0·025 ml.*	High 0·033 ml.	High 0·00055 ml.
25	Low >0·025 ml.*	High 0·03 ml.	Low 0·02 ml.

* >0·025 = no antitoxin demonstrable at this level.

Attempts to measure the relative potencies of antisera against Hughes press extracts before and after absorption with whole cells to remove antibacterial antibodies were generally unsuccessful because of the development of toxicity in the absorbed sera. This toxicity was due to a substance liberated from the bacterial cells by the serum during the absorption, and was produced whether living, formalin-killed or thiomersalate-killed organisms were used in the process. These toxic sera were lethal in an intraperitoneal dose for mice of 0·1–0·4 ml. and so could not be used for *IP/IC* tests. They could be used in the *IN Mix* test, because the serum dose 0·025 ml. is below the lethal level.

PROTECTIVE POTENCY OF ANTISERA WITH HIGH AND LOW HAEMAGGLUTININ NEUTRALIZING TITRES

Masry (1952) showed that a cell-free preparation of haemagglutinin failed to protect mice actively or passively against both intracerebral and intranasal challenges, though

TABLE 8

LACK OF CORRELATION BETWEEN SOME IN VITRO TESTS AND IN VIVO TESTS WITH INTRACEREBRAL (*IP/IC*) AND INTRANASAL (*IN Mix*) CHALLENGES

Antiserum batch No.	*In vitro tests*		*In vivo tests*	
			% protection against*	
	Haemagglutinin neutralizing titre (reciprocals)	*Agglutinin titre (reciprocals)*	*Intracerebral challenge*	*Intranasal challenge*
B875	1,024	6,400	2	62
P4	512	< 200	5	63
B731	512	12,800	6	5
B733	256	< 200	0	10
P15	256	25,600	87	85
P15 abs.†	< 2	25,600	86	85
C348	< 2	800	60	3
C141	< 2	400	35	46

* % protection calculated from number of survivors/number of mice given maximum dose of serum 0·04 ml. *IP/IC* or 0·02 ml. *IN Mix*.

† Serum P15 absorbed with cell-free haemagglutinin until haemagglutinin neutralizing titre reduced to zero.

Keogh and North (1948) had claimed that the antihaemagglutinin titres of antisera could be correlated with their protective potency. It appears that Keogh and North used sera in which the protective potency and the antihaemagglutinin happened to run parallel; they did not carry out any absorption tests.

There was no association between the protective antibodies and the antihaemagglutinin titres in a series of eight sera. (Table 8.)

PROTECTIVE POTENCY OF ANTISERA AGAINST OTHER *Bordetella* SPP.

The relative protective potencies of antisera to smooth and rough *B. pertussis*, *B. parapretussis* and *B. bronchisepticus* are shown in Table 2. Smooth *B. pertussis* serum is about ten times more potent than the others. This difference is reflected in both tests so that the ratios for the PD50 by the two routes is similar.

TABLE 9

COMPARISON OF ANTISERA TO *Bordetella pertussis* AND TO FRACTIONS OF *B. Pertussis*

Antigen used to immunize rabbits	*PD50 in ml. against*	
	Intracerebral challenge (IP/IC)	*Intranasal challenge (IN Mix)*
Whole cell	0·031	0·003
K1 fraction	0·044	0
Topping fraction ..	0	0·0052

0 = no protection at highest dose of serum used in titration, 0·4 ml. in *IP/IC* and 0·02 in *IN Mix*.

PROPERTIES OF THE TWO PROTECTIVE ANTIGENS

In the preparation of these rabbit antisera, two factors have to be taken into account; (*a*) the different rates of production of the two antibodies (Table 3), the *IN Mix* antibody being produced before the *IP/IC* antibody where both are produced, and (*b*) the purity of the antigen used to immunize the rabbits. Although several different schemes of chemical fractionation were tried, a really satisfactory method giving 100 per cent differentiation between the two antigens was not found. Most antigenic preparations were mixed.

Most of the 26 sera equally potent by *IP/IC* or *IN Mix* assay were prepared by immunizing rabbits with whole bacterial cells, killed or living. The antigens stimulating *IP/IC* and *IN Mix* antibodies fell into two fairly clearly demarcated groups (Table 9).

Antigens producing IP/IC Antibody

Antigens prepared by Cruickshank and Freeman's (1937) F68 method, by a modification of Goebel's phenol extraction (Jesaitis and Goebel, 1952), and by J. Dolby's (to be published) methanol precipitation method (K1 antigens), all gave antisera with only traces of 'intranasal' antibodies, the potency of the 'intracerebral' antibodies depending on the purity of the preparation.

