THE HISTAMINE-SENSITIZING PROPERTY OF *HAEMOPHILUS PERTUSSIS*

By H. B. MAITLAND, R. KOHN and A. D. MACDONALD

Bacteriological and Pharmacological Departments of the University of Manchester

(With 2 Figures in the Text)

Parfentjev & Goodline (1948) were the first to record that mice injected with pertussis vaccine became more susceptible to the lethal effect of histamine. Parfentjev, Goodline & Virion (1947*a–c*) had shown that pertussis vaccine and extracts of *Haemophilus pertussis* could cause anaphylactic shock in mice, which are relatively resistant to anaphylactic sensitization, and as mice are also resistant to histamine the effect of vaccine on susceptibility to histamine was tested. It was then found that 5 days after receiving vaccine they succumbed to a fraction of the dose of histamine required to kill normal mice. This phenomenon has been referred to as 'sensitization' to histamine but the term does not denote sensitization in an anaphylactic sense; there is a reduction in the normal resistance of mice to histamine and they therefore become more susceptible. For convenience of expression the term 'sensitization' will, however, be retained. The interest attaching to the study of the histamine-sensitizing factor (HSF) of *H. pertussis* is twofold; its remarkable action on a physiological mechanism concerned with resistance to histamine and its possible importance in pertussis infection and immunity.

As HSF has not been specified as an entity or differentiated from other components of *H. pertussis*, the experiments reported here were directed to analysing the phenomenon of sensitization, and characterizing the HSF. The relation of HSF to immunity has also been considered and some points relating to the mechanism of sensitization are discussed.

MATERIALS AND METHODS

Male mice from one strain, weighing 18–20 g. at the beginning of an experiment were used.

Solutions of histamine acid phosphate (BDH) were freshly prepared, sterilized at 10 lb. for 20 min., and injected intraperitoneally. Doses and results are stated as weights of this salt in mg. per kg. of body weight of the animal.

A large batch of reference vaccine (RV) was stored at 5° C. It was made from recently isolated strains grown on a blood medium, killed with formalin and preserved with merthiolate. Before use it was deposited by centrifuging, washed in saline to remove the preservative and resuspended in saline to contain 20,000 million organisms per ml.

A number of whole bacterial vaccines labelled V5–V15 were tested. The stromata protective antigen (SPA) described by Pillemer, Blum & Lepow (1954) is

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

referred to as V 17. These vaccines were prepared for the Medical Research Council's field trials and are mentioned by Evans & Perkins (1953, 1954).

The bacterial content of vaccines was estimated according to the U.S. National Institutes of Health opacity standard. For many sensitization experiments the dose was 16,000 million organisms in 0·8 ml. and this is to be understood when the size is not stated. The corresponding dose of V 17 was 0·8 ml. as it was arbitrarily considered to be the equivalent of reference vaccine.

Determination of histamine-sensitization by estimating the LD_{50} *of histamine.* Mice were injected intraperitoneally with vaccine or other preparations, and 5 days later sensitization was determined by injecting them intraperitoneally with graded doses of histamine, using eight mice per dose. Usually four doses were given but sometimes five or six, the range being adjusted to suit particular experiments. Deaths of mice were recorded after 18 hr. and included mice that were moribund. The LD_{50} was calculated by the method of Reed & Muench (1938) and expressed as mg. per kg.

Production of rabbit antiserum against the histamine-sensitizing factor. Intravenous injections of vaccine were given on three consecutive days of each week for 3 weeks and thereafter once weekly. The dose was 10,000 million organisms or 0·5 ml. of V 17.

Prevention of histamine-sensitization by antiserum. The anti-HSF action of antiserum was demonstrated by the method of passive protection. Two groups, each of twenty-four mice, were used. The mice in one group were given 0·4 ml. of serum intraperitoneally and 1 hr. later both groups were injected intraperitoneally with 16,000 million organisms of reference vaccine. After 5 days the sensitivity of both groups was determined by giving 800, 400 and 200 mg. histamine using eight mice for each dose. The effect of serum was shown by differences in the number of deaths in the two groups. The method served also to indicate the strength of a serum.

The agglutinin production test in mice. The procedure of Evans & Perkins (1954) was followed. Three groups, each of fifteen mice, were used. Each mouse was injected intraperitoneally with 0·5 ml. containing respectively for the three groups 5000, 1250 and 312·5 million organisms or 0·5 ml. of V 17 diluted $\frac{1}{2}$, $\frac{1}{8}$ and $\frac{1}{32}$. The dose was repeated after 14 days. Ten days later the mice were bled and individual sera titrated in doubling dilutions from 1/25 with a living suspension of *H. pertussis* from a 48 hr. culture on Bordet-Gengou medium, containing 30,000 million organisms per ml. Tests were incubated at 37° C. for 1 hr. and kept overnight at room temperature. Controls of the suspension and a serum of known strength were included.

Agglutination tests with rabbit sera were also done in this way.

FACTORS AFFECTING THE DEVELOPMENT AND DEMONSTRATION OF HISTAMINE SENSITIZATION

Number of mice used in determining the LD_{50}

The increase in the accuracy of LD_{50} determinations that might accrue through using larger numbers of mice was examined. Three similar experiments were made; mice were given 16,000 million organisms of reference vaccine and challenged

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

5 days later with 800, 400, 200 and 100 mg. of histamine. Each dose was given to three groups of eight, twelve and sixteen mice respectively; by combination a fourth group of thirty-six mice was obtained. The LD_{50} values were of the same order for the four different sized groups and for all three experiments (Table 1). For determining the LD_{50} eight mice have therefore been used.

