HAEMAGGLUTINATION AND ADHESION OF BORDETELLA PERTUSSIS

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Presented for the degree of Doctor of Philosophy

in the Faculty of Science, University of Glasgow

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December, 1981

Declaration

This thesis is the original work of the author.

Kathleen A. Burns

To mum, dad and Charlie

with thanks for everything

ACKNOWLEDGEMENTS

I would like to thank Dr. J.H. Freer for his help and advice throughout my time in the department.

I am also grateful to the M.R.C. for providing financial support.

Thanks are also due to other members of the department especially Mr. A. Ellis for his assistance with electron microscopy and for allowing me to clutter up his bench space. I would also like to thank Dr. R. Parton for his help in preparation of SDS-polyacrylamide gels and for many helpful discussions.

In preparation of this thesis I would like to thank Dr. Freer and Professor A.C. Wardlaw for reading this manuscript and for their valuable advice, Mrs. A. Strachan for fast and efficient typing, Mr. I. McKie and Mr. A. Ellis for their photographic services.

Finally I would like to thank my family and my husband for their support, without which this thesis would never have been completed.

SUMMARY

The object of this work was to define the role of surface structures of Bordetella pertussis, particularly the fimbrial haemagglutinin (F-HA), in the virulence of the bacteria and in their ability to adhere to the epithelium of mouse lung. Preparatory to this, it was first necessary to investigate the production of F-HA by different strains and under different cultural conditions.

In agreement with recently published work, it was shown that B. pertussis produces two haemagglutinins (HA), one of which is associated with the toxic factor pertussigen and the other with the fimbriae. The F-HA is distinguished from pertussigen in that haemagglutination (HA) caused by it is inhibited by cholesterol.

Cells grown in shaken liquid culture showed little evidence of F-HA as determined by HA tests and electron microscopy of the bacteria.

In static cultures, both the cell-associated and cell-free HA were cholesterol-sensitive, and were associated with fimbriae visible in the electron microscope.

Highly purified F-HA was obtained by passing the supernate of a 5 day static culture through CM-Sepharose CL6B, concentrating the eluted protein and adsorbing the F-HA specifically onto cholesterol-containing liposomes. This liposome adsorbed protein (LAP) was used as a vaccine in mouse protection tests, and to raise specific antiserum. The fraction left after adsorbing the F-HA onto the liposomes had a high histamine sensitising activity (HSF).

The purified F-HA (LAP) when given to mice intraperitoneally (i.p.) two weeks before intracerebral (i.c.) challenge with approximately 200 $LD_{50's}$ of live B. pertussis strain 18323 showed protective properties. The fraction with high HSF activity also protected the mice. This HSF

or pertussigen fraction was toxic at around 6μg/mouse. Antiserum raised against the LAP (F-HA) was also found to protect mice passively against i.c. and intranasal (i.n.) challenge.

An adhesion assay for measuring the ability of B. pertussis to adhere to (or be retained in) the airways within mouse lungs was developed. Adhesion was found to be rapid over the first few minutes of contact, followed by a slower rate and with a maximum level of adhesion after 30 min. C mode, phase IV and large-colony variants of B. pertussis, which were of low virulence, showed a low level of adhesion to the lungs. There was also difference between strain 77/18319 which is i.n.-virulent but not i.c.-virulent, and strain 18323 which is i.c.-virulent but less virulent i.n., in each case there was a parallelism between the adhesion levels and lung virulence. This may indicate that F-HA and adhesion are more important in lung infections than in brain infections of mice.

Although HA by fimbriae was inhibited by cholesterol, adhesion and virulence were not inhibited, as judged by failure of cholesterol in the bacterial suspension to influence the results of these tests. Also cells from 48 h shake cultures were virulent, yet were HA-negative. Therefore there does not appear to be a simple direct relationship between the presence of fimbriae, as indicated by HA, and adhesion and virulence.

In conclusion, it seems that fimbriae are involved in adhesion, perhaps by rendering the cells more hydrophobic, although the evidence is not fully conclusive. When used as an antigen, fimbriae protected mice against both i.n. and i.c. infections, and may therefore protect man. Pertussigen was found to be a very potent protective

factor against i.c. challenge, and a reliable method for detoxifying it should be developed.

It is suggested that a possible improved pertussis vaccine for human use might consist of purified F-HA mixed with detoxified pertussigen.

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B-G	Bordet Gengou agar
CAD	casamino acids diluent
cfu	colony forming units
F-HA	fimbrial haemagglutinin
HA	haemagglutinin, haemagglutination and haemagglutinating
HAI	haemagglutination inhibition
HLT	heat labile toxin
HIC	hydrophobic interaction chromatography
HSF	histamine sensitising factor
IAP	islets activating protein
i.c.	intracerebral
i.n.	intranasal
i.p.	intraperitoneal
k Bq	kilo becquerel
LAP	liposome adsorbed protein
LPF	lymphocytosis promoting factor
LPS	lipopolysaccharide
LSP	liposome supernate protein
o.u.	opacity units
PA	protective antigen
PAGE	polyacrylamide gel electrophoresis
PEI	protein eluted from ion exchange column
PB	phosphate buffer
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate

INTRODUCTION

A. Introduction to pertussis

1) A short history

According to Olson (1975) the first written description of what is now known as pertussis or whooping Cough appeared in 1578 when an epidemic occurred in Paris (Olson, 1975). No further accounts appeared for almost a century, by which time the disease had become epidemic in Europe. The word pertussis, meaning a violent cough, was first used by Sydenham in 1670. This term is now preferred by many to whooping cough since not all patients with the disease whoop (Lapin, 1943; Olson, 1975).

Around 1900, Bordet and Gengou (1906) observed coccobacilli in such abundance and purity from Bordet's infant daughter suffering from whooping cough that its association with the infection could hardly be doubted. A few years later Bordet and Gengou (1906) described a medium suitable for the cultivation of the pertussis bacillus and in a series of articles the morphology, cultural characteristics, virulence and antigenicity of the organisms and the serological reactions between different isolates were described (Bordet & Gengou, 1906, 1907, 1909; Bordet & Sleeswyck, 1910). In 1908 and 1909, Klimenko (cited by Lapin, 1943) reported that he had produced whooping cough in monkeys and puppies by inoculation of Bordetella pertussis. There are other reports of animal infections, but it was the findings of Williams (1914), who isolated pure cultures of B. pertussis from many cases of whooping cough, that confirmed the etiological significance of B. pertussis. The MacDonalds (MacDonald & MacDonald, 1933) provided direct evidence for B. pertussis as the causative agent by infecting their own children with the organisms and observing the development of typical pertussis in two

of the children. The other two, who had been previously immunised with killed bacteria, did not develop the disease.

B. parapertussis isolated by Eldering & Kendrick (1938) has been associated with the pertussis syndrome and, when implicated, the infection usually takes the form of a mild case of pertussis. Although apparently quite rare in the United Kingdom and the U.S.A., B. parapertussis contributes much more significantly to pertussis outbreaks in other parts of the world (Olson, 1975).

B. bronchiseptica is capable of causing the pertussis syndrome but very rarely does so. B. bronchiseptica is, however, the cause of a natural respiratory tract infection of rodents and other animals (Switzer, Mare & Hubbard, 1966).

There are still doubts that all cases of pertussis are necessarily caused by one of the three Bordetella species; other agents, in particular adenoviruses, have been implicated (Olson, 1975).

As noted above, there are three organisms in the genus Bordetella, B. pertussis described by Bordet & Gengou (1906) as the cause of pertussis, B. parapertussis isolated by Eldering & Kendrick (1938) also from cases of whooping cough and B. bronchiseptica isolated by Ferry (1911) from respiratory tracts of dogs suffering from distemper. B. pertussis and B. parapertussis were initially included in the genus Haemophilus while B. bronchiseptica had been included in the genus Brucella and Alcaligenes. It was Lopez in 1952 who proposed that they be collected into a new genus, Bordetella.

Members of the genus Bordetella are small, rod-shaped organisms (0.5-1.0 by 0.3-0.5 μm), arranged singly, in pairs or small groups. B. bronchiseptica is flagellate and B. pertussis, when freshly isolated, is capsulated. B. pertussis is not dependent on either V or X factors, from blood, for growth although complex medium containing

blood is usually used for primary isolation. The optimal growth temperature is 35° to 37°C, with no growth anaerobically. The colonies are smooth, dome-shaped, and glistening with an entire edge. The development of the colonies may take 48 to 72 h. B. pertussis is killed at 55°C during 30 min; it ferments no sugars, renders litmus milk slightly alkaline, forms catalase but not indole and does not reduce nitrates. Haemolysis is observed around the growth on B-G medium.

2) Course of the disease in man

Infection occurs mainly by direct contact with patients suffering from the disease. The organisms gain access to the body by inhalation of material expelled in cough spray. The patient is most infectious during the catarrhal stage (Lawson, 1933). There does not seem to be any healthy carrier, nor any animal host as far as is known. Pertussis is a localised respiratory infection of humans with specific manifestations of paroxysmal coughing, lymphocytosis and neurological symptoms (Pittman, 1970).

(i) Diagnosis. Diagnosis of the disease is often difficult as the true nature may not be evident for a fortnight or so, by which time the organisms may have become very difficult to isolate from the nasopharynx. For rapid diagnosis, fluorescent antibody can be used and according to Linnemann et al (1968), offers the same degree of sensitivity as cultural techniques.

Cultural diagnosis can be carried out by three methods:
(a) Cough plates. Bordet-Gengou (B-G) medium is coughed onto, incubated for 48 to 72 hours and examined for characteristic colonies.
(b) Postnasal or nasopharyngeal swab (Maclean, 1937) onto B-G plates with penicillin added to reduce the growth of other organisms.

(c) Pernasal swab (Bradford & Slavin, 1940.)

B. pertussis can be cultivated from such swabs in 60 to 80% of cases during the catarrhal stage of the disease.

(ii) Disease. After an incubation period of 10 to 16 days (Lapin, 1943; Gordon & Hood, 1951; Court et al, 1953) there is the catarrhal stage which lasts for 1 to 2 weeks and resembles an ordinary, upper respiratory tract infection with occasional sneezing, mild cough and slight fever. It is during this acute phase that the isolation of bacteria is easiest. After the catarrhal stage, the paroxysmal stage develops, which is the essence of pertussis. A coughing attack consists of a series of forceful coughs, saliva and mucus streaming from the nose and mouth. As the final cough appears to clear offending secretions or mucus from the upper airway, air rushes into the lungs against a still narrowed glottis and the patients can whoop. At the end of the paroxysm, patients frequently vomit (Olson, 1975). The paroxysmal stage lasts for 1 to 4 weeks but may be prolonged for as long as 5 to 6 months (Laurence, 1965).

(iii) Lung Pathology. It is difficult to determine the pathology of the initial lesion as it is not until later stages, by which time the lesions are often complicated by secondary infection, that cases come to autopsy. B. pertussis is believed to multiply rapidly on the mucus membrane of the respiratory tract causing initially a necrotising inflammation of the bronchi, larynx and nasopharyngeal cavity. As infection extends to deeper structures, a diffuse bronchopneumonia with marked desquamation of the alveolar epithelium develops. A distinctive feature is the localisation of the bacteria between the cilia of the epithelial cells of the respiratory tract. As infection progresses, the bacteria may appear in the alveoli (Pittman, 1970). Secondary complications may occur during the paroxysmal stage which include

haemorrhagic events, pulmonary complications and, as pertussis bronchopneumonia may impair the normal defence mechanisms of the lung, the patients (particularly infants) may be vulnerable to other bacterial invaders (Strangert, 1970).

(iv) Neurological complications. Symptoms usually appear at the peak of the paroxysmal cough, being found most often in children with bronchopneumonia. Zellweger (1959) found that a third of the patients showing neurological symptoms fully recover; one third show varying neurological sequelae and the other third remain incurable. All the disorders found concern the brain exclusively and include convulsions, coma, paralysis and later epilepsy, mental retardation and other disturbances (Nelson, 1939). With the introduction of pertussis vaccine, reports of pertussis encephalopathy have been superceded by the rare reports of vaccine encephalopathy.

3) Vaccination

The Whooping Cough Immunisation Committee of the Medical Research Council conducted controlled trials of different pertussis vaccines which gave clear evidence of a substantial order of protection (M.R.C. Reports, 1951, 1956). Immunisation with pertussis vaccine was practiced nationally in the United Kingdom from 1957 onwards.

Whether the decline in whooping cough since 1957 can largely be attributed to immunisation is disputed by some authorities (Dick, 1975; Bassili & Stewart, 1976) who consider the general improvement in the environment and of the welfare of children during these years to be more important factors than immunisation in leading to the decline of whooping cough.

The vaccine used at present is prepared as whole bacterial suspensions and is given in conjunction with diphtheria and tetanus

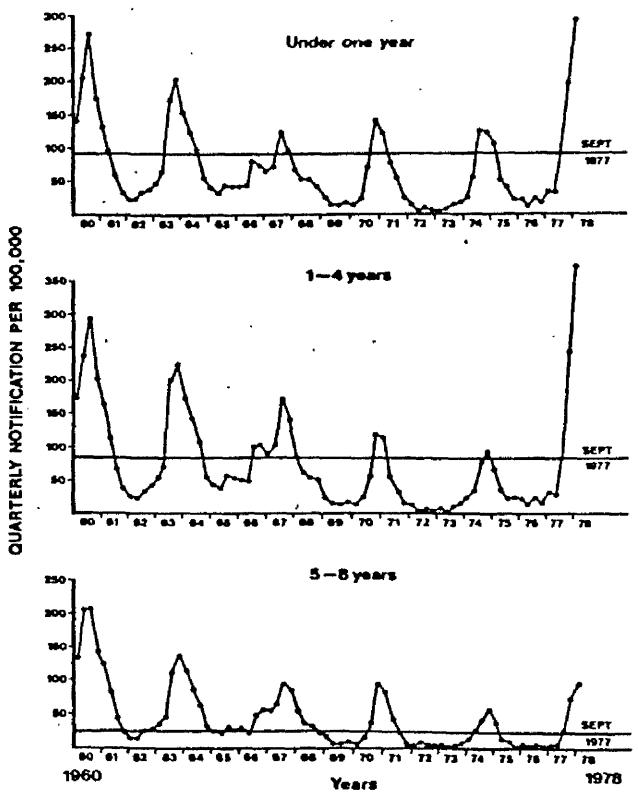
toxoids. The major difficulty in preparing an entirely satisfactory pertussis vaccine is the inability to identify precisely the antigen or antigens necessary for the protection of children. Some doctors have advocated the withdrawal of pertussis vaccine (Barrie, 1977; Stewart, 1977). However the Joint Committee on Vaccination and Immunisation (J.C.V.I., 1977) recommended otherwise, stressing the risk to children from pertussis, particularly young infants, and stating that many of the reported complications were not specific to the pertussis vaccine. Therefore no reliable estimate of the incidence of neurological complications after pertussis can be made, but the J.C.V.I. evidence supporting the continued use of the vaccine, is substantiated by recent data showing an increased incidence of whooping cough as the numbers accepting the vaccine falls mainly due to the fear of possible brain damage (Stuart-Harris, 1979). Fig 1 shows the increase in the number of cases of whooping cough in England and Wales particularly in children under 5 years of age since 1977. This increase in the number of cases of whooping cough, probably due to decreased use of the vaccine, illustrates the protective effect of the vaccine but also the necessity to produce a safer vaccine more acceptable to parents of young children.

4) Mouse assays for virulence and protective activity

B. pertussis is not, so far as is known, a natural pathogen for any animal other than man. However there have been successful infections of a number of species, including monkeys (Culotta et al, 1935; Sprunt et al, 1935; North et al, 1940; Huang et al, 1962), dogs, rabbits (Sprunt et al, 1938) and chicken embryos (Gallavan & Goodpasture, 1937; Shaffer & Shaffer, 1946). However the mouse has been used more extensively than any other species, with three routes of infection being adopted.

Fig 1: Pertussis quarterly notification rates for England and Wales.

(Copied from Stuart-Harris, 1979)


Under one year
1—4 years
5—8 years
SEPT
1977
QUARTERLY NOTIFICATION PER 100,000
1960
1978
Years

(i) Intraperitoneal (i.p.) - After infection by this route the bacteria do not multiply and are killed rapidly. Any lethal effects on the mouse are due to toxaemia (Gray, 1946; Proom, 1947).

(ii) Intracerebral (i.c.) - This route was introduced by Kendrick et al (1947). After intracranial injection, the bacteria appear to lodge on the ciliated ependymal cells in the ventricle of the brain. There they multiply on the ventricle wall giving rise to choroiditis and meningitis but there is no evidence of invasion of the bacteria into the substance of the brain. The mouse usually dies within 10 days (Berenbaum et al, 1960; Iida et al, 1962, 1966). The localisation of organisms is very similar to the localisation of bacteria among the bronchial and tracheal ciliated epithelial cells in the human infection. I.c. challenge was shown (M.R.C. 1959) to provide values for mouse protective potencies of vaccines which correlated fairly well with the human efficacy of these vaccines. For this reason that third route is prescribed for the potency assays of pertussis vaccines (W.H.O. 1964).

In i.c. infections the virulence of organisms can be divided into two groups, high virulence and low virulence (Adams, 1970). Low virulence strains are much more common and produce only transient infections even with initial inocula as large as 10^6 viable organisms. The relatively rare high virulence strains are lethal in inocula of as few as 10^3 viable organisms. The eradication of low virulence strains of B. pertussis from the ventricles seems to be effected by local phagocytes (Adams & Hopewell, 1970). The cells involved in phagocytosis and subsequent intracellular bacterial destruction seemed to be the ependymal cells and the underlying neuroglia (Hopewell et al, 1972).

Protection against highly virulent strains of B. pertussis in passively or actively protected mice requires the breakdown of the blood brain barrier and the leakage of specific gamma globulins into

the brain (Dolby & Standfast, 1961). The nature of the antigens which protect mice against the lethal effect of intracerebrally virulent B. pertussis is not known. The bactericidal antibody (anti LPS) produced in the mouse after vaccination with heat killed bacteria (Dolby, 1965; Dolby & Vincent, 1965) was found not to be responsible for i.c. protection (Ackers & Dolby, 1972). The nature of the i.c. protective antigen is discussed with reference to other pertussis antigens in section G .

(iii) Intranasal (i.n.) - Infection of mice by this route results in infection of the lung producing a patchy or diffuse interstitial pneumonia, leukocytic infiltration around vessels and bronchioles with proliferation of the bronchiolar epithelium and mucous secretion in the bronchioles containing masses of bacteria (Burnet & Timmins, 1937; Bradford, 1938). The histological picture resembles that of the lung in human pertussis. Sublethal doses of bacteria increase in number for 7 to 14 days (Cooper, 1952; Proom, 1947) and then gradually decline but infection may last 3 to 4 weeks (Andersen, 1953; Pittman, 1951; Standfast & Dolby, 1961). The duration of infection in the mouse is similar to that in the child, and in addition there is leukocytosis (Cooper, 1952). Parallelism of duration of histamine sensitivity in lung infected mice and of paroxysmal coughing in human infection was observed (Pittman, 1951; Geller & Pittman, 1973). The pathophysiological reactions associated with the respiratory tract infection in mice (leukocytosis, hypoglycemia hyperinsulinemia and histamine sensitivity, Pittman et al, 1980) and those reported in human infections (leukocytosis, Cooper, 1952, hypoglycemia, Regan & Tolstoouhov, 1936, and the attenuation of epinephrine hyperglycemia, Badr-El-Din et al, 1976) suggest that there is an analogy between the mouse reactions to respiratory infection and the clinical course of whooping cough in man.

Although there are close similarities in the mouse lung infection and human infections, Standfast (1958) found during MRC trials of different vaccines that the immunity response to a lethal i.n. challenge in mice showed little correlation with the protective effect of a vaccine in children; the i.c. challenge correlated fairly well. However Andersen & Bentzon (1958) found that when using the same highly virulent B. pertussis strain for both i.c. and sublethal i.n. challenge the orders of vaccine potencies obtained by the two routes were similar, which was not the case when a lethal i.n. dose was used as the challenge.

A recent report (Sato et al, 1980) has shown that by inoculating mice by aerosol inhalation a predictable and uniform respiratory infection with colonization of the lower respiratory tract can be accomplished.

B. In vitro growth of B. pertussis

1) Growth media

The organism that causes whooping cough was first grown on artificial media by Bordet & Gengou(1906) This medium (B-G) contains glycerinated potato extract and 50% blood. Until Hornibrook (1939) described a liquid medium containing amino acids, cysteine and starch, B-G medium was the only one suitable for the isolation and continued cultivation of B. pertussis. With the introduction of Hornibrook's and related media, the growth requirements of B. pertussis and its metabolism have been investigated, and various inhibitors of its growth identified. The development of solid and liquid media up to 1957 is well reviewed by Rowatt (1957).

(i) Solid media. Workers attempting to develop a medium other

than B-G for the cultivation of B. pertussis did not realise that cells grown, for example, on nutrient agar (Rowatt, 1957) were antigenically different from fresh isolates. In 1931, Leslie & Gardner showed that freshly isolated strains were antigenically distinct from cells grown on simplified media, these cells being phase IV or degenerate strains. Several different media were devised (Barksdale & Simpson, 1934; Cruickshank & Freeman, 1937; Silverthorne & Cameron, 1942; Dawson et al, 1951), none of which were superior to B-G medium.

Pollock (1947) showed that blood in the medium could be replaced by serum, serum albumin or charcoal and that isolates maintained on such media retained phase I characteristics. He found that growth of B. pertussis was inhibited by unsaturated fatty acids, the inhibition being overcome by the inclusion of albumin, charcoal or blood. Liquid media solidified with agar were not satisfactory for growth of B. pertussis unless blood was also added (Verway et al, 1949; Mazloum & Rowley, 1955). More recently it has been shown that agar itself may be inhibitory to the growth of B. pertussis and when a defined solid medium was being developed based on the defined liquid medium described by Stainer & Scholte (1971), agarose was used as a solidifying agent (Parker, 1976). Despite much time and effort being expended in attempts to develop better solid media for the isolation and growth of B. pertussis, the medium of choice for primary isolation is still B-G medium. The solid defined medium of Parker (1976) was developed for use mainly in genetic studies of B. pertussis.

(ii) Liquid media. The first liquid medium was that of Hornibrook (1939), containing hydrolysed casein, starch, salts, cysteine and yeast extract. Most liquid media used subsequently have been based on Hornibrook's. In a later modification Hornibrook (1940) replaced the

yeast extract of the original medium with nicotinamide which is the only essential growth factor of *B. pertussis* yet identified. Cohen & Wheeler (1946) modified Hornibrook's medium by increasing the buffering capacity, adding ferrous or copper sulphate and increasing the starch content. Sutherland & Wilkinson (1961) and Morse & Bray (1969) have further modified Cohen & Wheeler (1946) medium. All these liquid media contain casein hydrolysate with various salts, growth factors and either starch, charcoal or an anionic resin added. By studying the growth requirements of *B. pertussis* several recipes for chemically defined media have been devised (Jebb & Tomlinson, 1955, 1957; Wilson, 1963; Goldner *et al*, 1966). The semisynthetic medium used by Vajdic *et al* (1966) was completely defined apart from a liver co-enzyme preparation but was very complex. Stainer & Scholte (1971) published a much simplified defined medium giving improved growth yields containing L-proline, L-glutamic acid, L-cysteine, salts, Tris. buffer, ascorbic acid, niacin and glutathione.

As well as solid and liquid media necessary for isolation and production of bacterial cultures for vaccination and experimental infections an *in vitro* growth system which allows specific investigation of the mechanisms involved in pathogenicity is useful. One such system is that of organ culture.

2) Growth in organ culture

An *in vitro* model, used in studying the pathogenesis of *B. pertussis* is that of infected tracheal organ culture. The technique of organ culture permits the maintenance, *in vitro*, of fragments of organs in a viable, organised, differentiated condition. Kendrick *et al* (1947) successfully demonstrated the i.c. route of infection in mice but the first reported use of an analogous culture system was that by

Felton et al (1954) where brain tissue was used. Small pieces of human foetal brain or cat brain in a plasma clot were injected with whole bacteria or heat killed bacteria after sufficient growth of the brain fragment was observed. Immune serum or normal serum was added to some cultures. More tissue damage was observed with live B. pertussis than with heat killed organisms and specific antiserum markedly reduced this damage whereas normal serum had no protective effect. Damage was relatively tissue specific since none was observed in infected chick embryo heart muscle, leg muscle or skin and only minimal changes were seen in 18 day old chick embryo brain. It has since been shown that the apparent tissue specificity resulted from a marked specificity for ciliated cells (Iida & Ajiki, 1974; Muse et al, 1977).

Carlton (1925) was the first to develop respiratory tract organ cultures, but it was not until 1957 that these cultures were used to study pathogenicity of microorganisms (Barski et al, 1957). Since then organ cultures of respiratory tract have been used in studies of viral (Hoorn, 1966), mycoplasmal (Collier & Baseman, 1973) and other bacterial infections (Denny, 1974).

The respiratory tract is kept almost sterile by the defence mechanisms of the host, but some organisms including B. pertussis colonise the mucous membranes of the respiratory tract. Tracheal organ cultures have been utilised by several workers to study the local circumstances of B. pertussis infection.

Holt (1972) demonstrated adhesion of B. pertussis to monkey tracheal organ cultures after spraying the organisms through tracheal rings. He also showed that immune serum prevented attachment to trachea.

Chick tracheal organ culture in serum free medium supported excellent growth of phase I B. pertussis while phase I bacteria would

not grow in the serum free medium alone (Iida & Ajiki, 1974). Fluorescent antibody techniques were used to visualise the bacteria adhering to the ciliated epithelium. The localisation of the bacteria was strikingly similar to that seen in infected mouse brain or lung. Ciliary beating ceased in the tracheal fragments when the viable count exceeded 10^8 in the tracheal fragment and surrounding medium. They also noted that the efficiency of bacterial attachment was low with increasing numbers of bacteria appearing in the medium, as organisms growing on the surface of the tracheal fragment were released. Iida & Ajika (1974) concluded that the growth of B. pertussis was not dependent on growth factors excreted by the tissue fragments but required an unknown milieu intimately related to the tracheal fragment. In a later report Iida & Ajiki (1975) showed that the oxidative phosphorylation uncoupler, 2,4-dinitrophenol, at levels non-toxic for B. pertussis or the ciliary motility of tracheal epithelium, inhibited bacterial growth on the organ culture. They postulated that the growth of B. pertussis on the tracheal organ culture depended upon a high energy-requiring process in the tracheal fragments through which the physiological integrity of cells or fragments was maintained.

In 1977 two other groups of workers reported using tracheal organ cultures, rabbit trachea (Matsuyama, 1977) or hamster trachea (Collier et al, 1977; Muse et al, 1977). They found, as did Iida & Ajika (1974), that phase I organisms attached to the surface of the ciliary epithelium. There are differences in the amount of damage caused by B. pertussis which may be accounted for by differences in methodology. The techniques used and results obtained in these studies are summarised in Table 1.

3) Modulation of B. pertussis

Modulation is defined as a change of phenotype occurring in

TABLE 1 : Tracheal organ cultures infected with B. pertussis: a comparison of methods and results

	Iida & Ajika (1974)	Collier et al (1977) Muse et al (1977)	Matsuyama (1977)
Source of organ culture	Chicken	Hamster	Rabbit
Medium	199; Earles & Eagles MEM	Equal parts SSM + 2x concentrated MEM	1.5 to 2% Agar in L-15 medium
Antibiotics	100 µg/ml streptomycin (strep.)	Rings incubated with 10 U penicillin/ml before use	None
B. pertussis strains	phase 1: Tohama; 18323 or strep resistant SM : 18323 phase III : Tohama	No 114	phase 1 : Tohama; 35/2 18323. phase III : Tohama, Sabairi & Sugata
Method of inoculation	Suspension with $10^{1.3}$ to $10^{6.3}$ $0.1\ ml^{-1}$ added to 1 ml of culture medium	B. pertussis grown in SSM to give O.D. of 0.03. Rings immersed in 0.5 ml for 3 h at 37°C, washed	Point inoculation with bacterial suspension (600 cfu)
Attachment phenomena	Phase I adhered to the surface of the ciliated epithelium	Phase I adhered to the ciliated epithelial cells	Phase I adhered to ciliated epithelium and were resistant to clearance. Phase III were cleared.
Cytopathic effects	Disappearance of ciliary beating when viable count exceeded 10^8 in each tracheal fragment culture	Loss of ciliary beating and gradual extrusion or destruction of ciliated epithelial cells	None

Abbreviations: MEM - Minimum essential medium; SSM - Stainer & Scholte medium; O.D. - optical density

all or almost all members of a population as the expression of a reversible and continuously environmentally dependent change in metabolism' (Lacey, 1960). Modulation is quite different from the relatively stable and almost certainly mutational phase variation described by Leslie & Gardner (1931) and Lacey (1951, 1960). The three modes are called X-, C- and I-mode. I-mode will not be discussed here. Envirnomental conditions determine the mode, high temperature (35° to 37°C) and ions (e.g. sodium, potassium and halides) favouring X-mode. C-mode is favoured by low temperatures (25°C), magnesium sulphate and mono- and dicarboxylic acids. The two modes are very different. C-mode cells, unlike X-mode, do not haemagglutinate (HA), are not haemolytic nor are they agglutinable by heavy metal salts. They lack the surface antigen common to B. parapertussis and B. bronchiseptica. The change from one mode to another occurs without mutation or selection and is complete within 7 to 15 cell divisions (Lacey, 1960). As well as modulation brought about by temperature and salts, X to C modulation can be induced by high nicotinic acid concentrations (Pusytai & Joo, 1967), and is accompanied by a fall in the levels of activity of protective antigen (PA) and histamine sensitising factor (HSF). Holt & Spasojevic (1968) showed that C-mode cultures had less protective activity than X-mode cultures when assayed by the i.c. mouse protection test. Parton & Wardlaw (1974) and Wardlaw et al (1976) showed that X- to C modulation is accompanied by loss of two envelope peptides as well as PA and HSF. Similarly, Parton & Durham (1978) showed loss of adenylate cyclase and Wardlaw et al (1979) loss of IgE adjuvanticity. Livey et al (1978) also reported the loss of heat-labile toxin in magnesium sulphate induced modulation.