Antigens producing IN Mix Antibody

Antigens prepared by acid precipitation of an alkaline extract (Topping, 1934) elicited sera predominantly 'intranasal'. Rabbits given a single subcutaneous injection of S.P.A. produce almost pure 'intranasal' antibody, though further injections rapidly bring up the titre of the 'intracerebral' antibody.

DISCUSSION

In considering passive protection tests with *Bordetella* (*Haemophilus*) *pertussis* antisera in mice it is essential to take into account the type of antisera used and the route of infection. Three antibodies active *in vivo* are known—(*a*) antitoxin; (*b*) intranasal antibodies *IN Mix*; (*c*) intracerebral antibodies *IP/IC*—and three routes of infection, (1) intraperitoneal, (2) intranasal, and (3) intracerebral, with rather different characteristics.

From the work of Anderson and North (1943), Evans (1944), Ospeck and Roberts (1944), Proom (1947) and others, the role of antitoxin is clear, against both large and small infecting doses given intranasally or intraperitoneally. The role of antitoxin against intracerebral challenge has not been previously reported, except for one experiment by Ospeck and Roberts (1944, Table 6). Unfortunately in this experiment a relatively enormous infecting dose of 100 million live bacteria of a toxigenic strain 33A was used; against this dose their antitoxic serum gave up to 40 per cent protection on the ninth day and their antibacterial serum no protection after three days. These results suggest that they were not dealing with a true intracerebral infection of the type initiated with a small number of organisms but with a toxic effect due to the enormous dose used, which would explain why their antibacterial serum did not work. Experiments (Table 7) in this paper show that protection against a small but lethal dose of *B. pertussis* strain, e.g. 18–323 (dose 50,000 containing *c.* 500 LD50) is independent of antitoxin content.

The failure to find an *in vitro* test for either the 'intranasal' antibody or the 'intracerebral' antibody has underlined the distinction between these and other antibodies. Of course,

this distinction had been noted by Mishulow *et al.* (1939), though her mouse protection test with an intraperitoneal challenge was really a measure of antitoxin, and by North *et al.* (1939). In spite of this, Smolens *et al.* (1947) recommend an agglutinin absorption test to replace the mouse protection test for the assay of vaccines, and MacPherson *et al.* (1952) implied that the lack of correlation between the concentration of agglutinin N and the protective power of the sera as measured by mouse protection tests was due to the difficulties of the nitrogen determinations and not to the fact that she was measuring two different antibodies. In most smooth strains of *B. pertussis* such as are used for vaccine production the three antigens—agglutinogen, 'intranasal' protective and 'intracerebral' protective—are found together in close association, so that Evans and Perkins (1953, 1954) could suggest a mouse-agglutination-production test which gave a good general correlation between protection in the field and agglutinin response (M.R.C. Report, 1956). Smooth strains will elicit all three antibodies and rough strains will produce none, but this is not evidence of the identity of these antibodies, and hence the antigens and the presence and potency of one cannot be used as an accurate measure of the presence and potency of another in an unknown strain or antiserum. The evidence offered in the M.R.C. Report, (1956) that the active intracerebral mouse protection test showed a close correlation with the results in children, and the evidence in the previous paper (Standfast, 1958) that there is no correlation between the active intranasal mouse protection test and the field results, stresses the importance of the 'intracerebral' antigen and hence the 'intracerebral' antibody. The fact that one antibody can be present without the other (Table 1) indicates their importance in tests on human sera from vaccinated children or from convalescents, and indeed in all tests of human vaccines. Three papers show this: Taylor *et al.* (1956), comparing methods for assessing the antigenic response to pertussis vaccines, used a passive protection test in mice giving the serum intraperitoneally and the challenge intracerebrally; McGovern (1950), studying the response to pertussis vaccination in children, used a passive mouse protection test with an intraperitoneal challenge in mucin; and Winter (1953, 1956), also studying the response of children to pertussis vaccination and the development of antibodies in convalescent children, used a passive protection test in mice challenged intranasally. It will be difficult to evaluate these results.

In the light of the findings of North and his colleagues and Mishulow and hers in 1939, it seems surprising that many workers still continue to measure the efficacy of pertussis vaccination in human populations by the agglutinin titres, for if this is to continue or if such titres are to mean anything then the Licensing authorities should require that all vaccines for human use pass not only a mouse protection test (M.R.C. Report, 1956) but also an agglutinin production test—the two are not the same.

