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Rabbit nasopharyngeal colonization by *Bordetella pertussis*: the effects of immunization on clearance and on serum and nasal antibody levels

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SUMMARY

Two *Bordetella pertussis* antigen preparations, outer membrane protein (OMP) and filamentous haemagglutinin (FHA), and a standard vaccine were used to immunize rabbits, and the effects on nasopharyngeal colonization by the organism were determined. Antibodies were measured in serum and in nasal washes by ELISA before and after challenge of the rabbits with 10^6 bacteria of strain M2. Recoveries of *B. pertussis* in nasal washes were used to assess colonization, which in controls persisted for at least 65 days. Some rabbits of all the immunized groups showed enhanced clearance, but there was no correlation between the elimination of *B. pertussis* and serum antibodies to OMP, FHA, lipopolysaccharide, lymphocytosis-promoting factor or agglutinogen 3. In contrast, nasal IgA antibody to FHA showed significant inverse correlation with bacterial persistence. Such antibody was induced by the OMP preparation as well as by FHA, but to different extents depending on the immunization schedule and adjuvant used.

INTRODUCTION

Rabbits become colonized with *Bordetella pertussis* administered as a large droplet aerosol, provided they have not been infected previously with *Bordetella bronchiseptica* (Druett & MacLennan, unpublished work; Irons & MacLennan, 1979*a*). Unfortunately the colonization is asymptomatic and cannot serve as a model for whooping cough. Nevertheless, it might provide a system in which to test potential vaccine constituents and analyse protective mechanisms in the upper respiratory tract, the principal site of whooping cough (Linnemann, 1979). In this respect and in others (Preston, Timewell & Carter, 1980), the relevance of the mouse intracerebral challenge system is questionable. Respiratory infection of the mouse (Pittman, Furman & Wardlaw, 1980; Sato *et al.* 1980) is not wholly satisfactory as it involves the lower as well as the upper respiratory tract.

This study extends the work in the rabbit reported by Irons & MacLennan (1979*a*), but in contrast infection was by intranasal instillation of *B. pertussis* and

not by aerosol inhalation. Two *B. pertussis* fractions, filamentous haemagglutinin (FHA; also termed fimbrial haemagglutinin) and outer membrane protein (OMP), have been used to immunize rabbits parenterally. These fractions are believed to contain distinct protective antigens on the basis of the mouse potency assay (Robinson, Hawkins & Irons, 1981). Each was administered to rabbits in two different ways and compared with a standard whole-cell vaccine for its effect on the colonization of the nasopharynx by *B. pertussis*. Serum antibody responses to the defined antigens FHA, lipopolysaccharide (LPS), lymphocytosis-promoting factor (LPF) and agglutinogen 3, and to OMP, have been determined. Antibody to FHA has also been measured in nasal washes in an attempt to correlate protection against colonization with the local response following immunization.

METHODS

B. pertussis *strains*

Strains were obtained from the following sources: strain M2 (1, 3 serotype) from Dr N. Preston, Department of Bacteriology, University of Manchester; strain Tohama (1,2) from Dr C. R. Manclark, Bureau of Biologics, Food and Drug Administration, Bethesda, Maryland: strain B106 (1, 3) from the Lister Institute (Bronne-Shanbury & Dolby, 1976); strain wellcome 28 (1,2,3) from Dr P. Novotny, Wellcome Research Laboratories, Beckenham. All strains were maintained as freeze-dried stocks and recovered by growth on charcoal agar (Oxoid) containing 10% defibrinated horse blood and 0·2 u./ml penicillin.

Preparation of bacteria

(*a*) Strain Tohama for absorption of sera was grown in shake culture for 48 h at 36 °C in the medium of Sato, Arai and Suzuki (1974), centrifuged and treated overnight with 0·1% (v/v) formaldehyde in water.

(*b*) For bacterial agglutination stock suspensions were made from 48 h shake cultures in the modified medium of Cohen & Wheeler (Lane, 1970). The bacteria were killed with 0·1% (v/v) formaldehyde, washed with water and resuspended in PBS with 0·02% (w/v) merthiolate and 0·01% formaldehyde as preservatives.