4) Colonial variation

Andersen (1952), in the course of serial passage of strains

of B. pertussis intracerebrally through mice, noted that in primary subcultures there were often marked differences in the size of colonies. Smaller colony variants tended to be more virulent, more toxic and more easily agglutinable than bacteria from large colonies. Colony size differences were lost on further subculture, but differences in virulence, toxicity and agglutinability were retained even after numerous subcultures or freeze drying. Cameron (1976), after extensive studies on the colony variants found many different types of colony present in laboratory strains and in fresh isolates and noted variability in haemolytic activity and agglutinogens. Different colony types varied in the amounts of PA and HSF produced, and also in the ability to induce weight loss in mice. He did not suggest that any particular colonial or serological variant was associated with these properties except that the serotype 1 variants appeared to produce relatively little protective antigen and HSF and were considerably less toxic. He, however, noted the possibilities of clone selection for high protective activity with low toxicity.

C. Pathophysiological properties associated with B. pertussis

Bordet & Gengou (1907, 1909) and Bordet & Sleeswyck (1910) showed that freshly isolated strains were serologically similar but that many laboratory strains failed to agglutinate in the presence of antiserum prepared against freshly isolated strains. Bordet & Gengou (1909) also found that B. pertussis cells contained a heat-labile substance which, when given intraperitoneally or intravenously, killed guinea pigs and rabbits but when given subcutaneously produced necrosis of the skin. These early observations demonstrated two biological

properties of B. pertussis, the agglutinogen and the heat-labile toxin. Since then many other biological activities have been described which include many agglutinogen factors (Andersen, 1953; Eldering et al, 1957), a histamine-sensitising factor (Parfentjer & Goodline, 1948), a heat-stable toxic lipopolysaccharide (Flosdorf & Kimball, 1940) and the haem-agglutinating (HA) substance (Keogh et al, 1947). Also, as early as 1897, Frohlich noted a lymphocytosis reaction in many cases of whooping cough and work by Morse (1965) showed that this occurred when B. pertussis was injected into mice. The HA property of B. pertussis will be discussed in section E.

1) Heat-labile toxin (HLT)

The HLT is a cytoplasmic protein released upon cell lysis and inactivated at 56°C within 15 min (Bordet & Gengou, 1909; Wood, 1940; Munoz et al, 1959; Banerjea & Munoz, 1962). HLT causes a dermonecrotic lesion when injected subcutaneously and is lethal for mice within 24 to 48 h by intravenous or i.p. injection. When introduced i.n., the toxin is approximately 100 times less active than by the other routes of injection (Anderson & North, 1943). Although the toxin is cytoplasmic it may appear in the culture supernate during the early exponential phase (Lane, 1968). Lane (1968) proposed that the cell surface blebs observed by electron microscopy (also reported by Morse & Morse, 1970) may have been the mechanism by which cytoplasmic elements were being released. Munoz & Bergman (1977) found HLT in the culture supernate of stationary phase rather than exponential phase cultures.

The methods of obtaining HLT involve disruption of cells under conditions which do not generate heat. Several groups of investigators have attempted to purify HLT with varying degrees of success (Robbins & Pillemer, 1950; Onoue et al, 1963; Nakase et al,

1969; Iida & Okonogi, 1971). Although HLT content of B. pertussis did not correlate with virulence (Standfast, 1951) it is suggested that the HLT must play some role in the pathogenesis of whooping cough (Munoz, 1971; Munoz & Bergman, 1977), since (i) in chick embryos the toxin produces lesions in the epithelial cells of the lungs (Gallavan & Goodpasture, 1937), (ii) when given intracerebrally lesions are observed in the meninges of guinea pigs (Munoz & Bergman, 1977).

Standfast (1958) suggested that HLT produces ciliostasis which would represent a significant contribution to the overall pathogenesis of the whooping cough syndrome since such an impairment of the host defence mechanisms would facilitate adhesion of the organisms to the ciliated epithelium. Iida & Ajiki (1974), and Collier et al (1977) when growing B. pertussis on organ cultures also noted ciliostasis. However in a later paper, (Muse et al, 1979) the possible role of a released pertussis toxin on host cell function was examined by immersing uninfected rings in culture filtrates and no effect on ciliary activity was noticed. They also reported that C mode cells induced ciliostasis at times similar to that induced by normal phase I organisms. Little if any HLT activity was detected in C-mode cells (Livey et al, 1978).

The reported absence of HLT antibodies in convalescent serum (Anderson & North, 1943; Evans, 1947) argues against an active role for HLT in the pertussis syndrome. Antitoxin is not protective against i.c. infections in mice (Verwey & Thiele, 1949) although this does not necessarily imply no protective role for antitoxin in i.n. infections. However Standfast (1951) found no correlation between HLT production and lethal i.n. virulence.

2) Agglutinogens and serotypes of B. pertussis

The agglutinogens of B. pertussis are surface antigens which are easily extracted from the cell (Munoz, 1963). Also when cells are disrupted ultrasonically the agglutinogen is released almost entirely (Flosdorf et al, 1939). The substances are known only functionally and except for factor 1 which is protein (Onoue et al, 1961) little is known about their chemical nature. Nevertheless they are useful in classification and identification of B. pertussis strains.

Bordet & Sleeswyck (1910) described the agglutination of cells by B. pertussis specific antiserum and found all freshly isolated cultures agglutinated in the serum. For many years it was assumed that agglutination reflected the presence of a single agglutinogen. The complexity of the agglutinogen was not fully realised until Andersen (1953) and Eldering et al (1957) showed that smooth strains have a common heat-stable O antigen and one or more thermolabile surface antigens. Eight different agglutinogens have been reported for B. pertussis (Eldering et al, 1962). Numbers 1 to 6 are species specific and numbers 7 and 13 are shared by other species of Bordetella. Agglutinogen 1 is common to all strains of B. pertussis whereas 2 to 6 are found in various combinations as strain specific antigens.

A relationship was found between antibodies to agglutinins in children and their resistance to whooping cough (Medical Research Council, 1959), but mice immunised with agglutinogen 1 were not protected against i.c. challenge (Schuchardt et al, 1963). Strains of different agglutinin types were found not to differ significantly in immunising efficiency or in lethality for mice immunised with factor 1 strains. Using a passive transfer test, Eldering et al (1966) did not find any association between serotypes and mouse protection.

3) Endotoxin (LPS)

Little attention has been given to the structure and function of B. pertussis endotoxin when compared to those of enterobacteria. Recent reports show the endotoxin extracted from B. pertussis to possess a structure different from that of the enterobacteria (Le Dur et al, 1978; Chaby et al, 1979). The LPS is made up of two different polysaccharides of low molecular weight and two different lipids (Lipid X and Lipid A). The two polysaccharides are bound to lipid A, polysaccharide 1 through a single molecule of nonphosphorylated KDO and polysaccharide through a single molecule of phosphorylated KDO. The point of attachment of Lipid X is unknown. The LPS has all the usual biological activities of enterobacterial endotoxins. Lipid X is responsible for toxic, and pyrogenic properties caused by B. pertussis LPS and for the local Shwartzman reaction.

4) Pertussigen

The name "pertussigen" was proposed by Munoz (1976) to describe a factor with numerous properties isolated from B. pertussis. This reflected the inability to separate the lymphocytosis promoting factor (LPF) from the HSF and from other properties associated with these two activities. These include Islets Activating Protein (IAP) which causes hypoglycemia and an adjuvant activity for various classes of antibody including IgE. Pertussigen as defined by Munoz (1976) included the protective antigen (PA) which had not been separated from the HSF or LPF activities. Following the reported separation of PA and LPF activities (Arai & Sato, 1976), Pittman (1979) proposed the name pertussis toxin to include pertussigen activities other than the PA. However in this review the term pertussigen is used when the LPF, HSF,

IAP complex is discussed. As most of the previous work done on pertussigen was described under the titles of LPF, HSF or more recently IAP activities, the pathophysiological reactions which led to their names will be described separately. The evidence for these different activities being caused by a single component and evidence for and against it being a protective antigen will also be discussed.

(i) Lymphocytosis - B. pertussis stimulates lymphocytosis (a high level of lymphocytes in the blood) in most patients over 6 months of age. The underlying mechanism was studied in detail by S.I. Morse and his colleagues.

The cells are for the most part normal, mature, small lymphocytes although a degree of polymorphonuclear leukocytosis may also occur. This effect occurred in mice when B. pertussis cells (Morse, 1965) or culture supernate (Morse & Bray, 1969)were inoculated intravenously, the lymphocytes reaching a maximum after 3 to 5 days and declining to a base line level in 2 to 3 weeks. With autoradiography Morse & Riester (1967) showed that the lymphocytosis was not caused by an increase in production of lymphocytes but was primarily due to the entry into the circulation of mature cells from tissue pools. The accompanying polymorphonuclear leukocytosis was due to proliferation of myeloid elements and entry of mature cells from tissue reserves. An impairment of the normal recirculation of lymphocytes was suggested as the reason for the maintained high level of lymphocytes in the blood. Athanassiades & Morse (1973) showed a pronounced decrease in the number of cells crossing capillary venules through which cells traverse to re-enter lymphoid tissue. Abnormality of lymphocytes exposed to LPF was shown by Morse & Baron (1970) who found that lymphocytes isolated from pertussis-treated mice, subsequently labelled with tritiated

uridine and transferred into normal mice, entered the lymph nodes at a significantly lower level than did normal lymphocytes. Lymphocytes incubated in vitro with B. pertussis supernate or purified LPF showed the same effect (Iwasa et al, 1970; Taub et al, 1972). Emigration is impaired probably by a change in the lymphocyte surface (Taub et al, 1972). LPF combines with the surface of different cells types, including lymphocytes (Adler & Morse, 1973).

(ii) Histamine sensitization - This effect was first described by Parfentjer & Goodline (1948) who demonstrated that pertussis vaccinated mice became highly sensitive to the lethal effects of histamine where previously they were resistant. It was later found that these mice were more susceptible to serotonin, bradykinin, endotoxin and various other shock treatments (Munoz, 1963). The mechanism involved is not known but at the cellular level it is thought that HSF may either potentiate or inhibit the activity of regulatory substances. Support for this suggestion came from work by Bergman & Munoz (1966, 1971) who showed that adrenalin protects HSF-treated mice from lethal histamine challenge. The results of Fishel and co-workers (Fishel & Szentivanyi, 1963; Fishel et al, 1962, 1968), strongly suggested an effect by B. pertussis on adrenalin action. They also showed that β-adrenergic blocking agents can produce histamine-sensitivity in mice and that HSF blocks the hyperglycemic effect of adrenalin. However, Hewlett et al (1978) showed that B. pertussis does not cause β blockade and they propose that B. pertussis has an effect on insulin secretion.

(iii) Islets activation - Rats, when given a single i.p. injection of pertussis vaccine, show enhanced hyperinsulinemia in response to insulin secretagogues such as glucose (Sumi & Ui, 1975). The active principle, a protein, was purified from culture supernate

and was termed islets activating protein (IAP) (Yajima et al, 1978). Ui et al (1979) proposed that IAP potentiated insulin secretion by interacting directly but slowly with the pancreatic islet B cells causing sustained activation of native calcium ionophores in the cell membrane. This in turn, results in enhanced secretion by making more calcium available to the stimulus-secretion coupling mechanism.

(iv) Interrelationship between biological activities - Many attempts have been made to separate LPF and HSF activities (Lehrer et al, 1974, 1976; Morse & Morse, 1976; Arai & Sato, 1976; Irons & MacLennan, 1979b) without success. One rather diffuse band is obtained in polyacrylamide gel electrophoresis (PAGE) of various 'pertussigen' preparations, when run in 5% acrylamide at approximately pH 4.5 (Arai & Sato, 1976; Morse & Morse, 1976; Irons & MacLennan, 1979b; Ui et al, 1979). On boiling in sodium dodecyl sulphate (SDS) for 5 min and running in a higher acrylamide concentration (approximately 10%) four bands were obtained. The molecular weights of these bands are approximately 23,500, 19,300, 17,400 and 13,400 (Morse & Morse, 1976). The chemical nature of the 'pertussigen' preparations differs between workers. Morse & Morse (1976) found their LPF preparation to be protein as did Ui et al (1979) who showed only 1 to 1.5% carbohydrate and no detectable lipid in their IAP preparation.. However Arai & Sato found their preparation of LPF was only 47.7% protein with 25% carbohydrate and 23.9% lipid. Lehrer et al (1974) also found a large percentage of lipid in their HSF preparation. All the preparations described LPF, HSF or IAP had activities other than that for which they were being purified. A HA activity was also associated with this complex (Arai & Sato, 1976; Irons & MacLennan, 1979a). The HA activity of pertussigen is distinct from the fimbrial haemagglutinin (F-HA) as it is not sensitive to papain (Arai & Sato, 1976) nor is it inhibited by cholesterol.

(v) Pertussigen as a protective antigen - Much controversy exists over whether pertussigen is a protective antigen. Attempts to purify

protective antigen resulted in fractions with pertussigen activities (Munoz & Hestikin, 1963; Robinson & Manchee, 1978). By the i.c. protection test, Sato et al (1979) showed that formalised pertussigen (LPF) was not protective. Irons & Maclennan (1979a) showed that non-formalised pertussigen was toxic at 4 μg per mouse when injected i.p. and non-protective at lower doses. More recently Sato et al (1981) protected mice from aerosol challenge (Sato et al, 1980) by injecting antiserum to pertussigen i.p. 30 min beforehand. In contrast to Sato et al (1979), Munoz et al (1981) demonstrated that pertussigen, free of F-HA and detoxified by gluteraldehyde treatment actively protected mice against intracerebral challenge. Munoz et al (1981) also found that antiserum to pertussigen, given intraperitoneally 2 h before i.c. challenge, protected mice. If, as proposed by Pittman (1979), pertussigen acts as an exotoxin and is responsible for the pathogenic effects of pertussis infection, then it would be expected that an antibody to it would be protective. The difference in the results obtained by Sato et al (1979) and Munoz et al (1981) may be due to the method used to detoxify pertussigen.

D. Role of attachment in microbial ecology

1) General Background

Bacteria in many environments have a predeliction for colonising surfaces. As with colonisation of other surfaces, organisms before they can colonise human and animal bodies must, first, become firmly attached and, second, they must be able to proliferate under the existing conditions. Specificity of attachment is proposed as a mechanism whereby the bacteria only become located on a surface suitable for proliferation and colonisation. The specificity of different

bacteria for different surfaces in the nose and mouth were described (Gibbons et al, 1976; Aly et al, 1977), who showed the adhesion to different surfaces correlated with their natural site of infection.

The importance of the initial colonisation of mucosal surfaces is realised for a very varied selection of pathogenic microbes as Table 2 (adapted from Mims, 1977) illustrates.

Mechanisms of attachment may involve production of extracellular polysaccharide, as for adhesion of lactobacilli (Brooker & Fuller, 1975) or filaments produced on the cell surface. Although B. pertussis produces a capsule, there is no indication that this has a role in adhesion. Attachment may be mediated by fimbriae and for this reason further discussion is restricted to organisms with fimbriae-mediated adhesion.

The presence of fimbriae or filaments other than flagella were first observed in 1949 and 1950 (Anderson, 1949; Houwink & van Iterson, 1950). Anderson stated that the appendages he observed on the E. coli cell surface in the electron microscope were artifacts but Houwink proposed that they functioned as organs of attachment. Duguid et al (1955) agreed with Houwink & van Iterson that the filaments existed as appendages on living bacteria. In support of the proposed role in attachment, Duguid et al (1955) found that strains possessing what he called fimbriae were distinguished by their ability to agglutinate red blood cells.

2) Adhesion of pathogens to mucosal surfaces

(i) Enterobacteriaceae - Many of the Enterobacteriaceae produce type 1 fimbriae as defined by Ottow (1975) but their role in adhesion has not been clearly shown. Duguid & Gillies (1957) and Duguid et al (1966) failed to show any relationship between the production of type I

TABLE 2 : Microorganisms whose attachment has a role in pathogenicity

(adapted from Mimms, 1977)

Microorganism	Disease	Attachment site	Mechanism
Influenza virus	Influenza	Respiratory epithelial cells	H.A. reacts with neuraminic acid receptor on cell
Polio virus	Poliomyelitis	Susceptible tissue cell (neurone)	Viral capsid protein reacts with specific receptor on cell
Chlamydia	Conjunctivitis	Conjunctival epithelium	Unknown-sialic acid receptor on epithelial cell
Mycoplasma pneumonia	Atypical pneumonia	Respiratory epithelial cells	'Foot' attaches to neuraminic acid receptor on cell
Neisseria gonorrhoeae	Gonorrhoea	Urethral epithelium	Unknown
Vibrio cholerae	Cholera	Intestinal epithelium	Unknown
Escherichia coli	Diarrhoea	Intestinal epithelium	Requires specific bacterial surface component eg K88
Streptococcus mutans	Dental caries	Tooth	Dextran 'glue' synthesised by bacteria
B. pertussis	Whooping cough	Respiratory epithelium	Unknown
Plasmodium knowlesi	Malaria	Erythrocyte of susceptible host sp.	Unknown

fimbriae and pathogenicity in the gut. However the role of other fimbriae-like adhesins found on the surface of enteropathogenic E. coli has been investigated. These other filaments are distinguished from type 1 fimbriae, which produce a mannose-sensitive HA by their mannose-resistant HA. The fibrillar K antigen of E. coli, K88, was shown to be produced in vivo and to be responsible for the colonisation of pathogenic E. coli to the intestinal mucosa of neonatal piglets (Jones & Rutter, 1972). Bacteria without the antigen failed to adhere to the mucosa and hence failed to colonise and cause disease (Jones & Rutter, 1972). Also there is clear evidence that a similar mannose-resistant adhesin, K99, promotes intestinal infection in calves and lambs (Orskov et al, 1975; Burrows et al, 1976). Ninety-eight percent of entero-toxigenic strains of E. coli from human adults also have a mannose-resistant HA either Colonization Factor 1 or 11. As the name suggests, these are also involved in colonization of the small intestine (Evans & Evans, 1978; Evans et al, 1975; Evans et al, 1978). The role in pathogenesis of diarrhoeal disease of CFA/1 was demonstrated in human volunteers given a virulent strain of E. coli which was either CFA^+ or CFA^-. Only those given CFA^+ strains contracted diarrhoea and showed prolonged excretion of bacteria.

Fimbriae-mediated adhesion also occurs in urinary tract infections (UTI) caused by E. coli. Here type 1 fimbriae may be involved. Svanborg-Eden & Hansson (1978) showed that 11 out of 12 isolates from urine of UTI patients gave mannose-sensitive haemagglutination of guinea pig erythrocytes. However it is not clear whether the fimbriae involved are of type 1 only as mannose did not inhibit the adhesion of these strains to uroepithelial cells in vitro. Kallenius & Mollby (1979) proposed that adhesins other than type 1 fimbriae were involved. More recently Hagberg et al (1981) indicated that the

surface antigens of E. coli responsible for attachment to human urinary tract epithelial cells also induce mannose-resistant agglutination of human erythrocytes although many of these showed mannose-sensitive haemagglutination of guinea pig erythrocytes. This suggested that both type 1 fimbriae and a specific mannose-resistant adhesin were present on the cells.

(ii) Neisseria - Gonorrhoea is another disease where fimbriae are implicated in bacterial pathogenesis. This was first indicated when it was shown that there were four colony types of N. gonorrhoeae; the organisms from colony types 1 and 2 being virulent and fimbriate, colony types 3 and 4 being associated with avirulent and non-fimbriated organisms (Kellogg et al, 1963; Swanson et al, 1971). Fimbriae promote adhesion of in vitro grown gonococci to cultured human amnion cells (Swanson, 1973). Many adhesion experiments are done using human buccal epithelial cells (Tramont, 1977; Lambden et al, 1979). Using human fallopian tube organ culture (Ward et al, 1974), which is highly relevant for the study of gonococcal adhesion, a four fold greater adhesion of fimbriated gonococci to the mucosal surface over a non-fimbriated variant of the same strain was shown (Watt & Ward, 1980). Fimbriate gonococci not only bind in greater numbers to human cells than do non-fimbriate cells but also they show an increased rate of attachment. This may be critical in the transmission of the natural infection where flows of mucus or urine would tend to flush the bacterium from the mucosal surface (Watt & Ward, 1980). However as well as fimbriae outer membrane proteins play an important role in neisserial adhesion. Recently Virji & Everson (1981) using variants of N. gonorrhoeae strain P9 which differ in outer membrane proteins (Swanson, 1978; Lambden & Heckels, 1979), showed that one varient (P9-16) with no fimbriae but protein IIb in the outer membrane had high avidity of attachment, for

a Chang conjunctiva epithelial cell line. They also found that protein II increased the final level of adhesion compared to the prototype P9-1 which is non-fimbriate and lacking any protein II in the outer membrane. The rate of these non-fimbriate variants was however slow compared to a varient such as P9-20 which was fimbriate as well as containing protein II (Virji & Everson, 1981).

Lambden et al (1980) showed that gonococci may possess what they term either α or β fimbriae. They found that isolated α fimbriae attached to a greater extent to buccal epithelial cells than did β fimbriae at pHs above 5 with maximum adhesion occurring at pH 6.5. Both fimbriae types adhered to similar levels to erythrocytes. Virji & Everson (1981) with variants of P9, P9-2 which has α fimbriae and P9-20 a β fimbriate variant, showed that the β-fimbriate variant attached to a higher level than did P9-2 to Chang epithelial cells. However P9-20, the β fimbriate variant also contained protein II in the outer membrane which also increases the level of adhesion. These results indicate the necessity of extending these attachment studies to other types of tissues so that the relative importance of the various components involved in attachment may be assessed under different circumstances of infection.

(iii) Vibrio cholerae - This organism differs from the two described previously in that it is not so restricted in habitat. Populations can be found in the human intestine as well as a variety of surface waters. Several adhesins may be synthesised by V. cholerae, the specificity and nature of which may depend on the habitat (Jones, 1980).

Haemagglutination by different strains of V. cholerae is inhibited by carbohydrates to different extents, some strains being inhibited by D-mannose (Barua & Mukherjee, 1965) whereas others are not

(Jones & Freter, 1976). Jones & Freter (1976) found that the HA-caused by classical V. cholerae cells grown in broth culture was inhibited by L-fucose but not D-mannose while other workers (Finkelstein et al, 1977) have described strains producing HAs insensitive to D-mannose and L-fucose. These observations suggest that strains of V. cholerae produce several HAs and it is not known which, if any, of those described is involved in colonization of the intestine (Jones, 1980). The nature of the HAs from V. cholerae is uncertain. V. cholerae produces fimbriae and the more adhesive el tor strains are more fimbriate than classical cholerae vibrios and non-cholerae vibrios (Tweedy et al, 1968). However it is not certain that these fimbriae are the adhesive organelles responsible for the attachment of vibrios to brush border surfaces. Finkelstein et al (1977) have characterised a cell free haemagglutinin of a classical biotype of V. cholerae which has a very low M.W. (68,000) in the absence of depolymerising agents. Most adhesins described are long filaments of high M.W. but how this small HA is organized on the bacterial cell surface is unknown. There is evidence that this HA is involved in adhesion as it inhibits the attachment of an el tor vibrio strain to intestinal epithelium of infant rabbits (Finkelstein et al, 1977). All work on V. cholerae adhesion to intestinal epithelial cells is complicated by the fact that colonisation is dependent on three stages - a) organisms detect and move towards the mucosa by following gradients of chemo-attractants (Allweiss et al, 1977), b) organisms then penetrate into and through the mucus gel (Freter et al, 1978) and finally c) the organisms adhere to the brush border surface of the epithelial cells (Nelson et al, 1976). Interference with any of these steps may disrupt the process of association (Freter et al, 1978).

The three groups of organisms discussed were chosen to illustrate the complexity of bacterial adherence to epithelial surfaces. The overall mechanisms involved require further elucidation before reliable anti-adhesive vaccines can be produced.

3) Interaction of bacteria with animal cell surfaces

Both bacterial and mammalian cells have a net negative charge. Bacterial adhesion to animal cells illustrates an apparent paradox where two bodies of the same surface charge can attract and adhere to one another. This can be explained in part by application of the DLVO theory (Derjaguin & Landau, 1941; Verwey & Overbeek, 1948). This theory considers that the energy of interaction of two charged particles of like sign and magnitude is the resultant of the electrostatic energy of repulsion and the energy of attraction provided by London-van der Waals forces. The forces of repulsion and attraction between approaching surfaces vary with the distance of separation in such a manner that the surfaces at two distances attract one another. One energy level that favours surface-surface interaction occurs at the close approach of the particles and is known as the primary minimum. A further attraction occurs at the secondary minimum which involves greater distances of separation. The secondary minimum (Curtis, 1973) occurs at a separation distance determined by the nature and ionic environment of the contacting surfaces. In an electron microscope study Ward & Watt (1975) estimated a separation between the gonococcus envelope and the mucosal surface of a fallopian tube organ culture of 13 nm, a distance suggesting adhesion at the secondary minimum. In the interaction of the gonococcus with the mammalian cell surface the DLVO theory alone cannot explain the adhesion as there is a specificity of attachment indicated by the ability of the gonococcus to adhere to

and invade the moving surface of the human fallopian tube but not the rabbit oviduct (Taylor-Robinson et al, 1974). However secondary minimum adhesion may be a preliminary stage in gonococcal attachment permitting subsequent specific primary adhesion. The importance of surface charge on gonococcal adhesion was illustrated by Heckles et al (1976) who showed that neutralising the negative charge of the gonococcal surface increased the adhesion of fimbriate gonococci and that of non-fimbriate bacteria to a similar level.

Gonococcal fimbriae appear to reduce the forces of repulsion between the bacterial and mammalian cell surface since fimbriated cells adhered to a greater extent than non-fimbriated ones. It is possible that by their hydrophobic nature and small diameter they allow the organisms to come close enough to the epithelial cells to allow secondary minimal adhesion.

(i) Hydrophobic interactions - There is a preponderance of non-polar side chains in the amino acids of type 1 fimbriae, K88 fimbriae of E.coli and gonococcus fimbriae (Jones, 1977; Watt & Ward, 1980). Smyth et al (1978) showed that K88 antigen were solely responsible for the binding of smooth enteropathogenic E. coli to octyl and phenyl-Sepharose gels, K88 -ve strains showing no adsorption to hydrophobic columns. Smyth et al (1978) suggested that there is a role for hydrophobic interaction in fimbriae mediated adhesion in E. coli. They suggest that reduction in the surface potential due to the masking of the charge contribution of polysaccharide K antigens and lipopolysaccharide by certain fimbriae with hydrophobic characteristics probably promotes adhesion.

Although gonococcus fimbriae avidly adhere to octyl or phenyl sepharose, fimbriated gonococci were only approximately 20% more

efficient at binding to hydrophobic sepharoses than non-fimbriate cells. The outer membrane proteins of gonococcus play a part in hydrophobic binding, as variants with different outer membrane proteins differ in adhesion to phenyl sepharose (Watt & Ward, 1980).