The separation of agglutinins and protective antibodies has been reported before. Topley in 1936 at the International Congress in London reported that he and his colleagues had obtained two fractions of *Bacterium aertrycke* (*Salmonella typhimurium*), one when injected into rabbits stimulated the production of antibodies that gave characteristic o agglutination and a significant passive protection in mice. The rather more purified second fraction, when injected into rabbits, failed to stimulate the production of detectable agglutinins, but the serum of these rabbits conferred on normal mice a passive protection of the same order as that conferred by the first serum which contained agglutinins in considerable amount.

We have separated agglutinins from protective antibodies by cross absorption tests (Tables 4 and 6), but so far have not separated *IP/IC* sera from *IN Mix* sera. Owing to a

lack of sufficient monospecific antigen preparation, we have had to depend on the chance occurrence of one or two small samples of monospecific rabbit serum (groups 2 and 3, Table 1).

The findings of the passive protection tests reported in the paper corroborate and complement the active immunization test reported in the previous paper (Standfast, 1958).

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REFERENCES

ANDERSEN, E. K. (1953). 'Serological studies on *H. pertussis*, *H. parapertussis* and *H. bronchisepticus*.' *Acta path. microbiol. scand.*, **33**, 202–24.

ANDERSON, G. and NORTH, E. A. (1943). 'The relation of pertussis endotoxin to pertussis immunity in the mouse.' *Aust. J. exp. Biol. med. Sci.*, **21**, 1–8.

BURNET, F. M. and TIMMINS, C. (1937). 'Experimental infection with *Haemophilus pertussis* in the mouse by intranasal inoculation.' *Brit. J. exp. Path.*, **18**, 83–90.

CRUICKSHANK, J. C. and FREEMAN, G. G. (1937). 'Immunising fractions isolated from *Haemophilus pertussis*.' *Lancet*, ii, 567–70.

EVANS, D. G. (1940). 'Production of pertussis antitoxin in rabbits and the neutralization of pertussis, parapertussis and bronchisepticus toxins.' *J. Path. Bact.*, **51**, 49–58.

EVANS, D. G. (1944). 'The protective properties of pertussis antisera in experimental infection.' *J. Path. Bact.*, **56**, 49–54.

EVANS, D. G. and MAITLAND, H. B. (1937). 'The preparation of the toxin of *H. pertussis*: its properties and relation to immunity.' *J. Path. Bact.*, **45**, 715–31.

EVANS, D. G. and PERKINS, F. T. (1953). 'An agglutinin-production test in the study of pertussis vaccines.' *J. Path. Bact.*, **66**, 479–88.

EVANS, D. G. and Perkins, F. T. (1954). 'An improved method for testing the ability of pertussis vaccines to produce agglutinin.' *J. Path. Bact.*, **68**, 251–7.

FISHER, S. (1950). 'The haemagglutinin of *Haemophilus pertussis*. II. Observations on the structure of the haemagglutinating complex of culture supernatants.' *Aust. J. exp. Biol. med. Sci.*, **28**, 509–16.

FLOSDORF, E. W., BONDI, A. and DOZOIS, T. F. (1941). 'Studies with *H. pertussis*. VI. Antigenicity of the toxins and the relation to other cellular components from the several phases.' *J. Immunol.*, **42**, 133–48.

HORTON, J. M. and STANDFAST, A. F. B. (1953). 'The separation of the protective and agglutinating antibodies of *Haemophilus pertussis* (phase 1) rabbit antisera.' *Atti VI Congr. int. Microbiol.*, **2**, 335.

HUGHES, D. E. (1951). 'A press for disrupting bacteria and other micro-organisms.' *Brit. J. exp. Path.*, **32**, 97–109.

JESAITIS, M. A. and GOEBEL, W. F. (1952). 'The chemical and antiviral properties of the somatic antigen of phase II *Shigella sonnei*.' *J. exp. Med.*, **96**, 409–24.

KASUGA, T., NAKASE, Y., UKISHIMA, K. and TAKATSU, K. (1953). 'Studies on *Haemophilus pertussis*. Part 1. Antigen structure of *H. pertussis* and its phases.' *Kitasato Arch.*, **26**, 121–33.