(*c*) For infection of rabbits strain M2 was grown on charcoal agar plates at 36 °C for 3 days and suspended in saline.

Preparation of antigens

All antigens were prepared from the Wellcome 28 strain. FHA was prepared from static liquid culture supernatants as described previously (Irons & MacLennan, 1979*a*; Robinson, Hawkins and Irons, 1981).

OMP was extracted from cell envelopes with Empigen BB as described by Robinson & Manchee (1979), except that the isolated envelopes were not heated to 56 °C. The preparation used for immunization contained LPS. This was removed by gel exclusion chromatography on Sephadex G-75 in the presence of sodium deoxycholate before the preparation was used in ELISA tests.

LPF was prepared by affinity chromatography on haptoglobin-Sepharose 4B (Irons & MacLennan, 1979*b*).

LPS was extracted from bacteria by the phenol method as applied to *B. pertussis* by MacLennan (1960).

Antibodies and conjugates

Secretory IgA was prepared from rabbit milk by the method of Cebra & Robbins (1966) except that gel filtration was done on Ultrogel ACA 34 (LKB). The purified IgA reacted with class-specific anti-IgA in immunoelectrophoresis, but not with anti-IgG (Miles Laboratories, Ltd). A sheep was hyperimmunized with the rabbit IgA, and IgG prepared from its serum by DEAE-cellulose chromatography. This sheep IgG was used either as general anti-rabbit Ig(SAR) or was made class-specific for rabbit IgA (SARA) by affinity purification on rabbit IgG coupled to cyanogen bromide-activated Sepharose 4B.

Horseradish peroxidase (HRPO: Sigma Type VI) was coupled to SAR and SARA by the technique of Nakane & Kawaoi (1974) using sodium periodate at a final concentration of 25 mM. Conjugates were separated from free enzyme on Sephadex G-100 and stored at −20 °C. The ratios of HRPO:IgG were 3·5:1 and 3:1 for HRPO-SAR and HRPO-SARA, respectively.

Antibody assays

ELISA: an indirect microplate assay was used to measure antibodies to FHA, OMP, LPS and LPF. PBS containing 0·1 % Tween 20 as a blocking agent was used as diluent and wash solution. Washing was carried out using a modified cell harvester (Mash II, Dynatech). Flat-bottomed 96-well plates (Dynatech M 129A) were coated overnight at room temperature with antigen (10 μg/ml) in 0·05 M carbonate buffer pH 9·6. Serial dilutions of sera or nasal washes were made in plates (Dynatech M 29AR) and transferred to the corresponding wells of washed antigen-coated plates which were covered and shaken for 2 h at room temperature. Plates were then washed, appropriate conjugate added, and shaking continued for 2 h, after which the plates were washed and freshly prepared substrate added. The colour developed after 20 min shaking was measured using a Titertek Multiskan photometer with 450 nm filter. The substrate was 5-aminosalicylic acid in water (0·8 mg/ml) adjusted to pH 6, to which H_2O_2 was added to 0·007 %.

A working standard serum, titrated several times within each assay, provided a dose–response curve with which titrations were compared. Results were expressed as the reciprocal of the $\log_2$ dilution, obtained by interpolation, giving 50 % of the maximum absorbance reading of the standard above that given by a conjugate-only blank. The volumes of nasal washes were related to an arbitrary set volume (2 ml) and titres corrected accordingly.

Bacterial agglutination: purified agglutinogen was not available for an ELISA, therefore serotype 3 agglutinins were measured in a microplate bacterial agglutination assay using strain B 106 (serotype 1, 3) cells. Sera were tested after being absorbed twice with *B. pertussis* of 1, 2 serotype, and titres were expressed as the reciprocal of the highest $\log_2$ dilution giving definite agglutination.

Infection of rabbits

New Zealand rabbits of either sex and of approximately 2·5 kg body weight were obtained from CDE Farm (Porton Down, Salisbury). All the rabbits were free of *B. bronchiseptica*, shown by per-nasal swabbing, and had no detectable antibody to *B. bronchiseptica* LPS as shown by passive haemagglutination tests using goose erythrocytes coated with alkali-treated *B. bronchiseptica* LPS.