E. The HA's of B. pertussis

In 1947 Keogh et al observed that erythrocytes of man, fowl and other animals were agglutinated by saline suspensions of B. pertussis and also by filtrates and supernatant fluids of liquid cultures. The degree of cell-association and time of maximum release of the HA into the culture medium was dependent on the size of the inoculum and the suitability of the medium. Keogh & North (1948) found that strains of B. pertussis with maximum HA content showed maximal virulence. In contrast Standfast (1951) found no correlation between virulence and viable count, presence of toxin or HA. Keogh & North also showed that the supernates from liquid cultures of B. pertussis with 'adequate' HA content protected mice against i.n. infection as efficiently as did vaccines prepared from B-G cultures. Following these early reports suggesting a protective role for HA, Masry (1952) found no protective activity with an isolated HA preparation as immunogen. After an interval of almost 20 years further work on the HA of B. pertussis began in the 1970s when the role of adhesins in pathogenicity of several organisms became clear.

1) Growth of B. pertussis and associated HA activity in vitro

(i) Solid Medium - There does not seem to be any correlation between incubation time and the HA content of B. pertussis grown on solid medium. Ungar (1949) stated that cultures grown for 24 or 48 h on B-G showed

HA whereas those grown for 72 h had very low HA activity. Masry (1952) obtained no such variation with B-G cultures and reported no difference in the HA titre of cell suspensions from 24, 48, 72 or 96 h cultures.

(ii) Liquid Medium - Liquid medium is convenient for direct comparison between growth and production of HA in different strains. Shallow layers of static liquid culture were most commonly used for production of HA. According to recent reports where growth and HA production in both static and shake cultures is compared, static culture favours the production of fimbrial-like protrusions on the cell surface (Irons & MacLennan, 1979a; Arai & Munoz, 1979b).

Fisher (1948) showed that neither the rate of multiplication nor the final yield of B. pertussis was influenced by the size of the inoculum and the final yield of organisms in different cultures was between 10 and 20 x 10^9 ml^{-1}. Similar maximum counts were obtained by other workers (Keogh & North, 1948; Masry, 1952; Morse & Morse, 1970; Sato et al, 1974). Fisher (1948) showed that the HA content of the cells fell with the increase in total count and the HA titre of supernates first rose and then declined. The rise coincided with the rise in pH, the decrease reflecting destruction of the HA. Masry (1952) and Sutherland & Wilkinson (1961) obtained similar results. However other workers did not obtain the same HA patterns with growth. Keogh & North (1948) showed an increase in both cell and supernate HA activity with growth whereas Sato et al (1974) observed an increase in cellular HA activity which started to decrease as the cells moved into stationary phase. The HA titres of the supernate ran almost parallel with that of the cells. Differences in medium and in the HA assay systems may account for conflicting results. Arai & Munoz (1979b) compared the growth and HA production of two strains of B. pertussis in two different media (Sato et al, 1974; Munoz et al, 1978). Very little or no HA

was detectable with either strain in either medium when in shaken culture. However in stationary culture high HA titres were obtained with both strains in the medium used by Sato et al (1974) but not in that used by Munoz et al (1978). In the Sato medium Arai & Munoz (1979b) found that the HA activity associated with both the cells and supernate increased during logarithmic growth of the organisms.

In an ultrastructural study Morse & Morse (1970) followed changes in the appearance of B. pertussis when grown in static liquid medium. During the first 48 h round particles of heterogeneous size, a small number of filaments, and long dumbell-shaped particles appeared in the culture supernate. Larger numbers of filaments were seen attached to the cell surface after 48 h culture but after 4 days virtually no cell associated filaments were observed. The early appearance of these filaments on the cell and then later in the supernate follows the pattern of haemagglutination associated with the bacterial cells and with the supernate described previously by Fisher (1948). The appearance of similar filaments on the cells and in the supernate were observed by Sato et al (1974). However, they observed filaments on both the cells and in the supernate during logarithmic growth of the cultures.

2) Purification of the HA

The first attempt to elute HA from cells of B. pertussis was made by Warburton & Fisher (1951), using 1M NaCl at pH 8.5. The cell free HA was then precipitated with 1M NaCl and adsorbed onto Al PO_4, pH 5.5, this being used to immunize mice. Protective activity was shown after the mice were challenged with a lethal intranasal dose of B. pertussis. The significance of this result was unclear since the

HA preparation also contained agglutinogen. A further attempt at purification of HA was made by Masry (1952) who extracted HA from cells grown for 24 h on B-G medium with 2M NaCl or with 1M sodium acetate followed by methanol precipitation. The NaCl extract contained agglutinogen whereas none was detected in the methanol precipitated sodium acetate extract. Both extracts were toxic, though differing in potency, producing necrotic lesions similar to those described by Evans & Maitland (1937) for the thermolabile toxin. In protection tests the methanol precipitate was adsorbed onto fowl red blood cells before i.p. injection into mice. This vaccine showed little or no protective activity against an i.c. challenge with 200 LD50's of B. pertussis cells.

Following these early efforts at purification of HA no further attempt was made until that of Sato & Arai (1972) who noted filamentous structures in partially purified LPF. The preparation had a high histamine sensitising activity as well as high HA activity. Later Sato et al (1974) reported that the partially purified LPF was protective against i.c. challenge. Nakase et al (1975) described a purification procedure which yielded HA free from histamine sensitising activity, lymphocytosis promoting activity, heat labile toxin, K and O agglutinogens and had no protective activity. Both the HSF and LPF were isolated and recovered in other fractions.

Morse & Morse (1976) separated HA and LPF, the HA preparation having no lymphocytosis promoting activity and being mainly filaments. The LPF preparation had minimal HA activity and consisted of round particles. At about the same time, Arai & Sato (1976) reported a rapid decrease in HA activity after treatment with papain, whereas lymphocytosis promoting activity was much less sensitive to this enzyme. Fractionation of the LPF preparation (Sato & Arai, 1972) on 6% sepharose yielded two fractions, one being filaments the other spherical particles. The

filaments were free of lymphocytosis promoting activity and contained most of the papain sensitive HA. LPF was associated with the spherical particles which possessed papain resistant HA activity. More recently Arai & Munoz (1979a) and Irons & MacLennan (1979a) described purification procedures. Arai & Munoz (1979a) used ion exchange chromatography followed by fractionation on 6% sepharose, Irons & MacLennan (1979a) ammonium sulphate precipitated culture supernate, the precipitate was then extracted with 50 mM Tris/HCl buffer with 1M NaCl pH 8.0. The F-HA was adsorbed from the extract by being adsorbed onto cholesterol containing liposomes. A summary of purification procedures for F-HA is summarised in Table 3.

3) Properties of the HAs

Cells of B. pertussis agglutinate many different types of red cells (Ungar, 1949) with no evidence of species specificity. The HA is antigenic and although 97% of HA activity of whole cells or the purified filaments responsible for haemagglutination was destroyed at 56°C, a large proportion of antigenic potency remained (Fisher, 1950). The properties described in this section are those associated with the purified F-HA. Properties of the pertussigen HA are described in section C4.

Electron microscopy showed the F-HA to consist of homogeneous filaments (fimbriae) about 2 nm in diameter and about 40 nm in length (Nakase et al, 1975; Arai & Sato, 1976; Morse & Morse, 1976; Irons & MacLennan, 1979a). The fimbriae were insoluble in water but disaggregated in high salt or alkaline solvents pH 10 to 11 (Nakase et al, 1975; Arai & Sato, 1976). The molecular weight of the disaggregated filaments, determined by gel filtration was approximately 70,000 (Nakase et al, 1975). In SDS-PAGE of the F-HA, Arai & Sato (1976) obtained a major band

Author(s)	B. pertussis strain	Medium and growth conditions	Purification Procedure	Electron microscopy
Warburton and Fisher, 1951	Various	Cohn & Wheeler (1946) liquid medium 4 day static culture	HA extracted from cells with 1 M NaCl and absorbed onto $ALPO_4$ (10 mg ml^{-1})	-
Masry 1952	Various	24 hr B-G cultures	1) 2M NaCl extraction of supernate 2) M Na Acetate extraction of supernate followed by methanol precipitation	-
Nakase, Doi and Kasuga, 1975	Maeno phase I	48 hr charcoal agar cultures	HA extracted from cells with 1M NaCl, passed through a sepharose 4B column, peak of HA activity recycled through column.	Homogeneous filaments approx 2x40nm
Arai & Sato, 1976	Tohama phase I	Morse & Bray (1969) liquid medium 5 day static cultures	Supernate precipitated with ammonium sulphate, precipitate in P.B. with 1M NaCl pH 8.0 separated in starch block electrophoresis. Cathode peak separated on sucrose density gradient. First peak separated further on 6% agarose. First peak F-HA(A) third LPF-HA(B).	(A) filaments 2x40 nm (B) spherical molecules 6 nm diameter
Morse & Morse, 1976	NIH 14 (3779B)	Sutherland & Wilkinson (1961) 5 day static culture	Supernate precipitated with 90% saturated ammonium sulphate. Water insoluble fraction in 0.1M Tris 0.5M NaCl pH 10 centrifuged on CsCl gradient load volume separated on sephadex G100. First and second peaks HA, third LPF.	(1) filaments and membrane particles (2) filaments (3) small round particles
Arai & Munoz 1979	$3779B1_2$ S4	Sato et al (1974) 5 day	Supernate adjusted to pH 6.5 passed through CM-Sepharose. HA eluted with 0.5M NaCl.	filaments

corresponding to a molecular weight of 126,000 and several minor bands from 120,000 to 52,000 which were thought to be degraded products. Irons & MacLennan (1979a) obtained similar gel patterns with two major bands at 127,000 and 95,000 and several minor bands. Chemical analyses of the F-HA by Nakase et al (1975) and Arai & Sato (1976) yielded very similar results with about 85% protein, 5% carbohydrate and 4% lipid. However Arai & Munoz (1979) found their F-HA preparation to be at least 97% protein.

F. Attachment of B. pertussis

1. Requirement for attachment

Air contains a variety of suspended particles including microorganisms. Efficient cleansing mechanisms in the lungs remove inhaled particles and keep the respiratory tract clean.

A mucociliary blanket covers most of the surface of the lower respiratory tract. It consists of ciliated cells together with mucus secreting cells (goblet cells) and subepithelial mucus secreting glands. Foreign particles deposited on this surface are entrapped in mucus and borne upwards from the lungs by ciliary action to the back of the throat before being swallowed. The upper respiratory tract has a similar clearing system. The terminal portions of the lower respiratory tract are the alveoli which have no cilia or mucus but are lined by macrophages.

For a microorganism to initiate infection in the respiratory tract it must circumvent the mucociliary clearance mechanism and if it reaches the alveoli it must resist phagocytosis by alveolar macrophages or if phagocytosed, must survive and multiply rather than be killed and digested. Microorganisms have little chance unless the mucociliary

mechanisms are defective or unless they have a special device for attaching firmly to epithelial cells. An example of this is seen with influenza virus, which has a surface HA which reacts specifically with sialic acids on epithelial cells (Hirst, 1965). A firm union is established and the virus infects cells. As B. pertussis infects the lower respiratory tract it is proposed that some similar mechanism involving attachment to the epithelial layer contributes to the ability of the organism to infect a normal lung.

2. Site of attachment

B. pertussis is found localised on the ciliated epithelium of the respiratory tract. Arnheim (1903) and Mallory and Horner (1912) showed that in cases of pertussis, bacteria were found mainly among the bronchial and tracheal cilia. Gallavan and Goodpasture (1937) showed that, in chick embryo infected with B. pertussis, the organisms grew among the cilia of the respiratory tract and of the transiently ciliated pharynx and oesophagus. The specificity of the organisms for the ciliated cells is illustrated in scanning electron microscope pictures of organisms attached to the ciliated cells in tracheal organ culture (Muse et al, 1977). In i.c. infections of mice it has been shown that the organisms again attach specifically to the ciliated ependymal cells (Hopewell et al, 1972). Berenbaum et al (1960) showed that bacterial multiplication in the mouse brain was limited to the cranial cavity in which it was practically confined to the ciliated layer over the ependyma. Whether the sites of adhesion are on the cilia themselves or the microvilli of the ciliated cells is unclear. In the mouse brain infections (Hopewell et al, 1972) bacteria appeared to be held between microvilli of ependymal cells and no evidence was obtained for bacterial attachment to cilia. Muse et al (1977) showed by

scanning electron microscopy the apparent attachment of organisms to cilia of tracheal organ cultures infected with B. pertussis.

3. Proposed mechanism of attachment

The identity of filamentous HA with the filamentous appendages on the cell surface was shown by the uniform attachment of the specific HA antibody molecules to the bacterial fimbriae (Sato et al, 1979). Avirulent phase III organisms lacked fimbriae and did not attach to HeLa cells (Sato et al, 1979) or to rabbit tracheal organ cultures (Matsuyama, 1977). Antibody to phase III organisms did not prevent phase I bacteria adhering to HeLa cells whereas homologous phase I antibody or antibody to isolated HA largely prevented attachment (Sato et al, 1979). The weight of evidence suggests that fimbriae play a part in pathogenesis of B. pertussis by being involved in attachment of the organism. Whether or not the HA property of the pertussigen component (Arai & Sato, 1976; Irons & MacLennan, 1979a) reflects a role in adhesion in vivo is not known. Also the possibility of other outer membrane proteins being involved in adhesion cannot be ruled out.

4. Cell surface receptors for the HAs of B. pertussis

The most common receptors suggested for bacterial adhesins on cell surfaces are carbohydrate such as α-D-mannose which inhibits the HA of common type I fimbriae from the Enterobacteriaceae (Duguid et al, 1966; Tweedy et al, 1968; Old, 1972). The evidence for mannose being the receptor in vivo is less clear. Schaeffer et al (1979) reported the adhesion of E. coli, isolated from urine, to uro-epithelial cells as being completely inhibited by α-D-mannose. However in an earlier report Svanborg-Eden & Hansson (1978), again using strains of E. coli isolated from urine, found no inhibition of the adhesion of

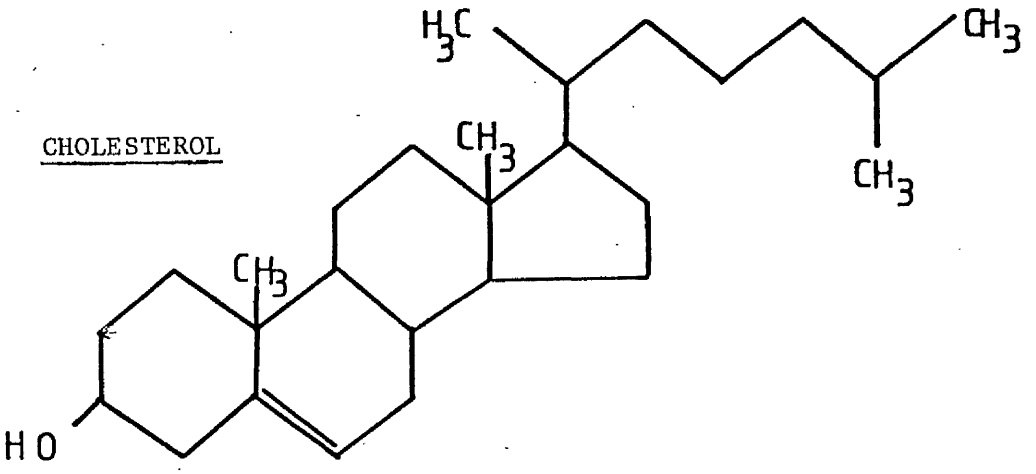
these strains to uroepithelial cells by D-mannose. For some strains of V. cholerae both the adhesion to brush-border membranes and HA are inhibited by L-fucose (Jones & Freter, 1976). The HA of turkey erythrocytes by Mycoplasma gallisepticum does not occur if the erythrocytes are pretreated with neuraminidase (Gesner & Thomas, 1965), implicating sialic acid residues in the receptor complex.

Pertussigen induced HA is inhibited by the sialoproteins haptoglobin and ceruloplasmin type III. Removal of the sialic acid residues from the proteins by pretreatment with neuraminidase reduced their inhibitory effects to less than 1% of its former value indicating sialic acid as the receptor (Irons & MacLennan, 1979a). As isolated sialic acid did not inhibit the HA by pertussigen (Irons & MacLennan, 1979a) the receptor is probably more complex requiring a specific orientation of the sialic acid residues.

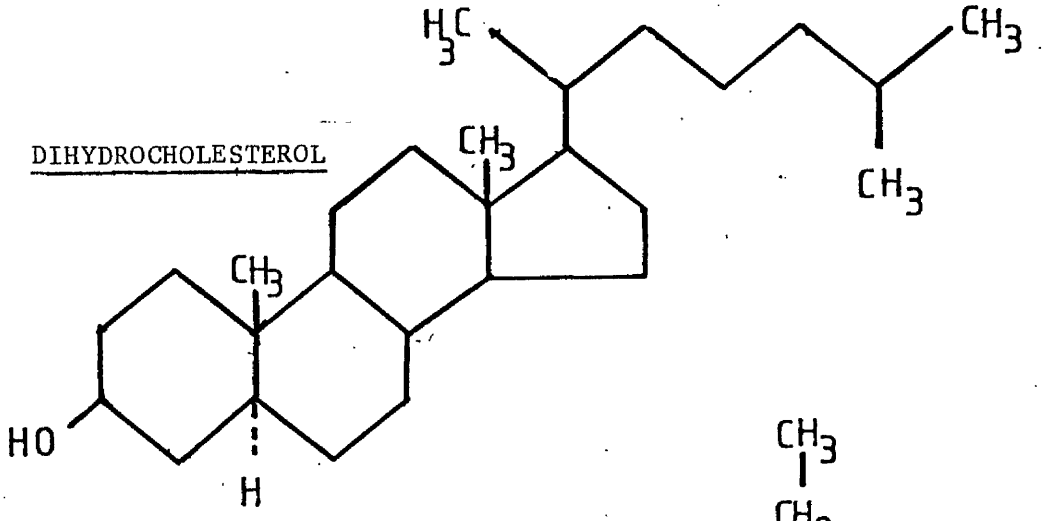
Sato et al (1979) and Irons & MacLennan (1979a) showed that F-HA activity was inhibited by sterol containing liposomes. Sato et al (1979) demonstrated inhibition of F-HA by cholesterol, concluding that the fimbrial receptor involved cell membrane sterols with a cis-hydroxyl at the C3 position. In a comparative study of the effects of sterol structure on F-HA inhibiting activity Irons & MacLennan (1979a) used a liposome system to show that only those containing cholesterol, dihydrocholesterol or β-sitosterol as the sterol moiety were effective in binding fimbriae from cell extracts, stigmasterol and epicoprostanol being ineffective. Binding of fimbriae was confirmed using SDS-PAGE to demonstrate the presence of two proteins with molecular weights of 127,000 and 95,000 on the liposomes.

Whether or not the sialoproteins and sterols, proposed receptors for the two haemagglutinins of B. pertussis, have a role in vivo has not been investigated.

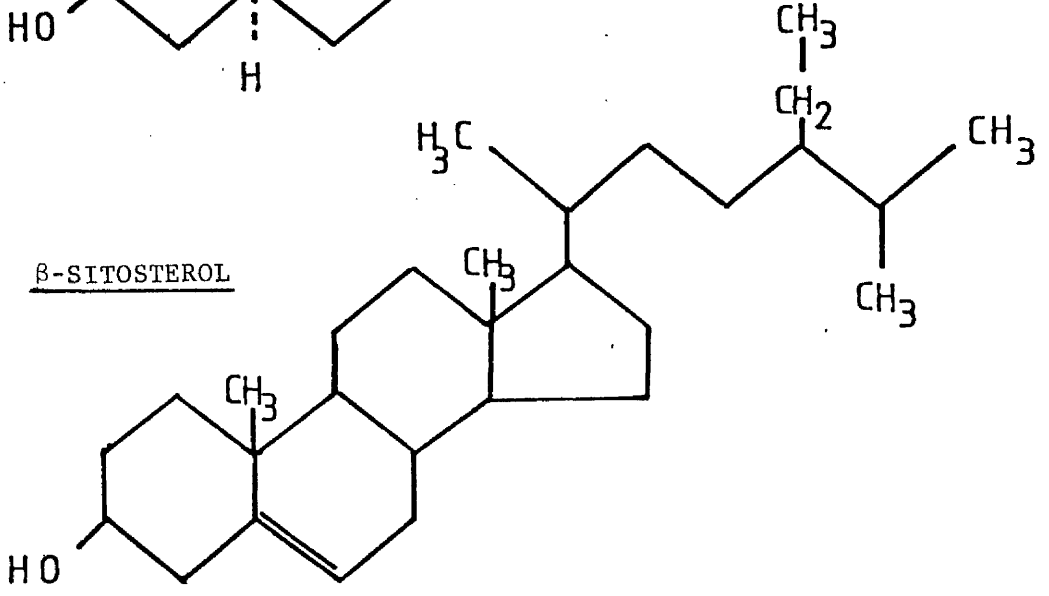
CHOLESTEROL



DIHYDROCHOLESTEROL



β-SITOSTEROL



G. Protection against B. pertussis infection

1. Protective antigen

Of great interest is the nature of the antigen(s) that protects mice against i.c. challenge. The laboratory assay for i.c. protection has proved to be the most reliable indicator of the protective value of whooping cough vaccines in man (Standfast & Dolby, 1961). Standfast (1958) showed that the lethal i.n. protective factor was different from the i.c. factor, but Andersen & Bentzon (1958) found the protective potency of vaccines as assayed using a sublethal i.n. challenge were similar to i.c. protective potencies.

Many attempts have been made to purify the protective antigen with only partial success (Billaudelle et al, 1960; Barta, 1963; Munoz & Hestikin, 1963; Sato & Nagase, 1967; Robinson & Manchee, 1978). Pittman (1979) proposed a protective role for the F-HA, and suggested that pertussigen is an exotoxic factor, responsible for many of the disease symptoms of whooping cough, and therefore antibodies against pertussigen will also be protective. Controversy exists as to whether pertussigen is a protective antigen and whether or not another non-toxic protective antigen exists. Pertussigen (as indicated by the presence of one of its activities) and PA have been shown to be present in similar quantities in different strains of B. pertussis and in more purified fractions (Pittman, 1951; Maitland et al, 1955; Munoz & Hestikin, 1963; Pieroni et al, 1965) but several observations indicate they are not identical. Though HSF activity may parallel that of PA (Joo & Pusytai, 1960) the response of mice to pertussigen as measured by histamine sensitisation and protection against i.c. challenge is distinguishable (Fishel, 1956). Nagel (1967) claimed separation of the two active components.

There are reports which claim protective activity associated with purified pertussigen preparations (Munoz et al, 1981; Sato et al, 1981) and also with F-HA preparations (Sato et al, 1979; Irons & MacLennan, 1979a). These reports indicate that there is not one PA but at least two proteins which can protect against B. pertussis infections in mice.

Pertussigen as a potential protective factor has been discussed earlier. In the following sections (a) the stimulation of a local immune response possibly an antiadhesion response, and (b) antiadhesion or anti F-HA as a potential protective factor will be discussed.

2. Local immune response induced by B. pertussis

Cheers and Gray suggest a role for cellular immunity in the recovery of mice from lung infections (Cheers, 1969; Cheers & Gray, 1969; Gray & Cheers, 1967; Gray & Cheers, 1969). They found an initial rapid multiplication of organisms followed by a decline in viable organisms to about 1% of this level after invasion by phagocytic granulocytes. At this stage bacteria were found within alveolar macrophages. Many of the bacteria survived within the phagocytes with the net result that steady state was reached and the number of viable organisms neither increased nor decreased (complaisant phase). To eradicate all the organisms, a protective humoral response was necessary and it would seem that the final cure and subsequent immunity was associated with a humoral response.

The role of local immunity, secretory IgA and IgG, in the mucous membrane seems to be important. Holt (1972) suggested a locally produced IgA response prevented the adhesion of organisms to the mucous membrane, this being the type of immunity the infection itself induces whereas parenteral vaccination induced a separate type of immunity, involving polymorphonuclear leukocytes and an opsin. Geller & Pittman

(1973) followed the production of different classes of antibody in serum and nasal secretions during the course of a sublethal i.n. infection in mice. B. pertussis specific IgG and IgA (but no IgM) appeared in respiratory tract secretions by day 15 and increased with time. IgA was the most abundant antibody present. In serum the most significant observation was the rise in serum IgA in i.n. infected mice after 30 days. However their study does not clarify how parenterally administered pertussis vaccine prevents the development of whooping cough. They propose that trace amounts of immunoglobulins in respiratory tract secretions of some intraperitoneally vaccinated mice suggest that serum immunoglobulins may pass the lung barrier. In a recent report using an enzyme-linked immunosorbent assay (Goodman et al, 1981) antipertussis IgA was detected in secretions during natural human infection usually appearing during week 2 or 3 of illness. It was most frequently found in patients from whom the organisms could no longer be recovered.

IgA is important in protection against other pathogens. Adinolfi et al (1966) found that antibody to E. coli in colostrum was mainly IgA. The serum and cervicovaginal secretions of women with gonococcal cervitis contained IgA antibodies to N. gonorrhoeae (O'Reilly et al, 1976). Secretory IgA and IgG prevented adhesion of E. coli to human urinary tract epithelial cells (Svanborg-Eden & Svennerholm, 1978). In studies with V. cholerae, protection by secretory IgA was associated with decreased bacterial attachment to intestinal mucosa (Fubara & Freter, 1973) and saliva containing IgA reduced the adhesion of streptococci to the lining of the oral cavity (Williams & Gibbons, 1972).

Parenterally administered vaccines can elicit a secretory response but the levels of local antibody are usually low and a much more pronounced antigenic stimulus can be achieved by direct application of a vaccine on the mucosal surface (Perkins et al, 1969). This was illustrated

in pertussis infections by North & Anderson (1942) who showed that i.n. vaccination of mice gave better immunity to i.n. infection than i.p. or subcutaneous vaccination.

3. Role of fimbriae or HA's in protection

In infections where fimbriae or fimbriae-like appendages have a role in the pathogenesis, a degree of protection has been elicited with fimbrial vaccines (Buchanan et al, 1977; Brinton et al, 1978). Fimbriae vaccines of N. gonorrhoea have induced some protection, although the antibody response was only weakly bactericidal. The maximum inhibition of attachment occurred when the antibodies were directed against fimbriae antigenically identical to the fimbriae mediating the attachment (Buchanan et al, 1977; Brinton et al, 1978). With E. coli intestinal infections of animals, purified fimbriae have been used as successful vaccines. Jones & Rutter (1974) and Rutter et al (1976) showed that piglets suckled by dams vaccinated into the mammary glands with purified K88 fimbriae were significantly more resistant to lethal neonatal diarrhoea after challenge with K88-positive enteropathogenic E. coli. Similar work using colostral transfer of antibodies induced by purified fimbriae to protect against E. coli infection has been done with K99 and 987 fimbriae in calves (Sojka et al, 1978; Nagy et al, 1978; Morgan et al, 1978).

With B. pertussis, Keogh & North (1948) found that protective potency of sera given i.p. against i.n. infection was directly related to their anti-HA titres. Masry (1952) using a more highly purified preparation of HA absorbed to chicken erythrocytes as vaccine found little or no protection against i.c. infection when compared with a control whole cell vaccine. Pillemer (1950) prepared an antigen, absorbed onto

red blood cell stromata, from B. pertussis which was protective but not identical with the HA although it was later shown to have histamine sensitising activity (Pillemer et al, 1954). Sato (1974) showed that red cell stromata added to a sonic extract of strain 134 by Pillemer's method (1950) absorbed LPF, HSF and HA activities suggesting that Pillemer's stromata-absorbed protective antigen was formed by adsorption of these factors onto the stromata. Sato et al (1974) with a formalin treated preparation of pertussigen and F-HA showed both active and passive protection in mice against i.c. challenge. In 1976 Arai & Sato separated the two components, and subsequently showed that the protection against i.c. challenge was associated with the F-HA (Sato et al, 1979). Sato et al (1979) also showed that anti F-HA antibodies prevented attachment of B. pertussis to HeLa cells. Munoz et al (1981) found no protective activity against i.c. challenge with their preparation of F-HA which was free of detectable pertussigen activity. However their pertussigen preparation was protective. Sato et al (1981) showed passive protection in mice against a sublethal i.n. challenge with both antiserum to pertussigen and to the F-HA. Further work is required to determine whether or not the F-HA is a protective antigen, and the effects of purification methods on protective properties. If the F-HA is protective it is likely to act by preventing adhesion and therefore colonization of B. pertussis in the respiratory tract. To inhibit adhesion at the mucosal surface an antibody response at the lung surfaces would be required.

OBJECTS OF RESEARCH

It is generally accepted that an important early event in B. pertussis infection of the mammalian respiratory tract is the adhesion of the bacteria to the ciliated epithelium. However the bacterial components involved in this adhesion have not been identified with certainty, although the F-HA is believed to play a role.

The primary object of this investigation was to explore this possibility by (a) studying the involvement of the F-HA in B. pertussis adhesion and virulence,

(b) testing the ability of a purified F-HA vaccine to protect mice against B. pertussis infection given by the i.n. and i.c. routes,

and (c) examining the effect of an F-HA inhibitor on the adhesion and virulence of the organism.