Table 1. *Effect of number of mice per dose of histamine in determining the* LD_{50}

	Number of mice per dose			
	8	12	16	36*
Expt.	LD_{50} (mg. histamine)			
1	234	231	207	218
2	266	400	236	283
3	238	352	275	286

* Total of the first three columns

Reproducibility of results

Although no special attempts have been made to establish the most suitable technique for assaying the histamine-sensitizing potency of vaccines or other preparations, replicate experiments have indicated that the LD_{50} values were reasonably consistent. Results for sensitization produced by intraperitoneal injection of reference vaccine have been collected from experiments made over a period of more than 2 years: the LD_{50} figures for seventeen determinations were: 76, 120, 142, 160, 168, 168, 180, 184, 196, 200, 200, 234, 234, 238, 266, 283 and 321. The average was 198; twelve results fell within $\pm 30\%$ of this figure and three other results within $\pm 50\%$. There was no trend in the results to indicate deterioration of the vaccine during storage.

Similarly, the results from 124 control tests with normal mice showed consistent LD_{50} values which ranged from 2040 to 2680. The great majority of the values fell between 2200 and 2400, and all but one result fell within $\pm 10\%$ of the average value of 2332.

Sex and weight of mice

In comparative experiments female mice were always slightly more susceptible to histamine than males. This applied both to vaccinated and unvaccinated mice. The difference between the sexes was somewhat greater for vaccinated (up to 20%) than for unvaccinated mice (up to 10%). These figures were smaller than those given by Pittman (1951*a*).

With the strain of mice used for these experiments there was no difference in sensitization between male mice weighing 14–16, 18–22 and 25–28 g. at the time of vaccination.

Kind (1953) found that mice weighing 20–30 g. were more readily sensitized than those weighing 10–15 g. He noted a difference between strains in the development of sensitivity but did not find any difference between the sexes.

It is clear from the work of Munoz & Schuchardt (1953) that strains of mice differ markedly in the degree of sensitization which they develop in response to pertussis vaccine as well as in the effect of age on this process, and this may explain some of the differences on these points noted by various workers.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Dose of vaccine

The degree of sensitivity in relation to dose of reference vaccine is shown in Table 2. A dose of 800 million organisms produced detectable sensitization; with larger doses, up to 32,000 million, there was a progressive increase in sensitivity. Doses greater than this were not well tolerated and were apt to kill some mice. For many experiments a dose of 16,000 million was used as it caused no deaths from vaccination and produced a marked sensitivity.

Table 2. *Sensitization of mice in relation to dose of vaccine*

Dose of vaccine (millions)	LD_{50} mg. histamine: Vaccinated		Normal	
32,000	116*	100	2160	2320
16,000	168	160	2400	2160
8,000	220	200	2400	2200
4,000	520	650	2400	2400
1,600	1080	—	2360	—
800	1600	—	2400	—
240	> 1600	2320	2400	2400

* Each figure and the corresponding control represent one experiment.

It is clear from subsequent work and reports of others that the sensitizing potency of *H. pertussis* can be affected by the strain, the medium on which it is grown and the method of killing it. Pittman (1951*a*, *b*) obtained substantial sensitization with 300–1000 million killed organisms. Others have had to use much larger doses: Parfentjev & Goodline (1948) 5000 million; Halpern & Roux (1950) 50,000 million; Kind (1953) by giving 18,000 million on three consecutive days produced a high degree of sensitivity, a dose of 50 mg. histamine per kg. being fatal to all mice. The method used for estimating the bacterial content of a suspension has not always been stated, and may account in part for some of the differences reported and the strain of mice may also be a factor.

Onset and duration of sensitivity

After a dose of 16,000 million organisms of reference vaccine sensitivity was not apparent after 24 hr. but in 3–4 days it reached a maximum which was maintained for about 2 weeks and then gradually disappeared during the next 2–3 weeks (Table 3).

In another experiment there was a considerable degree of sensitivity after 48 hr. Mice were challenged with 400 mg./kg. histamine at intervals after receiving vaccine. None died at once or after 24 hr.; 19 % died after 48 hr.; 50 % after 3 days and 63 % after 5 and 10 days.

Pittman (1951*b*) showed that sensitization developed more slowly after intranasal infection than after an injection of vaccine, reaching its height in 10–14 days, and lasting longer.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Table 3. *Time of onset and duration of sensitivity in mice after* 16,000 *million organisms of reference vaccine intraperitoneally*

Days after vaccine	LD_{50} (mg. of histamine)	
	Vaccinated	Normal
1	>1600	2400
3	180	2200
4	80	2200
5	184	2320
9	56	2200
18	112	2160
27	950	2320
30	1900	2540
36	2400 >1600	2540 2360

Route of injection

There was little difference in the sensitivity produced when vaccine was given by intraperitoneal, intramuscular or subcutaneous routes, but intravenous injection was more effective. The lowest LD_{50} was obtained by giving both vaccine and histamine intravenously. Vaccine given by mouth produced only a little sensitivity; double the usual dose gave LD_{50} readings of 1050 and 1300 mg./kg. histamine compared with 2350 and 2400 mg./kg. for the controls.