Strain M2 *B. pertussis* organisms were instilled in a volume of 0·1 ml into each nostril. Viable counts on the inoculum, which were approximately equal to the total counts, were estimated using a standard Miles and Misra technique. The infection was monitored by swabbing both sides of the nasopharynx or by nasal washing at intervals. Swabs consisted of absorbent cotton wool wound tightly around the roughened ends of lengths of fine brass wire. Washing involved injection of 2·5 ml saline into each nostril via a small-diameter plastic tube inserted to a depth of 30 mm, and collection by gravity. A microscopic check was made for blood contamination. *B. pertussis* from swabs or washes was recognized by morphology, colony form and by slide agglutination with pertussis-specific antiserum (Wellcome).

Immunization experiment

Six groups of rabbits were studied. I, nine control rabbits. II, five rabbits injected with increasing doses of Standard vaccine without adjuvant (British reference preparation of Pertussis vaccine; National Institute for Biological Standards and Control, Holly Hill, London). The doses, given in alternate flanks, were 1 international unit on day 0, 5 units on days 4 and 8, and 10 units on days 11, 15 and 18. The dose of test material injected into animals of the remaining groups was distributed equally between four sites, two in the hind limbs (i.m.), and two on the neck (s.c.). III, five rabbits injected with 1 ml doses of OMP (100 μg/ml) on day 0 and again on day 18. IV, five rabbits given one dose of 100 μg OMP absorbed onto Alhydrogel (Superfos, Denmark). V, six rabbits injected with 112 μg FHA in 1 ml of emulsion in incomplete Freund's adjuvant (IFA). VI, six rabbits given 107 μg FHA absorbed onto Alhydrogel.

The rabbits were bled on day 32 or 33 and nasal washes performed on days 33–35. On day 36 they were challenged intranasally with 10^6 M2 strain organisms.

RESULTS

Infection of rabbits with B. pertussis

An infectious dose 50% value of approximately 10^4 viable organisms was determined from two experiments in which groups of 6 rabbits were given doses ranging from 10^3 to 10^6 bacteria of strain M2. Infection of the nasopharynx involved proliferation of the organism and persisted for at least 65 days after administration of 10^6 bacteria, the dose selected for challenge in the immunization experiment. Tissue sampling revealed *B. pertussis* associated with the nasal mucosa, tonsils and lungs and, sporadically at low levels, the cervical lymph nodes.

Table 1. *The effect of immunization on colonization of the rabbit nasopharynx with* B. pertussis *after intranasal challenge**

Group and immunization	Days post challenge: 13 (swab)	18 (wash)	28 (wash)	52 (wash)
I Controls	9/9	9/9 8·3 ± 1·1	8/8† 6·8 ± 1·0	8/8 4·5 ± 0·8
II Standard vaccine, six increasing doses	5/5	5/5 3·7 ± 0·7	5/5 4·0 ± 0·5	0/5 0
III OMP, days 0 and 18	5/5	5/5 7·2 ± 1·7	4/4† 5·6 ± 2·2	0/5 0
IV OMP in Alhydrogel, day 0	5/5	5/5 4·7 ± 1·0	3/5 2·6 ± 2·4	1/5 0·7
V FHA in incomplete adjuvant, day 0	6/6	6/6 6·6 ± 0·6	6/6 5·5 ± 1·1	2/6 1·3
VI FHA in Alhydrogel, day 0	6/6	3/6 2·6 ± 2·7	3/6 1·7 ± 1·9	1/6 0·3

* Results expressed as the proportion of animals from which *B. pertussis* was recovered, together with the group means of $\log_{10}$ colony forming units recovered ± S.D., following challenge with 10^6 organisms.

† Two rabbits died of enteritis.

Histological examination of these tissues did not show any pathological changes associated with the infection.

The effect of immunization on colonization

The mean recoveries of *B. pertussis* from the nasopharynx of control and immunized rabbits are given in Table 1.

Initially, 13 days after challenge, the animals were swabbed per-nasally, care being taken to minimize local trauma, and all had *B. pertussis* in one or both sides of the nasopharynx.

