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INVESTIGATIONS INTO
THE IMMUNOLOGY, PHYSIOLOGY AND EPIDEMIOLOGY
OF PERTUSSIS

by

MARK GREENSLADE THOMAS
MB ChB, FRACP

A collection of papers submitted for the degree of Doctor of Medicine of the University of Auckland, 1994.
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Among many very generous friends I especially wish to thank Diana and Stephen Mellor, Rob Davison and Mary Rutherford, Harold and Joan Lambert, George and Daphne Griffin, and Mark and Inge Wansbrough-Jones.
ABSTRACT

This volume contains an introduction to the topic of pertussis followed by twelve papers on pertussis arising from research conducted while I was a research fellow at St. George's Hospital Medical School from 1985 to 1988.

**Paper 1** provides a qualitative description of the serum antibody responses to several *Bordetella pertussis* antigens. It demonstrates that the serum antibody response to a specific *B. pertussis* antigen, following either natural infection or vaccination, is dependent both on the antigen and on the subject. Some *B. pertussis* antigens appear to consistently evoke strong serum antibody responses following either natural infection or vaccination, while other *B. pertussis* antigens appear to consistently fail to evoke any significant serum antibody response. Other *B. pertussis* antigens induce an antibody response in a variable proportion of subjects.

**Paper 2** describes the serum IgG, IgA and IgM responses to *B. pertussis* antigens in patients with pertussis. These antibody responses are compared with those in the family contacts of patients with pertussis and with those in infants immunised with a whole cell pertussis vaccine. It demonstrates that both pertussis and pertussis vaccination produce marked serum antibody responses to three *B. pertussis* antigens. In contrast, family contacts who are exposed to a patient with pertussis, but who themselves fail to develop disease, have a minor antibody response to the same antigens. Immunity to disease in these family contacts is associated with high titres of antibody to *B. pertussis* antigens at the time of exposure to infection.

**Paper 3** demonstrates the serum antibody responses to *B. pertussis* antigens in two subjects immunised with new acellular pertussis vaccines. The antibody responses are compared with those in subjects who had been immunised with a whole cell pertussis vaccine, and with those in subjects who had suffered natural infection. The new acellular pertussis vaccines are shown to induce antibody responses solely to two purified *B. pertussis* antigens, and not to other potential contaminating antigens.
Paper 4 demonstrates that the IgA antibody response in nasal mucus in patients with pertussis is less pronounced than the serum antibody responses. It also demonstrates that titres of antibody in nasal mucus are not especially strongly correlated with immunity to pertussis in family contacts.

Paper 5 compares the serum antibody responses to *B. pertussis* antigens in breast fed infants with pertussis with those in bottle fed infants with pertussis. It also compares the levels of IgA to *B. pertussis* antigens in breast milk from mothers of infants with pertussis with the levels in breast milk from mothers of healthy infants. This paper provides further evidence that *B. pertussis* infection does not produce an especially strong secretory immune response.

Paper 6 provides evidence that *B. pertussis* infection does not produce a significant impairment of immunoglobulin synthesis.

Paper 7 compares the responses of peripheral blood lymphocytes, from patients with pertussis and two control groups, to *in vitro* stimulation with *B. pertussis* antigens. This study is the first to show that *B. pertussis* infection induces cell mediated immune responses.

Paper 8 describes the respiratory physiology of six infants with severe pertussis. It demonstrates that abrupt severe hypoxaemia may be due to prolonged apnoea or may occur despite continued breathing movements and respiratory airflow. It postulates that these findings are due to a ventilation perfusion mismatch secondary to alveolar atelectasis caused by a defect in lung surfactant synthesis, secretion or function.

Paper 9 describes the effects of *B. pertussis* infection on the ciliary function and electron microscopic appearances of human nasal epithelial cells. These findings are extended by similar investigations performed on human nasal epithelial cells exposed to *B. pertussis* toxins in vitro.

Paper 10 attempts to explain the large-scale epidemiology of pertussis by a simple mathematical formula. This formula is then used to derive an estimate of the proportion of the population susceptible to pertussis. Finally the relationship between the incidence of pertussis and the level of vaccine uptake is illustrated using data from several countries during the last century.
Paper 11 describes the small-scale epidemiology of pertussis and demonstrates that pertussis is usually transmitted by patients with clinical disease rather than by persons with either atypical disease or asymptomatic infection.

Paper 12 briefly discusses the use of erythromycin and other antimicrobial agents in the treatment and prophylaxis of pertussis.
STATUTORY DECLARATION

I, Mark Greenslade Thomas of Auckland, Candidate for the Degree of Doctor of Medicine of the University of Auckland hereby state and declare as follows:

1. THAT in the research described in the paper entitled "Human serum antibody responses to Bordetella pertussis infection and pertussis vaccination" the execution of the study, the interpretation of the results and the preparation of the manuscript were largely my own work. Prof. Harold Lambert initiated the collaboration with Dr. Keith Redhead and contributed advice on the final wording of the manuscript. Dr. Keith Redhead assisted with the design of the study and contributed advice on the final wording of the manuscript.

2. THAT in the research described in the paper entitled "Serum IgG, IgA and IgM responses to pertussis toxin, filamentous hemagglutinin, and agglutinogens 2 and 3 after infection with Bordetella pertussis and immunization with whole-cell pertussis vaccine" the design and execution of the study, the interpretation of the results, and the preparation of the manuscript were largely my own work. Prof. Harold Lambert initiated the collaboration with Dr. Ted Ashworth and Dr. Liz Miller and contributed advice on the interpretation of results and the final wording of the manuscript. Dr. Ted Ashworth performed the antibody assays and contributed advice on the interpretation of the results and the final wording of the manuscript. Dr. Liz Miller provided the sera from healthy vaccinees and contributed advice on the interpretation of the results and the final wording of the manuscript.

3. THAT in the research described in the paper entitled "Human serum antibody responses to acellular pertussis vaccine" the design and execution of the study, the interpretation of the results, and the preparation of the manuscript were largely my own work. Prof. Harold Lambert suggested the topic and contributed to the final wording of the manuscript. Dr. Patrick Olin provided the serum samples from two Swedish vaccinees. Dr. Keith Redhead contributed to the interpretation of the results and the final wording of the manuscript.
4. THAT in the research described in the paper entitled "Nasal immunoglobulin A responses to pertussis toxin, filamentous hemagglutinin, and agglutinogens 2 and 3 after infection with *Bordetella pertussis* and immunization with whole-cell pertussis vaccine" the design and execution of the study, the interpretation of the results, and the preparation of the manuscript were largely my own work. Prof. Harold Lambert initiated the collaboration with Dr. Ted Ashworth and Dr. Liz Miller and contributed advice on the interpretation of results and the final wording of the manuscript. Dr. Ted Ashworth performed the antibody assays and contributed advice on the interpretation of the results and the final wording of the manuscript. Dr. Liz Miller provided the nasal swabs from healthy vaccinees and contributed advice on the interpretation of the results and the final wording of the manuscript.

5. THAT in the research described in the paper entitled "The contribution of breast feeding to immunity to pertussis" the design and execution of the study, the interpretation of the results, and the preparation of the manuscript were largely my own work. Prof. Harold Lambert initiated the collaboration with Dr. Ted Ashworth and Dr. Liz Miller. Dr. Ted Ashworth performed the antibody assays and Dr. Liz Miller provided the serum samples and nasal swabs from healthy vaccinees and also the breast milk samples from the mothers of healthy vaccinees.

6. THAT in the research described in the paper entitled "*Bordetella pertussis* infection does not suppress human serum immunoglobulin responses" the design of the study, the interpretation of the results, and the preparation of the manuscript were my own work. Prof. Harold Lambert initiated the collaboration with Dr. Pamela Riches. Dr. Pamela Riches performed the antibody assays and contributed to the final wording of the manuscript.

7. THAT in the research described in the paper entitled "Human cellular immune responses to *Bordetella pertussis* infection" I made a major contribution to the design of the study, provided the blood samples from patients with pertussis, and contributed to the interpretation of the results and the final wording of the manuscript.

8. THAT in the research described in the paper "Severe hypoxaemia in pertussis" the execution of the study, the interpretation of results, and the preparation of the manuscript were largely my own work. Prof. Harold Lambert initiated the collaboration with Dr. David Southall and contributed advice on the final wording of the manuscript. Dr. David Southall designed the study, and assisted with the interpretation of results and the preparation of the manuscript.
9. THAT in the research described in the paper entitled "Effects of *Bordetella pertussis* infection on human respiratory epithelium in vivo and in vitro" I made a major contribution to the design of the study, provided the nasal epithelial samples from patients with pertussis, and contributed to the interpretation of the results and the final wording of the manuscript.

10. THAT in the research described in the paper entitled "Epidemiology of pertussis" the conception, and execution of the study were my own work. Dr Norman Noah and Prof. Harold Lambert contributed advice on the final wording of the manuscript.

11. THAT in the research described in the paper entitled "From whom do children catch pertussis?" the execution of the study, its interpretation and the preparation of the manuscript were my own work. Prof. Harold Lambert suggested the topic and contributed advice on the interpretation of results and on the final wording of the manuscript.

12. THAT the paper entitled "Antibiotics in whooping cough" was the result of my own research into this topic. Prof. Harold Lambert and the editors of the Drug and Therapeutics Bulletin contributed to the final wording of the manuscript.

I further state and declare THAT none of the work identified as being my own in (1) to (12) above has previously been accepted for a degree or diploma awarded to me by any University.

Signed by me **MARK G. THOMAS**

this **11th** day of **July** 1994.

Witnessed by **PETER HUGGARD**

**MANAGER**

**AUCKLAND, NEW ZEALAND**

**JUSTICE OF THE PEACE**
RESEARCH AIMS

1. To investigate the immune responses to purified components of *B. pertussis* following natural infection and to compare these responses with those produced by immunisation with the killed whole-cell pertussis vaccine. The *B. pertussis* antigens selected for study were those regarded as most important in conferring immunity to pertussis and were those included in new acellular pertussis vaccines manufactured by a variety of pharmaceutical companies.

2. To compare the immune responses to *B. pertussis* antigens found in patients with pertussis with those found in family contacts of patients with pertussis in an attempt to identify determinants of immunity to disease.

3. To investigate the value of immune responses to component antigens as a diagnostic test for *B. pertussis* infection.

4. To investigate the clinical and pathophysiological features of pertussis particularly with regard to the mechanisms responsible for apnoea and hypoxaemia.

5. To investigate the epidemiology of pertussis particularly with regard to the frequency with which persons with asymptomatic infection or atypical illness transmit the infection to susceptible children.
INTRODUCTION

Whooping cough or pertussis is an important disease of childhood. It has a worldwide distribution and is estimated to be responsible for 500,000 to one million deaths globally each year (1). Most of these deaths are in poorer countries where malnutrition and a high incidence of other childhood illnesses exacerbate the effects of pertussis. In more affluent societies it is rarely fatal but is responsible for a prolonged debilitating illness which causes great anxiety and disruption of family life (2).

In susceptible persons the disease has a characteristic and easily recognised presentation. Following inhalation of infected droplets *Bordetella pertussis* multiplies on the ciliated respiratory epithelium. After an incubation period of approximately one week symptoms commonly start with rhinorrhea and an intermittent productive cough. This catarhal phase is closely followed by the onset of episodes of repetitive paroxysmal cough. The cough is generally nonproductive and worse at night and is frequently followed by an inspiratory whoop or vomiting or episode of apnoea which may progress to cyanosis and convulsions. The average duration of cough is approximately six weeks but may be much longer and episodes of viral upper respiratory tract infection may precipitate brief recurrences of a typical paroxysmal cough in the months following apparent recovery from pertussis.