A second objective was to compare the hydrophobicity of virulent and avirulent B. pertussis, and also fimbriated and non-fimbriated virulent cells, to determine possible correlations.

Before investigating the above, it was necessary to study the influence of cultural conditions on the production of F-HA so as to obtain B. pertussis cells with different amounts of this component.

MATERIALS AND METHODS

A. Growth and cultivation

1. Strains

Several freshly isolated strains of B. pertussis were used viz: 77/18319, 77/19110, 77/35296, 77/25171 all supplied as fresh isolates from cases of pertussis by Dr. R. Fallon (Ruchill Hospital, Glasgow). Strain 18323 was the mouse virulent strain used in the mouse protection test (Kendrick et al, 1947) and was supplied by Dr. F. Sheffield (National Institute for Biological Standardisation, London). Strain Taberman, an isolate from postmortem material was supplied by Prof. G.T. Stewart (Ruchill Hospital, Glasgow). Strains D30042 phase I and D30042 phase IV were obtained from Dr. J. Dolby (Clinical Research Centre, Harrow, London), and strains 44122/7S and 44122/7R were from Dr. P. Branefors (Dept. of Bacteriology, University of Goteborg, Goteborg, Sweden). Strain 44122/7 transformant X was supplied by Mr. Salah al Salami of the Dept. of Microbiology, University of Glasgow. Intracerebrally, strain 44122/7S was avirulent whereas strains 44122/7R and 44122/7 transformant X were virulent.

2. Media

Bordet-Gengou (B-G) medium (Gibco Biocult Ltd) supplemented with 20% (v/v) horse blood (Gibco Biocult). The liquid media were (i) X mode "Hornibrook's medium", a modification of that described by Hornibrook (1939) as used by Wardlaw et al (1976), (ii) a modification of Hornibrook (1939) as used by Sato et al (1974). Both these media were used to grow X mode cells; C mode cells (Lacey, 1960) were grown by replacing the NaCl with an equal amount of $MgSO_4$. The compositions of the media are in Appendix I.

3. Growth and storage

Freeze dried organisms were reconstituted from ampoules and plated onto B-G agar plates. After about five days incubation at 35°C in plastic boxes, the resultant confluent growth was resuspended in a 1% (w/v) casamino acids (Difco) diluent (CAD) pH 7 (Appendix 2) containing 10% (v/v) glycerol and stored as a cell suspension at -70°C. These cell suspensions were used for subsequent inoculations of B-G plates when required.

Inocula for flasks of liquid media were prepared by resuspending the growth from 24 h B-G plates in a small volume of the liquid medium. Static cultures were grown in shallow layers of medium in Roux bottles (100 ml) or in Erlenmeyer flasks (400 ml of medium per 2 litre flask, 50 ml per 250 ml flask). The flasks were incubated at 35°C for 4 to 5 days without shaking. Shake cultures were grown using similar volumes to static cultures but in dimpled Erlenmeyer flasks at 35°C on an orbital shaker at 100 cycles min^{-1} for up to 72 h. Liquid cultures were harvested by centrifuging at 10,000 g for 30 min at 4°C, and the pellet resuspended in either CAD or CAD plus 10% (v/v) glycerol when low temperature storage was required.

4. Standardisation of bacterial suspensions

Bacteria for infection experiments and adhesion assays were resuspended in CAD to 10 opacity units (ou), the same opacity (as judged by eye) as the International Opacity Standard (W.H.O. International Lab. for Biol. Standards, Nat. Inst. for Biological Standardisation and Control, Holly Hill, Hampstead, London). Suspensions of 10 ou are equivalent approximately to a total count of 10^{10} bacteria ml^{-1}. Suspensions were further diluted depending on the number of viable

organisms required. For some HA assays more dense bacterial suspensions were used which were standardised by diluting a sample to 10 ou and correcting the original suspension to a standard number of ou.

B. HA activity

1. HA assay

Most of the HA assays were made in a Cooke microtitre system (Cooke Engineering Ltd.). To each well in a row except the first was added 0.05 ml of phosphate buffered saline (PBS, Appendix 2). The putative haemagglutinin was added to the first two wells in each row (0.05 ml into each well) and doubling dilutions made from the second well in each row with microtitre diluters. After addition of 0.05 ml of a 2% red blood cell suspension (usually horse red cells unless stated otherwise), the contents of the wells were mixed and the trays incubated for 1 to 2 h at 37°C or left to settle for several hours at 4°C. The HA titre was recorded as the reciprocal of the last dilution giving complete HA. To distinguish between HA caused by F-HA and by pertussigen, HA assays were done in 50 mm x 13 mm tubes, as recommended by Irons & MacLennan (1979a), using ten fold in volumes of reagents. F-HA induced HA was completely dispersed on gentle tapping of the tubes whereas that caused by pertussigen remained in clumps even when quite vigorously shaken.

2. Inhibition of HA

Two methods were used, the first involved mixing the inhibitor (either cholesterol or haptoglobin) with the HA and then proceeding with the HA assay as described above and measuring inhibition as the reduction in titre. The second method was by titration of the inhibitor, *B. pertussis* antisera, and adding a constant amount of HA. This was done

by adding PBS 0.05 ml to each well except the first, inhibitor 0.05 ml was added to the first two wells in each row and doubling dilutions done along the rows from the second well. Eight HA units (HA suspension with HA titre of 8) were mixed with the contents of each well and the trays left at room temperature for 30 min. After this time, 2% horse red cells 0.05 ml were added to each well and the trays incubated as in the HA assay. The highest dilution of inhibitor to show complete inhibition of HA was the HA inhibition titre (HAI titre).

3. Cholesterol dispersions

Aqueous dispersions of cholesterol for use in the HAI tests were made as follows: 25 mg of cholesterol dissolved in 5 ml of acetone was added to 25 ml of rapidly stirred distilled water heated at about 98°C on a hot plate/stirrer. Once the acetone had completely evaporated the dispersion was filtered through glass wool to remove large crystals. Fresh dispersions were always used.

4. Blending to remove F-HA

In order to remove fimbriae from B. pertussis cells 5 day static cultures were treated in an MSE blender at 14,000 rpm at 0°C for 2 min. The cells (10 ml of a suspension of 50 ou) are blended in a square bottomed universal attachment. Blending has been used to remove fimbriae from E. coli (Novotny et al, 1969). After blending, the cells were washed in CAD by centrifugation at 10,000 g and resuspended in CAD. Viability and HA titres of the cells before and after blending were compared.

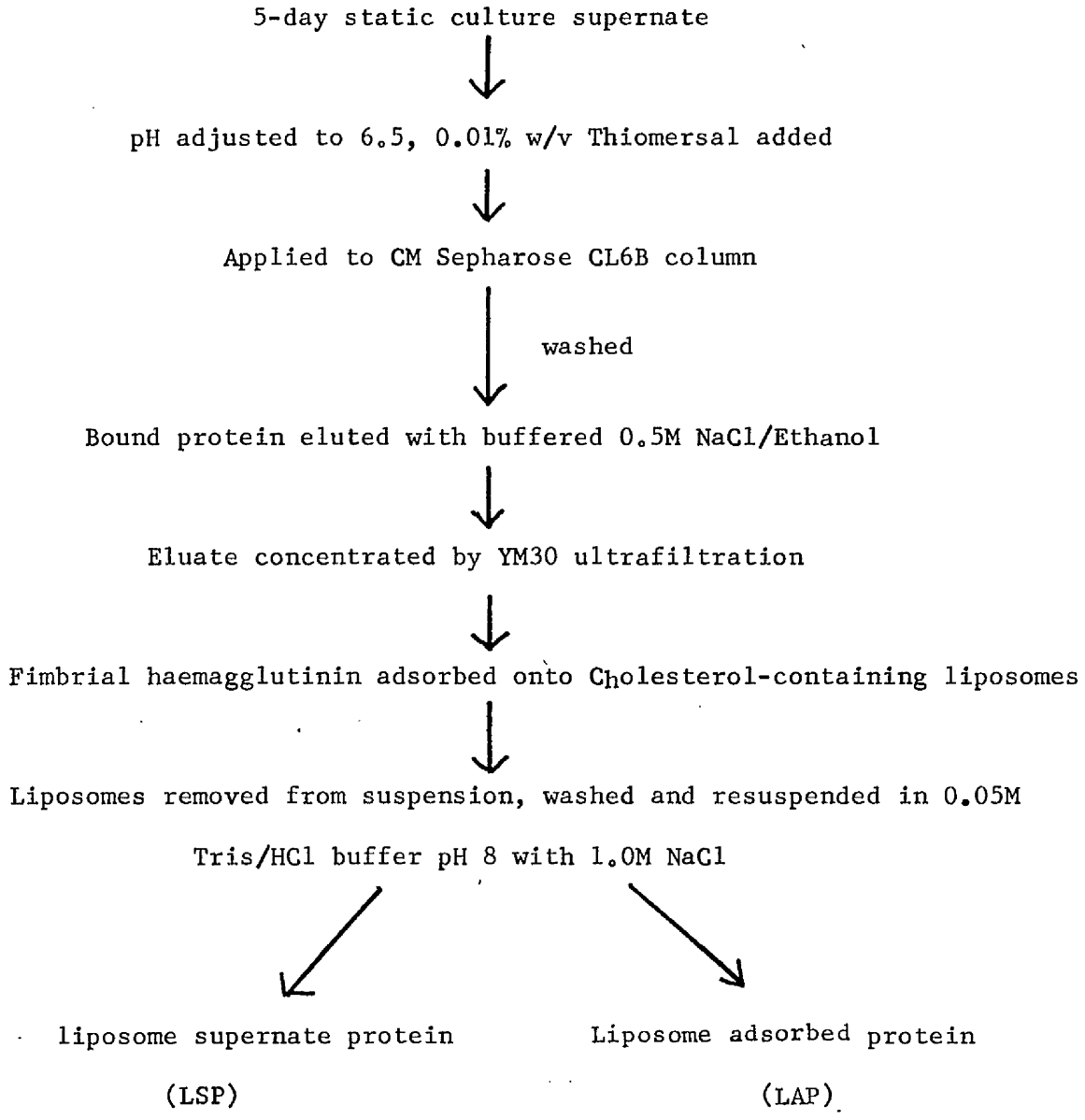
C. Purification of the F-HA

1. Purification procedure

Five litres of B. pertussis strain 77/18319 were grown in static liquid culture as used by Sato et al (1974) (Appendix 1). After centrifugation at 10,000 g for 30 min at 4°C the pH of the culture supernatant fraction was adjusted with glacial acetic acid to pH 6.5 and thiomersal added to a final concentration of 0.01% (w/v). The total volume of fluid was passed through CM Sepharose CL6B ion exchange resin (Pharmacia) in a column (2 x 40 cm) previously equilibrated with 20 mM sodium phosphate buffer pH 6.5. The flow rate was approximately 60 ml h^{-1}. Next, the gel column was washed with approximately 1 litre of buffer to remove unbound material. The retained protein was eluted with 0.5M NaCl in 20 mM phosphate buffer pH 6.5 containing 5% v/v ethanol (Arai & Munoz, 1979a), and concentrated by ultrafiltration (Amicon Ultrafiltration Cell Model 52) with a YM30 hydrophobic membrane (Amicon). The concentrated protein (PEI) (approximately 200 μg ml^{-1}) was mixed with equal volumes of a liposome suspension (Irons & MacLennan, 1979a) and allowed to stand overnight at 4°C. The suspension was then centrifuged at 26,000 g for 1 h at 4°C which yielded a firm white pellet containing the lipid and adsorbed HA. The pellet was resuspended in 50 mM Tris/HCl buffer 1.0M NaCl to half the volume of PEI added.

2. Liposome preparation

Liposomes were prepared as described by Irons & MacLennan (1979a). Chloroform solutions of egg yolk lecithin (28.8 mg, gift from Dr. J.H. Freer), dicetyl phosphate (3.18 mg, Sigma) and cholesterol (11.4 mg, Fisons) were dried as a thin film in a 100 ml round bottomed flask on the rotary evaporator (Rotavapor-R, Buchi). The dried lipids were resuspended by vigorous hand shaking in 8 ml of 50 mM Tris/HCl buffer pH 8 with 1.0M NaCl and allowed to swell at room temperature for 5 h with intermittent shaking. After 5 h, 8 ml of the PEI (200 μg ml^{-1}) was added to the liposome suspension.


5-day static culture supernate
pH adjusted to 6.5, 0.01% w/v Thiomersal added
Applied to CM Sepharose CL6B column
washed
Bound protein eluted with buffered 0.5M NaCl/Ethanol
Eluate concentrated by YM30 ultrafiltration
Fimbrial haemagglutinin adsorbed onto Cholesterol-containing liposomes
Liposomes removed from suspension, washed and resuspended in 0.05M
Tris/HCl buffer pH 8 with 1.0M NaCl
liposome supernate protein
(LSP)
Liposome adsorbed protein
(LAP)

3. Polyacrylamide gel electrophoresis

The method used was based on that of Laemmli (1970) as modified by Ames (1974). Electrophoresis was done in the presence of sodium dodecyl sulphate (SDS). Separating and stacking gels contained respectively 11% (w/v) and 5% (w/v) acrylamide.

Both the gels and the buffers contained 0.1% w/v SDS. The sample was prepared by mixing 1 volume of sample with 1 volume of solubilizing buffer (Appendix 3) and heating at 100°C for 5 min. Samples of 50 or 100 µl were added to each track in the gel. The buffers and gels are described in Appendix 3.

4. Protein estimation

Protein estimations were done by the method of Lowry et al (1951) using Bovine Serum Albumin (Sigma) as the protein standards. Whole cell protein was estimated using a derivation of the Lowry method described by Herbert et al (1971).

5. Assay for HSF activity

The component of B. pertussis called pertussigen (or pertussis toxin) is thought to be one of the toxic factors of pertussis vaccines (Pittman, 1979). Contaminating pertussigen may be responsible for the protective activity of F-HA preparations (Munoz et al, 1981). For these reasons the histamine sensitising activity (which is a property of pertussigen) was estimated for fractions obtained in the purification procedure. Groups of 5 HAM/ICR mice of 7 weeks or older were used (under 7 weeks of age mice of this strain are much less responsive to HSF). Animals in each group were injected intraperitoneally (i.p.) with 0.5 ml of one of a series of graded doses of one of three fractions from the purification procedure, PEI, LAP and the LSP.

Five days later each mouse was injected i.p. with 3 mg of histamine dihydrochloride (Sigma) contained in 0.5 ml of saline. Survivors were counted 3 h after this challenge. The HSD_{50} dose was calculated as described in section J.

D. Antiserum production

1. Antiserum against whole cells

A five day static broth culture of B. pertussis 77/18319 was harvested, washed and resuspended in saline to approximately 100 ou. The bacterial suspension was mixed with an equal volume of Freund's incomplete adjuvant until a stable water in oil emulsion was obtained. The emulsion (1 ml) was injected into the muscle of each hind limb of a rabbit and on the fourth week 1 ml of the same antigen mixture was administered by i.p. injection. The rabbit was bled from the ear on the sixth week and the serum titrated for agglutinins. Two further i.p. injections were given at four weekly intervals before the agglutination titre reached 8,000 when the rabbit was bled out by cardiac puncture. The blood was allowed to clot for 1 h at room temperature and then stored at $4^{o}C$ overnight before the clear serum was collected and stored at $-20^{o}C$.

2. Bacterial agglutination test

The method used was based on that of Preston (1970). Using the microtitre system, serial dilutions of serum were made in PBS as described for HA tests. To each well, 0.05 ml of a bacterial suspension (30 ou ml) was added and the trays shaken at room temperature at 200 rpm on an orbital shaker for 5 min. The trays were then heated at $55^{o}C$ for 30 min which allowed the trays to be read.

immediately. The titre was recorded as the reciprocal of the highest dilution of serum to cause complete bacterial agglutination.

3. Antiserum to the F-HA

LAP (see purification procedure, p. 56) was mixed with an equal volume of Freund's incomplete adjuvant to form a stable water in oil emulsion. The emulsion (0.1 ml) was injected intradermally into each side of the rabbit above the scapula at two weekly intervals until each animal had received four pairs of injections. During the period of immunisation, HAI titres were determined from test bleedings taken from the marginal ear veins. Eight days after the final injections the rabbits were bled out by cardiac puncture and serum collected as described above.

4. Ouchterlony double diffusion in gels

The medium used for gel diffusion consisted of 1% agarose in 50 mM Tris/HCl buffer pH 8 with 1.0M NaCl (Munoz et al, 1981). The molten agar (20 ml) was poured into scratch free glass plates and allowed to harden and dry. The template was then cut which consisted of 5 mm wells 5 mm apart, 1 drop of molten medium was then added to each well to prevent leakage of reagents under the agar layer. Antiserum was added to the outer wells and incubated at 37°C for 30 min. The F-HA which was the antigen used was sonicated in an MSE ultrasonic disintegrator at an amplitude of 7 peak to peak microns for a total time of 2 min in 20 sec bursts. The F-HA was kept on ice during the sonication. Immediately after sonication the antigen was added to the centre well and the plates incubated at 37°C for 24 h after which time precipitin lines were obtained. The F-HA was sonicated to disperse aggregates which tend to form in the suspension giving diffuse precipitin lines in the gel.

E. Mouse infection and protection tests

1. Sublethal intranasal infection

Bacterial suspensions were diluted to approximately 10^7 colony forming units (cfu) ml^{-1}. In the case of five day static cultures this required a 1/10 or 1/100 dilution of a 10 ou suspension. Usually both dilutions were used. Mice of either sex from a randomly bred closed colony originally derived from the Ham/ICR strain (Charles River, U.K. Ltd.), were anaesthetised with ether. Two drops of bacterial suspension (0.05 ml containing approximately 10^5 viable organisms) were placed on the nose of the mouse and the mouse inhaled the inoculum. On day 14 after the instillation of bacteria the mice were sacrificed, and the lungs examined for pathological changes and samples taken for culture of B. pertussis. The lung pathology was scored on a scale of 0 to 4, 0 being no gross change in lung appearance, 4 being almost complete consolidation with all the lobes infected. A small piece of lung from the middle lobe on the left side was removed from each mouse and spread onto a B-G plate. As with the pathology, the lung culture was scored on a scale of 0 to 4, 0 being negative, 4 being confluent growth.

2. Passive protection against sublethal i.n. infection

Passive protective properties of antiserum against sublethal i.n. challenge and inhibition of infection by cholesterol was examined by mixing the antiserum or cholesterol dispersion with the challenge dose 30 min before infecting the mice. The infection was then carried out as above.

3. Intracerebral infection

To titrate i.c. virulence, challenge suspensions

were made by suspending cells of B. pertussis strain 18323 in CAD to 10 ou and dilutions made (10^4, 10^5, 5×10^5, 2.5×10^6) to give suspensions with approximately 10^4 cfu, 10^3 cfu, 200 cfu and 40 cfu in 0.03 ml. Each of the four dilutions were injected by the i.c. route into groups of 10 mice, viable counts were also done on the suspensions. The injection technique involved anaesthetising the mice with ether, and using a fine needle (26 g 3/8 inch), 0.03 ml of suspension was injected through the parietal bone into the brain slightly to the right of the mid line and about midway between the eye and the ear. The mice were allowed to recover from the anaesthetic and inspected daily for the next 14 days. Dead animals were removed and those showing unsteady gait or lethargy were killed. At the end of fourteen days the number of survivors in each group was noted and LD_{50} values calculated.

4. Passive protection against i.c. challenge

As with the passive protection against i.n. challenges, the highest i.c. challenge dose (10^4 cfu) was mixed with antiserum before the mice were injected. The infections were done as described previously. In each experiment the challenge was titrated using the dilutions described above.

5. Active protection against i.c. infection

The method used was a modification of the method described by Kendrick et al (1947). Groups of three week old mice were vaccinated i.p. with one of a series of graded doses of one of three vaccines tested. The vaccines tested were the Glasgow standard vaccine which is a whole cell vaccine, LAP and the LSP (p.56). Two weeks after vaccination the mice were challenged with approximately 200 LD_{50s} (10^4 cfu)

of 18323 the i.c. challenge strain. The PD_{50} values of the vaccines were calculated as described in section J.

F. Adhesion of bacteria to mouse lung

1. Assay by viable counting

Mice used for the adhesion assays were killed immediately before use by cervical dislocation, dipped in disinfectant and pinned out on a board. The rib cage was opened to expose and allow expansion of the lungs. The trachea was carefully exposed by dissection, taking care not to cause bleeding and a small incision was made to allow insertion of a cannula through which 0.5 ml of bacterial suspension was instilled into the lungs. The cannula attached to 1 or 2 ml plastic syringes consisted of small tapered pieces of teflon tubing which fitted into the trachea but which were wide enough to seal the upper part of the trachea so that the instilled fluid did not escape via the mouth. After the suspension had been in the lungs for the stipulated time (30 min in most cases), as much of the fluid as possible was removed from the lungs via the cannula. The lungs were then washed out *in situ* with 2 x 1 ml volumes of sterile CAD before being removed for further processing.

The percentage of the inoculated organisms retained by the lungs was determined by viable counts on the lungs after homogenisation. The excised lungs were washed in sterile CAD and then homogenised for 30 sec in 10 ml of CAD in a Silverson multipurpose mixer-emulsifier. The homogeniser blade assembly was sterilised before the start of the experiment and immersed in boiling water for 2 min followed by rinsing in cold distilled water after processing each set of lungs in a single experiment. This sterilization procedure was devised to prevent

carryover of organisms from lung to lung. Viable counts were done on dilutions of the whole lung homogenates and derived values were expressed as "percent of the original inoculum".

2. Assay with radiolabelled bacteria

The bacteria used in these experiments were grown in the medium described by Sato & Arai (1974) as previously described but containing 37 k Bq (^{14}C)-glutamic acid (Radiochemical Centre, Amersham, England) for 10 ml of medium. The radiolabelled organisms were used in the adhesion assay as with nonlabelled bacteria except that the excised lungs were put directly into 3 ml of Soluene 350 (Packard) and heated at 50°C for 2 to 4 h to dissolve them. After cooling to room temperature each solution was decolourised with 0.6 ml of propan-2-ol followed by 0.6 ml of hydrogen peroxide. After gentle shaking, the samples were left at room temperature for 15 min and then heated at 40°C for 15 min followed by cooling to room temperature. Scintillant (10 ml) was added to each sample before counting. Duplicate samples of the inocula were counted (approximately 2000 counts), and to measure recovery, the radioactivity in the suspensions removed from the lungs and the lung washings was also determined.

3. Variables in the adhesion assays

(i) Time - The relationship between time of exposure and extent of retention was determined by leaving the bacteria in contact with the lungs for times up to 1 h.

(ii) pH - pH 7 was the pH used in all the assays but the effect of varying the pH on retention was examined by suspending the bacteria in CAD at pH 6.5, pH 7.0 and pH 8.0. These other pHs were chosen because at pH 6.5 the F-HA adheres to a cation exchanger (Arai & Munoz, 1979a)

and at pH 8.0 the F-HA is soluble in the presence of high salt concentrations.

(iii) Mouse sex - Groups of male and female mice were used in adhesion assays to see if sex would influence the result.

(iv) Inhibition of adhesion - The bacteria were mixed with whole cell antiserum, antiserum raised against the F-HA or with cholesterol dispersions which inhibit HA caused by F-HA. Each mixture was then used in the assays as before. To study the ability of vaccination to cause inhibition of B. pertussis adhesion to the mouse lung, mice were vaccinated with a standard vaccine (Glasgow Standard supplied by Prof. A.C. Wardlaw) i.n., i.p., or with F-HA both i.n. or i.p. To see if infection results in active protection against adhesion of bacteria, mice were given a sublethal i.n. infection and adhesion assays were done on the mice at various times after infection.

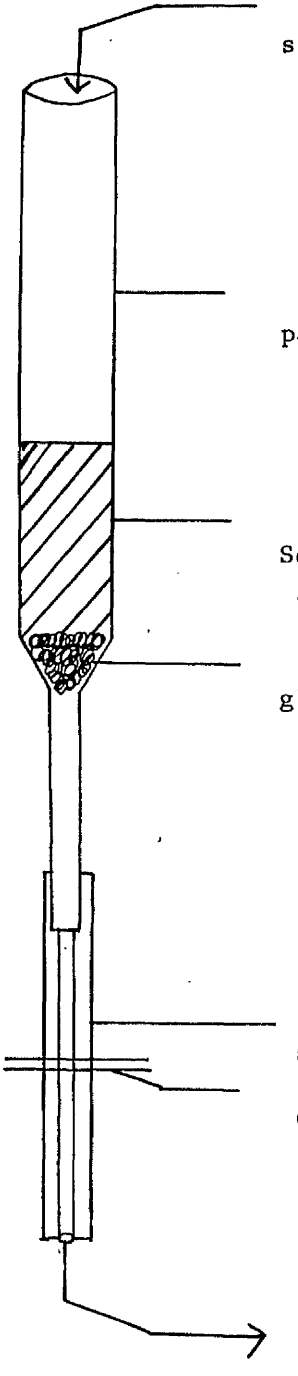
(v) Alteration of surface charge - A postulated role for fimbriae in bacterial adhesion to epithelial cells is to allow the repulsive effects of like negative charges on the bacterial and epithelial cell surfaces to be overcome. The effect of reduction of the net charge of avirulent cells of B. pertussis (C mode and phase IV cells) on adhesion was investigated. The method of Heckels et al (1976) was used to remove carboxyl groups from avirulent non-fimbriated gonococci. Heckels et al showed that the treatment to modify carboxyl groups reversed the original surface charge, increasing the pI value from 5.3 to 8.2. They also showed the change in surface charge using cationised ferritin which reveals acid groups as electron dense regions, ferritin coating being heavy on normal cells but scanty or absent on those with blocked carboxyl groups. The reagent used to modify carboxyl groups consisted of 500 mg of 1 ethyl-3-(dimethylaminopropyl)carbodiimide (EDC Sigma), 600 mg of methylamine hydrochloride (Sigma) and 5 mg of $MgCl_2H_20$ made up to 5 ml

with the pH adjusted to 4.5 with 1.0M HCl. A B. pertussis radiolabelled suspension was made in CAD to an optical density at A600 of approximately 1.0 and 5 ml of this suspension was added to the EDC solution. The mixture was stirred at 25°C and the pH readjusted to 4.5 with NaOH. When the pH stopped falling the suspension was stirred for a further 16 h before the bacteria were recovered by centrifugation and washed in CAD. The washed bacteria were resuspended in CAD and stored at -70°C.

G. Hydrophobic interaction chromatography

Surface hydrophobicity of B. pertussis strains was measured as described by Smyth et al (1978) but with minor changes. As with the adhesion assay, bacterial numbers were assessed by viable counting and by use of radiolabelled organisms. Suspensions of octyl sepharose (Pharmacia) were autoclaved and used sterile. Small columns were made by loading a pasteur pipette with 1 ml of the gel suspension (see Fig 4). The gel was washed in the pipette with 4M NaCl prior to addition of the bacteria (0.1 ml of 10 ou) which were allowed to drain into the gel. The column was then eluted with approximately 5.0 ml of 4.0M NaCl in 0.02M Na-Phosphate buffer (P.B.), pH 6.5, followed by 5.0 ml of 0.02M Na-P.B. pH 6.5.

When unlabelled organisms were used, the different eluates were collected and viable counts done on each as well as the starting material. If the bacteria were labelled then a sample of the material loaded onto the column was counted along with small volumes of each eluate. With the 0.02M P.B. 1 ml was counted in 10 ml of scintillant (see Appendix 4) but with the 4.0M NaCl eluate it was necessary to reduce the salt concentration before counting. Therefore 0.25 ml of eluate was diluted to 1.0 ml with distilled water before



sample and buffers added

pasteur pipette (I.D. = 5mm.)

Sepharose gel bed (approximately 0.6 ml gel bed volume)

glass wool

silicone rubber tubing

clamp

eluates collected and viable counts or radioactive counts obtained

addition of scintillant and counting. The percentage of the organisms retained on the gel was calculated for each strain.

H. Assay of radioactivity

All samples were counted for 10 min in a Packard Tri Carb 300 scintillation counter in 10 ml of Triton-Toluene scintillant (Appendix 4), which was aged for 10 h before use. Samples containing Soluene 350 were left in the dark for several hours before counting and counted until stable counts were obtained.

I. Electron microscopy

The specimen and the negative stain which was 2% ammonium molybdate were mixed before being applied to a copper grid (mesh size 400) coated with a formvar film 15-20 nm thick and the excess liquid was removed with a paper point. The specimens were examined in a Phillips 300 microscope. Whole cells were chromium shadowed at an angle of 45^{o} in an Edwards model E12 coating unit.