The capture of *B. pertussis* organisms on per-nasal swabs is non-quantitative. Therefore nasal washes were taken on days 18, 28 and 52 post-challenge to obtain an estimate of the relative numbers of organisms in different animals, although it was recognized that washing might redistribute or remove a large proportion of the bacterial load. On day 18 post-challenge all groups, apart from III, gave mean recoveries of *B. pertussis* significantly lower than the control group, evidence of some protection. However only group VI contained rabbits with no detectable infection. After a further 10 days the organisms recoverable by nasal washing had fallen in all groups apart from group II, and two animals in group IV yielded no *B. pertussis*. On day 52 post-challenge no organism was recovered from groups II and III, whereas one animal from each of groups IV and VI, and two from group V still carried *B. pertussis*. Two rabbits died of enteritis during the course of the experiments.

Serum antibody responses

Serum antibodies to four nominally different antigens, FHA, OMP, LPS and LPF were titrated in ELISA. Although the same HRPO-SAR conjugate was used throughout, titres for the different antibodies in any serum were not directly comparable. Titration curves were sigmoidal and the 50% end-point was selected as the most reliable. Sera which failed to yield 50% of the maximum OD obtained in a test, even when they yielded low colour over a range of dilutions, were considered as negative for the major antigenic specificities represented on the plates. This was encountered with OMP rather than FHA, the former being a more heterogeneous preparation, with minor antigens giving rise to the low coloration.

The bacterial agglutination test was shown to measure only type 3 agglutinins since, after absorption with organisms of Tohama strain, none of the sera agglutinated bacteria of serotype 1, 2.

Table 2 gives the mean antibody responses 32 days after the first immunizing dose and also 25 days later, i.e. 21 days after challenge with live *B. pertussis*, strain M2 (serotype 1,3). Group II, given six injections of a whole cell vaccine, developed good antibody titres before challenge to all the antigens tested. Rabbits in group IV, immunized with OMP in Alhydrogel, responded similarly, but gave a slightly higher titre to LPS and lower titres to LPF and agglutinogen 3. The OMP preparation obviously contained immunogenic amounts of all the antigens examined, but in the absence of adjuvant much poorer antibody responses were obtained (group III). Incomplete adjuvant was slightly more efficient than Alhydrogel in those rabbits (groups V and VI) immunized with FHA, not only for this antigen, but also for the LPS which the immunizing dose of FHA must have contained. Most importantly, no antibody to LPF was detected in these groups and only 3/12 rabbits had any type 3 agglutinins. Overall, by 21 days after challenge, the responses of immunized rabbits were relatively uniform and greater (with the exception of type 3 agglutinin in group V) than those of control animals responding to infection. Comparison of the serum antibody titres of individual rabbits (excluding controls) prior to challenge, with the corresponding bacterial recoveries 18 and 28 days after challenge, showed no significant inverse correlation. Twenty-one days after challenge antibody titres of individuals (including controls) showed significant inverse correlation ($P < 0{\cdot}02$) with bacterial recoveries 18 and 28 days post-challenge. Correlation coefficients for antibodies to OMP were $-0{\cdot}593$ and $-0{\cdot}628$, respectively, and for antibodies to FHA were $-0{\cdot}515$ and $-0{\cdot}457$, respectively, with antibodies to agglutinogen 3 and to LPS yielding intermediate values. These findings after challenge make it impossible to draw conclusions about the role of any of the individual serum antibodies in enhancing clearance of *B. pertussis*.

Antibody to FHA in nasal washes

An ELISA specific for IgA antibody was developed for tests on nasal washes taken from rabbits 33–35 days after immunization commenced (i.e. 1–3 days before challenge) and 18 days after challenge. The HRPO–SARA conjugate gave no

Table 2. *Serum antibody responses of rabbits after immunization and after challenge with* B. pertussis*