While the prolonged and repeated episodes of paroxysmal cough may be exhausting for both the patient and his family, it is the associated symptoms of vomiting and apnoea which are responsible for the major adverse effects of the illness. Failure to gain weight during the period of severest symptoms is common in infants, and some may require admission to hospital and a brief period of intravenous fluid therapy for dehydration due to persistent vomiting. Apnoeic episodes also may be severe enough to warrant admission to hospital. In older patients apnoeic episodes usually follow a paroxysm of coughing and rarely cause cyanosis. However in infants apnoeic episodes may occur either following a paroxysm of coughing or during normal breathing and may cause hypoxaemia severe enough to precipitate convulsions (3). Severe episodes are responsible for many of the deaths in infants with pertussis and may be responsible for permanent intellectual impairment in infants who survive the illness (4).
Despite the severe and occasionally life threatening nature of the symptoms in patients with pertussis there is usually little evidence of systemic illness. Between paroxysms of coughing patients usually appear well and this commonly leads to delays in diagnosis. Fever is rare and when it does occur is usually mild and brief. Bacteraemia does not occur, the erythrocyte sedimentation rate is normal (in the absence of secondary infection), and the serum biochemistry is usually normal (allowing for the effects of vomiting if present). A marked lymphocytosis (>10 X 10^9/L) which is present in approximately 50% of patients with pertussis (5) is the only systemic effect which has been repeatedly demonstrated. It is frequently stated that secondary bacterial infections, particularly bronchitis, pneumonia, and otitis media are common following an episode of pertussis but there is little published evidence to support this claim. At present there is no effective treatment for patients with pertussis. Erythromycin eradicates B. pertussis infection within three days of the start of treatment but has little (6,7) or no (8) effect on the severity or duration of symptoms. A variety of other agents including corticosteroids, and salbutamol have been recommended but there is little evidence that they are beneficial (9). Administration of hyperimmune globulin containing high titres of antibody to B. pertussis virulence factors reduces the duration of whooping but has no effect on the duration or severity of coughing or vomiting or on the duration of hospital admission in young children with pertussis (10). In the absence of pharmacological remedies treatment relies on careful continuous observation and prompt assistance to restore breathing and reverse hypoxaemia when apnoea occurs.

Pertussis is a highly contagious disease and unless protected by vaccination most children will suffer the disease before the age of ten years (11). The infection is transmitted by inhalation of droplets coughed out by a patient. The organism adheres to the ciliated cells of the respiratory epithelium and replicates there. In a susceptible host the average duration of infection is about four weeks (12). Symptoms usually begin one week after the acquisition of infection and persist for an average of three weeks after the resolution of infection. Patients are most infectious during the first week of symptoms when the diagnosis has not usually been considered (13). Despite the ability of B. pertussis to infect the ciliated ependymal cells of mice and the ciliated respiratory mucosa of a number of laboratory animals it does not appear to naturally infect species other than man.
MICROBIOLOGY

While pertussis has been recognised since at least the sixteenth century, it was not until 1906 that the responsible organism was recovered in the laboratory. Bordet and Gengou who had previously seen small gram negative bacilli in the sputum of a patient with pertussis, then isolated *B. pertussis* from sputum using agar supplemented with potato starch infusion, glycerol, and defibrinated blood (14). This medium has subsequently been known as Bordet-Gengou or BG medium. *B. pertussis* grows slowly on this, and other media and incubation for three to four days is commonly required before the small raised glistening colonies are visible. The isolation of *B. pertussis* from clinical specimens may be improved by supplementing the medium with charcoal, starch, anion exchange resins, blood cells, albumin, or methyl-B-cyclodextrin. These agents adsorb unsaturated fatty acids and other substances which may be present on laboratory glassware or be produced by *B. pertussis* and which would otherwise inhibit its growth (15). A further improvement in isolation of *B. pertussis* may be achieved by supplementing the medium with an antibacterial which will inhibit the growth of other bacteria present in respiratory secretions and which might otherwise overgrow any *B. pertussis* colonies. Cephalexin 40 μg/ml is commonly used for this purpose (16). Another refinement of culture technique has been the development of the Stainer Scholte synthetic liquid medium which may be used to grow large amounts of *B. pertussis* for vaccine production etc. (17).

The fastidious requirements of *B. pertussis* make it a difficult organism to isolate from patients with disease and despite careful handling of specimens isolation rates are usually less than 40% (18,19). Early investigators incubated cough plates which had been held in front of the patient during a paroxysm, but it is now more usual to use a swab introduced into the nasopharynx to collect mucus which is then inoculated onto the isolation medium. Aspiration of nasopharyngeal mucus using a soft flexible cannula is a less commonly used alternative to swabbing the nasopharynx with a calcium alginate tipped flexible wire swab. While these two methods have not been compared in a single study, isolation rates appear to be higher for aspirated mucus (approximately 80%) (17), than for swabbed mucus (approximately 30-40%) (18,19) possibly because a larger volume specimen is collected using a cannula.

The low rate of isolation of *B. pertussis* using conventional culture techniques has prompted attempts to identify *B. pertussis* in specimens using other techniques. A
fluorescent antibody technique to detect *B. pertussis* in nasopharyngeal mucus has been found to be less sensitive than culture (23-25). However because the result is rapidly available a positive fluorescent antibody test may be useful while awaiting the results of culture.

Improved knowledge of the *B. pertussis* genome has enabled the development of diagnostic tests based on the detection of *B. pertussis* DNA in nasopharyngeal mucus. The identification of a repeat sequence present in the *B. pertussis* genome (26) was followed by the development of a gene probe for this repeat sequence (27). Initial evaluation of this diagnostic test suggested that it did not provide improved sensitivity when compared with routine culture techniques. Subsequently the polymerase chain reaction has been used to amplify target *B. pertussis* DNA in an attempt to improve diagnostic sensitivity (28). When nasopharyngeal aspirates from children with suspected pertussis were tested a *B. pertussis* specific PCR product was detected in 19/25 culture positive and 5/50 culture negative samples in one study (29), and in 63/66 culture positive and 35/266 culture negative samples in another study (30). In both studies there was strong epidemiologic, clinical or serologic evidence of *B. pertussis* infection in the patients from whom the PCR positive but culture negative samples were obtained. These results suggest that, especially in the research setting, PCR detection of *B. pertussis* DNA may be a useful addition to isolation of the organism for the confirmation of infection.

Counterimmunoelectrophoresis has been used to detect *B. pertussis* antigens in serum or urine from patients with pertussis and was found to be more sensitive (10/17 patients positive) than culture (8/17 patients positive) (31). These results are surprising as *B. pertussis* causes surface infection of the respiratory epithelium without tissue invasion. A possible explanation is that the antiserum reacted with an antigen transported from the surface infection into the patient's circulation.

Two tests attempting to detect *B. pertussis* proteins in nasopharyngeal specimens have been developed to diagnose *B. pertussis* infection. An enzyme-linked dot blot immunoassay which used a monoclonal antibody to pertussis toxin (see below) was tested on a small number of nasopharyngeal aspirates and swabs from patients with pertussis (32). While the test appeared to have similar sensitivity to culture it was marred by a significant false positive rate in healthy controls. *B. pertussis* is almost unique among bacterial species in its ability to synthesise and secrete large amounts of adenylate cyclase (AC). An assay for the presence of this enzyme in nasopharyngeal
secretions has been found to be a useful rapid test which correlates well with the results of *B. pertussis* culture. In one study *B. pertussis* was isolated from 23/28 nasopharyngeal swabs with high levels of AC (positive predictive value 82%) and was isolated from only 11/92 swabs with low levels of AC (negative predictive value 88%) (33). In a second study high levels of AC were found in 37/56 culture positive nasopharyngeal aspirates (sensitivity 66%), but were found in only 8/145 culture negative nasopharyngeal aspirates, and in only 6/91 nasopharyngeal aspirates from patients with negative serology (specificity 93%) (34).

**PATHOGENESIS**

The pathogenesis of pertussis is poorly understood. In 1912 Mallory and Hornor described the histological changes in the respiratory tracts of children who had died from pertussis (35). They observed masses of bacteria between the cilia of the epithelial cells, loss of ciliated cells, and a cellular immune response to infection. There was no evidence of tissue invasion or necrosis. There are few other reports of the histopathology or of the pathophysiology of the respiratory tract in pertussis (13). This dearth of information about human pertussis is mirrored by the absence of a good animal model of disease. While a variety of laboratory animals may be infected with *B. pertussis*, in none does the infection induce a syndrome similar to that in man. *B. bronchiseptica*, a closely related species causes kennel cough in dogs and atrophic rhinitis in pigs (36), but neither of these diseases has been widely used as an animal model of human pertussis.

The animal models most commonly used to investigate *B. pertussis* infection have been the mouse intracerebral infection model, and the mouse respiratory tract infection model. The mouse intracerebral infection model was developed to provide a convenient method of assessing the potency of pertussis vaccines. In this assay mice are given an intracerebral injection of a laboratory strain of *B. pertussis* and the mortality over the next fourteen days determined. Protection of mice against death by prior intraperitoneal immunisation with pertussis vaccine has been shown to correlate with the protective effect of the same vaccine in children (39). In the absence of another reliable laboratory test for vaccine efficacy, this test has remained widely used. However efforts to elucidate the pathogenesis of human *B. pertussis* infection and the mediators of
immunity to disease have led to the development of the mouse respiratory tract infection model which more closely reflects the disease in humans. In this model mice are inoculated with \textit{B. pertussis} either by nasal instillation of a suspension of organisms or by exposure to an aerosol generated from such a suspension. Depending on the age of the mice and the size of the inoculum, infected mice suffer a variety of effects including an increase in lung weight and \textit{B. pertussis} bacterial density, lymphocytosis, splenomegaly, fall in body temperature, weight loss, sensitisation to the effects of an intraperitoneal injection of histamine, potentiation of insulin secretion, and death (38,39). This model has been used to study the effects of infection with mutant \textit{B. pertussis} strains deficient in one or more virulence factors (40), intraperitoneal immunisation with purified \textit{B. pertussis} antigens (41,42), and infusion of antibodies to purified \textit{B. pertussis} antigens (41-43).

A variety of features of pertussis infection in humans and in the animal models suggest that pertussis is a toxin mediated disease. The prolonged duration of symptoms following resolution of infection, the recurrence of typical symptoms upon acquisition of a viral respiratory infection, and the presence of a marked lymphocytosis early in the illness have been regarded as evidence of a circulating toxin in human pertussis. The recognition that many of the features of \textit{B. pertussis} respiratory infection in mice can be simulated by administration of purified \textit{B. pertussis} toxins (44) provides further support for this conclusion. Table 1. summarises the available information about those \textit{B. pertussis} toxins which have been most fully investigated (45-49).

Pertussis toxin (PT)[ synonyms: pertussigen, lymphocytosis promoting factor (LPF), islet activating protein (IAP), histamine sensitising factor (HSF)] is responsible for a multitude of effects in-vitro and in animal models. Pittman has postulated that the harmful effects of pertussis are caused almost entirely by this toxin (45,46). It is presumed that PT is responsible for the striking lymphocytosis which is common in the early phase of human pertussis. (see below.) An attenuated hypoglycaemic response to adrenaline has been reported in one series of patients with pertussis (50) and may be another result of PT action in human infection, but apart from these effects there is little direct evidence that PT is important in the pathogenesis of human disease.

Tracheal cytotoxin (TCT) is a muramyl peptide fragment of cell wall peptidoglycan which causes slowing of ciliary motility and extrusion of ciliated respiratory epithelial cells in hamster tracheal explants (51,52. These effects are similar to those seen when \textit{B. pertussis} is incubated with hamster tracheal epithelial cells (53,54) and may be
important in the pathogenesis of pertussis in man (55). It appears that TCT causes ciliated respiratory epithelial cells to produce, but not release, interleukin-1 which then is responsible for inhibition of replication and destruction of these cells (56). Adenylate cyclase appears to cause apoptosis of alveolar macrophages (57) and may contribute to the evasion of host defences by *B. pertussis*. The importance of the other identified toxins is even less certain.