Sensitivity following intranasal infection has been mentioned; Pittman (1951*b*) also noted that vaccinated mice which survived intracerebral challenge with living *H. pertussis* were more sensitive than mice which had been vaccinated only.

Rats and guinea-pigs

Rats could be sensitized similarly to mice. If a rat of about 200 g. was given intraperitoneally 10 times the dose of vaccine given to a 20 g. mouse (160,000 million organisms) and tested 5 days later with histamine, also given intraperitoneally, the LD_{50} was about 10 times that for the mice. Thus weight for weight rats and mice were similar with regard to sensitivity.

Guinea-pigs, on the other hand, which normally are about 200-fold more susceptible to histamine, weight for weight, than rats and mice did not show any increase in susceptibility after receiving proportionate doses of vaccine or even larger doses. Thus it would appear that the 'sensitization' of rats and mice to histamine is due to the removal or counteracting of some normal mechanism which protects them from the lethal effect of histamine. Guinea-pigs appear not to possess this mechanism and hence pertussis vaccine has no effect on their reaction to histamine.

PROPERTIES OF THE HISTAMINE-SENSITIZING FACTOR (HSF)

A number of the properties of HSF have been determined with a view to its identification. It appears, however, to differ from any of the known main components of *H. pertussis*.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Species specificity of HSF

The histamine-sensitizing action of the haemophilus species appears to be limited to *H. pertussis.*

H. parapertussis did not sensitize. Thirteen strains were tested; eight had been freeze-dried shortly after isolation and five were freshly isolated strains. All were grown on Bordet-Gengou medium suspended in saline and killed at 56° C. for 30 min. The dose was 16,000 million organisms.

Three strains of *H. bronchisepticus* failed to sensitize. Strain 1 was avirulent, but strains 2 and 3 were virulent for guinea-pigs. Strains 1 and 2 were grown on blood agar, Cohen & Wheeler (1946) solidified medium and nutrient agar; they were suspended in saline, killed with 0·5 % formalin overnight at 4° C., and washed to remove the formalin. Strain 3 was grown on blood agar, killed similarly with formalin or by heating at 60° C. for 30 min. The dose of each of the eight vaccines was 32,000 million organisms.

Two strains of *H. influenzae* did not sensitize. They were capsulated meningeal strains, grown on a blood extract medium and chocolate agar and killed at 60° C. for 30 min., or with formalin as described above. Doses of 16,000 and 40,000 million organisms were given.

Table 4. *Effect of heat on the histamine sensitizing potency of* Haemophilus pertussis *vaccine*

Control unheated	Vaccine heated for 40° C., 1 hr.	60° C., 1 hr.	70° C., 1 hr.	80° C., ½ hr.	100° C., ½ hr.
	LD$_{50}$ mg. histamine 5 days after 16,000 million vaccine				
200	256	300	520	2360	2360
200	280	240	372	> 1600	—
—	—	200	520	> 1600	—
—	—	200	—	—	—

The failure of phase IV *H. pertussis* to sensitize has been reported by Halpern & Roux (1950) and Kind (1953). Halpern & Roux (1950) found that pneumococcus and typhoid vaccines and Parfentjev & Goodline (1948) that typhoid vaccine and horse serum failed to sensitize.

Malkiel & Hargis (1952) reported that *Brucella abortus* sensitized significantly but less than *H. pertussis* and that a number of other gram-negative bacteria did not sensitize.

Heat lability of HSF

The effect of heat on the histamine-sensitizing potency of reference vaccine is shown in Table 4. Heating for 1 hr. at 40° C. or 60° C. had no significant effect; at 70° C. there was slight deterioration; at 80° C. for ½ hr. the HSF was destroyed. Vaccines killed by heating at 56° C. sensitized satisfactorily.

Lability of HSF when bacteria were disrupted by shaking or grinding

HSF was rapidly destroyed when bacteria were shaken with glass beads or ground in a dried state in order to disintegrate them.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Shaking. In attempting to extract HSF by shaking, 15 ml. of reference vaccine was put into a 35 mm. square-based glass bottle holding 130 ml., adding 15 g. of glass beads about 100μ in diameter, and subjecting it to 620 double excursions per minute through a distance of 35 mm. At intervals the amount of bacterial destruction was noted and the histamine-sensitizing potency determined. With vaccine before shaking the LD_{50} was 196 mg.; after 30 min. shaking bacterial destruction was marked and the LD_{50} 540 mg. (two experiments); after 2 hr. destruction was almost complete and the LD_{50} 1600 and >1600 mg. (two experiments). It is perhaps not surprising that such treatment rapidly destroyed HSF, as shaking is known to denature proteins, to inactivate enzymes and toxins and to kill bacteriophage and virus.

Grinding. Reference vaccine was deposited by centrifuging, resuspended in a small amount of water and freeze-dried; it was then rubbed into a smooth paste with an agate pestle and mortar, made into a suspension and the equivalent of 16,000 million organisms given to mice. This material sensitized them, the LD_{50} being 154 mg. compared with 76 mg. for unaltered vaccine. Thus drying from the frozen state had little or no effect on the HSF. When the dried organisms were lightly ground with an agate pestle and mortar in order to break them up the HSF was soon destroyed. After grinding, the powder was made into a suspension and mice vaccinated with the equivalent of 16,000 million organisms. The LD_{50} after grinding for 5 min. was increased from 76 to 500 mg.; after 15 min. to 1380 mg.; and after 30 min., the mortar being kept cold in a salt and ice mixture, to 1500, 2200 and 2500 mg. in three separate experiments.