	Group and vaccination	Post-immunization†/pre-challenge					21 days post-challenge			
		Anti-F HA	Anti-OMP	Anti-LPS	Anti-LPF	Type 3 agglutinin	Anti-F HA	Anti-OMP	Anti-LPS	Type 3 agglutinin
I	Controls	< 1·0	< 1·0	< 1·0	n.t.	< 2	7·1 ± 1·8	2·1 ± 1·9 (7/9)	5·1 ± 1·7	3·5 ± 2·0
II	Standard vaccine, six increasing doses	11·5 ± 0·6	7·5 ± 0·7	6·7 ± 1·0	8·0 ± 1·5	8·9 ± 3·0	11·2 ± 1·5	8·3 ± 0·5	7·6 ± 0·8	10·6 ± 2·2
III	OMP, days 0 and 18	4·9 ± 4·4	3·4 ± 2·1 (4/5)	3·4 ± 2·1 (4/5)	1·5 (2/5)	3·2 ± 1·9 (4/5)	9·8 ± 1·7	6·7 ± 0·8	7·1 ± 1·2	8·6 ± 1·5
IV	OMP in Alhydrogel, day 0	11·0 ± 1·8	8·8 ± 1·3	9·9 ± 1·4	5·7 ± 4·0 (4/5)	4·5 ± 3·2	11·5 ± 0·5	9·1 ± 1·3	8·6 ± 1·9	9·5 ± 2·9
V	FHA in incomplete adjuvant, day 0	14·3 ± 0·6	5·7 ± 1·7	7·9 ± 0·3	< 1·3 (0/6)	0·3 (1/6)	13·6 ± 0·7	7·6 ± 1·7	9·6 ± 0·8	3·9 ± 1·8
VI	FHA in Alhydrogel, day 0	12·4 ± 0·7	4·0 ± 1·4	4·4 ± 1·5	< 1·3 (0/6)	0·7 (2/6)	12·8 ± 1·2	8·2 ± 1·4	9·0 ± 0·8	6·9 ± 1·2

* Results expressed as mean titre for each group (proportion of animals, where this was less than 100 %, with a detectable response). Type 3 agglutinin measured by bacterial agglutination: other antibodies measured by ELISA. n.t. signifies not tested.

† 32 days after the first immunizing dose.