In order to produce disease *B. pertussis* must first adhere to host cells at the site of infection. Adherence of *B. pertussis* to ciliated respiratory epithelium in experimental infection prevents clearance of the infecting organism by the mucociliary stream (58). *In vitro* evidence suggests that PT, filamentous haemagglutinin (FHA), agglutinogens 2 and 3 (Agg 2/3), and pertactin (69kD antigen) may be important in mediating adherence. Phase I *B. pertussis* adheres to hamster (58) and human (59) ciliated epithelial cells whereas Phase III organisms do not. This difference is presumably due to failure of Phase III organisms to produce sufficient adherence factors. Phase III organisms do not produce PT, FHA, pertactin or fimbriae (60). (Expression of other toxins including adenylate cyclase, dermonecrotic toxin and haemolysin is also lost (49).) Mutants of *B. pertussis* deficient in either PT or FHA have reduced adherence to human ciliated respiratory epithelial cells. This effect is more pronounced in PT deficient mutants than in FHA deficient mutants (60). Furthermore anti-FHA raised in mice (61), goats (62), and rabbits (63), inhibits adherence of *B. pertussis* to HeLa, WiDr, and Vero cells respectively, and monoclonal antibodies to FHA, PT and Agg 2/3 inhibit adherence of *B. pertussis* to Vero cells (64). PT and FHA appear to mediate adhesion of *B. pertussis* to ciliated respiratory epithelial cells via carbohydrate recognition domains which bind to ciliary lactosamines (65), and pertactin mediated adhesion appears to utilise a similar mechanism (66,67). Attachment to and invasion of macrophages by *B. pertussis* via FHA binding to the integrin CD11b/CD18 (68) and or PT binding to cell surface carbohydrates (69) may also play a role in the evasion of host defences and persistence of infection. Electron microscopy suggests that fimbriae may be important in the adherence of *B. pertussis* to ciliated epithelial cells (59). Such fimbriae appear to be composed of agglutininogen 2 (70-73) or agglutininogen 3 (71,72).

The production of several *B. pertussis* virulence factors, including pertussis toxin, filamentous haemagglutinin, adenylate cyclase-haemolysin, and pertactin is regulated by the products of a separate genetic locus termed *vir* (74) (Figure 1). This locus codes for the production of three gene products termed BvgA, BvgB and BvgC (75). BvgB and BvgC together comprise a transmembrane sensor protein which conveys a message to
the cytoplasmic regulator protein which binds to DNA and can positively or negatively regulate the expression of multiple genes either directly (eg fha and vir) or indirectly (eg ptx and ac-h) via a second activator protein (76).

*B. pertussis* may become avirulent with coordinate loss of all the *vir* regulated proteins as a result of a reversible frameshift mutation in the *vir* gene (77). This change is called phase variation with virulent organisms termed phase I and avirulent organisms termed phase III. A similar change from virulence to avirulence may be achieved in the laboratory by alteration in growth conditions (78). Thus a phase I virulent strain will repress production of all *vir* regulated virulence factors if it is cultivated in the presence of MgSO4 (20mM) or nicotinic acid (5mM) or at reduced temperatures (22-27°C). The mechanism of this effect, termed antigenic or phenotypic modulation, is by an action on the transmembrane BvgB-BvgC sensor protein which inhibits the transmission of the phosphorylation message from the sensor protein to the BvgA regulator protein (75,76,79). Virulent organisms grown in the absence of these modulators are said to be in X mode while avirulent organisms grown in the presence of these modulators are said to be in C mode. The significance of phase variation and phenotypic modulation on the growth of *B. pertussis* in the human respiratory tract is unknown.

**SEROLOGIC RESPONSES TO BORDETELLA PERTUSSIS IN ANIMAL MODELS**

Antibodies to FHA, PT and Agg 2/3 protect animals against the effects of experimental infection with *B. pertussis*. Most studies have investigated the effects of active or passive immunisation on *B. pertussis* aerosol infection of 7-10 day old mice. Such animals, if unprotected, have a persistent infection, develop a lymphocytosis, lose weight, and die 14-28 days after infection. The protection provided by active or passive immunisation results in enhanced clearance of infection from the lungs, failure to develop a lymphocytosis, or to lose weight, and survival for more than 28 days after infection. This protection may result from immunity to the antigens responsible for adherence and/or from immunity to the antigens responsible for the toxic systemic effects. Sato and co-workers found that passive immunisation of mice with rabbit (63), goat or mouse (80) anti-FHA IgG prevented the weight loss and mortality normally induced by aerosol *B. pertussis* infection. Active immunisation of mice with FHA resulted in elevated anti-FHA IgG and IgM titres, reduced lung colonisation with *B.*
pertussis and reduced mortality (81). However in a different animal model Ashworth et al (82) found that there was no correlation between the elimination of nasopharyngeal B. pertussis colonisation and serum antibodies to FHA, following immunisation of rabbits with FHA.

Passive immunisation of mice with mouse polyclonal (80), goat polyclonal (80,81), or mouse monoclonal (83) anti-PT IgG prevented mortality from aerosol B. pertussis infection. Active immunisation with PT (81) resulted in elevated anti-PT IgG and IgM, reduced colonisation with B. pertussis and reduced mortality. Elevated serum antibodies to PT following active immunisation did not however protect rabbits against nasopharyngeal colonisation following intranasal instillation (61). Passive immunisation of mice with goat or mouse anti-FHA plus anti-PT IgG resulted in increased survival (compared with either anti-FHA or anti-PT given alone) following B. pertussis aerosol infection of mice (80). When given alone anti-PT was more effective than anti-FHA in protecting mice against aerosol infection (80,81). Preston and his co-workers have studied the effect of varying the serotype of B. pertussis used for active immunisation, and for inducing respiratory infection in experimental animals. They found that immunisation of marmosets or rabbits with a vaccine lacking agglutinogen 3 was associated with prolonged respiratory infection when agglutinogen 3 containing serotypes were used as the infecting organism (84,85). A general conclusion from all these studies is that humoral immunity to FHA, PT, and Agg2/3 is important in protecting animals from the effects of B. pertussis infection, but that the relative importance of antibodies to each virulence factor is not apparent.

**SEROLOGIC RESPONSES TO BORDETELLA PERTUSSIS IN HUMAN INFECTION**

Bordet and Gengou detected agglutinating and complement fixing antibodies in convalescent sera from patients with whooping cough in 1906 (14). Since then the humoral immune response and its relationship to resistance to infection has been extensively investigated. Donald (86) studied 123 patients with culture positive pertussis and found that the proportion of patients with complement fixing antibody increased from 25% in the third week of illness to 89% in the eighth week of illness. Antibody titres were highest in the eighth week of illness. Evans and Maitland (87) detected agglutinating antibodies in sera from 23 patients with whooping cough. A titre of 1:160
or greater was found in 15/18 sera collected three or more weeks from the onset of illness. All four sera collected less than two weeks from the onset of illness had a titre of less than 1:10. Table 2 shows the proportion of patients with "positive" serology in relation to duration of illness for a number of studies (86-90). In general agglutinating and complement fixing antibodies could be detected in most patients by the third week of illness. Infants frequently had lower titres of antibody than older children (86,88,91,92), and in children of all ages antibody titres declined within one to two years after recovery from the illness (88). Asymptomatic adults, including those in close contact with pertussis patients, rarely had detectable antibody (86,88,93). Following vaccination, complement fixing (88,93) and agglutinating antibodies (94,95) could be detected in most individuals. The titre of these antibodies remained elevated for up to six years (94,95), and the titres of antibodies in vaccinated older children were equivalent to those in vaccinated infants (93).

The presence in serum of agglutinating antibodies following vaccination is associated with protection against subsequent development of clinical illness. Miller et al (96) followed over 500 children for up to five years following vaccination with 80 billion phase 1 \textit{B. pertussis} cells. Seventy-nine episodes of exposure to whooping cough were recognised in 77 children during the post vaccination followup period. Ten cases of whooping cough occurred, all but two following a recognised exposure to whooping cough. All children who developed whooping cough had an agglutinin titre of less than or equal to 1:160 before exposure while 46/69 children exposed to whooping cough who did not develop disease had agglutinin titres of at least 1:320 before exposure. Sako (97) observed clinical whooping cough following familial exposure in 89.7% of 438 unimmunised children, all of whom had an agglutinin titre of less than 1:10 before the onset of clinical illness. In contrast none of 149 immunised children, with an agglutinin titre of at least 1:320, and only 18.8% of 344 immunised children with an agglutinin titre of less than 1:320, developed whooping cough following familial exposure. The Medical Research Council (98) investigation also found a strong correlation between the agglutinin titre following vaccination and the degree of protection against home exposure to whooping cough. The development of complement fixing antibodies, in parallel with agglutinating antibodies, following disease or vaccination suggests that they may also be correlated with protection against subsequent illness but this has not been prospectively studied.

Immunity to pertussis following disease or vaccination is associated with antibodies directed against a wide range of \textit{B. pertussis} antigens (99). The antigen responsible for
complement fixing antibody is a soluble antigen not thought to be exposed on the cell surface (100). The antigens responsible for agglutinating antibody are surface antigens which vary between strains of *B. pertussis*. Agglutinogen 1 is common to all strains of *B. pertussis* while agglutinogens 2 - 6 occur in various type specific combinations (101,102). Antibody directed against agglutinogens 2 and 3 appears to be important in providing immunity to disease caused by strains expressing these antigens. Preston tested strains of *B. pertussis* isolated in Manchester before 1958 and found they mostly contained agglutinogens 1 and 2 while those isolated in Leeds and elsewhere in England after 1959 mostly contained agglutinogens 1 and 3 (103). He suggested that the use of vaccines which contained mostly agglutinogens 1 and 2 had resulted in inadequate protection against strains containing agglutinogens 1 and 3, and subsequently confirmed that these strains comprised 85% of clinical isolates (104). The 15% of strains which contained agglutinogens 1 and 2 were only isolated from unvaccinated subjects. These changes in the serotypes of clinical isolates of *B. pertussis* were associated with evidence that the efficacy of pertussis vaccination declined during this period but improved again when the vaccine was reformulated to contain significant amounts of both agglutinogens 2 and 3 (105). Further studies comparing clinical isolates of *B. pertussis* from children with pertussis in England in 1977-79 and 1981-83 have confirmed the importance of immunity to the agglutinogens in the prevention of pertussis (106). Similar evidence of the importance of the agglutinogens was obtained in Finland where the use of a vaccine shown to be ineffective in inducing antibodies to agglutinogen 2 was associated with outbreaks of infection almost exclusively due to strains containing agglutinogen 2. When the vaccine was reformulated to stimulate antibodies to agglutinogen 2, as well as agglutinogen 3, the incidence of pertussis declined dramatically (107).

Further evidence that PT, FHA, and Agg 2/3 are important in the pathogenesis of human pertussis is provided by the results of recent trials of Japanese acellular vaccines (108-110). Immunisation of Japanese children from the age of two years with three doses of vaccines containing PT, FHA, and agglutinogens resulted in mean titres of anti-PT and anti-FHA which were comparable to or exceeded those observed in convalescent sera, and a high degree of protection against pertussis. While there is considerable evidence that these vaccines contain large amounts of PT, FHA, and very small amounts of lipopolysaccharide, there is no certainty that their protective effect is not partially dependent on antibodies induced to "contaminating" antigens. A trial with highly purified vaccines, shown not to induce an antibody response to other antigens, has demonstrated that immunisation with PT alone, or with PT plus FHA, provides
considerable, but not complete, protection against pertussis (111). A surprising, and at present unexplained, finding from this trial was that the titres of antibodies to PT and FHA in the vaccinated children did not correlate with the degree of protection against pertussis.