It is difficult to understand why grinding should destroy HSF as the thermolabile toxin can be extracted by this method (Evans & Maitland, 1937) and this toxin is more susceptible to heat than HSF.

Release of HSF from cells by autolysis and by sonic oscillation

Bacteria-free supernatant liquid from an old and partly autolysed vaccine was found to have definite sensitizing activity; a dose of 0·8 ml. (a volume equivalent to 16,000 million organisms of the original suspension) sensitized so that the LD_{50} of histamine was 850 mg.

Pillemer, *et al.* (1954) described a method for breaking up *H. pertussis* by sonic oscillation at a low temperature which preserves the HSF. A small fraction of the disintegrated organisms was adsorbed on red cell stromata, the adsorbed material representing probably less than 1 % of the nitrogenous material in the suspension. This preparation (stromata-protective-antigen or SPA) used as a vaccine is known to protect mice against intracerebral challenge. It also sensitizes them to histamine. Using the SPA vaccine V 17 it was found that a dose of 0·8 ml. sensitized mice so that the LD_{50} of histamine was 120 and 140 mg. in separate tests, thus sensitizing as effectively as potent vaccine containing 20,000 million of whole killed organisms per ml. (Table 5).

The heat lability of HSF in V 17 was similar to that of a whole bacterial vaccine; heating at 70° C. for 1 hr. had little appreciable effect, whereas 80° C. for $\frac{1}{2}$ hr. abolished sensitizing potency.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Table 5. *Pertussis vaccines tested in mice for histamine sensitization and agglutinin production*

Vaccine	LD_{50} (mg. histamine)	Agglutinin response
V5	>1600,* >1600	+
V6	>1600, >1600	+
V7	>1600, >1600	+
V8	500, 550	+ + +
V10	1277, >1600	+ +
V11	<100, 33	+ + + +
V12	324, 400	+ + + +
V14	923	+ + + +
V15	724	+ + + +
V17	119, 140	+
RV	198†	+ + + +

* Each figure represents a separate test.
† Average of 17 tests with reference vaccines.

Antigenicity of HSF

Preparation of anti-HSF serum in rabbits. Immune sera prepared by inoculating rabbits (see section on methods) with sensitizing preparations of *H. pertussis*, including reference vaccine, organisms killed at 56° C. and the SPA vaccine V 17, possessed, along with other antibodies, the property of preventing the development of sensitization to histamine. This was a specific effect of antibody against HSF (anti-HSF) and was not shown by normal serum. Anti-HSF developed relatively slowly during immunization (Figs. 1, 2). Preparations of *H. pertussis* in which the HSF had been inactivated by heat, shaking or grinding did not produce anti-HSF in rabbits.

Neutralization of HSF by antiserum. The anti-HSF property of serum was demonstrated by passive protection in mice (see section on methods). In one experiment, as an example, 0·4 ml. antiserum prevented sensitization by reference vaccine so that no mice died after receiving 800 mg./kg. histamine whereas 200 mg./kg. killed about half the controls which had vaccine only. The effect of 0·2 ml. antiserum was distinct though weaker and 0·1 ml. had little if any effect. Antiserum prepared against reference vaccine prevented sensitization by V 17. Quantitatively the effect of the serum against 0·8 ml. of V 17 and 16,000 million organisms of reference vaccine was similar.

Route of administration of serum and vaccine. Serum was equally effective in preventing sensitization whether given by the intraperitoneal, intravenous or subcutaneous routes. Similarly, it was effective when reference vaccine was given by any of these routes. Thus in preventing the development of sensitization the effect of antiserum did not depend upon its action on bacteria within the peritoneal cavity.

Action of anti-HSF in vitro. That anti-HSF combined with bacteria was shown by *in vitro* experiments. Standard vaccine was deposited in the centrifuge and to the deposit 0·4 ml. anti-pertussis rabbit serum was added to every 16,000 million organisms. After incubation at 37° C. for 1 hr. and further periods at room temperature the bacteria were again deposited in the centrifuge; the serum was

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

removed and tested for anti-HSF and the deposit, made up to the original volume of the vaccine, was tested in mice for its ability to induce sensitization.

After contact for 1 hr. at 37° C. and overnight at room temperature, the bacteria had lost most of their capacity to sensitize and the serum most of its anti-HSF. This preliminary observation thus indicated that anti-HSF combined with HSF *in vitro* and thereby prevented HSF from exerting its sensitizing effect. A similar combination is the rational explanation for the action of anti-HSF *in vivo*.

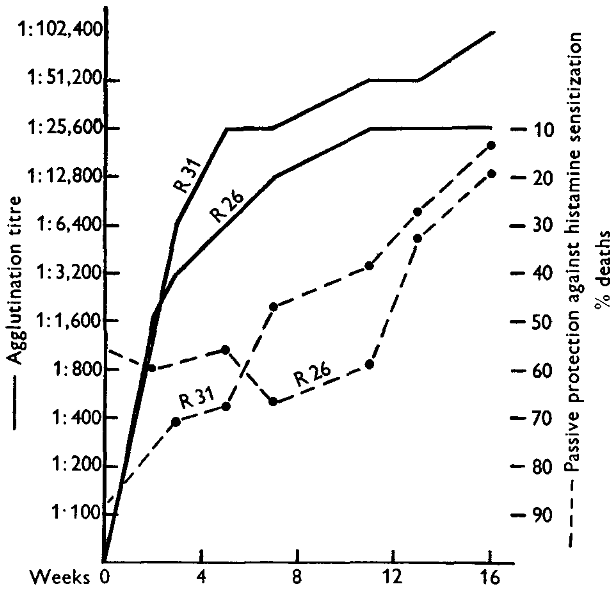

Fig. 1. Antibody production in rabbits nos. 26 and 31 inoculated with reference vaccine.