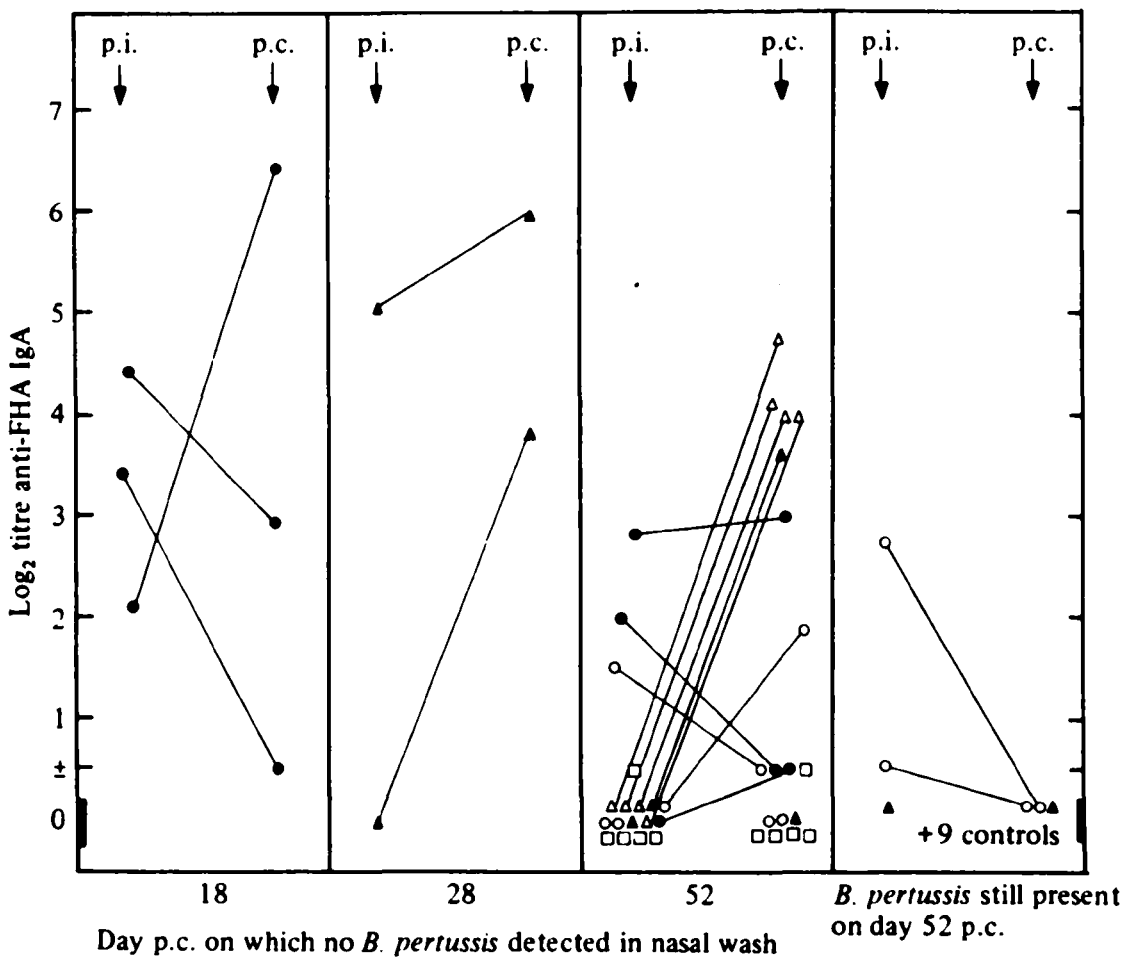

Fig. 1. Nasal anti-FHA IgA titres of individual rabbits (p.i.: post immunization: p.c.: post-challenge) related to colonization with *B. pertussis* expressed in terms of the days when no organism was recovered in nasal washes. Group II □; III △; IV ▲; V ○; VI ●.

reaction in ELISA even with the highest titre sera, confirming that all anti-IgG specificity had been absorbed and showing that there was no detectable IgA contribution to the serum antibody titres. Unfortunately IgG antibody in nasal secretions could not be assessed because too few of the washes were completely free of blood, as determined by the absence of erythrocytes, and the high levels of serum antibody made correction for contamination impossible.

Fig. 1 shows the titres of nasal wash IgA antibody to FHA measured by ELISA in relation to the period of colonization by *B. pertussis*. The titres of immunized rabbits just prior to challenge (p.i.) and 18 days after challenge (p.c.) are presented. Only 2 out of 10 rabbits with detectable nasal anti-FHA IgA on the first occasion did not show enhanced clearance compared to controls. It is interesting that these two animals no longer had this antibody 18 days after challenge, despite the local stimulus the challenge should have provided. Of three rabbits which eliminated the *B. pertussis* by day 18, and in which the local stimulus was therefore relatively short-lived, two had decreases in nasal anti-FHA whereas the other had an increase in titre.

In contrast to the serum picture, anti-FHA in nasal washes from immunized rabbits prior to challenge correlated inversely with bacterial recoveries. Thus

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coefficients of $-0{\cdot}499$ ($P < 0{\cdot}05$) and $-0{\cdot}553$ ($P < 0{\cdot}02$) were obtained for correlations of pre-challenge nasal wash anti-FHA with organisms in the washes 18 and 28 days after challenge, respectively. The corresponding coefficients for anti-FHA in washes taken 18 days post-challenge were $-0{\cdot}338$ and $-0{\cdot}405$ ($P < 0{\cdot}05$). Those animals showing traces of possible antibody (fig. 1: denoted $\pm$) were assumed negative for the purpose of these calculations.

The nasal washes taken prior to challenge were also tested for antibody to OMP but none was detected.

DISCUSSION

Intranasal instillation of *B. pertussis* in small volumes proved to be a reliable technique for infecting rabbits, and bacterial recoveries showed that the organisms proliferated in the nasopharynx. Preston, Timewell & Carter (1980) also used this technique for infecting rabbits. With an inoculum 50000-fold greater than that used as a challenge dose in the present experiment, it is not surprising that in their study infection persisted four times longer, that is for about 10 months.