If antibodies to PT, FHA, and Agg 2/3, alone or in combination, provide immunity to pertussis by inhibition of adherence and thus prevention of infection, or by neutralisation of toxin action and thus prevention of disease, it should be possible to detect elevated titres of these antibodies in sera collected following disease or vaccination. Granstrom et al (112) used an ELISA to detect serologic responses to FHA in 25 subjects with culture positive pertussis. A twofold or greater increase in antibody titre was detected in 17 subjects and 7 subjects had significantly elevated titres in the first sample collected. In general, previously vaccinated children and adults had higher titres both at the outset of illness and during convalescence than unvaccinated young children.

Granstrom et al also reported the results of an in vitro toxin neutralisation assay for anti-PT antibodies in 38 patients with culture positive pertussis (113). Titres of anti-PT were initially low (except in some adults), but rose by the 4th week of illness and remain elevated for up to 2 years. Ashworth et al (114) found that children immunised with adsorbed pertussis vaccine (Wellcome DTP vaccine) had elevated titres of serum antibodies to FHA, PT and Agg 2/3 compared with a group of unimmunised children with no history of clinical illness. A small group of children with no history of immunisation or pertussis had antibody titres similar to those found in the immunised group and presumably had suffered unrecognised B. pertussis infection.

SERUM CLASS ANTIBODY RESPONSES

At birth infants have titres of IgG to FHA (115-118), antibody to PT (118), antibody to agglutinins (118), and serum IgG anti-adherence activity (120), similar to those in maternal serum. These titres decline to undetectable levels at about four to six months of age, and rise again following vaccination or infection (115,116,118,119). IgG to whole B. pertussis (90,120,121), IgG to FHA (112,115,116,120,122), IgG to PT (116,120,122), and serum IgG anti-adherence activity (119), all rise in titre following
infection. IgG to PT (116,120,122), and IgG to FHA (116,122), and serum IgG anti-adherence activity (98) also rise in titre following vaccination, but the increases in these titres have been found to be of lesser magnitude than those induced by infection (119,120,122). The IgG response to infection is potentiated by prior vaccination (112,123).

Serum IgA to whole B. pertussis (90,120,121), IgA to B. pertussis supernatant (124), IgA to FHA (112,116,120,122), IgA to PT (116,120), and serum IgA anti-adherence activity (119), all rise in titre following disease but in several studies were not found to rise following vaccination (116,119,120,124). Titres of IgA to B. pertussis antigens declined within six months of onset of disease in some studies (90,112,1212,122) but remained elevated for up to one year after the onset of disease in other studies (116,124). Serum IgA to whole B. pertussis (99), IgA to B. pertussis supernatant (103), and serum IgA anti-adherence activity (119), could be detected in a high proportion of adults with no recognised recent episode of pertussis.

Titres of serum IgM to whole B. pertussis (90,120,121), IgM to FHA (112,116,120,122), and IgM to PT (116,120,122), rise following disease and vaccination, but decline within six months (90,112,116,121,122).

**LOCAL SECRETORY IgA ANTIBODY RESPONSES**

The above results confirm that raised titres of serum antibodies are common following either disease or vaccination. However it is possible that locally secreted IgA on the surface of the respiratory epithelium is at least as important as circulating serum antibodies in immunity to pertussis. Such local immunity has been much less fully investigated.

Intranasal immunisation of mice with pertussis vaccine (compared with intraperitoneal administration of the same vaccine) is associated with a similar level of antibody in the serum but with a greater degree of protection against subsequent B. pertussis infection (125). This suggests that intranasal immunisation stimulates local immunity and significantly supplements serum immunity. This hypothesis is supported by the finding that high titres of IgG and IgA to B. pertussis are present in tracheobronchial
washings from mice following experimental *B. pertussis* infection. The development of these surface antibodies is coincident with the clearance of *B. pertussis* from the lungs (126). In rabbits enhanced clearance of experimental nasopharyngeal *B. pertussis* infection is associated with the presence of IgA to FHA in nasal secretions (82).

Immunity to pertussis in humans following disease may be due in part to antibodies inhibiting *B. pertussis* adherence to the respiratory epithelium. Tuomanen et al demonstrated that IgG and IgA in the serum of patients convalescing after culture positive whooping cough inhibited adherence of *B. pertussis* to ciliated human tracheal epithelial cells in vitro (119).

In adult human volunteers following intranasal vaccination, titres of serum antibody to *B. pertussis* did not change, but titres of antibody in respiratory secretions were elevated (127). In contrast intramuscular vaccination resulted in elevated titres of serum antibody, but no change in the titres of antibody in respiratory secretions. IgA to *B. pertussis* becomes detectable in nasal secretions from children with pertussis approximately three weeks after the onset of symptoms, coincident with the clearance of nasopharyngeal infection, and is present for at least 12 weeks after the onset of symptoms (128).

**CELLULAR RESPONSES TO BORDETELLA PERTUSSIS**

A variety of effects on the cells of the immune system may be detected in humans with disease, in animal models of infection, and following treatment of animals or cell suspensions with *B. pertussis* toxins. A marked lymphocytosis is common in the first few weeks of pertussis in humans and in experimental animal infection (5,13,38,39). This lymphocytosis is due to an expansion of the circulating T and B cell populations (129,130). Conflicting reports have suggested that the T cell lymphocytosis is due to either an increase in both the CD4 and the CD8 subsets (131), or an increase in the CD4 subset alone (132). The lymphocytosis appears to result from a PT induced inhibition of lymphocyte migration out of the peripheral blood into lymphatic tissue (133), rather than from increased lymphocyte proliferation. The inhibition of lymphocyte migration occurs despite unimpaired binding of lymphocytes to the high endothelial venule within the lymph node, the usual site of lymphocyte egress from the peripheral blood (133), and is mirrored by a PT induced impairment of lymphocyte migration.
between reticulo-endothelial cells in tissue culture (134). PT also inhibits macrophage random migration and chemotaxis in vitro (135) and in mice induces an increase in the numbers of circulating mononuclear cells together with a reduction in mononuclear cell migration to sites of inflammation (136). The PT induced inhibition of lymphocyte, macrophage and mononuclear cell migration is not associated with any effect on lymphocyte (133) or macrophage (135) viability.

As well as its effects on lymphocyte migration, PT has several effects on cellular immunity. It is a potent mitogen for murine lymphocytes in vitro (137), and in vivo acts as an adjuvant, enhancing and prolonging T lymphocyte dependent inflammatory reactions in a variety of animal models (138,139). PT enhances antibody production following administration with a variety of non-related antigens (140) and in some strains of mice PT enhances IgE mediated fatal anaphylaxis following exposure to protein antigens (1141).

PT enhances γ interferon release by lymphoid cells (142,143) and stimulates production and release of interleukin-1 by human monocytes and murine macrophages, interleukin-2 by human lymphocytes and rodent splenocytes, and interleukin-3 by murine splenocytes (1144).

PT also affects neutrophil and macrophage function. In vitro, PT inhibits chemotaxis (145), Fc receptor mediated phagocytosis (146), lysosomal enzyme release (147), and generation of superoxide anion (148). These effects are mediated by the inhibitory action of a regulatory protein, following its ADP ribosylation by the active protomer of PT, (Figure 2) and are independent of the actions of the regulatory protein on intracellular adenylate cyclase synthesis (146,148). Similar inhibition of chemotaxis, generation of superoxide anion, and bactericidal activity, may be induced in human neutrophils and alveolar macrophages (149, and human peripheral blood monocytes (150), by treatment with adenylate cyclase, another toxin produced in large amounts by *B. pertussis*. Adenylate cyclase and PT alone or in combination inhibit human natural killer cell cytotoxicity, apparently by different mechanisms (151).

The importance of the various effects, induced by PT, adenylate cyclase or other *B. pertussis* toxins, on the cellular responses to *B. pertussis* infection in humans is unknown. A number of authors have suggested that phagocyte impotence induced by these toxins is responsible for a significant impairment of host defences, thus enhancing survival and replication of *B. pertussis*, and contributing to an increased susceptibility
to secondary infections (49,149,150). There are however very few studies of cellular immunity in *B. pertussis* infection which might support this suggestion. Hoidal et al found that alveolar macrophages from rabbits with natural *B. bronchiseptica* infection failed to increase their rates of oxygen consumption, chemiluminescence, or glucose oxidation when stimulated with heat killed bacteria or the chemical agent phorbol myristate acetate (152). *B. bronchiseptica* produces adenylate cyclase (153) but little or no pertussis toxin (15) and thus the effects of infection with this organism on alveolar macrophages presumably was due to adenylate cyclase acting alone or in concert with some toxin other than PT.

Gray and Cheers studied the sequence of immunologic events leading to clearance of experimental murine *B. pertussis* lung infection (154-156). Logarithmic multiplication of bacteria "lying free in bronchiolar mucus" occurred for 2 1/2 weeks after inoculation. This was followed by infiltration of the lung with polymorphs and macrophages and rapid killing of 99% of the infecting organisms. A steady state of "immunological complaisance" then ensued with no change in the number of infecting organisms, which apparently survived within macrophages. Development of bactericidal antibody at approximately six to eight weeks after inoculation allowed complete resolution of the infection. Gray and Cheers suggested that cell mediated immunity, in the absence of specific antibody during the "complaisant phase" was bactericidal only when the density of infection exceeded a threshold level. When this threshold was exceeded by superinfecting with additional *B. pertussis*, effective phagocytosis and intracellular killing restored the equilibrium. Similarly a reduction in bacterial density below the threshold (after treatment with chloramphenicol) was followed by a prompt return to the threshold level. In vitro assessments of mononuclear cell activity did not reveal any impairment of phagocytosis or intracellular digestion. It therefore appeared that while a small proportion of the infecting organisms evaded intracellular killing, there was no gross impairment of cellular defences. Subsequent work has confirmed that *B. pertussis* can invade and survive, but not usually multiply, within macrophages (157), and the detection by immunofluorescence staining of *B. pertussis* within pulmonary alveolar macrophages from patients with HIV infection (158) suggests that the organism may proliferate in this site in some patients.

Redhead and colleagues have recently presented evidence which suggests that a CD4 Th1 lymphocyte response is necessary for elimination of *B. pertussis* infection in the experimental mouse model of respiratory tract infection (159). They found that nude mice or sublethally irradiated mice, deficient in T cells, either failed to clear or had
delayed clearance of *B. pertussis* infection. In contrast, adoptive transfer of CD4 positive lymphocytes from convalescent mice into these T cell deficient mice could mediate rapid clearance of *B. pertussis* respiratory infection in the absence of a detectable serum antibody response. Subsequent experiments demonstrated that clearance of an experimental respiratory infection correlated well with the magnitude of the cell mediated immune response and correlated poorly with the serum IgG or lung IgA responses following either previous infection or vaccination (160). These results are consistent with an earlier report which demonstrated that clearance of infection from the lungs of experimentally infected mice occurred simultaneously with the development of a vigorous proliferative response to whole killed *B. pertussis* in lymphocytes extracted from tracheobronchial lymph nodes or from the spleen, and well before the development of a serum antibody response to *B. pertussis* antigens (161).