KEOGH, E. V. and NORTH, E. A. (1948). 'The haemagglutinin of *Haemophilus pertussis*. I. Haemagglutinin as a protective antigen in experimental murine pertussis.' *Aust. J. exp. Biol. med. Sci.*, **26**, 315–22.

MCGOVERN, J. P. (1950). 'Passive intraperitoneal mouse protection test in a study of immune response to *H. pertussis* vaccination.' *Pediatrics*, **5**, 38–44.

MACPHERSON, C. F. C., MAURER, P. H., ALEXANDER, H. E. and REDMAN, W. (1952). 'A method for the quantitative measurement of agglutinin nitrogen in antisera to *Haemophilus pertussis*, phase I.' *Canad. J. med. Sci.*, **30**, 284–93.

MASRY, F. L. G. (1952). 'Production, extraction and purification of the haemagglutinin of *Haemophilus pertussis*.' *J. gen. Microbiol.*, **7**, 201–10.

Medical Research Council (1956). 'Report on vaccination against whooping cough.' *Brit. med. J.*, **2**, 454–62.

MISHULOW, L., KLEIN, I. F., LISS, M. M. and LEIFER, L. (1939). 'Protection of mice against *H. pertussis* by serum. Comparison of protection with agglutination.' *J. Immunol.*, **37**, 17–32.

NORTH, E. A., KEOGH, E. V., ANDERSON, G. and WILLIAMS, S. (1939). 'Passive immunity in experimental pertussis.' *Aust. J. exp. Biol. med. Sci.*, **17**, 275–86.

OSPECK, A. G. and ROBERTS, M. E. (1944). 'Pertussis antitoxin: its relationship to protection in actively and passively immunised mice and rabbits.' *J. infect. Dis.*, **74**, 22–31.

PILLEMER, L., BLUM, L. and LEPOW, I. H. (1954). 'Protective antigen of *Haemophilus pertussis*.' *Lancet*, i, 1257–60.

POWELL, H. M. and JAMIESON, W. A. (1937). 'Further studies on the immunology of *Hemophilus pertussis*.' *J. Immunol.*, **32**, 153–6.

PROOM, H. (1947). 'Immunological aspects of experimental *Haemophilus pertussis* infection.' *J. Path. Bact.*, **59**, 165–80.

REED, L. J. and MUENCH, H. (1938). 'A simple method of estimating fifty per cent endpoints.' *Amer. J. Hyg.*, **27**, 493–7.

SCHERP, H. W., BRADFORD, W. L. and WOLD, M. (1939). 'Protective value of immune rabbit serum and its globulin fraction against experimental murine infection with *Haemophilus pertussis*.' *Proc. Soc. exp. Biol. N.Y.*, **42**, 172–6.

SMOLENS, J., MCLAREN, C. S., MCALEAR, D. S. and FLAVELL, E. H. (1947). 'The estimation of specific antigen in strains of *Haemophilus pertussis*.' *J. Immunol.*, **57**, 361–7.

REFERENCES (*continued*)

SMOLENS, J. and MUDD, S. (1943). 'Agglutinogen of *Haemophilus pertussis*, phase I, for skin testing.' *J. Immunol.*, **47**, 155–63.

STANDFAST, A. F. B. (1958). 'The comparison between field trials and mouse protection tests against intranasal and intracerebral challenges with *Bordetella pertussis*.' *Immunology.*, **1**, 135–43.

TAYLOR, E. M., MOLONEY, P. J. and REID, D. B. W. (1956). 'Comparison of methods for assessing the antigenic response to pertussis vaccine.' *Canad. J. Microbiol.*, **2**, 94–101.

TOPLEY, W. W. C. (1936). 'Immunity reactions in relation to antigenic structure.' *Report II Congr. int. Microbiol.*, pp. 354–56.

TOPPING, L. E. (1934). 'Carbohydrate and nucleoprotein fractions isolated from Brucella group.' *J. Path. Bact.*, **39**, 665.

WINTER, J. L. (1953). 'Development of antibodies in children convalescent from whooping cough.' *Proc. Soc. exp. Biol., N.Y.*, **83**, 866–70.

WINTER, J. L. (1956). 'Studies in pertussis immunity. III. Immunization of children with live and killed vaccine.' *Proc. Soc. exp. Biol., N.Y.*, **92**, 832–5.

WOOD, M. L. (1940). 'A filtrable toxic substance in broth cultures of *B. pertussis*.' *J. Immunol.*, **39**, 25–42.