All the vaccination schedules induced some degree of immunity to the colonization. It had been felt that successive nasal washings might alter the course of infection more than swabbing. However, this did not appear to happen, as recoveries of organisms from control rabbits showed the progression obtained in previous experiments using only swabs. The proportion of the total bacterial load removed by washing is not known, but the technique allowed relative numbers of organisms from individual animals to be compared. Moreover, the washes could be examined for antibody, and the most significant finding of the study is that anti-FHA IgA in nasal washes following immunization correlated well, although not completely, with the inhibition of colonization.

At present we can only speculate about the origins of this IgA antibody. As immunization was parenteral it could have been produced either locally, by antibody-forming cells seeded to the nasal mucosa from some distant site, or at such a site and then transported in the blood. Transport of oligomeric IgA by the blood followed by uptake onto cells bearing secretory component has been postulated for lacrimal, salivary and mammary glands (reviewed by Bienenstock & Befus, 1980), but it is not known whether this type of mechanism contributes to IgA in respiratory secretions. The finding that rabbits had no detectable serum IgA antibody to FHA does not rule out transport in the blood (Halsey, Johnson & Cebra, 1980).

Thomas (1975), using bacteria as antigen in a radioimmunoassay, measured anti-pertussis IgA in nasal secretions of human volunteers given vaccine either as an aerosol via the nose, or parenterally. He found, in keeping with studies in other systems (reviewed by Waldman & Ganguly, 1974), that local administration was more effective in stimulating nasal antibody. In the present study better nasal antibody responses to FHA were obtained after immunization with FHA in Alhydrogel than with FHA in incomplete adjuvant, although the latter induced higher serum titres of anti-FHA. The immunization schedules of groups II and IV

gave rise to serum anti-FHA titres of the same order as for group VI, but overall yielded poor local responses to FHA. Obviously work is needed to determine the best mode of immunization for inducing antibody in the upper respiratory tract either for prolonged periods or anamnestically. In terms of a common mucosal immune system (Bienenstock & Befus, 1980; but see Waldman & Ganguly, 1974) oral immunization would seem to be a rational approach, and there have been a number of studies with whole-cell pertussis vaccines given by this route to mice (Hof *et al.* 1976) and to humans (Stickl, Schweier & van Thiel, 1976; Maurer *et al.* 1979). Maurer *et al.* found salivary agglutinins following oral vaccination of very young babies againt *B. pertussis*, but it is not known whether such antibodies would be protective.

The finding that serum antibody levels after vaccination were unrelated to bacterial recoveries was not unexpected. Respiratory colonization of the rabbit by *B. pertussis* is asymptomatic and produces no histological lesion. Eradication of a non-invasive organism like *B. pertussis* must be mediated by mechanisms able to operate at the mucosal surface of the respiratory tract. The partial protection seen in some immunized rabbits without detectable nasal anti-FHA antibody could be due to secretory responses to one or more of a variety of pertussis surface antigens. Before this possibility could be examined, the sensitivity of the ELISA would have to be increased and antigens other than FHA more fully characterized.

Evidence from *in vivo* (Mallory & Horner, 1912; Sato *et al.* 1980) and *in vitro* (Muse, Collier & Baseman, 1977) studies with *B. pertussis* indicates that organisms tend to be associated with the cilia of respiratory epithelium. Holt (1972) postulated that prophylactic immunity to whooping cough is mediated by local antibody that can prevent adhesion of *B. pertussis*. More recently it has been suggested that FHA might be the surface antigen to which anti-attachment antibody is directed (Pittman, 1979; Sato *et al.* 1981).

Munoz, Arai & Cole (1981) proposed that any protective activity of FHA in the mouse intracerebral challenge test was due to LPF present at low levels in the FHA. Immune response to LPF, considered as a second line of defence (Pittman, 1979), is unlikely to be involved in preventing colonization in the rabbit, but it is interesting to note that the FHA preparation failed to elicit any anti-LPF response. The protection afforded by FHA to rabbits of group VI is therefore independent of LPF as antigen. Moreover, the poorer protection afforded group V animals, which received the same dose of FHA (together with any LPF present), indicates that LPF is unlikely to be exerting a marked adjuvant effect in this system. Thus the view (Preston, Timewell & Carter, 1980) that results from the mouse intracerebral challenge test can be quite misleading is further strengthened.

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