There is very little information about the effects of pertussis on cellular immunity in humans. Tsukimoto and Lampkin found that lymphocytes from children with pertussis respond normally to a variety of mitogens in vitro (129). In contrast Pieroni et al found that children with pertussis and a history of BCG vaccination had a lower prevalence of positive tuberculin skin test responses than would be expected in the Yugloslav population (162). This finding was confirmed and extended by Hewlett et al who found that Mexican children with pertussis had impaired delayed hypersensitivity responses to tuberculin and six other skin test antigens when compared either with control children or with their own responses one to three months later (163). In one small study of adults who had never been vaccinated against pertussis the *in vitro* peripheral blood mononuclear cell proliferative responses to killed *B. pertussis* were significantly greater in nine subjects who had a memory of pertussis as a child than they were in five subjects with no memory of childhood pertussis (164).

**CONCLUSIONS**

In 1985 the authors of a WHO review of developments in pertussis vaccines stated that "the host-pathogen relationships in pertussis are still poorly understood. In particular, relatively little is known about the bacterial structures and extracellular products that are important in pathogenesis and in the establishment of immunity" (165). Despite considerable scientific effort in the last few years this statement remains largely true today. The general direction of most investigations of immunity to pertussis has
been influenced by prevailing concepts of microbial pathogenicity. Thus improved knowledge of the nature and mode of action of the antigens responsible either for bacterial adherence to the host cell, or for toxic effects in host tissues; and a widespread expectation that immunity to these antigens would provide effective protection against infection and the symptoms of disease and be safer than the presently used whole cell vaccines, have led to the formulation of new acellular component vaccines containing large amounts of a very few antigens.

The suggestion that PT is the primary toxin of *B. pertussis* and is responsible for the symptoms of disease (45,46), and that a vaccine composed purely of pertussis toxoid would be highly protective has been followed by the development and trial of acellular vaccines composed of PT alone or combined with FHA (111) and/or other antigens (166). It has been suggested that immunity to PT alone might prevent the symptoms of disease but not inhibit colonisation and thus not prevent transmission of infection, and that antigens thought to be important in adherence, such as FHA and Agg 2/3, should be included in a purified component acellular vaccine (167-169). Only careful comparison of the protective efficacy of vaccines composed of differing combinations of these antigens will determine their relative importance in human immunity to *B. pertussis* infection and disease.

The relative importance of circulating humoral antibody compared with local secretory antibody, and of circulating cellular immunity compared with local respiratory mucosal cellular immunity (eg. alveolar macrophages) is uncertain. It has been suggested that immunity to a local noninvasive infection such as that caused by *B. pertussis* is likely to be influenced by local cellular immunity and secretory antibody (167,169). Local immunity in human *B. pertussis* infection has not been widely investigated due to the difficulty of obtaining respiratory secretions from the site of infection. Available evidence does suggest that eradication of infection is associated with the appearance of secretory IgA to *B. pertussis* in nasopharyngeal secretions. The relative importance of these antibodies in eradication of infection, and in protection against the local and systemic effects of infection is however uncertain.
The papers included in this thesis do not define the determinants of immunity and thus cannot indicate which antigens are necessary and sufficient components of an acellular pertussis vaccine. They do, however, provide useful information on the serum and nasal antibody and circulating cellular responses to *B. pertussis* infection and pertussis vaccination. They also contribute to understanding the epidemiology and pathophysiology of pertussis. I humbly hope they will be interesting and useful to the reader who wishes to learn about pertussis.
### TABLE 1

Toxins of *Bordetella pertussis*

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Molecular Mass (kD)</th>
<th>Biological Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis Toxin</td>
<td>117</td>
<td>lymphocytosis, potentiation of insulin secretion, increased susceptibility to histamine, adjuvant.</td>
</tr>
<tr>
<td>(pertussigen, lymphocytosis promoting factor, islet activating protein, histamine sensitising factor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracheal Cytotoxin</td>
<td>&lt; 1.8</td>
<td>ciliostasis, extrusion of ciliated cells.</td>
</tr>
<tr>
<td>Heat Labile Toxin</td>
<td>140</td>
<td>dermonecrosis, splenic atrophy</td>
</tr>
<tr>
<td>(dermonecrotic toxin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenylate Cyclase</td>
<td>43</td>
<td>inhibition of granulocyte function</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>-</td>
<td>pyrogenesis, adjuvant</td>
</tr>
</tbody>
</table>
TABLE 2

Serum agglutinin and complement fixing antibodies in patients with pertussis

<table>
<thead>
<tr>
<th>Antibody Response</th>
<th>Agglutinin</th>
<th>Complement Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference</td>
<td>87 90</td>
<td>86 87 88 89</td>
</tr>
<tr>
<td>No. Subjects</td>
<td>21 22</td>
<td>123 21 75 112</td>
</tr>
<tr>
<td>Culture Positive *</td>
<td>NS NS</td>
<td>132/136 NS NS NS NS</td>
</tr>
</tbody>
</table>

Proportion of patients with antibody responses at varying durations of illness

<table>
<thead>
<tr>
<th>week</th>
<th>Agglutinin</th>
<th>Complement Fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>0/1 0/4</td>
<td>0/2 0/1 1/5 0/3</td>
</tr>
<tr>
<td>1-2</td>
<td>0/3 4/10</td>
<td>1/22 3/3 9/14 2/8</td>
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<tr>
<td>2-3</td>
<td>6/6</td>
<td>12/43 6/6 21/25 9/16</td>
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<td>3-4</td>
<td>6/7 5/10</td>
<td>35/54 7/7 11/16 20/29</td>
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<td>4-5</td>
<td>3/3</td>
<td>44/57 3/3 2/4 14/14</td>
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<td>27/35 1/1 5/6 13/13</td>
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<td></td>
<td>8/10 6/7</td>
</tr>
<tr>
<td>12-13</td>
<td></td>
<td>4/5</td>
</tr>
</tbody>
</table>

* NS = not stated
Figure 1: Regulation of expression of *Bordetella pertussis* virulence factors

Cytoplasmic membrane sensor proteins: BvgB, BvgC

DNA binding activator protein: BvgA

BvgA regulated upstream promoter: Act

Act regulated upstream promoter: ac-h

vir gene expresses BvgA, BvgB, BvgC

fha gene expresses FHA

act gene expresses Act

(activator protein)

ptx gene expresses PT

ac-h gene expresses AC
Figure 2
Pertussis Toxin: Structure and Effects

Active Protomer

Binding Pentamer

Complete Pertussis Toxin

phospholipase C

protein kinase C activation

fMet Leu Phe

Ri

Ni GDP ADP ribose

Ni GDP

Ni GTP

adenylate cyclase

cyclic AMP

ATP
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Human Serum Antibody Responses to *Bordetella pertussis* Infection and Pertussis Vaccination

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We used an immunoblotting technique to compare the serum antibody responses to pertussis toxin (PT), filamentous hemagglutinin (FHA), a 69-kilodalton (kDa) adenylate cyclase-associated protein (69 KD protein), and *Bordetella pertussis* outer membrane proteins (OMPs) following either *B. pertussis* infection or immunization with whole-cell pertussis vaccine. Infection and vaccination induced nearly equally intense antibody responses to PT and to FHA, but vaccination induced stronger antibody responses to the 69 KD protein and to many OMPs. The importance of serum antibody responses to the 69 KD protein and to *B. pertussis* OMPs other than PT and FHA in conferring immunity to pertussis after vaccination is unknown. Serum antibody responses to PT following either infection or vaccination were almost exclusively to the 28-kDa enzymatic subunit (S1) and only rarely and weakly to the lesser molecular weight binding subunits (S2-S5).

Recent efforts to produce a safe and effective acellular pertussis vaccine have stimulated interest in the pathogenesis of pertussis and in the determinants of immunity after disease or vaccination. Much attention has focused on the *Bordetella pertussis* components filamentous HA (FHA), pertussis toxin (PT), and adenylate cyclase (AC). FHA is a large-molecular-weight protein originally thought to be fimbrial in origin. Recent work, however, has shown that FHA is not a fimbrial component [1]. PT is an A-B type protein toxin composed of four binding subunits (S2-S5) and of adenosine diphosphate-ribosylase enzymic subunit (S1) [2, 3]. AC, a protein with a molecular mass of ~44 kilodaltons (kDa) [4] that is secreted in high concentration into the extracytoplasmic space, appears to be functionally related to a 69-kDa AC-associated protein (69 KD protein) that may function as a binding protein for the AC enzyme.

Serum antibodies to PT and FHA protect mice against intracerebral and aerosal infection with *B. pertussis* [12, 13], and antibodies to PT and AC ameliorate the effects of *B. pertussis* respiratory infections in mice [14, 15]. There is little information, however, on the presence of serum antibodies to these components in humans with *B. pertussis* infection.

We used an immunoblotting system to study the antibody responses to FHA, PT, outer membrane proteins (OMPs), and the 69 KD protein in patients with pertussis, family contacts of patients with pertussis, and recipients of whole-cell pertussis vaccine.

**Subjects and Methods**

*Serum.* Serum samples were collected from eight family contacts of patients with pertussis and from four family contacts of patients with pertussis during a pertussis epidemic between July 1985 and February 1987. All patients with pertussis had a prolonged paroxysmal cough associated with whoop and/or vomiting and/or apnea. Two family contacts had a brief nonparoxysmal cough without whoop, vomiting, or ap-
nea (atypical pertussis), and two family contacts remained asymptomatic. Sera were obtained at initial presentation and at follow-up assessments and were stored at −20°C until used. Consecutive sera from each patient were tested simultaneously. A postnasal swab cultured on charcoal agar supplemented with 10% sheep blood and 40 μg of cephalexin/mL was used to detect *B. pertussis* infection at presentation [16]. Infants and children with severe symptoms were treated with oral erythromycin for 10 d.

Table 1 shows the clinical details of the subjects and the duration of symptoms (or duration of exposure to pertussis in family contacts who remained asymptomatic) at serum collection. Six unvaccinated children (A–F; two months to seven years of age) with culture-positive pertussis had a total of 17 serum

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<th>Subject, age (y), vaccination history*</th>
<th>Symptoms</th>
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<th>Serum no.</th>
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NOTE. ND = not done; NK = not known.
* Number of immunizations with whole-cell pertussis vaccine given in the first year of life.
† Duration of cough for patients with pertussis or atypical pertussis, and duration of exposure to pertussis for asymptomatic family contacts.
‡ *B. pertussis* was isolated from another family member.
samples collected from 104 d before the onset of cough to 205 d after the onset of cough. Two family contacts (G, H; three and six years of age), who had received three pertussis vaccinations, with culture-negative, atypical pertussis had a total of six serum samples collected from 39 d before the onset of cough to 158 d after the onset of cough. Eleven serum samples were collected from two adults with pertussis (I, J) and two asymptomatic adult family contacts (K, L).

Sera were also collected from four children (M–P) three to nine months after completing a course of vaccination with absorbed diphtheria, tetanus, pertussis vaccine that was given at three, five, and nine months of age. None of these children had a history of exposure to pertussis or had symptoms suggestive of pertussis.

Preparation of antigens. B. pertussis strain W28 was maintained in 5% glycerol and 1% casamino acids in ampoules under liquid nitrogen. Cells from the ampoules were grown in 100 mL of Stainer and Scholte medium [17] in 250-mL Erlenmeyer flasks at 36 °C, either static for five days or agitated for three days. Crude OMPs were prepared by sonic disruption of B. pertussis cells, followed by Triton X-100® (Sigma, St. Louis) extraction of soluble cytoplasmic membrane proteins as described previously [18].

PT was purified by affinity chromatography from supernatants of three-day agitated cultures of B. pertussis [19], and FHA was obtained from supernatants of five-day static cultures [20]. The 69 KD protein was isolated by monoclonal affinity chromatography [21].

The protein concentrations of all antigens were determined by the method of Schacterle and Pollack [22].