J. Statistical analysis

Results from HSF, i.c. virulence titrations and mouse i.c. protection tests were analysed by the probit method (Finney, 1952) using a computer programme developed in the Dept. of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada. One sample was used as a standard and the potencies with 95% confidence limits of other samples relative to the standard were estimated. The ED_{50} values of samples were also calculated.

Results from sublethal i.n. experiments and passive protection experiments were analysed for significance at the 5% or 1% probability levels using tables for testing significance in 2 x 2 contingency tables (Finney et al, 1963).

RESULTS

A. Growth and production of HA of B. pertussis in vitro

The first step was to determine the conditions of the HA assay to be used throughout. The results from different incubation temperatures and different blood types on HA titres of strains 77/18319 and 77/19110 are shown in Table 4. Incubation temperature did not affect the titre within the range tested (4°C to 37°C). Therefore conditions for the HA assays were arbitrally set at 37°C for 1 to 2 h by which time the red cells had settled. Rat red cells gave slightly higher titres than human or horse red cells but the marginal increase in sensitivity was not sufficient to justify the inconvenience of using them in place of the readily available horse erythrocytes.

1. Solid medium

Two solid media were commonly employed for growth of B. pertussis, B-G agar with added blood and charcoal agar. In a preliminary study several strains of B. pertussis were grown on B-G plates and charcoal plates and HA titres recorded. The results obtained with B-G cultures and charcoal cultures were similar (Table 5). There was no obvious pattern of HA as the time of incubation of the cultures increased, the titre varying from day to day. This was probably due to lack of standardisation of inoculum and therefore the stage of growth from plate to plate would be varied. No consistent differences were found among the 9 strains studied. Differences were obtained however with the large and small colony types discernable in strain 77/18319, the large colony type having a lower HA titre than the small colony type (Table 7).

2. Liquid medium

(i) Shaken cultures - Cells were grown in Erlenmeyer flasks which were

TABLE 4 : Effect of erythrocyte species and incubation temperature on HA titres of *B. pertussis* strain 77/18319[1]

Species of Red Cell	HA titre at incubation temp (°C)		
	4°C	25°C	37°C
Horse	8	8	8
Chicken	2	2	2
Man	4	4	4
Rat	ND	ND	16

[1]Strains 77/19110 and 77/18319 at 60 ou gave similar results

ND - not done

TABLE 5 : HA titres of different strains of *B. pertussis* grown on B-G medium

Strain	HA titre of cells harvested from B-G plates after (h)		
	24	48	72
77/24833	32	4	4
77/26621	16	ND	8
77/19110	32	ND	8
77/18319	16	8	4
77/35296	16	ND	8
77/25171	16	ND	16
18323	2	8	ND
Maeno	4	8	ND
134	2	8	ND

Cell suspensions of 120 ou were used.

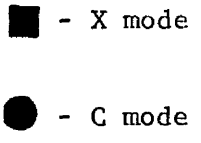
ND - not done

dimpled to increase aeration efficiency. Under these conditions cultures reached stationary phase in approximately 48 h (Fig 5). The inoculum in all cases consisted of 1.0 ml of a suspension of the growth from B-G plates resuspended in sterile CAD to approximately 40 ou. The HA titres in these liquid cultures fell quite quickly and by 48 h of incubation was reduced to < 1 in both the cells and the supernate (Fig 5). No significant differences in the HA titres of different strains of B. pertussis were detected (Table 6). However and large and small colony variants of 77/18319 retained their different HA properties when grown in liquid medium. Fimbriae were rarely seen on cells from shaken culture when they were examined by electron microscopy. PAGE of freeze dried shake culture supernate showed no, or very little evidence, of the high molecular weight bands associated with F-HA (Plate 1).

Cells of strain 77/18319 grown in C mode medium, where the NaCl was replaced by $MgSO_4$, had no HA activity associated with either cells or supernate at any point during growth. The growth curves obtained for X and C mode cells (Fig 5) are similar. However after 48 h, X mode cells tend to autoagglutinate which could be responsible for the subsequent reduction in adsorbance (A600) during stationary phase.

(ii) Static culture - In most purification procedures described for the F-HA, starting material came from static liquid culture. When cells of strain 77/18319 were grown in static culture for 5 days growth was obtained, although the adsorbance value for the stationary phase culture was only about half the value for stationary phase shaken cultures. Most of the growth in X mode cultures formed a surface pellicle or stuck to the glass of the culture vessel. In the electron microscope cells from these cultures showed fimbriae-like protrusions on the cell surface

Fig 5 : Growth and HA titres of X and C mode cells in shaken cultures.



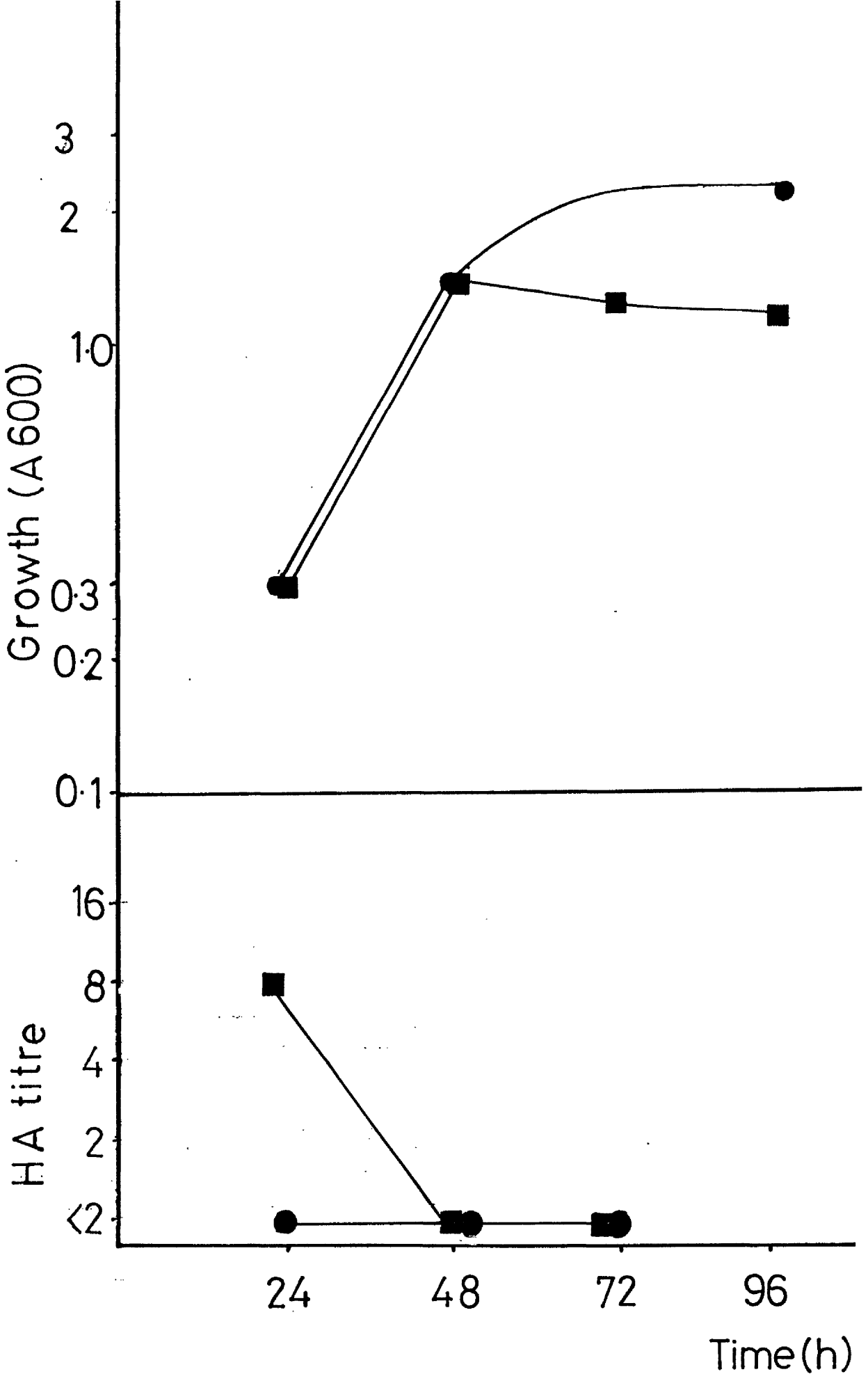

3
2
1·0
0·3
0·2
0·1
Growth (A 600)
16
8
4
2
<2
HA titre
24
48
72
96
Time (h)

TABLE 6 : HA titres of different strains of B. pertussis grown in shaken culture

Strain of B. pertussis	HA titre at 24 h
77/18319	16
18323	32
77/19110	16
77/25171	16
77/35296	32
77/24833	8

Cell suspensions were used at 240 ou

Plate 1 : SDS-PAGE, of freeze dried B. pertussis liquid culture supernates.

1 - X-mode 77/18319 static culture supernate

2 - X-mode 77/18319 shake culture supernate

3 - C-mode 77/18319 static culture supernate

4 - C-mode 77/18319 shake culture supernate

5 - X-mode 18323 static culture supernate

6 - C-mode 18323 static culture supernate

7 - partially purified F-HA

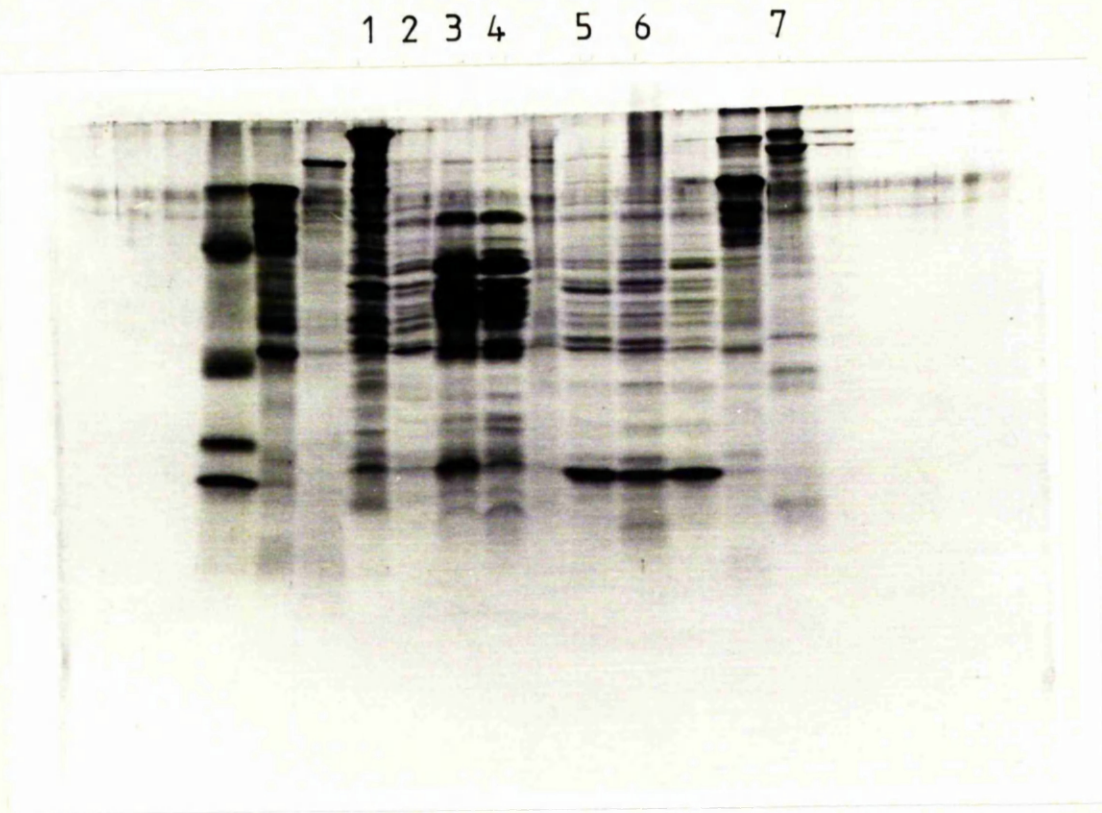

1 2 3 4 5 6 7

TABLE 7 : HA titres of large and small colony variants of strain 77/18319 grown under different conditions.

Culture	HA titre with colony variant	
	Large	Small
24 h B-G	< 2	16
18 h shake (Hornibrook X mode medium)	2	8
96 h static (Hornibrook X mode medium)	<1	8

(Plate 2). The HA titres of both the cells and the supernate from static cultures show an increase during the stationary phase between 72 and 120 h (Fig 6). Under C modulating conditions in static liquid culture, no HA was produced. The growth of C mode cells in static culture never reached the density of X mode cells and no surface pellicle was formed as it was with X mode cells. An HA positive culture supernate was found to have completely lost its HA activity after shaking for 12 h under the growth conditions of the shaken cultures. This indicates that F-HA may be produced in shaken culture but destroyed by the shaking.

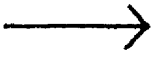
3. HAI by cholesterol

The final concentration of cholesterol used in these inhibition studies was approximately 100 μg/ml. The HA caused, by both cells and supernate of static cultures of strain 77/18319, was reduced when the same suspensions were titrated in the presence of cholesterol. With culture supernates, there was a 9/10 dilution of the original supernate after addition of cholesterol but this cannot account for the reduction in titre (Table 8). There was no significant reduction in the HA titres of either cells or supernate from 18 h shake cultures of strain 77/18319. There was no significant reduction in the HA titre of strain 18323 in the presence of cholesterol and as with shake cultures of strain 77/18319 PAGE of freeze dried culture supernates showed little evidence of the high molecular weight bands corresponding to the F-HA (Plate 1).

4. Removal of F-HA from the cell surface by blending

Blending cells from static liquid culture in an MSE blender at 14,000 rpm for 2 min caused a significant decrease in the HA titres of the cells after washing (Table 8). These cells while losing the ability to cause HA remained viable.

Plate 2 : Electron micrograph of chromium shadowed 5 day static cells of B. pertussis strain 77/18319 (Mag x 68,000)



Fimbriae

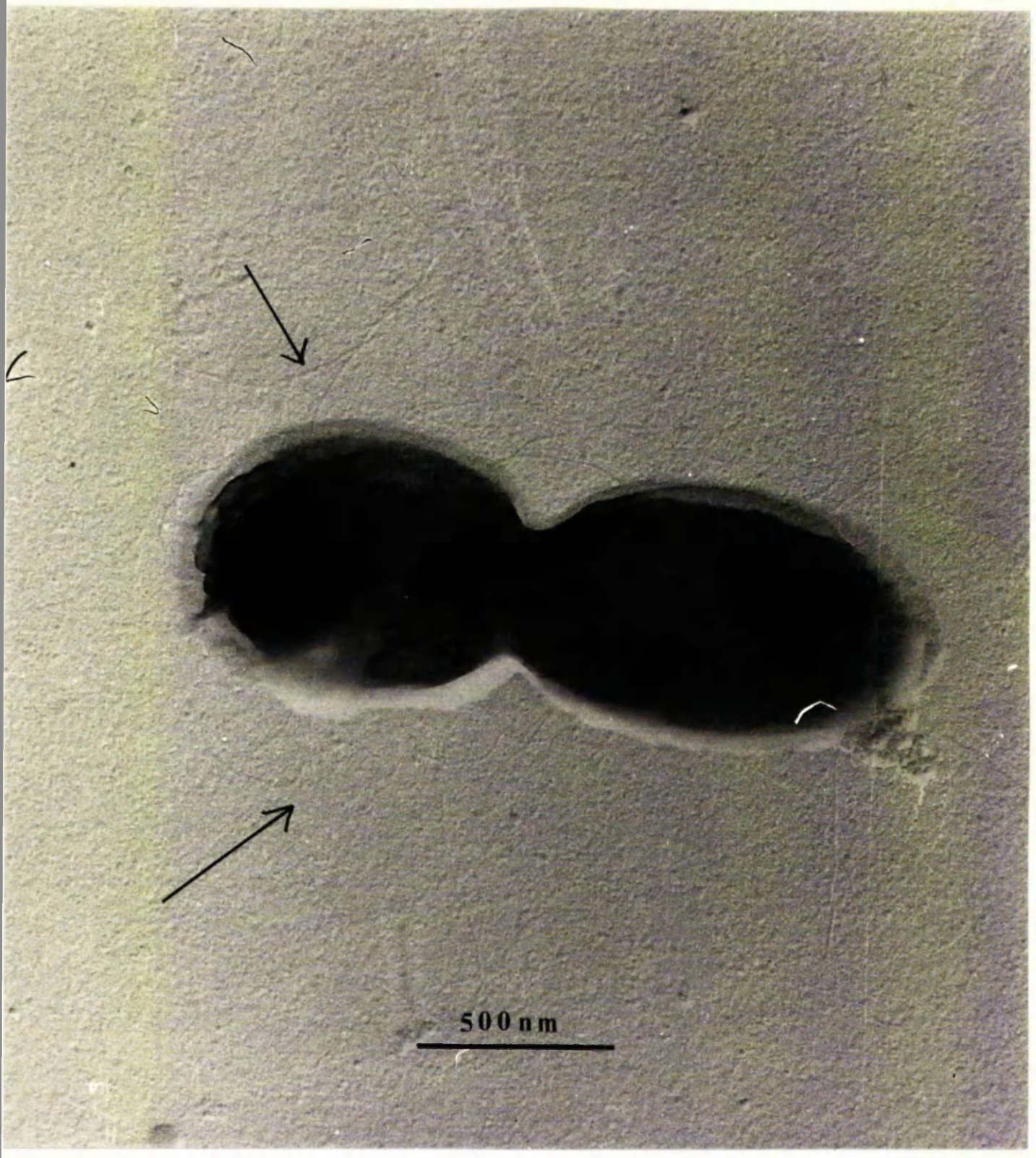

500 nm

Fig 6 : Growth and HA titres of X and C mode cells from static culture.

■ - X mode

● - C mode

HA titres calculated on cell suspensions of 120 ou.

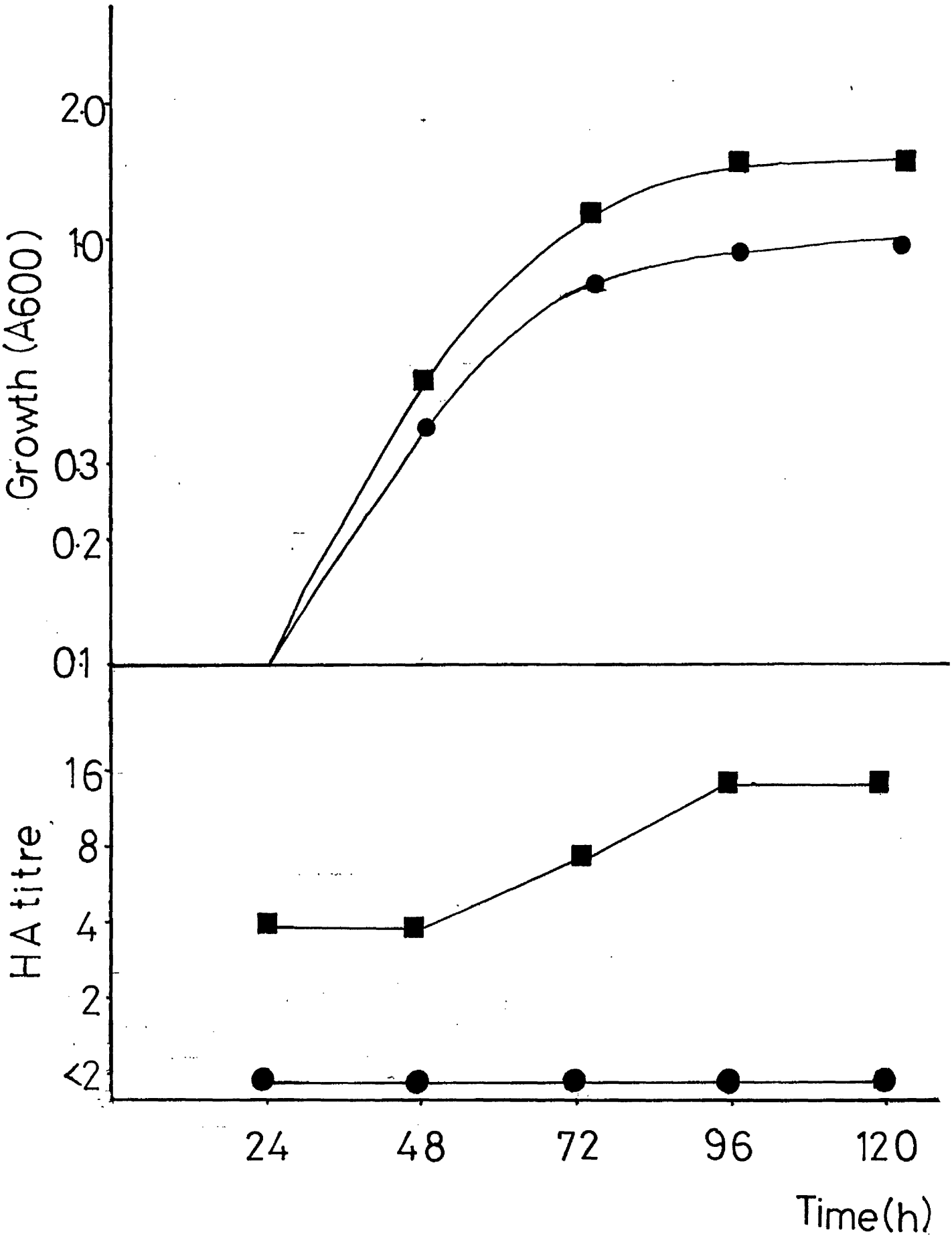

2·0
1·0
Growth (A600)
0·3
0·2
0·1
16
8
4
2
<2
HA titre
24
48
72
96
120
Time(h)

TABLE 8 : Effect of cholesterol on HA titres of static and shaken liquid cultures of strain 77/18319 and static cultures of strain 18323

Culture	Source of HA	HA titre with cholesterol	
		absent	present
120 h static	77/18319 cells	16	2
	77/18319 supernate	8	2
	77/18319 cells recovered after blending	1	<1
	18323 cells	32	16
	18323 supernate	2	1
18 h shake	77/18319 cells	8	4
	77/18319 supernate	2	1

Cell suspensions were used at 120 ou

B. Sublethal in. virulence of B. pertussis

The aim of these experiments was to assay the in. and ic. virulence of several strains of B. pertussis and to investigate the possible interrelationship with HA and adhesion ability. For this purpose the ic. virulent B. pertussis strains 44122/7R, 44122/7X and 18323 were used, with the ic. avirulent strains 44122/7S, 77/18319 and 18334 providing a contrast.

The assays showed that sublethal in. virulence of strains did not go hand in hand with the ic. virulence (Table 9). Strain 18323 although only tested in one experiment was less virulent by the in. route than any of the other strains tested except strain Taberman. With the variants of strain 44122/7 (44122/7R, 44122/7S and 44122/7X) which differed in ic. virulence, no difference was observed in their in. virulence. Strains 77/18319 and 18334 were as virulent by the in. route as the 44122/7 variants and more virulent than 18323 although they were both avirulent by the ic. route.

When the large and small colony variants of strain 77/18319 were tested for in. virulence the small colony type seemed as though it may be more virulent than the large type although the results showed no significant difference (Table 10).

1. Sublethal in. virulence of cells grown under different cultural conditions

HA activity was lost under C mode growth conditions, and when the cells were grown in shaken liquid cultures for 48 h (p 73). C mode cells, 48 h shake culture cells and 120 h static cells of strain 77/18319 were used in sublethal in. virulence experiments in an effort to determine the role of fimbriae in infection. The results from two

TABLE 9 : Sublethal in. virulence of different strains of B. pertussis

Strain	ic virulence	Inoculum cfu	Lung Pathology[1] in Expt No. 1	2	3	Total	Lung Culture[2] in Expt No. 1	2	3	Total
77/18319	-	10^5	5/10)	3/5)	3/5)			2/5)	4/5)	
			)12/20	)9/10	)8/10	26/40	ND	)6/10	)9/10	15/20
		10^6	7/10)	4/5)	5/5)			4/5)	5/5)	
18334	-	10^5			3/5)NS				4/5)NS	
			ND	ND	)7/10	7/10	ND	ND	)9/10	9/10
		10^6			4/5)				5/5)	
44122/75	-	10^5		3/5)NS	2/3)NS			5/5)NS	2/3)NS	
			ND	)7/10	)5/6	12/16	ND	)10/10	)5/6	15/16
		10^6		4/5)	3/3)			5/5)	3/3)	
Taberman	-	10^5			0/5) *				1/5)NS	
			ND	ND	)2/10	2/10	ND	ND	)4/10	4/10
		10^6			2/5)				3/5)	
18323	+	10^5	2/10) *							
			)5/20	ND	ND	5/20	ND	ND	ND	-
		10^6	3/10)							
44122/7R	+	10^5		4/5)NS	2/3)NS			4/5)NS	3/3)NS	
			ND	)8/10	)5/6	13/16	ND	)9/10	)6/6	15/16
		10^6		4/5)	3/3)			5/5)	3/3)	
44122/7X	+	10^5		2/5)NS	3/3)NS			2/5)NS	3/3)NS	
			ND	)7/10	)6/6	13/16	ND	)7/10	)6/6	13/16
		10^6		5/5)	3/3)			5/5)	3/3)	

ND - not done: 1 - number of mice showing greater than 2+ pathology/number of mice in group

2 - number of mice showing greater than 2+ culture/number of mice in group

NS - not significantly different than strain 77/18319

* - significantly different than strain 77/18319 at 5% probability level.
Although totals are given, significance of results given within experiments due to the differences in numbers in each total group.

TABLE 10 : Sublethal in, virulence of the large and small colony types of B. pertussis strain 77/18319

Colony Type	Inoculum cfu	Lung Pathology[1]	Total
Small	3×10^4	1/5	5/10
	3×10^5	4/5	
Large	3×10^4	1/5	$3/10^{NS}$
	3×10^5	2/5	

NS - no significant difference between large colony type and small colony type

1 - number of mice showing greater than 2+ pathology/number of mice in group

experiments are shown in Table 11. C mode cells were less virulent than the other types of cells tested. However in each group of mice at least one showed significant lung pathology. In experiments where infecting doses of around 10^7 cfu per mouse were used, some mice infected with C mode cells showed 3 and 4+ lung pathology. Static liquid culture cells and shake liquid culture cells were equally virulent, showing 3 and 4+ lung pathology with an inoculum of 10^5 to 10^6 cfu per mouse.

2. Effect of inhibitors and mechanical removal of fimbriae on mouse virulence of B. pertussis

Cholesterol inhibits HA by the F-HA of B. pertussis (Sato et al, 1979; Irons & MacLennan, 1979a). The HA activity of strain 77/18319 when grown in static culture was inhibited by cholesterol dispersions (section A.3). Irons & MacLennan (1979a) reported that the HA associated with pertussigen is sensitive to haptoglobin, a sialic acid containing glycoprotein. In early purification attempts made during this study, the F-HA and the pertussigen were partially separated on a sepharose 6B column, after the initial ion exchange column. Two peaks were obtained which both had HA activity. The protein from the first peak caused HA which was not sensitive to cholesterol. The red cell mat formed in this instance was very tight and when the tubes in which the assays were done were tapped, the mat of agglutinated red cells resuspended in clumps. Haptoglobin (supplied by Dr. L. Irons, Porton Down) inhibited the HA, and the remaining HA was as a fine mat which completely resuspended on tapping the tubes. The HA activity associated with the second peak was sensitive to cholesterol and the agglutinated red cells resuspended completely when the test tube was tapped. Since these inhibitors were specific for one type of HA they are proposed as receptors for these HAs on mammalian cells. These two inhibitors were

TABLE 11 : Sublethal in. virulence of B. pertussis strain 77/18319 grown in different ways

Culture of 77/18319	Inoculum cfu	Lung Pathology[1] in Expt No.			Lung Culture[2] in Expt No.		
		1	2	Total	1	2	Total
X-mode	10^5	3/5	3/5	6/10	3/5	4/5	7/10
static	10^6	4/5	5/5	9/10	4/5	5/5	9/10
	Total	7/10	8/10	15/20	7/10	9/10	16/20
C-mode	10^5	1/5	1/5	2/10	1/5	2/5	3/10
static	10^6	1/5	ND	1/5	1/5	ND	1/5
	Total	2/10	1/5	3/15**	2/10	2/5	4/15**
X-mode	10^5	4/5	4/5	8/10	3/5	4/5	7/10
shaken	10^6	4/5	5/5	9/10	5/5	5/5	10/10
	Total	8/10	9/10	17/20^{NS}	8/10	9/10	17/20^{NS}

1 - number of mice showing greater than 2+ lung pathology/the number of mice in group

2 - number of mice showing greater than 2+ lung culture/the number of mice in group

** - significantly different from X mode static cells at 1% probability level

NS - not significantly different from X mode static cells

therefore used in mouse virulence experiments to see if they might cause reduction of virulence *in vivo* as they do HA *in vitro*. Bacteria were mixed with the inhibitors and administered to the mice either intracerebrally (strain 18323) or intranasally (strain 77/18319) (Tables 12&13). Due to unavailability of purified haptoglobin, crude material (Cohn fraction IV from type 1 human plasma) was used as the inhibitor. Tables 12 and 13 show that Cohn IV inhibited both *in.* and *ic.* virulence but whether this was due to the haptoglobin content was not determined. However in experiment 4 in the *in.* series, purified haptoglobin was used with some inhibitory effect. Cholesterol was inactive in both types of infection.