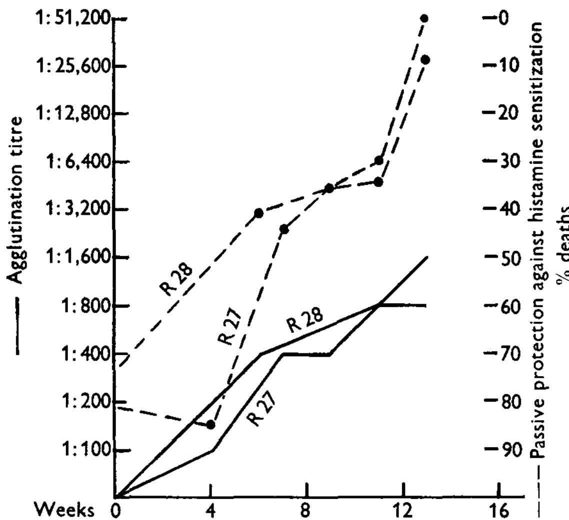

Fig. 2. Antibody production in rabbits nos. 27 and 28 inoculated with V 17.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Time relationships in passive protection. Antiserum was fully effective in preventing sensitization when given 48 hr. before reference vaccine; no deaths were caused by 800 mg./kg. of histamine, whereas 80 % of the controls died. Serum given 96 hr. before vaccine had somewhat less effect, but it was still markedly protective.

Serum prevented sensitization when given after the vaccine. Up to 24 hr. it was almost completely successful; only a small degree of sensitization developed. At 96 hr. after the vaccine the serum did not affect the state of sensitivity which was by that time well established. It appeared therefore that antiserum was able largely to overtake the sensitizing action of bacteria for at least 24 hr. after their injection.

Attempts to transfer histamine sensitivity passively in mice. Mice were given 16,000 million organisms of standard vaccine intraperitoneally and 5 days later were bled out. The serum obtained was given to mice, 0·4 ml. intraperitoneally, and after 24 and 96 hr. separate lots of mice were tested with histamine. None were killed by 800 mg./kg. Thus serum from mice at the height of their sensitivity did not in the dose used transfer sensitivity passively.

Discussion of antigenicity. These experiments lead to the view that HSF has two properties; it causes the animal to become more susceptible to histamine and it stimulates the production of antibody. Although the two properties appear together and are inactivated by the same procedures, there is no evidence anywhere in these investigations that histamine-sensitization depends upon antibody production.

Anti-HSF nevertheless, by combining with HSF, prevents it from exerting its histamine-sensitizing effect. This has been shown, in preliminary experiments, to occur *in vitro* and it may be assumed to be the explanation for the inhibiting effect of the serum *in vivo*. It is of interest that the serum could be given 24 hr. after the vaccine and still prevent sensitization.

Antiserum against HSF can be envisaged therefore as acting on the agent which induces sensitization, rather than on the mechanism by which sensitization is established, and the state of sensitivity which develops is not affected by antiserum.

The serum of a sensitive mouse did not passively transfer sensitivity which accords with the view that sensitization is not an anaphylactic type of phenomenon.

DIFFERENTIATION OF HSF FROM OTHER COMPONENTS OF *HAEMOPHILUS PERTUSSIS*

HSF is a separate entity differentiated from other components of *H. pertussis* by having different properties or lacking some of the properties which they possess. It is found in *H. pertussis* but not in *H. parapertussis*; it is inactivated at 80° C. in ½ hr. but is only slightly diminished by heating at 70° C. for 1 hr.; it is antigenic and neutralized by antiserum.

Thus HSF differs from heat-labile toxin on the grounds that (*a*) toxin is inactivated at 56° C. for ½ hr.; (*b*) it is found in *H. parapertussis* and *H. bronchisepticus*, (*c*) anti-HSF was produced in rabbits with preparations of *H. pertussis* which would not (Evans & Maitland, 1937) induce the formation of antitoxin and

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

(*d*) V 17 which contained HSF was non-toxic for guinea-pigs and rabbits (Pillemer *et al.* 1954).

HSF is not the heat-stable toxin described by Flosdorf & Kimball (1940) and Flosdorf, Bondi & Dozois (1941) as this toxin resisted heating at 100° C. for 1 hr., was present in *H. parapertussis* and appeared to be only weakly antigenic. The non-toxicity of V 17 is also relevant.

HSF can be differentiated from haemagglutinin as Masry (1952) showed that purified haemagglutinin was inactivated at 60° C. in a few minutes. It deteriorated rapidly on storage, whereas HSF was active after storage for long periods. Further, haemagglutinin is found in *H. parapertussis* and *H. bronchisepticus*. Pillemer *et al.* (1954) produced SPA (containing HSF) from strains which had little or no haemagglutinin.