Electrophoresis of antigens. FHA, OMPs, the 69 KD protein, and molecular weight standards (BDH Chemicals, Poole, England) were dissolved by heating to 60 °C for 15 min in 0.0625 M Tris-HCl (pH 6.8) containing SDS (3%), glycerol (10%), bromophenol blue (0.01%), and 2-mercaptoethanol (5%). PT was solubilized in a similar buffer, without 2-mercaptoethanol, that was heated to 37 °C for 60 min. The solubilized antigens were subjected to SDS-PAGE at a constant current of 50 mA, in slab gels (1.5 mm thick) containing 12.5% acrylamide and 0.1% SDS, prepared as described by Laemmli [23]. Five micrograms of FHA, PT, or OMP or 10 μg of 69 KD protein was applied per lane of gel. After electrophoresis, gels were either fixed and subjected to silver staining of proteins [24] or were used for immunoblotting.

Electrophoretic immunoblotting. Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose sheets (0.45-μm pore size; Schleider and Schuell, Dassel, West Germany) by using an electroblot system (Bio-Rad Laboratories, Richmond, Calif) at a constant current of 45 mA for 2 h in a cooled Tris-glycine-methanol buffer [25]. The nitrocellulose sheets were agitated for 2 h at room temperature in PBS containing 3% bovine hemoglobin and 0.02% azide and then incubated overnight at room temperature with 50 μL of serum per antigen lane diluted in 25 mL of fresh PBS hemoglobin solution. After five washes with PBS hemoglobin solution the nitrocellulose sheets were incubated overnight at room temperature with 125I-labeled antibody to human immunoglobulin (~10^4 cpm per an-

Figure 1. Protein profiles of the 69 KD protein, FHA, PT, and OMPs demonstrated by silver staining (lanes a–d, respectively) and by immunoblotting (lanes e–h, respectively) with the second serum specimen from subject B.
Figure 2. Antibody responses to FHA in serum specimens collected from subjects A-P. Clinical details of subjects are in table 1.

Results

Silver-stained SDS-polyacrylamide gels of *B. pertussis* antigens (figure 1) showed the gels were relatively pure and that the protein profiles of the 69 kD protein, FHA, PT, and the OMP preparation were similar to those we and others have obtained previously [19, 20, 26, 27]. The FHA preparation was resolved into four components with molecular masses of 136 kDa, 114 kDa, 108 kDa, and 92 kDa. The PT preparation contained four subunits with molecular masses of 28 kDa (S1), 23 kDa (S2), 22 kDa (S3), and <13 kDa (S4 and S5).

Immunoblotting of sera collected from subjects after pertussis infection or vaccination clearly demonstrated the presence of antibodies to the 69 kD protein; to the 136-kDa, 114-kDa, and 108-kDa proteins of FHA; to the S1 subunit of PT; and to several proteins in the OMP preparation. Figure 1 shows

Figure 3. Antibody responses to PT in serum specimens collected from subjects A-P. Clinical details of subjects are in table 1.
Serum Antibody Responses to B. pertussis

Figure 4. Antibody responses to the 69 KD protein in serum specimens collected from subjects A–P. Clinical details of subjects are in table 1.

typical immunoblot patterns produced using serum collected from a vaccinated child with atypical pertussis 33 d after onset of the disease.

Figures 2–5 show immunoblots of sera obtained from subjects A–P with FHA, PT, the 69 KD protein, and OMP antigen preparations, respectively. Antibodies to FHA and PT could not be detected in initial sera from unvaccinated children with pertussis (A–F) but were present in initial sera from vaccinated children with atypical pertussis (G, H) and in initial sera from adults with typical pertussis (I, J) or asymptomatic infection (K, L). Antibodies to PT were detected in sera collected more than four weeks after the onset of cough from all unvaccinated children with pertussis. Also, antibodies to FHA were found in sera collected more than four weeks after the onset of cough from five of six unvaccinated patients with pertussis. Antibodies to FHA and PT were also detected in sera obtained from three of four children who received three pertussis vaccinations but were not exposed to B. pertussis infection (M–P). When present, antibodies to FHA bind to the 136-, 114-, and 108-kDa proteins but never to the 92-kDa protein. Antibodies to PT bind almost exclusively

Figure 5. Antibody responses to OMPs in serum specimens collected from subjects A–P. Clinical details of subjects are in table 1.
to the S1 subunit and only rarely to the S2 and S3 subunits. None of the sera contained detectable antibodies to the S4 or S5 subunits of PT.

Antibodies reacting with the 69 KD antigen were detected in almost all sera tested. In sera from unvaccinated subjects (A-F), including a serum specimen obtained from subject A 104 d before the onset of pertussis symptoms, a very low level of antibody specific for the 69 KD protein was found. In contrast, sera from vaccinated subjects (G-P) frequently had strong antibody responses to the major 69 KD protein as well as weaker responses to some of the lower-molecular-weight proteins in the preparation.

Antibodies to OMPs usually could not be detected in the initial sera collected from unvaccinated children with pertussis (A-F). Even in sera collected from these children more than four weeks after the onset of cough, antibodies tended to react with few of the proteins in the OMPs, and then only weakly. In contrast both initial and late sera from vaccinated individuals (G-P) frequently reacted strongly with many proteins in the OMPs.

Discussion

Pertussis infection is thought to produce a more complete and longer lasting immunity than vaccination with whole-cell vaccine [28]. Thus, it is important to establish the differences between human immune response to disease and to vaccination, particularly with respect to the putative protective antigens of *B. pertussis*.

Most previous studies of human serum antibody responses to *B. pertussis* components after disease or vaccination have used ELISAs. Such systems enable antibody responses to be quantified, but their accuracy is dependent on the purity of the antigens involved; when mixed preparations are used it is not possible to identify the particular antigens to which the antibody response is directed. Similarly, if a purified material is composed of subunits or aggregates, the response to all or to only some component parts cannot be distinguished. We used the more qualitative method of immunoblotting to examine human serum antibody responses to *B. pertussis* antigens. The antigens were chosen from among those included in acellular pertussis vaccines now under investigation.

We found that the immunoblotting technique readily demonstrated serum antibodies to FHA, PT, and OMPs after pertussis infection or vaccination. These results agree with those from other studies, which used ELISAs to detect antibodies to FHA, PT, and OMPs in recipients of whole-cell pertussis vaccine [29] and to detect antibodies to sonicated *B. pertussis* cells [30], FHA [31], PT [32], and the 69 KD protein [33] in patients with pertussis.

Recognition of the roles of FHA and PT in the pathogenesis of pertussis and of the protection against pertussis provided by antibodies to these components has led to interest in the use of these proteins as the basis of new acellular pertussis vaccines. The ability to isolate the gene sequences coding for FHA [34] and PT [35] and to insert them in other bacteria [34, 36, 37] has given rise to suggestions that pertussis vaccines might be manufactured using recombinant DNA technology [38]. Such vaccines would be purer than those derived from *B. pertussis* cultures and could be designed to contain parts of the FHA and PT molecules thought most likely to be protective.

The PT subunits responsible for binding to cell surfaces, the S2 to S5 polypeptides, have been proposed as suitable candidates for such a vaccine [39]. The absence of the active S1 enzyme subunit would ensure that the vaccine was free from PT-induced toxicity. There is some evidence for the feasibility of a PT subunit vaccine. It has been found that both active immunization with the binding subunits of PT [39] or the use of a monoclonal antibody that reacts with the S2 and S3 subunits [40] will protect mice against the leukocytosis-promoting effects of injected PT. Caution must, however, be used when extrapolating from these results to protection against disease in humans. The present study and an earlier one [26] have shown that after whole-cell vaccination or pertussis infection, the human humoral response to PT is directed almost exclusively to the S1 subunit. If serum antibody responses to *B. pertussis* antigens accurately reflect immunity to pertussis after infection or vaccination, these results suggest that unless the binding subunits of PT can be modified to increase their immunogenicity in humans they are unlikely to fulfill their theoretical promise as components of a safe acellular vaccine.

We have also shown that following *B. pertussis* infection or pertussis vaccination, serum antibodies only react with three of the four polypeptides that make up FHA [41]. This result suggests that the form of FHA used in an acellular vaccine may significantly affect its efficacy. As both of the above findings are only readily detectable using immuno-
blotting, care must also be exercised in the choice of methods used to assess the serum immune responses to acellular pertussis vaccines.

Although acellular vaccines composed of FHA and PT have been shown to protect against pertussis respiratory infection in mice [42], their efficacy in children [43] may not be as great as that of currently used whole-cell pertussis vaccines. This suggests that responses to antigens other than FHA and PT also contribute to immunity after infection or vaccination. Our study has shown that immunization with whole-cell vaccine and, to a lesser extent, B. pertussis infection induces antibody responses to B. pertussis components other than FHA and PT, notably the 69 KD protein and several OMPs. These antibody responses may contribute significantly to immunity after vaccination or infection [21]. Although vaccination induced stronger serum antibody responses to a wider range of components than did infection, other constituents of the immune response may be stimulated more strongly by infection than by vaccination.

References

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Serum IgG, IgA, and IgM Responses to Pertussis Toxin, Filamentous Hemagglutinin, and Agglutinogens 2 and 3 after Infection with *Bordetella pertussis* and Immunization with Whole-Cell Pertussis Vaccine

Mark G. Thomas, L. A. Edward Ashworth, Elizabeth Miller, and Harold P. Lambert

Class antibody responses to pertussis toxin (PT), filamentous hemagglutinin (FHA), and agglutinogens 2 and 3 (Agg 2/3) were studied in 114 patients with pertussis, 49 family contacts of patients with pertussis, and 139 infants attending vaccination clinics. IgG responses to FHA and PT were similar in patients with pertussis and in infants immunized three times with whole-cell pertussis vaccine. IgA responses to FHA and PT were greater in patients with pertussis than in vaccinees, and a serum IgG titer of IgA to PT $>1.9$ was a useful discriminant of *Bordetella pertussis* infection. When compared with patients with pertussis, vaccinees had a greater IgG response to Agg 2/3 and an equivalent IgA response to Agg 2/3. Serologic responses to FHA, PT, and Agg 2/3 were common in family contacts but were smaller than the responses in patients with pertussis. Initial titers of IgG to FHA and IgA to FHA were significantly higher ($P < .005$ and $P < .05$, respectively) in family contacts aged 3–10 y than in patients with pertussis in an identical age group.

In experimental models of pertussis, antibody responses to pertussis toxin (PT) and filamentous hemagglutinin (FHA), and agglutinogens 2 and 3 (Agg 2/3) are strongly correlated with protection against *Bordetella pertussis* infection and its effects [1-3]. These findings have led to the development of highly purified and relatively simple acellular pertussis vaccines composed of PT, FHA, and fimbrial agglutinogens [4-7]. Recent trials in Japan [8] and Sweden [9] showed that acellular pertussis vaccines confer some protection against pertussis; however, they failed to demonstrate that high titers of serum antibodies to PT and FHA are sufficient to confer a high level of immunity to pertussis [9].

Both *B. pertussis* infection and immunization with whole-cell pertussis vaccine confer some degree of immunity to pertussis, but protection after infection appears to be more complete and of longer duration [10]. Enzyme-linked immunosorbent assay (ELISA) techniques have been used to measure antibody responses to PT and FHA following infection [11-19] and after immunization with either whole-cell [14, 18-20] or acellular [4-7, 21] pertussis vaccines. However, none of these studies compared class antibody responses to purified *B. pertussis* antigens in large numbers of patients with pertussis with those in vaccinees. Further, while raised titers of serum agglutinins in infants immunized with whole-cell pertussis vaccine have been correlated with protection against pertussis [22-24], there have been few studies [7, 20] of immune responses to the fimbrial *B. pertussis* antigens responsible for inducing serum agglutinins.