Blending of cells from static liquid cultures reduced the HA properties of the cells without reducing viability (Section A.4). These blended cells were also used in sublethal *in.* experiments but the blended cells were no less virulent than those which had not been blended (Table 13).

C. Purification and protective properties of F-HA

Five day static cultures of *B. pertussis* strain 77/18319, previously shown to produce fimbriae, were used as a source of the F-HA. By day 5, the HA was found in the culture supernate. The HA titres, protein estimations and recovery of HA throughout the purification procedure are given in Table 14. There are no values for HA titres or specific activity for the LAP (as the liposomes made reading impossible). The protein estimation for the LAP is derived by subtracting the protein content of the LSP from the PEI. On centrifugation at 26,000 g for 1 h at $4^{\circ}C$ the liposomes with adsorbed fimbriae formed a pellet and not a surface layer as described by Irons & MacLennan (1979a). However HA

TABLE 12 : Effect of HA inhibitors on the i.c. virulence of *B. pertussis* strain 18323

18323 challenge mixed with	Dose cfu	Deaths/ Total	Relative potency (95% conf.limits)	LD_{50} (cfu)
	7250	9/10		
no additive	725	10/10	1.0	22
	145	10/10		
	29	5/10		
	950	9/10		
Cholesterol	190	9/10	0.79 (0.16,3.8)	28
	38	6/10		
	900	8/10		
Cohn fraction IV	180	6/10	0.12 (0.022,0.49)	180
	36	1/10		

TABLE 13 : Effect of inhibitors of HA and mechanical removal of fimbirae on sublethal in. virulence of *B. pertussis* strain 77/18319-

77/18319 challenge treated by	Dose (cfu) in Expt No. 1	2	3	4	Lung Pathology[1] in Expt No. 1	2	3	4	Total	Lung Culture[2] in Expt No. 1	2	3	4	Total
untreated	$2x10^4$	$5x10^5$ $5x10^4$	10^6 10^5	$5x10^5$ $5x10^4$	4/7	9/9 7/9	4/5 3/6	4/5 2/5	33/46	6/7	ND ND	5/5 5/6	5/5 4/5	25/28
				Total	4/7	16/18	7/11	6/10		6/7	ND	10/11	9/10	
mixing with cholesterol	$2x10^4$	$5x10^5$ $5x10^4$	$7x10^5$ $7x10^4$	$5x10^5$ $5x10^4$	3/6	8/10 4/7	4/5 4/7	4/5 3/5	30/45[NS]	4/6	ND ND	5/5 4/7	4/5 4/5	21/28[NS]
				Total	3/6[NS]	12/17[NS]	8/12[NS]	7/10[NS]		4/6[NS]		9/12[NS]	8/10[NS]	
mixing with Cohn fraction IV[3]	$2x10^4$	ND	ND	$5x10^5$ $5x10^4$	1/7	ND	ND	2/5 0/5	3/17**	2/7	ND	ND	1/5 0/5	3/17**
				Total	1/7[NS]	ND	ND	2/10**		2/7*	ND	ND	1/10**	
Blending	ND	$5x10^5$ $5x10^4$	$8x10^5$ $8x10^4$	ND	ND	7/8 5/8	6/6 4/6	ND	22/28[NS]	ND	ND	6/6 3/6	ND	9/12[NS]
				Total	ND	12/16[NS]	10/12[NS]	ND		ND	ND	9/12[NS]	ND	

1 - Number of mice showing greater than 2+ lung pathology/no of mice in group
2 - Number of mice showing greater than 2+ lung culture/no of mice in group
3 - The infecting mixture 77/18319 + Cohn IV in Expt 4 was 77/18319 + haptoglobin
* - significantly different from untreated 77/18319 at 5% probability level
** - significantly different from untreated 77/18319 at 1% probability level
NS - not significantly different from untreated 77/18319

TABLE 14 : Purification of HAs from culture supernate

Sample	Protein Conc (μg/ml)	Volume (ml)	Total Protein (mg)	HA titre	Specific Activity (HAU/mg protein)	Recovery (%)
Dialysed Culture Supernatant	50	5,000	250	8	3×10^3	100
PEI	200	30	6	512	5×10^4	40
LSP	33	57	1.9	8	5×10^3	1.3
LAP	268	15	4	ND	ND	ND

activity was removed from the suspension by the liposomes as evidenced by the decrease in HA titre. Although a direct demonstration of HA activity on liposomes was not shown, PAGE (Plate 3) of the sedimented material showed that the high molecular weight bands characteristic of B. pertussis fimbriae (Irons & MacLennan, 1979a) were found mainly in the sedimented liposomes, whereas other proteins remained mainly in the supernatant fraction. The PAGE pattern for the LSP showed 4 bands about half-way down the gel similar to those described for purified pertussigen (Irons & MacLennan, 1979b). The bands in PAGE for the LSP are faint due to the low amount of protein added, 3.5 μg compared to that added to the tracks for the PEI and LAP of about 20 μg. PAGE of the LAP preparation revealed very faint bands corresponding to pertussigen. From PAGE evidence, chromatography on CM Sepharose CL6B retains mostly F-HA with some pertussigen. The liposomes adsorb almost exclusively the F-HA from the concentrated ion exchange column eluate (PEI). The large diffuse band which moved with the buffer front was probably lipid, since it was found only in preparations containing liposomes (LAP). Electron microscopy was done on the PEI and as can be seen from Plate 4 it consisted mainly of short filaments (approx 20 x 40 nm) similar to those described by Arai & Sato (1976) for the F-HA. Plate 5 shows only one band between F-HA antiserum (1) and PEI in Ouchterlony double diffusion.

1. Histamine sensitizing activity of fraction with HA activity

Histamine sensitisation is a property of pertussigen and was used to monitor the pertussigen content of fractions from the purification procedure. PEI, LAP and LSP were tested for their ability to sensitise mice to the lethal effect of histamine (HSD_{50}). Table 15 shows the doses of each sample administered and the number of deaths in each group 4 h after ip. injection of 3 mg of histamine five days later.

Plate 3 : SDS-PAGE of F-HA at different stages of purification

1. - PEI

2. - LAP

3. - LSP

3 2 1

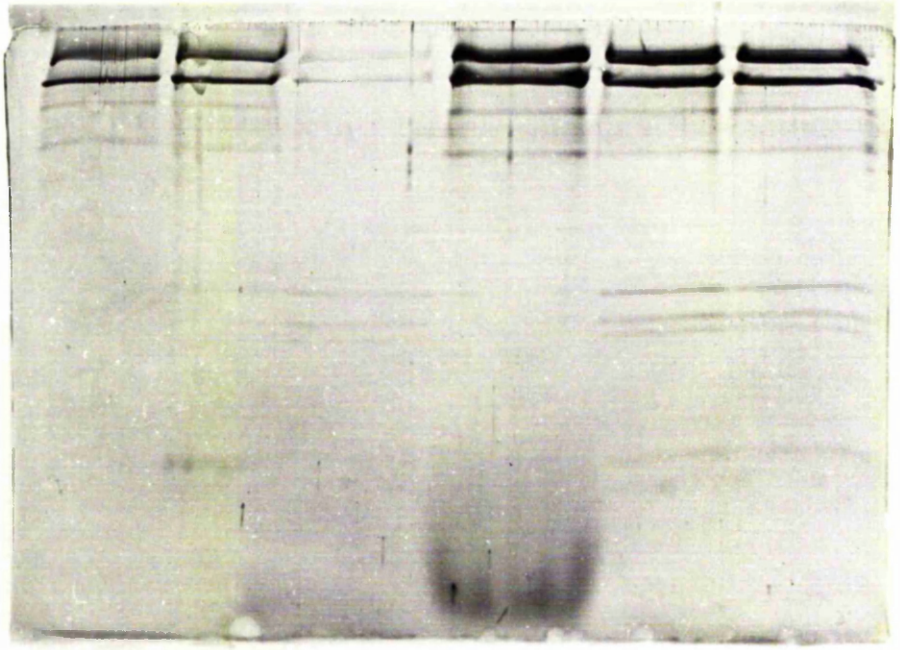


Plate 4 : Electron micrograph of a negatively stained sample of partially purified F-HA (PEI) (Mag x 107,000)

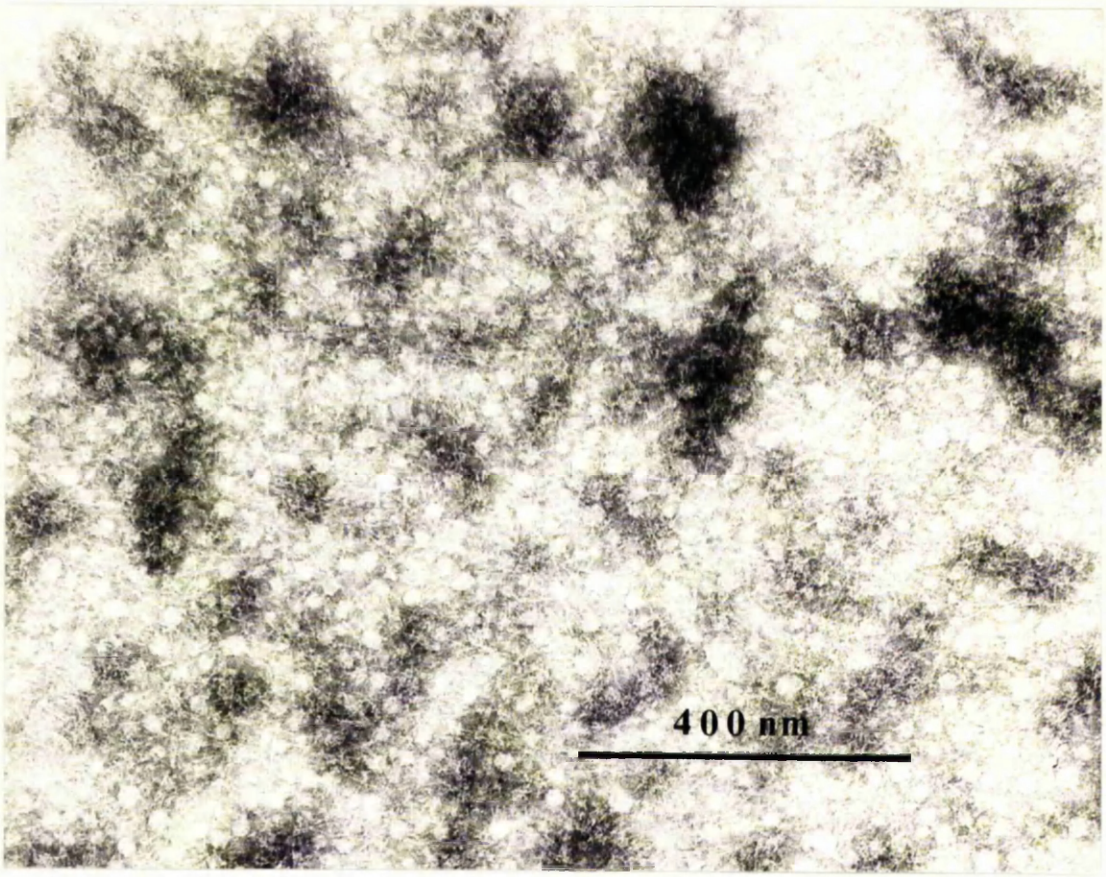

400 nm

Plate 5 : Ouchterlony double diffusion in agar of partially purified F-HA (PEI) against anti F-HA antiserum and whole cell antiserum.

1 - PEI

2 - F-HA antiserum 1

3 - pre immune serum

4 - Whole cell antiserum to strain 77/18319

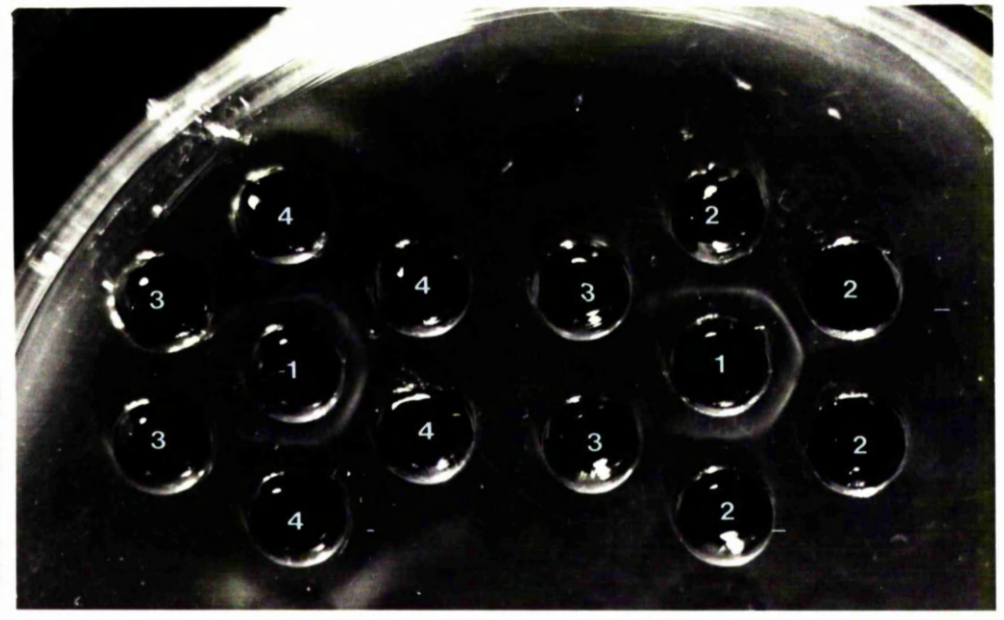

4
2
3
4
3
2
1
1
3
4
3
2
4
2

TABLE 15 : HSF activity of purified HAs from B. pertussis

Sample	i.p. Dose (μg)	Deaths/Total	HSD_{50} (μg)	Relative Potency (95% Confidence limits)
PEI	10	5/5		
	2.5	4/5	1.2	1
	0.625	1/5		
	0.156	0/5		
LAP	16	2/5		
	4	0/5	19	0.065 (0.019, 0.18)
	1	0/5		
LSP	3.3	6/6		
	0.825	5/5	0.26	4.8 (1.7, 13)
	0.21	2/6		
	0.052	0/6		

An HSD_{50} value is also given for each sample with 95% confidence limits. The results show that the LSP whose PAGE pattern indicated that it contained pertussigen had a very low HSD_{50} dose (0.26 μg). This indicates that some of the protein in the preparation was pertussigen. The LAP had a greatly increased HSD_{50} dose and at the highest dose tested (16 μg per mouse) some activity was detected but this dose was less than 1 HSD_{50} the value for that being calculated at 19.1 μg.

2. Active i.c. protective properties of HA fractions

Immunisation of mice with fimbriae from B. pertussis has been reported to protect against i.c. challenge, whereas immunisation with pertussigen does not (Sato et al, 1979). More recently these results have been disputed (Munoz et al, 1981). The F-HA (LAP) and pertussigen (LSP) preparations obtained from the purification procedure described were tested for their ability to protect mice against i.c. challenge. As with the histamine sensitising activity the LAP and the LSP were titrated for their ability to protect mice against approximately 200 LD_{50} units of B. pertussis strain 18323, a highly i.c. virulent strain. The results were computed using the same programme as for the HSF results to give protective $dose_{50}$ (PD_{50}) values. Results from two different liposome preparations are given in Table 16. A standard vaccine preparation was used for comparison. Protective activity was associated with both fractions.

3. Passive i.c. and in. protection by anti F-HA

The LAP preparation was used as an immunogen in rabbits for preparation of antisera to the F-HA. The anti HA properties of the F-HA antisera, whole cell antiserum to 77/18319 and US standard antiserum

TABLE 16 : Protective activity of purified fractions from B. pertussis as measured by the i.c. challenge

Antigen	Protein Dose/mouse (μg)	Survivors/Total in Expt Nos 1	Survivors/Total in Expt Nos 2	PD_{50} (μg protein) in Expt Nos 1	PD_{50} (μg protein) in Expt Nos 2	PD_{50} (μg protein) in Expt Nos Mean	Relative Potency (95% confidence limits) in Expt Nos 1	Relative Potency (95% confidence limits) in Expt Nos 2	Relative Potency (95% confidence limits) in Expt Nos Mean
Glasgow	28.8 (1 ou)	5/9	6/9	23.86	9.8	16.83	1	1	1
Standard	5.76 (0.2 ou)	1/9	5/10	(0.7 ou)					
Vaccine	1.15 (0.04 ou)	2/9	2/10						
	16	7/9	6/8						
LAP	3.2	5/9	3/10	4.72	4.89	4.8	5.05 (1.03,48.84)	2.01 (0.14,28.62)	3.53
	0.64	0/9	3/10						
	6.5	toxic	toxic						
LSP	1.3	5/9	5/9	0.88	0.88	0.88	27.17 (4.75,275.3)	11.14 (2.01,105.36)	19.15
	0.26	3/10	3/10						

were tested (Table 17). Antiserum (1) to F-HA showed the greatest ability to inhibit HA. To test the ability of anti F-HA to protect mice against pertussis infection, the antiserum (1) was mixed both with a sublethal in. challenge dose and with an ic. challenge dose. Passive protective activity against sublethal in. infection was estimated by the ability of antiserum to reduce or inhibit completely lung consolidation. Good protection against lung consolidation was observed with the highest dilution of F-HA antiserum (1) tested (1/50) (Table 18). With ic. challenge F-HA antiserum (1) showed protective activity when used undiluted or as a 1/2 dilution but at the 1/5 dilution the protective activity was already decreasing. F-HA antiserum (1) was less protective than either whole cell antiserum to strain 77/18319 or the US standard antiserum (Table 19). F-HA antiserum (2) which had an HAI titre equal to that for the whole cell antiserum was not protective even when undiluted. This indicates that although F-HA antiserum was protective, the anti HA is probably not the important protective antibody in whole-cell antiserum.

The ability of F-HA antiserum (1) to neutralize the histamine-sensitising property of a standard vaccine was examined in order to check the specificity of the antiserum. It was decided to check the neutralization of histamine sensitization as pertussigen may have been a small contaminating factor in the antigen preparation. The results show (Table 20) that F-HA antiserum (1) did not have the ability to neutralize approximately 1 HSD_{50} dose of a standard vaccine. In contrast, the antiserum to whole cells of strain 77/18319 did neutralize 1 HSD_{50} dose.

4. Ability of 77/18319 F-HA antiserum to protect against non-fimbriated 77/18319 and other strains of B. pertussis

TABLE 17 : HAI activities of different F-HA antisera, whole cell antiserum and the US standard antiserum

Antiserum	HAI titre	HAI units[1]
US standard	8	64
Whole cell antiserum to *B. pertussis* strain 77/18319	2	16
F-HA antiserum (1)	64	512
F-HA antiserum (2)	2	16

1 - HAI units are HAI titre x no of HA units the antiserum is inhibiting, i.e. 8.

TABLE 18 : Passive protective activity of antiserum to F-HA in the sublethal in. infection with B. pertussis strain 77/18319

Antiserum Dilution	Lung Pathology[1]	Lung Culture[2]
No antiserum	29/33	22/26
Undiluted	1/25**	1/22**
1/5	4/25**	7/24**
1/10	3/25**	7/25**
1/50	7/20**	9/20**

The values in the table are the additive results of 4 separate experiments.

The inoculum/mouse varied from 4.8×10^5 to 7×10^6 cfu.

1 - number of mice showing greater than 2+ lung pathology/ number of mice in group.

2 - number of mice showing greater than 2+ lung culture/ number of mice in group.

** - significantly different from no antiserum control at the 1% probability level.

TABLE 19 : Passive protective activity of F-HA antiserum (1) and two anti-whole cell sera against i.c. challenge with 150 LD_{50s} of *B. pertussis* strain 18323

Antiserum	Dilution	Survivors/Total
none	-	1/10
anti F-HA (1)	Undiluted	9/10**
	1/2	10/10**
	1/5	$4/10^{NS}$
Anti-whole cell	Undiluted	10/10**
77/18319 static	1/5	8/10**
	1/10	9/10**
US standard	Undiluted	8/10**
anti-pertussis sera	1/5	8/10**
	1/10	9/10**

** - significantly different from no antiserum control at 1% probability level

NS - not significantly different from no antiserum control

TABLE 20 : Anti histamine sensitising activity of F-HA antiserum (1)

Vaccine/mouse (ou of Glasgow Standard)	Antiserum	Dilution	Deaths/Total
1.3	None	-	10/10
0.65	None	-	7/10
0.325	None	-	0/10
0.65	F-HA (1)	1/2	$10/10^{NS}$
		1/4	$7/10^{NS}$
0.65	Whole cell antiserum to strain 77/18319	1/2	2/10*
		1/4	$7/10^{NS}$

* - significantly different from 0.65 ou of vaccine control at 5% probability level

NS - not significantly different from 0.65 ou of vaccine control

B. pertussis strain 77/18319 when grown in shaken culture for 48 h had no HA activity and there was no evidence of fimbriae on the cell surface. However these cells were found to be intranasally virulent and the ability of F-HA antiserum to protect against sublethal i.n. infection by these cells was examined. The results show (Table 21) the antiserum to be protective.

Whether or not there is any antigenic variation in fimbriae from different strains of B. pertussis is not known. Before a fimbrial vaccine could be used its effectiveness against a large number of strains would need to be tested. The strain used to challenge immunised mice intracerebrally (18323) was different from the strain from which the fimbriae were derived (77/18319) yet the mice are protected (Results Table 16). Similarly some passive protection was afforded mice challenged intracerebrally with 18323 where the anti F-HA was derived from strain 77/18319 (Results Table 19). Another strain commonly used in our laboratory, 18334, was used as a sublethal i.n. challenge in a passive protection test using anti F-HA from 77/18319. The results (Table 21) show that the antiserum protects against this challenge.

D. Adhesion of B. pertussis to mouse lung

An intra-tracheal infusion technique was used to study the affinity of B. pertussis for murine pulmonary epithelium.

The effect of various times of exposure on the adhesion or retention level of B. pertussis in mouse lung was studied over the period 0 to 60 min and using both viable counting and radioactive counting methods. Fig 7 shows that the percentage of infused bacteria retained in the lung increased up to 30 min after which it levelled off, both

TABLE 21 : Protection by antiserum to F-HA from static cultures of 77/18319, against sublethal in. infection by B. pertussis strain 77/18319 from static and shake cultures and against strain 18334 from static culture

Culture or mixture used for inoculum	Lung Pathology[1]	Lung Culture[2]
77/18319 static	16/17 (3 died)	11/12
77/18319 static with F-HA antiserum (1)	1/20**	1/17**
77/18219 shake	12/16 (4 died)	7/11
77/18319 shake with F-HA antiserum (1)	1/20**	3/15*
18334 static	16/17 (3 died)	11/12
18334 static with F-HA antiserum	0/20**	1/18**

Inoculum in cfu approximately 5×10^6 in all cases

1 - number of mice showing greater than 2+ lung pathology/number of mice in group

2 - number of mice showing greater than 2+ lung culture/number of mice in group. Discrepancy in numbers/group between pathology and culture due to contamination of plates

** - significantly different from no antiserum controls at the 1% probability level

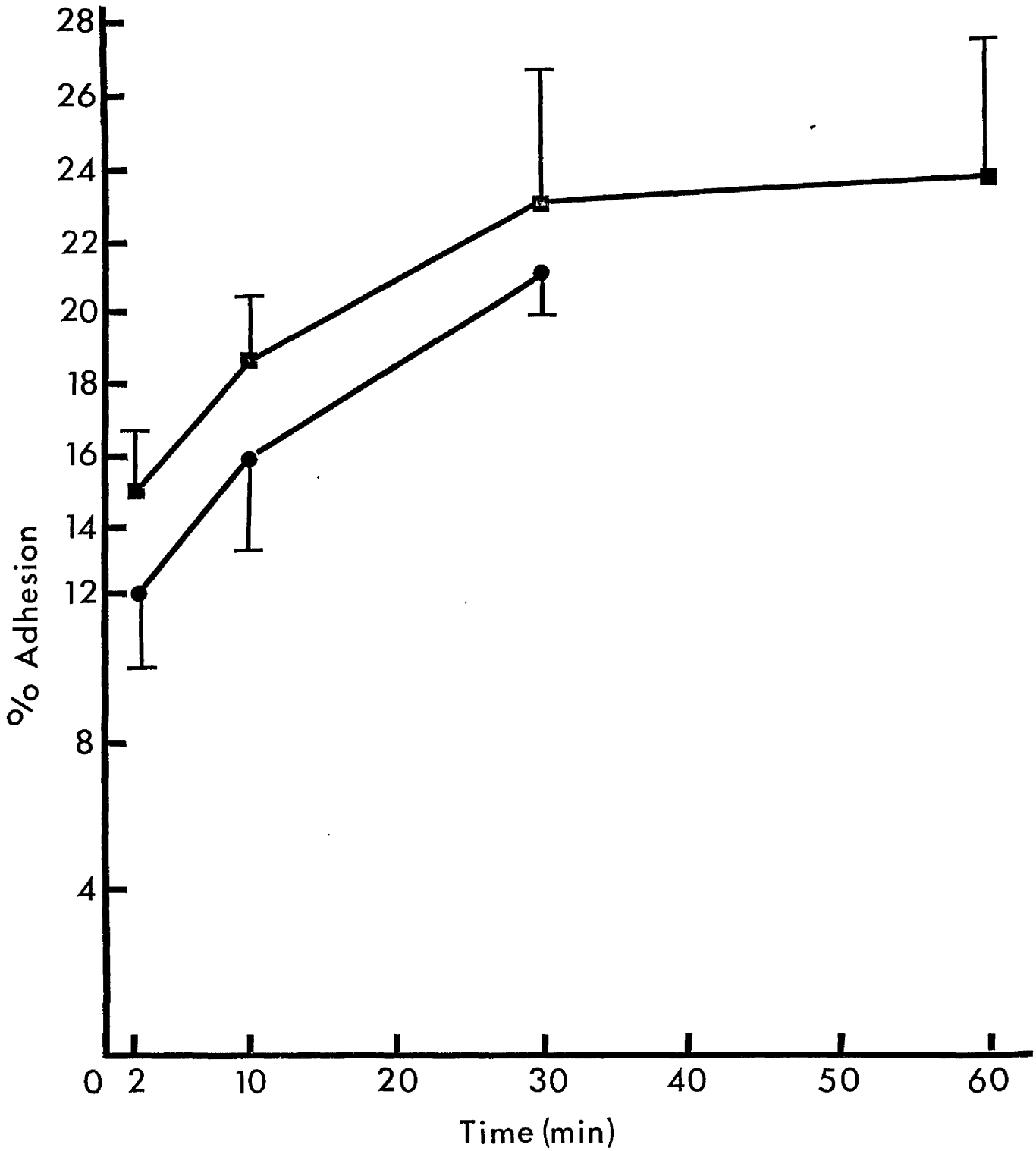
* - significantly different from no antiserum controls at the 5% probability level

Fig 7 : Percentage adhesion of *B. pertussis* strain 77/18319 to mouse lung as the time of exposure is increased

■ - Radioactive counting method

● - Viable counting method

Points are plotted with bars representing 1 SD.


28
26
24
22
20
18
16
14
12
8
4
% Adhesion
0
2
10
20
30
40
50
60
Time (min)

methods giving similar results. It was decided that 30 min was a satisfactory time and was therefore used in most subsequent experiments. From Figs 7 and 8 it can be seen that there seems to be two phases to the adhesion or retention of B. pertussis to mouse lung. An initial fast rate of adhesion in the first 0 to 2 min after which the rate of adhesion is much slower. The rate of adhesion of X and C mode cells is in two phases (Fig 8). The initial rate of adhesion between 0 and 10 min where the virulent X mode cells adhere at a much faster rate than the avirulent C mode. The second phase of adhesion being between 10 and 30 min where both X and C mode cells adhere at similar rates.

Strain 77/18319 on different occasions gave different values for percentage adhesion (Figs 7 & 8). This could possibly have been due to variations in the cells from culture to culture which caused the cells to have different abilities to adhere. To ensure variation in inoculum size was not responsible for this variation three ten-fold dilutions of strain 77/18319 were used in lung assays and the results are shown in Table 22. No difference in the percentage of organisms adhering was found, indicating that a culture of B. pertussis has a fixed proportion of organisms which can adhere.