There is evidence that HSF is not capsular material. Evans & Adams (1952) showed that the capsular material of *H. pertussis* could be removed by washing repeatedly in the centrifuge and collected by freeze-drying the washings. Using their technique, cultures in Bordet-Gengou medium were harvested in saline, killed at 56° C. for 30 min. and diluted to contain 20,000 million organisms per ml. The suspension was deposited in the centrifuge, washed by centrifuging 3 times from an equal volume of distilled water and resuspended in distilled water. It sensitized to the same degree as did the original suspension.

Several fractions of *H. pertussis* have been described which from their recorded descriptions cannot be said either to contain or not to contain HSF, and which have not been tested directly as histamine-sensitizing agents. These include the 'agglutinogen' described by Flosdorf & Kimball (1940) and the preparation with similar properties of Smolens & Mudd (1943); the 'F 68' fraction (Cruickshank & Freeman, 1937); a polysaccharide fraction (Eldering, 1942); and a number of extracts (Parfentjev *et al.* 1947*a*, *b*) which contained protein or nucleo-protein.

Differentiation of HSF and agglutinogen. HSF was compared with the composite agglutinogen of *H. pertussis* rather than with separate fractions such as those indicated by Lacey (1951) or Andersen (1953). The species specificity and heat lability of HSF differentiate it from agglutinogen which is common to *pertussis*, *parapertussis* and *bronchisepticus* and agglutinogen which resists 80° C. Thus the fraction with which HSF might possibly be identified is limited to specific agglutinogen labile at 80° C. The technique employed was adequate to detect such a fraction. Agglutination tests were made with living suspensions and sera were from rabbits or mice inoculated with organisms killed at 56° C. or by formalin or with the stromata-adsorbed fraction V 17. The evidence on which HSF can be differentiated from agglutinogen is as follows:

(*a*) Although the ability, or inability, of different preparations of *H. pertussis* to induce sensitization usually coincided with their capacity to stimulate agglutinin production in mice, this was not always the case. Reference vaccine unheated and heated at 60° C. or 70° C. sensitized well and produced high agglutinin titres. Vaccine shaken with beads for 2 hr. or dried and ground for ½ hr. was almost inactive in both respects. With V 17, however, there was a marked difference between histamine-sensitization and agglutinin production. Although V 17 repre-

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

sented only a small part of the components of *H. pertussis* it appeared to contain the greater part of the HSF. It was a potent histamine-sensitizer but a very poor agglutinin producer (Table 5).

(*b*) The anti-HSF property of immune rabbit sera did not always correlate with agglutinin titres. The majority of antisera which prevented sensitization by vaccine had an agglutinin titre of 1:12,000–1:50,000. But gross discrepancies in anti-HSF potency and agglutinin titre were noted with some sera. One such serum with an agglutinin titre of 1:100,000 was weak in anti-HSF, not protecting vaccinated mice against a dose of 400 mg. histamine, whereas a serum with an agglutinin titre of 1:25,000 had a good content of anti-HSF and afforded complete protection against a dose of 800 mg. histamine. Another serum which did not protect against 400 mg. histamine had an agglutinin titre of 1:12,000.

(*c*) The anti-HSF potency and the agglutinin titre of rabbit sera developed at different rates during immunization. Figs. 1 and 2 illustrate this for reference vaccine and V17. In assessing anti-HSF the percentage of deaths refer to mice that died after receiving serum followed by reference vaccine and tested 5 days later with 800, 400 and 200 mg. of histamine, using eight mice per dose. The effect of anti-HSF serum was to prevent sensitization and thus to reduce the deaths after histamine.

It will be seen from Fig. 1 that in rabbits inoculated with reference vaccine the agglutinin rose rapidly and reached a high titre in 5–7 weeks; the anti-HSF developed relatively slowly. The maximum agglutinin titre was reached in 11 weeks, but anti-HSF continued to increase up to 16 weeks when tests were discontinued. In rabbits inoculated with V17 (Fig. 2) agglutinin developed more slowly and to a much lower titre; the anti-HSF developed faster. In 13 weeks the anti-HSF titre had reached a higher level than was found after 16 weeks in rabbits inoculated with reference vaccine. These curves clearly differentiated between HSF and agglutinogen and explained how it was that the titres of anti-HSF and agglutinin in immune rabbit serum were not correlated.

THE POSSIBLE RELATIONSHIP BETWEEN HSF AND IMMUNIZING ANTIGEN

The antigen which immunizes children against whooping cough is not known with certainty, and consideration may therefore be given to the possibility that HSF itself is concerned in immunization though much further work needs to be done before the problem is settled. HSF is found in vaccines which are good immunizing agents and is not found, according to Halpern & Roux (1950) and Kind (1953) in phase IV strains. HSF can be differentiated from components which are known not to be the essential antigen concerned in immunity, particularly haemagglutinin, capsular material, heat-labile and heat-stable toxin. It can also be differentiated from agglutinogen but whether agglutinogen is the essential immunizing antigen may not be finally settled. The fact that V17 protects mice against intracerebral challenge but is a poor agglutinin producer suggests a lack of correlation. And Winter (1953) found that convalescent sera from twenty cases all contained a mouse protective antibody against intranasal infection which increased in titre

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

during the later stages of the disease, whereas only 40 % of these cases showed an increase in agglutinin. Others have noted a similar lack of correlation between mouse protective antibody and agglutinin.