We used purified PT, FHA, and the fimbrial antigens, agglutinogens 2 and 3 (Agg 2/3), to measure class-specific antibody responses to these antigens in patients with pertussis and their close contacts during a pertussis epidemic and compared the results with those obtained from healthy infants after immunization with whole-cell pertussis vaccine.

Subjects and Methods

Patients with pertussis were prospectively recruited between July 1985 and July 1987 from patients ad-
Pertussis Serology

Serum antibodies to PT, FHA, and Agg 2/3 were assayed by an ELISA similar to that previously reported [20]. Antigens used in ELISA were purified by techniques identical to those used to produce antigens for an acellular pertussis vaccine [26]. Although they were of comparable purity to those in the acellular vaccine, they were not toxoided. Microplates (96-well Nunc Immunoplate 1; Gibco Bio-cult, Paisley, Scotland) were coated with the appropriate antigen at 2 μg/ml. Horseradish peroxidase conjugates of affinity-purified goat IgG antibodies to human IgG, IgA, and IgM (North-east Biomedical Laboratories, Uxbridge, UK) were used at concentrations determined by preliminary titration.

Starting with a 36-fold dilution, threefold dilution series of serum samples were tested. Titers were expressed as the reciprocal of the dilution giving 50% maximum color development with tetramethylbenzidine as substrate [27]. Titers were standardized against a working standard assayed in duplicate on each plate. Differences between population means were tested by Student's t test.

Results

During the study, 125 patients with pertussis and 67 family contacts were seen; however, 11 patients and 18 family contacts did not give consent to be investigated. Thus, 114 patients with pertussis were studied, ages 1 mo-49 y, as were 49 family contacts, ages 3-44 y. Of 114 patients with pertussis, 72 (63%) were hospitalized because of the illness.

Seventy-two (63%) of the patients with pertussis had episodes of whooping, 94 (82%) had vomiting, 75 (66%) had apnea, and 33 (29%) had cyanosis in association with a prolonged paroxysmal cough. Of those with pertussis, 60 (52%) were female. Twenty-three family contacts had a nonparoxysmal cough that started within 3 w of the onset of pertussis in another family member, lasted <1 w, and was not associated with whoop, vomiting, or apnea. The other 26 family contacts remained asymptomatic. In 26 families clinical information was available for all family members [28]. In 25 of 26 families the onset of cough in the patient(s) with pertussis preceded the onset of cough in the family contacts. Figure 1 shows the relationship between the age of subjects and the pertussis vaccination status of patients with pertussis and family contacts.

B. pertussis was isolated from 30 (33%) of 91 patients with pertussis who had a postnasal swab collected <42 d after the onset of cough. B. pertussis was isolated from 26 (40%) of 65 patients with pertussis who were <4 y; 4 (15%) of 26 patients >4 y;
Figure 1. Number of patients with pertussis (P) and family contacts (C) who received 0, 1, 2, 3, or an unknown number of pertussis vaccinations.

and 2 (6%) of 31 family contacts of patients with pertussis. Twenty-two *B. pertussis* isolates from patients with pertussis were serotyped: 15 were serotype 1-2, 4 were serotype 1-3, and 3 were serotype 1-2-3.

An absolute lymphocyte count of >10 × 10⁹/l was found in 26 (31%) of 84 patients with pertussis who had a count performed <42 d after the onset of cough. No family contacts had an absolute lymphocyte count >5 × 10⁹/l.

Figure 2 shows mean log₁₀ titers of IgG, IgA, and IgM to FHA, PT, and Agg 2/3 in serum samples from 30 patients with culture-positive pertussis, 139 healthy infants before their first DTP vaccination, and 74 healthy infants after their third DTP vaccination. Antibody responses in serum samples from the 84 patients who did not have *B. pertussis* isolated (16 of whom did not have a postnasal swab collected) were nearly identical to those in serum from the 30 patients with culture-positive pertussis (data not shown). IgG to FHA, PT, and Agg 2/3 was detected in serum from all healthy infants before their first DTP vaccination and was thought to represent transplacentally acquired maternal IgG. In contrast, IgA to FHA, PT, and Agg 2/3 was detected in only 5% of healthy infants before their first DTP vaccination. IgA to FHA and Agg 2/3 was detected in ~80% of serum samples from healthy infants after their third vaccination, but IgA to PT was found in only 20% of the samples.

Figure 3 shows mean log₁₀ titers of IgG, IgA, and IgM to FHA, PT, and Agg 2/3 from onset of cough or exposure to pertussis for family contacts of patients with pertussis and for mothers of vaccinees. While patients with pertussis had large rises in mean log₁₀ titers of antibody, family contacts had much smaller increases. Mean log₁₀ titers of IgG and IgA to FHA, PT, and Agg 2/3 in serum from family contacts were similar to those in serum from mothers of vaccinees. (Titers of IgM were not measured in serum of mothers of vaccinees.)

Twenty-two patients with pertussis (11 of whom had *B. pertussis* isolated from postnasal swabs) and 14 family contacts had one serum sample collected <15 d after the onset of cough or exposure to pertussis and another serum sample collected between 28 and 84 d after the onset of cough or exposure to pertussis. The median fold rise in antibody titer was greater in patients with pertussis than in family contacts for each antibody tested except IgG to Agg 2/3 (table 1). Nineteen of 22 patients with pertussis and 11 of 14 family contacts had a threefold or greater rise in the titer of antibody to at least one antigen, and 16 of 22 patients with pertussis and 5 of 14 family contacts had a threefold or greater rise in the titer of antibody to at least two antigens.

Titers of antibodies in serum samples collected <15 d after the onset of cough or exposure to pertussis were lower in patients with pertussis than in family contacts. This difference was greatest for titers of IgG and IgA to FHA (figure 4). Fourteen patients with pertussis (ages 3-10 y) had significantly lower titers of IgG to FHA (P < .005) and IgA to FHA (P < .05) than five family contacts 3-10 y old.

A titer of IgA to PT >log₁₀ 1.9 was found in serum samples collected 28-84 d after the onset of cough from 17 (89%) of 19 patients with culture-positive pertussis and 34 (69%) of 49 patients with culture-negative pertussis but in only 1 (1%) of 72 vaccinees and 14 (17%) of 82 mothers of vaccinees.
Figure 2. Mean logₐ titers (±1 SD) of antibodies in serum collected from 30 patients with culture-positive pertussis, 139 healthy infants before their first diphtheria-tetanus-pertussis (DTP) vaccination, and 74 healthy infants after their third DTP vaccination. Solid lines show mean logₐ titers (±1 SD) in serum from patients with pertussis collected at 8, 28, 56, and >84 d after the onset of cough. Broken lines show mean logₐ titers (±1 SD) of IgG to filamentous hemagglutinin (FHA), pertussis toxin (PT), and agglutinogens 2 and 3 (Agg 2/3) in serum from vaccinees before their first and after their third DTP vaccination and mean logₐ titers (±1 SD) of IgA to FHA, PT, and Agg 2/3 in serum from vaccinees who had detectable specific IgA after their third DTP vaccination.
Figure 3. Mean log₁₀ titers (±1 SD) of antibodies in serum of family contacts at ≤8, 8-14, 15-21, 22-28, 29-42, 43-84, and >84 d after exposure to pertussis (or after onset of cough for family contacts who developed a brief non-paroxysmal cough). Broken lines represent mean log₁₀ titers (±1 SD) of IgG to filamentous hemagglutinin (FHA), pertussis toxin (PT), and agglutinogens 2 and 3 (Agg 2/3) and mean log₁₀ titers (±1 SD) of IgA to FHA, PT, and Agg 2/3 in serum from mothers of vaccinees.
Table 1. Median fold rises in antibody titers between serum collected <14 d after onset of cough or exposure to pertussis and 28–84 d after onset of cough or exposure to pertussis in patients with pertussis and family contacts.

<table>
<thead>
<tr>
<th>Antigen, antibody</th>
<th>Pertussis patients (n = 22)</th>
<th>Family contacts (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filamentous hemagglutinin</td>
<td>IgG 9.1 IgA 7.5 IgM 3.4</td>
<td>IgG 3.0 IgA 2.9 IgM 1.1</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>IgG 75.8 IgA 29.1 IgM 2.9</td>
<td>IgG 1.7 IgA 1.4 IgM 1.2</td>
</tr>
<tr>
<td>Agglutinogens 2 and 3</td>
<td>IgG 2.0 IgA 5.6 IgM 2.6</td>
<td>IgG 2.2 IgA 1.7 IgM 1.2</td>
</tr>
</tbody>
</table>

NOTE. The median interval between samples was 37 d for patients with pertussis and 41 d for family contacts.

Peak titers of IgA to FHA and to Agg 2/3 in serum from patients with pertussis were similar to titers of the antibodies in serum from vaccinees or from mothers of vaccinees.

Discussion

Previous studies of the serum antibody responses to B. pertussis antigens documented significant rises in serum antibodies to FHA or PT in patients with pertussis [11–19] or showed that titers of antibodies to these antigens are greater than those present in serum from healthy control subjects [11, 13, 15, 29, 30]. Our results confirm that, in patients with pertussis, mean titers of serum IgG to FHA, PT, and Agg 2/3 rise from initially low levels during the first week of cough to peak ~6 w later.

It has been postulated that immune responses to asymptomatic infection or atypical, and consequently unrecognized, pertussis are responsible for maintaining immunity to the disease in older children and adults [31]. Others studied contacts of patients with pertussis and documented elevated titers or significant rises in titer of serum antibodies to B. pertussis [32–34], to PT [16], and to an antigen described as fimbrial [16]. We found that rises in antibody titers were common in family contacts of patients with pertussis but such rises were much smaller than those found in patients with pertussis. As expected the antibody rise in family contacts was almost solely in the IgG and IgA classes (table 1). Peak titers of IgG and IgA to FHA, PT, and Agg 2/3 in patients with pertussis were similar to those in mothers of vaccinees (who had no history of recent respiratory illness). A possible explanation of this finding is that asymptomatic infection or atypical disease occurs frequently in adults in England at present.

Antibody titers were higher in serum collected from family contacts during the first 2 w of exposure to pertussis.
to pertussis than in serum collected from patients with pertussis during the first 2 w of cough. Although antibody titers in contacts generally showed modest increases, it is likely that the titers noted in early serum samples represented levels at the time of contact since they were similar to those in mothers of vaccinees.

We have also considered the possibility that the differences between patients with pertussis and family contacts were due to their age differences. A direct comparison was not possible in patients <3 y because all family members in this age group developed pertussis; few family members >10 y developed pertussis. In the group 3-10 y, a comparison was possible (figure 4), and the results suggested that low early titers of antibodies were associated with susceptibility to pertussis. Our results do not indicate an exclusive association between one particular antibody and protection against disease.

Others have found that titers of IgG to FHA [13, 14, 18] and PT [14, 18, 19] after pertussis disease are similar to or greater than those found after vaccination. We found that disease and vaccination induced similar rises in titers of IgG to FHA but that disease induced a greater rise in titers of IgG to PT and a lesser rise in titers of IgG to Agg 2/3 than did vaccination. The presence of antibodies to *B. pertussis* agglutinogens including Agg 2/3 is strongly associated with immunity to pertussis after vaccination [22-24]. If antibodies to Agg 2/3 are indeed protective, then whole-cell vaccines containing agglutinogens may be more effective than disease in stimulating this component of immunity.

Others have reported large rises in titers of serum IgA to *B. pertussis* [19, 23] and FHA [18] after pertussis but little or no rise after vaccination and have suggested that detection of serum IgA to *B. pertussis* is a useful serologic test for infection [35]. We previously reported that immunization with whole-cell pertussis vaccine induces modest serum IgA responses to FHA, PT, and Agg 2/3 in some infants [36]. Conley and Delacroix [37] found a serum IgA response to tetanus toxoid in infants following tetanus toxoid immunization, and serum IgA responses to vaccine antigens have been reported after immunization of adults with acellular pertussis vaccines [21, 36].