The sex of mice used in the adhesion assays had no effect on the adhesion of B. pertussis strain 77/18319 (Table 23). The mice were used at between 5 and 8 weeks of age, as at 3 weeks, the age the mice were used at for sublethal in. virulence experiments, they were too small to allow the cannula to be inserted into the trachea.

1. Adhesion of virulent and avirulent B. pertussis

C mode and phase IV cells lack HA activity and are avirulent. The ability of these variants to adhere to mouse lung was compared with their X mode phase 1 counterparts (Table 24). The C mode and phase IV

Fig 8 : Percentage adhesion of X and C mode cells of 77/18319 with different times of exposure to mouse lungs

■ - X mode

● - C mode

Viable counting method used

Points are plotted with bars representing 1 SD.

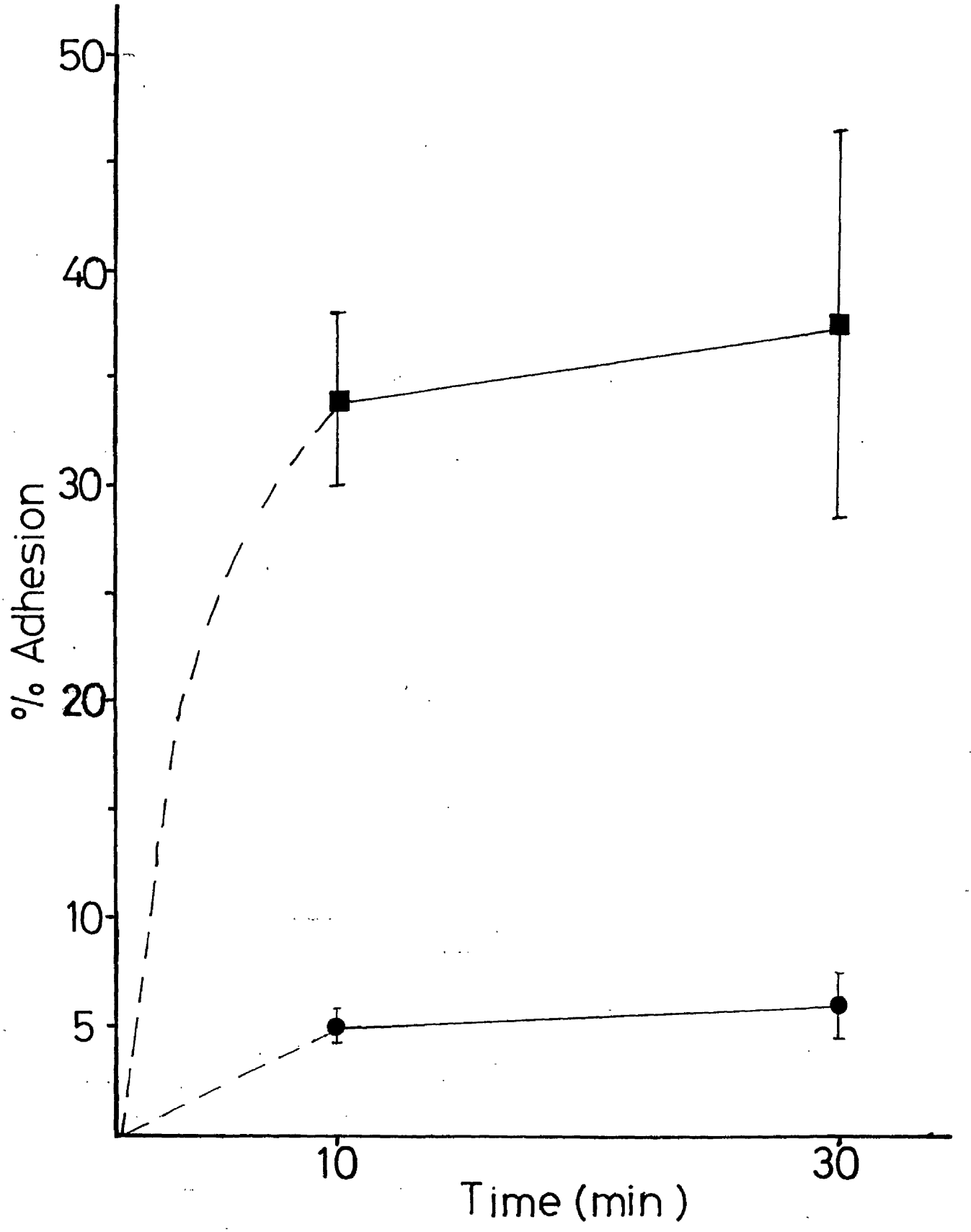

50
40
30
20
10
5
% Adhesion
10
30
Time (min)

TABLE 22 : Effect of 10 fold increases in inoculum size of strain 77/18319 on percentage adhesion in mouse lung assay

Inoculum size (cfu)	% Adhesion $\pm$ 1 SD
2×10^5	15.8 ± 3.25
2×10^6	19 ± 0.5
2×10^7	16.5 ± 5.5

Viable counting method used

TABLE 23 : Effect of mouse sex on adhesion of B. pertussis strain 77/18319 to mouse lung

Sex	% Adhesion $\pm$ 1 SD
Male	23.5 $\pm$ 5.06
Female	24.8 $\pm$ 4.72

Viable counting method used

TABLE 24 : Percentage of the inoculum of virulent and avirulent variants of *B. pertussis* strains 77/18319 and D30042 adhering to mouse lung

Inoculum	% Adhesion ± 1 SD
77/18319 X mode	31.3 ± 4.0
77/18319 C mode	6 ± 1.6
D30042 phase I	20.6 ± 0.3
D30042 phase IV	3.5 ± 1.8
77/18319 Small colony variant	22 ± 2
77/18319 Large colony variant	10 ± 2.6

Viable counting method used

cells were much less able to adhere than X mode phase 1 cells. Another set of HA positive and HA negative cells are the small and large colony variants of 77/18319. With these cells the HA negative, less virulent large colony type showed a lower level of adhesion than the small colony type (Table 24).

Other strains which differ in virulence properties were also used in adhesion assays. These strains were 44122/7R, i.c. virulent, and 44122/7S which is i.c. avirulent. Both these strains are equally virulent by the i.n. route. Strain 18323 was also used, this strain being i.c. virulent but less i.n. virulent than 77/18319. The values obtained for percentage adhesion to mouse lung do not reflect the i.c. virulence of the strains but seem to relate to the sublethal i.n. virulence (Table 25).

The values obtained for percentage adhesion for strain 77/18319 for the two experiments illustrated (Table 25) are very different although the same batch of cells were used for both experiments. This was probably due to the chemiluminescent effect of the soluene which had been allowed to decay for several days in the dark in the 44122/7R, 44122/7S, and 77/18319 experiment, but only for approximately 24 h in the 77/18319, 18323 experiment. This phenomena was found throughout the radioactive experiments when the vials had been left standing in the dark for several days the percentage adhesion to the mouse lung seemed to decrease. Each experiment was therefore treated individually and an internal standard always included usually strain 77/18319 X mode cells.

2. Effect of prior vaccination of the mice on adhesion of B. pertussis to mouse lung

Experiments where groups of mice were vaccinated i.p. before use in the adhesion assay with the Glasgow standard vaccine, or infected i.n.

TABLE 25 : Adhesion of different strains of B. pertussis to mouse lung

Strain	Percentage Adhesion ± 1 SD in Expt No. 1	2
77/18319	22.3 ± 4.8	12.3 ± 4.1
18323	11.7 ± 2.3	ND
44122/7R	ND	13.6 ± 2.9
44122/7S	ND	14.2 ± 3.7

Radioactive counting method used

were done. On day 14 after vaccination a group of mice were sacrificed and used in an adhesion assay. This was repeated on groups of mice on day 31, 33 and 43 after vaccination. The results obtained (Table 26) show no change in the percentage of the inoculum adhering to the lungs compared to control animals tested over this time period. Mice which had been sublethally infected were sacrificed on days 31, 33 and 43 for adhesion assays. A control group were sacrificed on day 14 to ensure the mice had been infected. The level of infection was very low, giving lung pathology scores of 1 or 2+. Infected animals on all three days showed evidence for some reduction in the adhesion level compared to control and vaccinated animals although this reduction was not significant.

The effect of vaccination with fimbrial preparations, as compared to standard whole cell vaccines, on adhesion was studied (Table 27). Groups of mice were vaccinated intraperitoneally or intranasally with either F-HA or the Glasgow standard vaccine. Although the results obtained were variable they do indicate 1) that F-HA given intraperitoneally inhibits subsequent adhesion of B. pertussis to the lungs; 2) although the level of protein administered was very low, the F-HA given intranasally also seemed to cause an inhibition of adhesion; 3) the whole cell vaccine showed little inhibitory activity against adhesion to lungs. With whole cell vaccine and F-HA administered intranasally very little can be said about the results as the dose administered by this route was only 10^{-1} of that administered intraperitoneally.

3. Effect on lung adhesion of mixing the bacteria with F-HA inhibitors

Passive inhibition of adhesion was studied by mixing rabbit antiserum to whole B. pertussis cells and antiserum to the F-HA preparation with the inoculum into the mouse lung. The results showed

TABLE 26 : Effect of prior infection or vaccination, with a standard vaccine, of the mice, on adhesion to mouse lung after 30 min exposure

Mouse treatment	Day after treatment	% Adhesion $\pm$ 1 SD
None	14	16.5 ± 1.8
	31	14.5 ± 3.4
	33	17.5 ± 4.7
	43	15.5 ± 2.4
Vaccinated	14	17.3 ± 2.3
2 ou ml of Glasgow	31	12.4 ± 2.1
Standard/mouse	33	17.1 ± 3.2
	43	14.3 ± 1.5
Infected sublethal	41	11.4 ± 1.8
in. dose (6×10^4 cfu)[1]	33	15.5 ± 1.0
	43	12.9 ± 2.6

1 - number of mice showing greater than 2+ lung pathology/total number of mice in group was only 1/10

Radioactive counting method used

TABLE 27 : Effect of prior vaccination by a purified F-HA antigen or a standard vaccine on subsequent adhesion to mouse lung

Antigen (μg protein/ ml)	Route of Antigen adminis-tration	Dose (ml) (μg protein)	% Adhesion ± 1 SD in Expt Nos	
			1	2
LAP (26)	ip.	0.5 (13)	5.5 ± 2.1	12.7 ± 2.8
	in.	0.05 (1.3)	9.5 ± 3.9	13.2 ± 2.4
Glasgow Standard Vaccine (60)	ip.	0.5 (30)	11.53 ± 3.6	14.7 ± 4.4
	in.	0.05 (3)	10.3 ± 4.6	15.4 ± 3.2
None	-	-	13.8 ± 3.1	17.0 ± 2.9

Radioactive counting method used

evidence of inhibition of adhesion by the F-HA antiserum but not with the whole cell antiserum (Table 28). The F-HA inhibitor cholesterol showed no ability to decrease the adhesion to mouse lung when mixed with B. pertussis before inoculation.

4. Effect of varying the bacterial cell surface charge on adhesion to mouse lung

The net charge of proteins can be altered by changing the pH of the suspending fluid. The pI value of the F-HA is not known but its retention on CM Sepharose at pH 6.5 indicates a positive charge at this pH. It is assumed the lung epithelial cells will, like other mammalian epithelial cells, be negatively charged. The effect of reducing the negative charge of B. pertussis by causing the fimbriae to be positively charged was examined. The percentage adhesion decreased when the pH was increased in the suspending fluid from 6.5 to 7.0 (Fig 9). This increase in adhesion cannot be directly attributed to the F-HA as other surface proteins may be affected by this pH and may be involved in adhesion.

Another method used to show that fimbriae are involved in adhesion by overcoming the repulsive forces of the like charges on the bacterial and mouse cells was by reducing the net negative charge of the bacterial cells. If fimbriae act to overcome these repulsive forces then non-fimbriated bacteria, when their negative surface charge has been reduced, should be able to adhere to the mouse lungs. In a preliminary experiment C-mode and phase IV cells were treated by the method of Heckels et al (1976). It was found that these treated cells adhered to a much greater extent than untreated C mode or phase IV cells.

E. Hydrophobic interaction chromatography of B. pertussis

Smythe et al (1978) using HIC showed that strains of E. coli

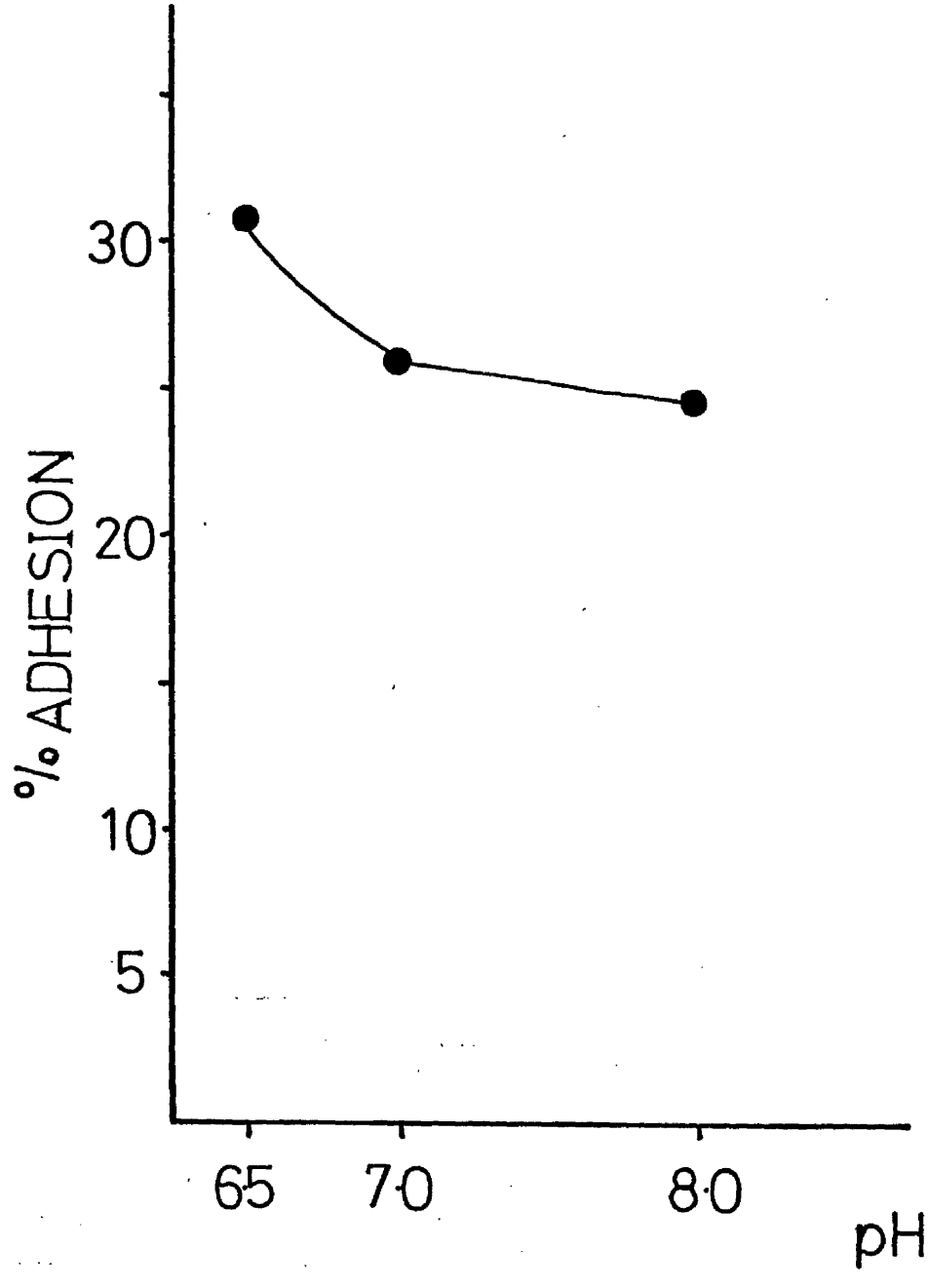
TABLE 28 : Effect on adhesion to mouse lung of mixing *B. pertussis* strain 77/18319 with antiserum or cholesterol before inoculation

Inhibitor	% Adhesion ± 1 SD
None	24.6 ± 4.1
Whole cell antiserum to *B. pertussis* strain 77/18319	24.7 ± 5.2
F-HA (1) antiserum	19.0 ± 4.5
Cholesterol	23.1 ± 3.9

Radioactive counting method used

Fig 9 : Effect of pH of suspending fluid on the percentage of cells (77/18319) adhering to mouse lung.

Radioactive counting method used


30
20
10
5
% ADHESION
6·5
7·0
8·0
pH

with K88 and K99 fibres on the cell surface were more hydrophobic than K88 and K99 negative strains. HIC is a chromatographic procedure based on hydrophobic interaction between nonpolar groups on a gel bed and nonpolar regions of proteins. The gels were run in high ionic strength buffer to inhibit any ionic binding, decreasing the ionic strength of the running buffer allows desorption of the less strongly adsorbed substances.

No data are available on the surface hydrophobicity of virulent and avirulent strains of B. pertussis. The two commonly used avirulent variants of B. pertussis are C modulated and phase IV variants. Two sets of cultures were used in HIC, X and C mode cultures of 77/18319 and phase I and IV of strain D30042. As indicated by the results (Table 29) virulent X mode or phase I organisms are much more hydrophobic than are the C mode or phase IV cells as evidenced by their increased retention on octyl sepharose. Even after washing with low ionic strength buffer (PB) a small percentage of X mode and phase I organisms were desorbed from the gel bed. The large colony variant of B. pertussis strain 77/18319 which was found to be less virulent than small colony types was also less hydrophobic by HIC as evidenced by the small percent of the cells which adsorbed to the gel bed.

The hydrophobicity of organisms varying in i.n. and in i.c. virulence (see Results Section B) was assessed on columns of octyl sepharose (Table 30). The strains used which were intracerebrally virulent were 44122/7R, and 18323, the other strains 77/18319, and 44122/7S were avirulent. The i.c. virulence of strain Taberman is not known. The i.n. virulence of the strains also varied and decrease in the order 77/18319, 44122/7R and 44122/7S > 18323 > Taberman. Strains 77/18319 and 44122/7R were the most hydrophobic followed by 44122/7S, 18323 and Taberman.

TABLE 29 : Retention of virulent and avirulent B. pertussis on octyl sepharose gel

Culture	Virulence	% Eluted by 4M NaCl	% Eluted by PB	% Retained on column
77/18319 X mode	+	10.4	2.2	87.4
77/18319 C mode	-	50	21.6	28.4
D30042 phase I	+	20.4	1.8	77.8
D30042 phase IV	-	64.1	3.5	32.4
77/18319 Small colony variant	+	10.2	ND	89.8
77/18319 Large colony variant	+ -	43	ND	57

All cultures were five day static cultures

TABLE 30 : Elution and retention by octyl sepharose gel of different strains of B. pertussis

Strain	Virulence i.c.	Virulence i.n.	% Eluted by 4M NaCl	% Eluted by PB	% Retained
77/18319	-	+	10.4	2.2	87.4
Taberman	ND	+ -	25.2	2.5	72.3
18323	+	+ -	22.7	0.2	77.1
44122/7R	+[1]	+	9.5	2.5	88
44122/7S	-[1]	+	19.0	3.5	77.5

1 - Personal communication from Mr. Salah al Salami

When B. pertussis cells are grown in shaken culture HA ability is lost and no fimbriae can be seen on the cell surface. However the cells had a similar level of i.n. virulence as statically grown cells. The hydrophobicity of cells from shaken cultures was compared with that of statically grown cells and as the results show (Table 31) there was a reduced ability to stick to octyl sepharose but the level was not as low as with avirulent C mode cells which also lack HA ability and lack fimbriae.

TABLE 31 : Effect of cultural conditions on the retention of *B. pertussis* by octyl sepharose gel

Growth Condition of strain 77/18319	% Eluted by 4M NaCl	PB	% Retained
Static	12.2	2.3	85.5
Shake	45.7	1.0	54.3
C mode	50.0	21.6	28.4

DISCUSSION

A. Production of HA and virulence of *B. pertussis*

1. Production of HA

The strains of *B. pertussis* tested from B-G cultures showed very little difference in HA titres obtained. Changes were found when the cells were grown under different cultural conditions. When grown in shaking cultures the HA titre was maximal at 18 h and was on most occasions completely lost by 48 h. In static culture however the HA titre of the cells increased up to 96 to 120 h incubation. These results agree with those obtained by Arai & Munoz (1979b) who examined the HA titres of cells grown in different media and in different cultural conditions.

The growth curve for X-mode and C-mode cultures in shake cultures are similar for the first 48 h. As the growth reaches stationary phase, the adsorbance (A600) of the X-mode cultures starts to drop slowly. This is probably due to the clumping of cells. In static liquid culture the X-mode culture grows more than the C-mode cultures. When a 4 day static X-mode culture was transferred to a dimpled Erlenmeyer flask and incubated on an orbital shaker the cells tended to form clumps. It is possible that the cells forming the clumps in shake cultures and those in the shaken static cultures are hydrophobic and fimbriated and due to these surface properties the cells stick together. Static culture supernate which was HA positive completely lost its HA titre after 12 h shaking in a dimpled Erlenmeyer flask at 37^{o}C. This indicated that HA released into the supernate in shake cultures was destroyed or inactivated in some way. It is possible that fimbriae are produced in shake cultures but most of the cell associated fimbriae and the HA released into the culture supernate are destroyed by the shaking.

2. Inhibition of HA

The HA activity of the fimbriae was inhibited by cholesterol whereas that caused by pertussigen was not inhibited (Irons & MacLennan, 1979a; Sato et al, 1979). The two HAs were distinguished in the present study by incorporating cholesterol into the HA tests. The results (Table 8) show that for strain 77/18319 in static culture both the cell free and cell-associated HA activity was sensitive to cholesterol. The cell-associated HA activity was also sensitive to mechanical shearing in a blender. With 18 h shake cultures cell-associated and cell-free HA activity was reduced only slightly when titrated in the presence of cholesterol. This perhaps reflects a lower level of F-HA in shake cultures compared to that in static cultures. Strain 18323 an intracerebrally virulent strain, does not even when grown in static culture show any significant reduction in HA titre in the presence of cholesterol.

3. Virulence of B. pertussis

The static and shake cultures of strain 77/18319 with different levels of HA activity were used in mouse sublethal i.n. virulence experiments (Table 11) along with blended static cells. The shaken cultures and blended cells with very little or no detectable HA titres were as virulent as the static cells which had positive HA titres. If the fimbriae are important in adhesion it would have been expected that the cells without fimbriae would be less able to adhere and be swept away by the clearing mechanisms in the lungs. The fact that non-fimbriated cells were as virulent as fimbriated cells would suggest either that (a) fimbriae are not necessary for adhesion and virulence in the mouse lung assay or (b) the cells are triggered by the lung environment to produce fimbriae. In E. coli there is an intracellular pool of common type 1 fimbriae subunit protein (Brinton, 1965). When

E. coli was blended to remove the fimbriae they regenerated very quickly, the numbers returning to control level 1 to 4 min after blending (Novotny et al, 1969). It is also possible that there is a second mechanism of adhesion, perhaps involving the HA property of the pertussigen component.

Strain 18323, which has a low cholesterol-sensitive HA titre when grown in static culture but a greater cholesterol-resistant HA property than strain 77/18319 (Table 8), is less virulent by the i.n. route than strain 77/18319 (Table 9). As strain 18323 is i.c. virulent and strain 77/18319 is not, it would seem that the F-HA is not of great importance in i.c. virulence. However the low level of fimbriae present on strain 18323 may be sufficient to allow some organisms to adhere and initiate infection. After this point another factor must be responsible for i.c. virulence. Strain 77/18319 may adhere in the mouse brain but either (a) not have a specific factor required to allow multiplication, (b) not have a toxic factor required to cause death or (c) it may multiply in the brain but be overcome by the mouse protective factors whereas 18323 evades these factors. As anti F-HA antiserum and vaccination with F-HA extracted from 77/18319 shows protective activity against i.c. infection with strain 18323 it would seem that the fimbriae are probably involved in adhesion in the brain.

In lung infections, the presence of fimbriae may be more important in virulence since the poorly fimbriate strain 18323 is less virulent than the more richly fimbriate 77/18319. However when static and shake cultures of strain 77/18319 were used, this correlation did not appear to hold. It is likely however that shaken cells have the ability to regenerate fimbriae when environmental conditions are favourable as may occur in the lung. The lower i.n. virulence of

strain 18323 may reflect the lower numbers of fimbriae in this strain, which in turn may reduce its adhesive capacity in the respiratory tract.

Adhesion would seem to be more important in mouse lung infections than in i.c. infections. This is supported by the large inoculum required for lung infection as compared to the i.c. LD_{50} value for strain 18323 of 22 to 68 cfu. In i.c. infection 90% of the inoculum into the brain is immediately lost into the blood stream (Dolby & Standfast, 1961). It is probable that 2 to 7 cfu retained in the brain are sufficient to cause eventual death of the mouse.

Two variants of strain 44122/7 differ in streptomycin resistance (7R being resistant and 7S sensitive) and also in i.c. virulence (7R virulent, 7S avirulent). A transformant of 44122/7S (transformant X prepared by Mr. S. al Salami, Dept. of Microbiology, Univ. of Glasgow) gained i.c. virulence along with streptomycin resistance. These three variants 44122/7R, 44122/7S and 44122/7X were all tested for HA activity. No difference was detected in HA activity or the sublethal i.n. virulence of these three variants of strain 44122/7 (Table 9). This provides additional evidence for the view that different cellular components are responsible for mouse virulence of B. pertussis by the i.n. and i.c. routes.

4. Hydrophobicity of B. pertussis

The technique of HIC was used to compare the hydrophobicity of different strains and cultures of B. pertussis. Strains Taberman, 44122/7S and 18323 were all slightly less hydrophobic than 77/18319 and 44122/7R. Strains 18323 and Taberman are also both less intranasally virulent than 77/18319 and this reduced surface hydrophobicity could account for this. However strain 44122/7S is as virulent by the i.n. route as strains 77/18319 and 44122/7R. The hydrophobicity of the cell

surfaces could be related to more than one surface factor. As strain 18323 is less fimbriate than strain 77/18319 it would seem possible that fimbriae play a role in surface hydrophobicity. However as 44122/7R is more hydrophobic than 44122/7S it is possible that a factor involved in i.c. virulence is a hydrophobic surface component.

Table 31 shows the degree of retention, of cells of strain 77/18319 grown in static culture (HA positive) and grown in shake culture (HA negative), on octyl sepharose. C mode cells were used as a comparison of cells with a low hydrophobic nature. The results show three levels of retention: (a) the static cells with high retention properties; (b) the C mode cells with a low level of retention and (c) the shaken cells with a percentage retention between these two values. Static and shake X-mode cultures were equally virulent but differed in HA properties and in hydrophobicity. This indicates that fimbriae confer hydrophobicity on the cells. However when the fimbriae were lost from virulent cells the level of hydrophobicity did not drop to the level of avirulent cells. This suggests that differences in outer membrane proteins may also affect the hydrophobic nature of the cell surface. A hydrophobic outer membrane protein could therefore be involved in adhesion.

Fimbriae have been shown to play an important role in adhesion of the gonococcus and various other bacterial parasites of mucous surfaces. Gonococcal fimbriae contain 46% non polar amino acid residues and, as such, are reported to play a dominant role in the gonococcal surface hydrophobicity (Watt & Ward, 1980). However, when whole gonococci were interacted with hydrophobic gels, fimbriate cells only showed a 20% increase in retention on the gel compared to non-fimbriate cells. Watt & Ward (1980) interpret this result as implicating the

surface of the gonococcal outer membrane itself in hydrophobic binding. Smyth et al (1978) reported that the fimbrial-like K88 antigen was solely responsible for binding of smooth enteropathogenic E. coli to hydrophobic sepharoses.

The possible relationship between i.c. or i.n. virulence, hydrophobicity, and the presence of fimbriae on different strains of B. pertussis was a central theme in this investigation. If the hydrophobicity of non-fimbriated cells is a reflection of virulence by either route, then hydrophobic outer membrane proteins may be responsible for virulence as would seem possible from the preliminary results obtained here. Pertussigen is a membrane protein and is a good candidate for both a virulence factor and a hydrophobic component of the cell surface. The hydrophobic nature of pertussigen is suggested by reports of its association with lipid (Arai & Sato, 1976) and its insolubility in water (Irons & MacLennan, 1979b). The hydrophobicity of pertussigen could be determined by the retention of the purified protein on hydrophobic gels. If found to be hydrophobic, then strains of B. pertussis differing in activities associated with pertussigen may differ in hydrophobicity. If this were so then pertussigen could be a contributary factor to cell surface hydrophobicity.