Histamine-sensitization as a test for the immunizing potency of vaccines. The accepted test for assessing the potency of vaccines in immunizing children is the intracerebral challenge of mice with living organisms 14 days after they have received a dose of vaccine intraperitoneally (Report, 1953). The mechanism of this test is however not fully understood, although it is clear that *H. pertussis* can multiply in the brain and that antibody to *H. pertussis* can passively protect against intracerebral challenge. Which antibody is responsible for this effect is not clearly established.

Evans & Perkins (1953, 1954) have shown that agglutinin production in mice can be used to differentiate vaccines and suggested that this may relate to their immunizing potency, also (1955) that SPA is a poor agglutinin producer.

Vaccines can also be graded by histamine-sensitization. The results of examining a number of vaccines for their histamine-sensitizing potency are shown in Table 5; it is evident that vaccines differed markedly in content of HSF.

Differences in histamine-sensitizing potency did not always correspond with differences in agglutinin production (Table 5). The results of tests for agglutinin production with vaccines V5–V15 shown in the table indicate the conclusions of Evans & Perkins (1954), regarding the potency of these vaccines, derived from numerical data given in their papers. The results for V17 and RV (reference vaccine) were from our own experiments.

Vaccines V5, V6 and V7 were poor both in HSF and in agglutinin production. Vaccines V8, V10, V11, V12, V14, V15 and RV all sensitized and produced agglutinin but at different levels. V10 was the poorest of this group by both tests. V17 showed a striking discrepancy having a high histamine-sensitizing value and a low rating by agglutinin production.

The two tests measure different components of *H. pertussis*, the HSF and the agglutinogen. It is possible that the two components usually occur and disappear together which would account for the instances when the histamine-sensitizing test and the agglutinin-production test, applied to vaccines of whole bacteria, gave corresponding results. But it is perhaps unlikely that the two components should always be similar quantitatively, which would account for instances when the tests differed. In the preparation of V17 differential adsorption of antigens by red cell stromata might well account for its high histamine-sensitizing value and low agglutinin production.

Whether the histamine-sensitizing value of vaccine is an index of the immunizing potency, either for mice or children, is not established. But V17 which is but a small fraction of *H. pertussis* is a good protective antigen when tested in mice by the method of intracerebral challenge, as well as a good histamine sensitizer.

A comparison between the histamine-sensitizing property of vaccines and their potency in protecting mice against intracerebral challenge has been examined by Pittman (1951*c*) who found that for six commercial vaccines of whole bacteria the ratios between the sensitizing dose and protective dose were similar although the

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

absolute values differed considerably. Nevertheless, because of lack of agreement between the two properties when toxic and non-toxic vaccines were compared or when a vaccine that had been kept at 37° C. for 30 days was tested, the opinion was stated that 'the histamine-sensitizing and protecitve activities are not interrelated'. This aspect of the subject clearly requires further investigation.

SUMMARY AND CONCLUSIONS

Mice and rats which are normally resistant to histamine become more susceptible to its lethal action after an injection of *H. pertussis*. This so-called sensitization to histamine is not an anaphylactic phenomenon. It is due to the action of a component of *H. pertussis*, the histamine-sensitizing factor (HSF), which in some unknown way overcomes the physiological mechanism in rats and mice that makes them more resistant than other species to histamine. Guinea-pigs which appear not to possess this mechanism and are about 200-fold more susceptible, weight for weight, than rats and mice, were not made more sensitive by *H. pertussis*.

After a stated dose of vaccine, sensitization was detectable in 48 hr., reached a maximum in 3–4 days, remained at this level for about 2 weeks and gradually disappeared. The effects of dosage, route of injection, and weight and sex of mice have been examined.

The HSF was found in strains of *H. pertussis*; it was not found in *H. parapertussis*, *H. bronchisepticus* or *H. influenzae*. It was only slightly affected by heating at 70° C. for 1 hr. but was destroyed at 80° C. in ½ hr. It was destroyed when bacteria were disintegrated by shaking with glass beads, or by grinding after being freeze-dried. It was found in the supernatant fluid of a partially autolysed vaccine.

HSF was antigenic. Antisera were prepared in rabbits. Anti-HSF combined with HSF and neutralized its histamine-sensitizing activity. Bacteria treated with antiserum *in vitro* absorbed anti-HSF and did not thereafter sensitize mice.

Antiserum protected mice passively against the sensitizing action of vaccine, presumably by combining with HSF. After sensitization had developed the sensitive state was not affected by antiserum.

Although HSF is an antigen there was no indication that histamine-sensitization was due to its antigenicity.

The HSF was differentiated from heat-labile and heat-stable toxin, haemagglutinin, capsular material and agglutinogen.

The preparation V17, which is a small fraction of the disintegrated bacteria, adsorbed on red cell stromata (Pillemer *et al.* 1954) had a high histamine-sensitizing value. Compared with whole bacterial vaccine it caused little production of agglutinin in mice; in rabbits it caused a slower and smaller production of agglutinin and a faster and greater production of anti-HSF. For this reason immune rabbit sera may have a high agglutinin titre and a low anti-HSF value or vice versa. Anti-HSF rabbit serum protected mice against sensitization by V17.