5. Effect of HA inhibitors on virulence

Cholesterol is an inhibitor of F-HA activity and haptoglobin inhibits the HA caused by pertussigen (Irons & MacLennan, 1979a). These two inhibitors were used in virulence experiments although the haptoglobin was not purified but used as Cohn fraction IV of human plasma, which is rich in haptoglobin. The results (Tables 12&13) show that cholesterol had no inhibitory effect on challenges by either the i.c. or i.n. route. Cohn IV however reduced both i.c. and i.n. virulence of

the strains used. As Cohn IV contained proteins other than haptoglobin, the significance of this result is less clear. It would be desirable to perform virulence and adhesion assays with purified haptolobin. If the purified material were to reduce virulence as did Cohn IV then it could act as a receptor for pertussigen *in vivo*.

B. Adhesion to mouse lung

This adhesion system was devised to allow direct measurement of adhesion (or more accurately retention) in one of the virulence assays for *B. pertussis*, namely the sublethal i.n. assay. Whether the assay measures adhesion or if it is more accurately retention is not easily determined. It is not certain that *B. pertussis* actually physically adheres to the ciliated epithelium. This assay measures retention in mouse lung some of which may be adhesion. Therefore the results should not be interpreted as actual values for percentage adhesion but comparisons between different levels of retention made. Adhesion however has been used throughout when referring to retention of *B. pertussis* in mouse lungs.

Two other factors must be considered when comparing levels of adhesion, these are (a) the possibility that the cells are killed and (b) the cells may be agglutinated in the mouse lung. Agglutination seems improbable as similar results were obtained in the time course experiments for both viable and radioactive counting methods. A killing effect is a possibility where the avirulent cells of *B. pertussis* (C mode and phase IVs) are killed more readily than virulent cells (X mode phase I cells).

The number of cells adhering increases for 30 min and this time was therefore used as the exposure time in most other experiments.

However from Figs 7 and 8 adhesion appears to be biphasic with the rate over the first 2 min exposure being much greater than the rate after the first 2 min.

The idea that in any one culture of any strain of B. pertussis there exists only a proportion of the total number of organisms which are capable of adhering was expressed in the Results. This was suggested because different percentages of the inoculum were found to adhere in different experiments using different batches of the same strain. In one experiment (Table 22), using the same batch of cells, 10 fold increasing amounts of inoculum did not change the percentage of cells adhering. Electron microscope evidence supported the idea that only a fixed percentage of the cells would be expected to adhere since in all the static cultures examined only some of the cells were fimbriate. This correlation with the presence of fimbriae assumes that fimbriae are essential for adhesion. It would be of interest to re-inoculate organisms removed after the initial exposure to the lung into fresh lungs to determine levels of adhesion in this fraction of the original inoculum. This would tell whether all those organisms which can attach have already done so after 30 min in the lungs. With the viable counting method, variations in the percentage adhering on different occasions with the same strain could be accounted for if slight variations in culture affects the percentage able to adhere.

With the radioactive experiments, batches of labelled cells were grown and stored at -70°C. Therefore the same batch of labelled cells of each strain was used throughout the experiments. However there are obvious areas which could account for the variation in percentages adhering for any one strain. The most important factor causing variation was the chemiluminescent effect caused by the tissue solubiliser, Soluene 350. Although vials were counted until consistent counts were

obtained, vials left for several days in the dark before recounting showed a reduction in counts, in some cases dropping to very low percentages indeed. The differences in adhesion levels were also greatly reduced in these instances.

It is probable that in a radiolabelled 5 day static culture, a considerable amount of the label was associated with dead cells and debris. Although the culture was washed, it is likely that label was present on dead cells. These labelled nonviable cells would interfere with calculations for levels of lung adhesion and also tend to mask any differences in adhesion of different cell types. Although much less convenient, the viable count method of assaying adhesion would avoid these complicating factors.

1. Inhibition of adhesion

The ability of prior vaccination of mice with either Glasgow standard vaccine or with F-HA (as LAP) to reduce adherence of B. pertussis to mouse lung was examined. The standard vaccine which is a whole cell vaccine showed little ability to inhibit adhesion (Tables 26 and 27). The F-HA preparation administered either i.p. or i.n. 2 weeks prior to adhesion assays being done on the mice caused reduced levels of adherence of B. pertussis. Vaccination by the i.p. route appeared to be marginally more effective in reducing adhesion but this may simply reflect the larger amount of vaccine given by this route. Further experiments would be desirable with mice vaccinated i.n. repeatedly with F-HA to see if better inhibition of adhesion might be obtained. North & Anderson (1942) found that i.n. vaccination of mice gave better immunity to i.n. infection than i.p. vaccination. However solid immunity was only found when mice had been vaccinated several times.

A low-level sublethal i.n. infection was also found to

inhibit subsequent adhesion of B. pertussis to the mouse lung. This indicates that infection stimulates the production of local anti-adhesion antibodies. However it is possible that the antibodies are not antiadhesive but rather act as opsins and therefore stimulate phagocytosis.

When the inoculum was mixed with whole static-grown cell antiserum, no reduction in adhesion was evident. When the inoculum was mixed with F-HA antiserum there was a reduction in the percentage of B. pertussis adhering to the mouse lung (Table 28). The ability of the two antisera to inhibit adhesion correlates with their different anti-HA titres (Table 17). This perhaps indicates that the reduction in adhesion after vaccination with F-HA is due to the production of antiadhesive antibodies.

2. Adhesion of different strains

Cells with different virulence properties were examined for their ability to adhere to mouse lungs. Strain 18323 (i.c. virulent, less i.n. virulent than strain 77/18319, which is avirulent i.c.), adhered less than strain 77/18319. This was expected as it had a lower cholesterol-sensitive HA titre than did 77/18319. In results not presented here, the strain Taberman which was less virulent than strain 77/18319 showed a level of adhesion similar to strain 77/18319 indicating that there is another factor involved in i.n. virulence additional to adhesion. This might, for example, be a factor that promoted the inflammatory response, leading to heightened consolidation of the lungs.

3. Effect of pH on adhesion

The effect of varying the pH of the adhesion assay was studied

(Fig 9). Greater adherence occurred at pH 6.5 than at pH 7.0 or pH 8.0. A pH value of 6.5 was chosen as at this pH the F-HA adhered to CM Sepharose CL6B indicating that at this pH it was positively charged. If fimbriae are involved in overcoming the repulsive forces of like charged surfaces of bacteria and epithelial cells (it is assumed that these are both negative), then giving them a positive charge should increase the ability of the bacteria to adhere. It is impossible to know the exact pH at the lung surface where the B. pertussis cells adhere, however the pH of lung washing was found to be about pH 7.0. It is possible that the pH at the epithelial surfaces may be slightly acidic due to the mucus and dissolved CO_2, which would make adhesion of B. pertussis cells easier. With gonococci, a maximum level of adhesion was obtained between pH 5.5 and 7.0. The pH of maximum adhesion corresponded to the pH of the endocervix and male urethra.

4. Effect of F-HA inhibitor cholesterol on adhesion

The F-HA inhibitor cholesterol, which had no effect on i.n. or i.c. virulence also had no effect on bacterial adhesion to mouse lung (Table 28). There are two possible explanations for the ability of cholesterol to inhibit HA but not adhesion to mouse lung. These possibilities are (a) the cholesterol dispersions act as receptors for the fimbriae but they themselves have an affinity for epithelial cells which they do not have for erythrocytes hence causing indirect adhesion of B. pertussis to mouse lungs, or (b) cholesterol is not a receptor for the fimbriae but has an affinity for mammalian cells which causes the fimbrial receptors to be blocked. If proposal (b) were the case then this could explain the difference between HA inhibition and inhibition of adhesion to mouse lung. In the case of HA there is a limited number

of erythrocytes present and the cholesterol present in the system may be sufficient to block enough receptor sites to inhibit HA. With the mouse lung adhesion system there should be a vast number of receptors for F-HA present and the chance of the concentration of cholesterol present in the lung blocking all the receptors is very slight.

An experiment which would help in discerning whether or not cholesterol is a receptor for the F-HA would be to add radiolabelled cholesterol to a bacterial cell suspension which would then be centrifuged. If cholesterol is recovered with the cell pellet then it is probably attached to the bacteria via the fimbriae. If radiolabelled cholesterol is not recovered with the cell pellet, but when instilled into mouse lungs is found attached to the lungs it would seem unlikely that cholesterol is a receptor for the fimbriae.

C. Modulation, phase and colonial variation

Lacey (1960) showed that C mode cells had lost the HA. Since then it has been shown that B. pertussis has two HAs, a F-HA and a HA property associated with pertussigen (Arai & Sato, 1976). Pertussigen is lost during modulation as indicated by the loss of histamine sensitising and other activities (Parton & Wardlaw, 1974). In this study B. pertussis was grown in C modulating conditions in static culture, which stimulates production of fimbriae, but no HA ability was associated with 5-day cells. No fimbriae were observed by electron microscopy and no high molecular weight bands were found on SDS-PAGE of freeze dried culture supernate of C-mode cells (Plate 1). Under static conditions, the C-mode cultures did not grow as well nor form a surface pellicle like X-mode cultures; lack of a pellicle suggested a more hydrophilic cell surface in C-mode than in X-mode cells. Similar

observations were made with both phase IV and large colony variants. These three cell types (C mode, phase IV and large colony variants) had similar virulence properties (although phase IV was not tested here). Although C-mode and large variants lacked HA and fimbriae they were not completely avirulent. However the low virulence cannot be attributed solely to the lack of HA, as these variants are also deficient in other factors (Andersen, 1953; Wardlaw et al, 1975).

These three variant types were all compared to X-mode or phase I cells in the mouse lung adhesion system. All three showed a very marked reduction in the percentage of inoculum adhering which may explain their lower virulence. Although these cells are of low virulence a level of lung pathology is observed with C-mode and large colony cultures. It is possible that infection results from reversion in the lung to virulent X mode type. This seems likely in the case of C-mode cells since environmental factors in the culture induce C-modulation, and the environment in the lung would not contain the high amounts of $MgSO_4$ required to maintain the C-mode. However with both C-mode and large colony types it is possible that the lung pathology is caused by outgrowth of a constant low level of virulent, X-mode type organisms present in the cultures.

The nature of the interacting surfaces is important in adhesion. The bacterial cell, which is presumed to have a net negative charge has to come in close contact with the mammalian epithelial cell surface, which also has a net negative charge, to allow adhesion to occur. Fimbriae from E. coli and N. gonorrhoea are thought to be hydrophobic (they have high levels of non-polar amino acids). It is possible that the hydrophobic nature of these appendages allows close contact between like charged cells by reducing the bacterial surface charge and conse-

quently the mutual repulsion between the bacterium and the animal cell. The hydrophobic nature of the cell surfaces of C-mode, phase IV or the large colony variant was compared to that of X-mode cells. It was found that these variants were much less hydrophobic than X-mode, phase I small colony type cells insofar as they showed a much lower affinity for octyl-sepharose than did the virulent cell types. As has been discussed previously (p. 127) the low level of retention of B. pertussis C-mode and phase IV cells on octyl sepharose would indicate not only the loss of fimbriae but the loss of other hydrophobic cell surface components. This is indicated by the level of retention of X-mode shake culture cells which although lower than X-mode static cells was higher than C-mode cells. The loss of hydrophobic surface components in C-mode, phase IV and large colony type cells could be contributory to the reduced ability to adhere, by causing the cells to express higher net negative charge. This would increase the repulsive forces between the bacteria and the mouse lung epithelia.

Non-fimbriate, avirulent variants (type III and IV) of N. gonorrhoeae adhere as well as the fimbriate types I and II if the cells are pretreated to remove the negatively-charged carboxyl groups on the cell surface (Heckels et al, 1976). This suggested that the role of the fimbriae in this case was to overcome the repulsive forces between the bacterial surface and the surface of the cultured cells. If fimbriae from B. pertussis are hydrophobic then they may reduce the cell surface charge and allow the cells to adhere. A preliminary experiment where the negatively charged carboxyl groups were removed from C-mode cells caused an increase in their ability to adhere to mouse lung. This indicates that fimbriae are probably involved in overcoming repulsive forces of like-charged surfaces.

D. Purification and protection

In recent years many attempts have been made to purify the protective antigen(s) of B. pertussis. This has become of great importance as the number of children being vaccinated has decreased due to publicity about the risk of brain damage.

Various investigators have purified the components responsible for different pathophysiological activites: HSF (Lehrer et al, 1974, 1976), LPF (Morse & Morse, 1976; Sato et al, 1974), HA (Sato, Arai, 1976), IAP (Yajima et al, 1978). These efforts eventually resulted in the suggestion that many of the toxic properties associated with B. pertussis were caused by a single component termed "pertussigen". The protective properties of these different fractions were tested in the mouse i.c. assay for pertussis vaccine potency (Kendrick et al, 1947) with much resultant controversy. An obvious problem with pertussigen is its toxic nature; indeed Pittman (1979) proposed that pertussigen or pertussis toxin (name preferred by Pittman) is the toxic factor in the disease. It would be expected that, if pertussigen was the toxic factor responsible for disease symptoms, that antitoxin would be protective in man, and if the mouse i.c. model assays human virulence, pertussigen would be protective. However according to Sato et al (1979) and Irons & MacLennan (1979a) prior vaccination with pertussigen afforded no protection against i.c. challenge in mice.

In the study of Sato et al (1979) pertussigen had been toxoided with formaldehyde and found not to be protective. Irons & MacLennan (1979a) used native pertussigen which they found to be toxic at higher levels and not protective at lower non-toxic levels. In contrast Munoz et al (1981) reported that vaccination with pertussigen protected mice against i.c. challenge. It is possible that the toxoiding

procedure used by Sato et al (1979) destroyed the protective activity of the pertussigen.

The results obtained in the present study agree with those of Munoz et al (1981) in that a fraction LSP with high HSF activity, which was toxic, showed protection against i.c. challenge at lower, non-toxic levels. In addition vaccination with LAPcontaining mainly the F-HA also conferred protection against i.c. challenge. This indicates the presence of two protective factors. From SDS-PAGE and histamine sensitisation tests both fractions are cross-contaminated. However the levels of contamination do not account for their protective properties; the PD_{50} value of the LAP (F-HA) corresponded to approximately 4.8 μg but the HSD_{50} value for the preparation was about 19 μg (Tables 15 and 16). With the LSP (pertussigen) the protective dose was approximately 4 to 5 HSD_{50s}. Similarly the level of protein giving protection in the pertussigen preparation was only 0.88 μg compared with 4.8 μg for the F-HA preparation. From these figures it is clear that protection conferred by the F-HA could not be due to contaminating pertussigen and vice versa, therefore there are at least two protective factors.

Munoz et al (1981) suggested that the protection noted with F-HA preparations was dependent on the presence of small amounts of pertussigen, possibly acting as an adjuvant, as they found that F-HA preparations with no detectable pertussigen activity were not protective. Since the F-HA preparation used in these experiments was partly contaminated with pertussigen then this explanation may be applicable. They also suggested that since pertussigen was a poorer antigen than F-HA in the presence of F-HA pertussigen became highly effective. The results obtained in this study disagree with this proposal. Both F-HA and pertussigen are cross-contaminated therefore the pertussigen

fraction should be highly effective due to the presence of F-HA. The effectiveness of the pertussigen in the presence of F-HA cannot account for the level of protective activity associated with the F-HA preparation.

The LAP was used to raise rabbit anti F-HA antisera. The ability of the two F-HA antisera to inhibit HA was shown (Table 17). One serum (2) was very poor, showing approximately the same ability to inhibit HA as did whole cell antiserum. However F-HA antiserum (2) showed different abilities to passively protect mice against i.c. challenge than whole cell antiserum (Table 21). F-HA antiserum (2) was not protective. F-HA antiserum (1) had a higher HAI titre than F-HA antiserum (2), whole cell antiserum to strain 77/18319 or the US standard antiserum. F-HA antiserum (1) was protective against i.c. challenge but not to the same extent as the two whole cell antisera. These results show that although anti F-HA may be protective it is not the major protective factor against i.c. challenge in whole cell antiserum.

F-HA antiserum (1) was also used in studies of passive protection against i.n. infection. Even at a 1/50 dilution of the antiserum a significant reduction in lung pathology was observed. The whole cell antiserum to strain 77/18319 also showed passive protective activity against lung infection but this antiserum was not titrated here. The passive protection results indicate that the F-HA antiserum was a much better protective agent against lung infection than against i.c. infection. This would seem to be true for other antisera including the US standard antiserum (L.Q. Stevenson, personal communication).

To check that the protection afforded by the F-HA antiserum (1) was not due to antibodies to contaminating pertussigen, an anti-HSF assay was done. When mice were injected with 1 HSD_{50} mixed 1:1 with undiluted F-HA antiserum (1) no reduction in deaths was observed when

the mice were challenged with 3 mg of histamine 5 days later (Table 20). This indicated either no or a low level of anti-pertussigen activity in the antiserum. However it cannot be concluded that protection was due only to anti-F-HA activity, as many more antibodies would be required to neutralise the large mass of bacterial substance involved in an HSF test, than to neutralise infection. Therefore there may be antibodies to pertussigen present in the antiserum.

With whole-cell antiserum, it is obvious that there are protective factors involved other than anti-HA activity, since the level of protection afforded by a whole-cell antiserum is not directly related to its anti-HA activity. What these other protective antibodies are directed against is not known. However the results (Table 20) show that whole cell antiserum to strain 77/18319 contains anti-HSF activity.

Recently Sato et al (1980) described an aerosol infection assay similar to the sublethal i.n. infection used in this study. In the aerosol infection, mice were kept in a sealed box and the atmosphere sprayed with droplets containing B. pertussis (Sato, 1980). Using this system Sato et al (1981) showed that antiserum raised against pertussigen with no detectable antibodies to F-HA, protected mice against i.c. challenge. Munoz et al (1981) also showed that antiserum to pertussigen with no detectable antibodies to F-HA, protected mice against i.c. challenge. They also obtained protection against i.c. challenge with a mixed antiserum (antiserum to pertussigen and F-HA) but not with an antiserum raised against pure F-HA. In this present study a low level of passive protective activity against i.c. challenge was found with F-HA antiserum. There are differences in the method used by Munoz et al (1981) and this present study in determining the passive protective activity of the F-HA antiserum. Munoz et al (1981) administered the antiserum i.p. 2 h before i.c. challenge. In this study the challenge was mixed with the F-HA antiserum before being

injected i.c. into the mice. With the method used here it is possible that the protective activity of the F-HA antiserum was not specific. This possibility was suggested by the ability of normal serum to reduce the hydrophobicity of static X-mode B. pertussis cells as much as F-HA antiserum. This might cause a reduction in the ability of the cells to adhere and therefore reduce infection. Normal serum was not tested here for ability to reduce i.c. virulence, but the results of the anti-adhesion assays support a specific effect since F-HA antiserum reduced the adhesion to mouse lung to a greater extent than whole cell antiserum to strain 77/18319.

In the method used by Munoz et al (1981) any non-specific reaction can be discounted as the antiserum is administered separately from the i.c. challenge, via the i.p. route. However there are possible explanations for the lack of protection observed with their F-HA antiserum. The antiserum was administered i.p. and therefore would have to reach the site of infection via the blood. This would require the breakdown of the blood brain barrier which may take several days (Dolby & Standfast, 1961). This means that the antibody to the F-HA is unlikely to be present in the brain when the infecting bacteria first encounter brain substance. The longer the delay in the antibodies reaching the site of infection the less likely antiadhesive antibodies are liable to be effective. This is particularly true for fimbrial adhesion which may only be a transient stage before closer cell-cell contact is established.

From the evidence presented by other workers (Sato et al, 1979; Irons & MacLennan, 1979a) and the results obtained in this study it seems that F-HA is a protective antigen. However from this study it would seem to be less effective than pertussigen, therefore further work should

focus on producing a detoxified pertussigen antigen while maintaining its protective properties.

No work has been done on the serospecificity of the F-HA from different strains of B. pertussis. The antigenic differences in fimbriae from N. gonorrhoeae, have caused problems for production of a fimbrial vaccine. Before a vaccine is prepared using the F-HA, this area should be investigated. In this study the F-HA was derived from strain 77/18319 yet protected against an i.c. challenge with strain 18323. Also antiserum raised from the F-HA preparation protected against sublethal i.n. infection with strain 18334. These preliminary results may indicate that the F-HA of different strains are antigenically similar. However the protective activity against further strains would have to be tested in order to generalize.

E. Conclusions and perspectives

1. Proposed model for adhesion

The following model for adhesion of B. pertussis is suggested by the present results together with other information. However some of the results do not fit the model but further experiments are suggested for resolving inconsistencies.

It is suggested that (1) fimbriae promote rapid adhesion through their hydrophobic structure which may interact with similarly hydrophobic structures on the mammalian cell, or simply mask the negative charge of the bacteria, thereby allowing the bacteria to approach the epithelial cell surface. The results from HIC and mouse protection experiments support this idea. (2) The epithelial cell receptor for the fimbriae is not cholesterol as suggested by its ability

to inhibit HA activity (Irons & MacLennan, 1979a; Sato et al, 1979). Probable explanations for the cholesterol inhibition of HA but not adhesion or virulence were discussed and further experiments which could help clarify the situation were also suggested (p. 134). Also the abundance of cholesterol in mammalian cells would make the specificity of B. pertussis for ciliated epithelial cells difficult to explain. (3) After the initial adhesion between the bacterial cell and the epithelial cell via the fimbriae, a second adhesion involving an outer membrane protein may operate. (4) This second adhesion step may facilitate the transfer of toxic factors, including pertussigen, from the bacterium to the mouse epithelium. (5) This secondary adhesion may occur more slowly than the first. Therefore non-fimbriate cells would be less effective in adhesion and consequently of lower virulence.

There is some evidence to support this secondary adhesion hypothesis (a) the ability of the haptoglobin-containing Cohn fraction IV of plasma to reduce the lung pathology in sublethal i.n. virulence experiments, (b) the ability of bacteria of strain 18323 with a low cholesterol-sensitive HA content to adhere, (c) the lack of protection conferred by F-HA antiserum when administered i.p. before challenge, which would delay its contact with the infecting bacteria (Munoz et al, 1981) and (d) if cholesterol is the receptor for the fimbriae then a second adhesion may provide the specificity of attachment site shown by the B. pertussis cells (Hopewell et al, 1972; Muse et al, 1977).

The ability of B. pertussis to adhere to epithelial cells would seem to be more important for virulence in the mouse lung, than in intracranial infections. In the lung larger numbers of organisms are required for severe lung pathology, than with the i.c. infection where only a few virulent organisms are sufficient for death of the

mouse to occur. As the human disease involves the respiratory tract, adhesion should be important as a method of by-passing the mucociliary clearance mechanisms. To understand the adhesion mechanism(s) is important if antiadhesive activity is to be used for protection. It would also be important to investigate the production of antiadhesion antibodies in the lung secretions. However there would be no point in injecting children with an antiadhesion vaccine if the antibodies remain in the serum. Efficient methods of producing a local antibody response should be further investigated.

2. Proposed future work

From the results in this study and the subsequent discussion there is evidence for an adhesion role for fimbriae in lung infections, but there is also the likelihood of a second adhesion mechanism being involved. Future work should include the search for F-HA negative mutants which have high levels of pertussigen. This would enable further clarification of the role of fimbriae in adhesion without the complication of possible regeneration of fimbriae as is the case with X-mode shake cultures. Adhesion studies should also be done on strains with constant fimbrial endowment but different pertussigen content. This might be achieved by screening strains for constant F-HA but widely different HSF activity. These experiments might show whether the level of pertussigen, which has HA activity, affects adhesion and would therefore be a candidate for a secondary adhesion mechanism.

Identification of the receptors for B. pertussis might help elucidate the possible role of cholesterol in adhesion. Purified haptoglobin should also be used in adhesion and virulence assays to see whether it inhibits these systems.

Another important point is the actual production of fimbriae. Do shaken cells lacking fimbriae produce the appendages in the mouse lung? This would seem to be so as these bacteria are virulent, and the virulence is protected against by F-HA antiserum. Electron microscope examination of organisms before infecting mouse lungs, and organisms removed from mice with lung pathology should help in determining whether or not the fimbriae are regenerated. Some *in vitro* experiments, where shaken cells or blended static cells, are transferred to fresh medium incubated statically, sampled and examined in the electron microscope could reveal the time needed for regeneration of fimbriae. Such a study has already been done with *E. coli* (Novotny *et al*, 1969).

F-HA is worthy of serious consideration as a component of a future improved pertussis vaccine with detoxified pertussigen as a second component. If F-HA is to be used in this way the purification method described here is simple and removes most of the toxic activity. Also there would seem no need to remove the F-HA as it seems to be a potent antigen when attached to the liposomes.

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APPENDICES

Appendix 1 - Liquid Culture Media (10 litre)

	Components of Hornibrook's (g)		Sato *et al* (1974) (g)	
	X medium	C medium	X medium	C medium
Casamino Acids (Difco Technical)	100	100	100	100
$CaCl_2$ anhyd.	0.02	0.02	0.1	0.1
$CuSO_4$ $5H_2O$	-	-	0.0075	0.0075
cysteine HCl	-	-	0.3	0.3
$FeSO_4$ $7H_2O$[1]	-	-	0.1	0.1
Glutamic Acid	-	-	2	2
Glutathione[1]	0.1	0.1	0.1	0.1
KCl	2	2	-	-
KH_2PO_4 anhyd	-	-	5	5
K_2HPO_4 anhyd	2.5	2.5	-	-
$MgSO_4$ $7H_2O$	-	50	-	25
$MgCl_2$ $6H_2O$	0.25	0.25	4	4
Nicotinamide	0.01	0.01	0.3	0.3
NaCl	50	-	25	-
Soluble Starch[2]	10	10	15	15
Tris(hydroxymethyl)amino-methane	-	-	60.7	60.7

All the components of the media except those labelled 1 are dissolved in distilled water and the pH adjusted to 7.2 with Na_2CO_3 in the case of Hornibrook's medium and HCl in the case of Sato *et al* medium. The components labelled 1 are dissolved in water, filter sterilized and then added just before use. The starch (2) is dissolved in boiling water before adding it to the bulk of the medium before sterilisation.

Appendix 2 - Diluents

1% Casamino Acids (C.A.D.) 1 litre -	Casamino Acids (Difco)	10 g
	$MgCl_2\ 6H_2O$	0.1 g
	$CaCl_2$	0.016 g
	NaCl	5 g

Adjust to pH 7.1 with 1.0M NaOH

Phosphate Buffered Saline (PBS) 1 litre -	NaCl	8 g
	KH_2PO_4	0.34 g
	K_2HPO_4	1.21 g

Appendix 3 - Gels and Buffer for P.A.G.E.

STOCK SOLUTIONS

1. Acrylamide (B.D.H.) 30.0 g)
) made up to 100 ml
   NN'methylenebisacrylamide (NN'bis) 0.8 g)

2. Running buffer (10x) pH 8.8

glycine	144.13 g)	
S.D.S.	1.0 g)	made up to 1 litre
Tris	30.28 g)	

3. Solubilizing buffer

0.5M Tris/HCl pH 6.8	25 ml
20% SDS w/v	20 ml
β mercaptoethanol	10 ml
glycerol	20 ml
0.1% bromophenol blue w/v	2 ml
H_2O	23 ml

4. Staining Solution

Coomassie Brilliant Blue R (Sigma)	1.25 g
50% Methanol	454 ml
glacial acetic acid	46 ml

5. Destaining solution

Methanol	50 ml
Acetic Acid	75 ml
H_2O	875 ml

Gels were run in 1/10 dilution of running buffer at a constant current of 15 mA for approximately 3 h, in a Shandon analytical PAGE outfit (Shandon Southern Instruments Ltd., Surrey, England).

Sample Preparation

Protein samples (approximately 200 μg ml^{-1}*) were mixed 1:1 with solubilizing buffer and heated at 100°C for 5 min.

Gel Preparation

(a) Separating gel (11% acrylamide)

Acrylamide/NN'bis	36.7 ml
1M Tris/HCl buffer pH 8.8	37.5 ml
20% (w/v) SDS	0.5 ml
TEMED	25 μl
Ammonium persulphate 0.8% w/v (freshly prepared)	10 ml
H_2O	15.3 ml

(b) Stacking gel (5% acrylamide)

Acrylamide/NN'bis	16.7 ml
0.5M Tris/HCl buffer pH 6.8	25 ml
20% w/v SDS	0.5 ml
TEMED	25 μl
Ammonium persulphate 0.8% w/v	10 ml
H_2O	47.8 ml

* - This was only the case when possible with the pertussigen preparation the protein concentration was much lower.

Appendix 4

Triton-Toluene Scintillant

Triton X-100	1 litre
Toluene (technical grade)	2 litres
2,5-Diphenyloxazole	8 g
1,4-Di-2-(4-methyl-5 phenyl-oxazolyl benzene	0.2 g