Vaccines could be graded according to their histamine-sensitizing activity. This did not always correspond to their grading by agglutinin production in mice. The

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

relation of HSF to the immunizing antigen of *H. pertussis* and the use of histamine-sensitization to indicate the immunizing potency of pertussis vaccines are discussed.

We wish to thank Glaxo Laboratories Ltd. for the supply of reference vaccine, and the Whooping Cough Immunization Committee of the Medical Research Council for vaccines and sera.

REFERENCES

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

Cohen, S. M. & Wheeler, M. W. (1946). Pertussis vaccine prepared with Phase-I cultures grown in fluid medium. *Amer. J. publ. Hlth*, **36**, 371.

Cruickshank, J. C. & Freeman, G. G. (1937). Immunizing fractions isolated from *Haemophilus pertussis*. *Lancet*, ii, 567.

Eldering, G. (1942). A study of the antigenic properties of *Haemophilus pertussis* and related organisms. II. Protection tests in mice. *Amer. J. Hyg.* **46**, 294.

Evans, D. G. & Adams, M. O. (1952). The inability of the capsular material of *Haemophilus pertussis* to produce protective antisera. *J. gen. Microbiol.* **7**, 169.

Evans, D. G. & Maitland, H. B. (1937). The preparation of the toxin of *H. pertussis*: its properties and relation to immunity. *J. Path. Bact.* **45**, 715.

Evans, D. G. & Perkins, F. T. (1953). An agglutinin-production test in the study of pertussis vaccines. *J. Path. Bact.* **66**, 479.

Evans, D. G. & Perkins, F. T. (1954). An improved method for testing the ability of pertussis vaccines to produce agglutinin. *J. Path. Bact.* **68**, 251.

Evans, D. G. & Perkins, F. T. (1955). Test for agglutinin production by stromata protective antigen (SPA) of *H. pertussis*. *J. Path. Bact.* **69**, 329.

Flosdorf, E. W., Bondi, A. & Dozois, T. F. (1941). Studies with *H. pertussis*. VI. Antigenicity of the toxins and relation to other cellular components from the several phases. *J. Immunol.* **42**, 133.

Flosdorf, E. W. & Kimball, A. C. (1940). Separation of the phase I agglutinogen of *H. pertussis* from toxic components. *J. Immunol.* **39**, 475.

Halpern, B. N. & Roux, J. (1950). Interférence entre l'immunisation par l'hémophilus pertussis et l'intoxication histaminique. *Sem. Hôp. Paris*, 26, 1806.

Kind, L. S. (1953). The altered reactivity of mice after immunization with *Haemophilus pertussis* vaccine. *J. Immunol.* **70**, 411.

Lacey, B. W. (1951). Antigenic modulation of *Haemophilus pertussis*. *J. gen. Microbiol.* **5**, xxi.

Malkiel, S. & Hargis, B. J. (1952). Anaphylactic shock in the pertussis-vaccinated mouse. *J. Allergy.* **23**, 352.

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

Munoz, J. & Schuchardt, L. F. (1953). Studies on the sensitivity of mice to histamine following injection of *Haemophilus pertussis*. I. Effect of strain and age of mice. *J. Allergy*, **24**, 330.

Parfentjev, I. A. & Goodline, M. A. (1948). Histamine shock in mice sensitized with *Haemophilus pertussis* vaccine. *J. Pharmacol.* **92**, 411.

Parfentjev, I. A., Goodline, M. A. & Virion, M. E. (1947*a*). A study of sensitivity of *Haemophilus pertussis* in laboratory animals. I. The hypersensitivity of laboratory animals to *Haemophilus pertussis*. *J. Bact.* **53**, 597.

Parfentjev, I. A., Goodline, M. A. & Virion, M. E. (1947*b*). A study of sensitivity to *Haemophilus pertussis* in laboratory animals. II. *Haemophilus pertussis* allergen and its assay on laboratory animals. *J. Bact.* **53**, 603.

Parfentjev, I. A., Goodline, M. A. & Virion, M. E. (1947*c*). A study of sensitivity to *Haemophilus pertussis* in laboratory animals. III. The formation of antibodies and the development of sensitivity in laboratory animals injected with *Haemophilus pertussis* antigens. *J. Bact.* **53**, 613.

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press

Pillemer, L., Blum, L. & Lepow, I. N. (1954). Protective antigen of *Haemophilus pertussis*. *Lancet*, i, 1257.

Pittman, M. (1951*a*). Influence of sex of mice on histamine sensitivity and protection against *Haemophilus pertussis*. *J. infect. Dis.* **89**, 296.

Pittman, M. (1951*b*). Sensitivity of mice to histamine during respiratory infection by *Haemophilus pertussis*. *Proc. Soc. exp. Biol.*, **77**, 70.

Pittman, M. (1951*c*). Comparison of the histamine-sensitizing property with the protective activity of pertussis vaccines for mice. *J. infect. Dis.* **89**, 300.

Reed, L. J. & Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Amer. J. Hyg.* **27**, 493.

Report (1953). Diphtheria and pertussis vaccination. *Technical Report Series, World Health Organization*, no. 61. Geneva.

Smolens, J. & Mudd, S. (1943). Agglutinogen of *Haemophilus pertussis*, phase I, for skin testing. Theoretical considerations and a simple method of preparation. *J. Immunol.* **47**, 155.

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

(*MS. received for publication* 31. xii. 54)

https://doi.org/10.1017/S0022172400000668 Published online by Cambridge University Press