In the present study we found that serum IgA responses to Agg 2/3 were nearly equivalent following disease and vaccination, but that serum IgA responses to FHA and PT were more common and of greater magnitude after disease. No doubt the frequency and the magnitude of vaccine-induced humoral IgA response is dependent on the vaccinee's prior exposure to the antigen, the nature of the immunizing antigen, and the dosage used.

In summary we showed that patients with pertussis mount brisk humoral responses to a variety of components of the organism including the type-specific agglutinogens. Immunity in family contacts is associated with high initial antibody levels and modest antibody responses. No easy distinction can be made between the serologic responses to vaccination and to disease, but the titers of IgA to PT are significantly higher in serum collected from patients with pertussis 28-84 d after the onset of cough than in serum collected from infants 6 w after their third DTP immunization. This difference in the serum IgA response to PT may provide a useful adjunct to culture in the confirmation of suspected pertussis, especially in recently vaccinated infants.

Acknowledgments

We thank the subjects and their families for their generous cooperation; the nursing and medical staffs of St. George's Hospital, Kingston Hospital, Brocklebank Health Centre, and the vaccination clinics of Hertfordshire Health Authority for valuable assistance; Andrew Robinson and Laurie Irons for providing the antigens used in the ELISAs; Andrew Day and Nichols Hornigold for assisting with ELISA measurements; and Valerie Dickinson and Andrew Tickle for data analysis.

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HUMAN SERUM ANTIBODY RESPONSES TO ACELLULAR PERTUSSIS VACCINE

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SUMMARY

We have used an immunoblotting system to qualitatively compare the antibody responses in sera from two children, one immunised with a vaccine (JNIH 6 acellular pertussis vaccine) containing filamentous haemagglutinin (FHA) and toxoided pertussis toxin (PT), and one with a vaccine (JNIH 7 acellular pertussis vaccine) containing only toxoided PT, with the responses in four children immunised with whole cell pertussis vaccine (Wellcome adsorbed DTP vaccine). Antibody responses in sera from two patients with pertussis, both previously immunised with whole cell pertussis vaccine, were also examined.

Both JNIH 6 and JNIH 7 vaccines induced strong serum antibody responses to PT, and JNIH 6 but not JNIH 7 vaccine induced strong serum antibody responses to FHA. Whole cell pertussis vaccine and Bordetella pertussis infection induced similar or lesser serum antibody responses to PT and FHA and also induced strong serum antibody responses to many outer membrane proteins (OMPs) and to a 69 kD adenylate cyclase associated protein (69kD protein). In contrast JNIH 6 and JNIH 7 vaccines induced only very weak antibody responses to very few OMPs and no antibody response to the 69 kD protein. These results suggest that JNIH 6 and JNIH 7 acellular pertussis vaccines do not induce significant antibody responses to B. pertussis antigens other than PT, and in the case of JNIH 6 vaccine FHA, and thus that the protection against pertussis conferred by these vaccines is unlikely to be due to immunity to other antigens.

INTRODUCTION

New acellular pertussis vaccines containing varying amounts of toxoided pertussis toxin (PT) and filamentous haemagglutinin (FHA) have been shown to provide a significant level of protection against pertussis to children in Japan (1) and Sweden (2,3). Considerable experimental evidence (4-7) supports the central roles of PT and FHA in the pathogenesis of pertussis but evidence that antibody responses to these antigens alone are sufficient for immunity to pertussis is lacking. Indeed while both a vaccine containing PT (JNIH 7 acellular pertussis vaccine) and a vaccine containing PT and FHA (JNIH 6 acellular pertussis vaccine) provided some protection against pertussis
to Swedish infants, titres of IgG to PT and IgG to FHA in serum collected two to four months after the second vaccination did not correlate with the subsequent incidence of disease (2). We have investigated the possibility that the vaccines used may have been contaminated with other antigens which induced a functionally significant immune response. We used an immunoblotting technique to detect antibodies to PT, FHA, outer membrane proteins (OMP), and a 69 kD adenylate cyclase associated protein (69 kD protein), in sera collected from a recipient of JNIH 6 vaccine, a recipient of JNIH 7 vaccine, and recipients of whole cell pertussis vaccine (Wellcome adsorbed DTP), and in sera collected from patients with pertussis.

MATERIALS AND METHODS

SERUM

Serum samples collected from two Swedish infants before their first immunisation and two to four months after their second immunisation with either JNIH 6 or JNIH 7 vaccine, were kindly provided by Dr P. Olin. These two infants had received their first pertussis vaccination when six to ten months old and their second pertussis vaccination two months later. These samples were selected for testing because ELISA testing, performed as a component of the acellular pertussis vaccine trial, indicated that their antibody responses were typical of the serum samples collected from vaccinated children (personal communication: P Olin). Serum samples were also collected from four English children, less than two years old, between three and nine months after their third immunisation with whole cell pertussis vaccine (given at three, five and nine months of age). None of the vaccinated children had a history of symptoms suggestive of pertussis or of recognised exposure to pertussis. A serum sample was also collected from a three year old child, thirty-three days after the onset of pertussis, and from a 21 year old nurse thirteen days after the onset of pertussis. Both these subjects had a prolonged (> 2 weeks) paroxysmal cough associated with post-tussive whoop. B. pertussis was isolated from a pernasal swab collected from the nurse but the three year old child did not consent to have a swab collected. Both these subjects had received three immunisations with whole cell pertussis vaccine in infancy.
PREPARATION OF ANTIGENS

*B. pertussis* strain Wellcome 28 was maintained in 5% (vol/vol) glycerol, 1% (wt/vol) Casamino acids, in ampoules under liquid nitrogen. Cells from the ampoules were grown in 100 ml of Stainer and Scholte medium (8), in 250 ml Erlenmeyer flasks at 36°C, either static for five days or agitated for three days. Crude OMPs were prepared by sonic disruption of *B. pertussis* cells followed by Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) extraction of soluble cytoplasmic proteins. PT was purified from three day agitated cultures of *B. pertussis* (9), and FHA from supernatants of five day static cultures of *B. pertussis* (10), by affinity column chromatography. 69 kD protein isolated by monoclonal affinity chromatography from *B. pertussis* strain CN 2992 (11), was kindly provided by P. Novotny. The protein concentrations of all antigens were determined by the method of Schacterle and Pollack (12).

SDS-PAGE

OMPs, FHA, 69 kD protein, and molecular weight standards were solubilised in a buffer containing 5% (vol/vol) B-mercaptoethanol, 10% (vol/vol) glycerol, 3% (wt/vol) SDS, 0.01% (wt/vol) bromphenol blue, and 0.0625 M Tris base (pH = 6.8) heated to 60°C for 15 minutes. PT was solubilised in a buffer which did not contain B-mercaptoethanol (but which was otherwise identical), heated to 37°C for 60 minutes. Fifty ug PT, 50 ug of FHA, 200 ug of OMP or 200 ug of 69 kD protein was applied to the single well of a polyacrylamide gel slab and electrophoresis of the four gels performed in parallel. Vertical slab gel electrophoresis was performed for approximately 2.5 hr at a constant current of 50 milliamperes using a 4.5% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, stacking gel, and a 12.5% (wt/vol) acrylamide, 0.1% (wt/vol) SDS running gel. Molecular weight standards used were cytochrome C (12,300), myoglobin (17,200), chymotrypsinogen A (25,700), ovalbumin (45,000), albumin (66,250), and ovotransferrin (76,000 - 78,000) (BDH, Poole, England). The purity of the PT, FHA and 69 kD protein preparations, and the relationships of these proteins to the protein components of the OMPs, were determined by silver staining the SDS-polyacrylamide gel (13).
IMMUNOBLOTTING

Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose sheets (0.45 μm pore size; Schleicher & Schuell, Inc.) by electrophoresis at a constant current of 45 milliamperes for 2 hr (Bio Rad, Richmond, Ca.) in a cooled Tris-glycine-methanol buffer (14). The nitrocellulose sheets were then cut into longitudinal strips approximately 1 cm wide and the position and density of transferred proteins was checked by gold staining (Aurodye, Janssen, Beers, Belgium) a single strip. The unstained nitrocellulose strips were agitated for 2 hr at room temperature in PBS containing 3% (wt/vol) bovine haemoglobin and 0.02% (wt/vol) azide and were then incubated overnight at room temperature with 50 µL of sera per strip diluted in 25 ml of fresh PBS haemoglobin solution. The nitrocellulose strips were then washed five times with fresh PBS haemoglobin solution and incubated overnight at room temperature with 125I-labelled anti-human immunoglobulin (ca. 10^6 cpm per strip diluted in 25 ml of fresh PBS haemoglobin solution). After washing in PBS and airdrying, the strips were autoradiographed for 10 days at -70 C with Hyperfilm-MP (Amersham, Buckinghamshire).

RESULTS

Silver staining of SDS-polyacrylamide gels (results not shown) and gold staining of nitrocellulose strips (Figures 1-4) revealed protein profiles for PT, FHA, OMPs and 69 kD protein similar to those obtained previously (9,10,15,16). No antibodies reacting with PT, FHA or OMPs were present in sera collected from infants before immunisation with either acellular vaccine (Figures 1-3, lanes 2 and 4). However antibodies reacting weakly with the 69 kD protein were present in preimmunisation sera from these two infants (Figure 4, lanes 2 and 4). Antibodies reacting strongly with the 28 kD enzymatic monomer (S1 subunit) of PT were present in sera from infants after immunisation with either acellular vaccine (Figure 1, lanes 3 and 5). Antibodies to FHA were present in sera from the infant who had received JNIH 6 acellular pertussis vaccine (Figure 2, lane 3) but not in sera from the infant who had received JNIH 7 acellular pertussis vaccine (Figure 2, lane 5).

While antibodies to many proteins present in the OMP preparation were present in sera
from children who had been immunised with the whole cell pertussis vaccine and in sera from two patients with pertussis (Figure 3, lanes 6-11), sera from infants who had received either acellular pertussis vaccine were essentially devoid of antibodies to proteins present in the OMP preparation (Figure 3, lanes 3 and 5). Antibodies to 69 kD protein were present in sera from children who had received the whole cell pertussis vaccine and in sera from patients with pertussis (Figure 4, lanes 6-11), but not in sera from infants who had received either acellular pertussis vaccine (Figure 4, lanes 3 and 5). Furthermore antibodies to proteins contaminating the FHA preparation were present in sera from children immunised with the whole cell pertussis vaccine and in sera from two patients with pertussis (Figure 2, lanes 6-11), but apart from antibodies to a 28 kD protein (presumably the S1 subunit of PT) no antibodies to proteins contaminating the FHA preparation were present in sera from infants immunised with either acellular vaccine (Figure 2, lanes 3 and 5).

Antibody responses to PT in the infants who had received acellular pertussis vaccines (and to FHA in the infant who had received JNIH 7 vaccine) were at least as strong as those found in either the children who had received whole cell pertussis vaccine or in the two patients with pertussis. Antibodies to the 22 kD (S2 subunit) and to the 23 kD (S3 subunit) of PT were present in sera from one patient with pertussis (Figure 1, lane 11), and were also present, though in smaller amounts, in sera from two vaccinated children (Figure 1, lanes 5 and 8). Antibodies reacting with the <13 kD (S4 and S5 subunits) of PT could not be detected in any serum specimen.

**DISCUSSION**

We found that neither of the two infants immunised with an acellular pertussis vaccine developed major antibody responses to *B. pertussis* antigens other than PT or FHA (as determined by immunoblotting). If these results are representative of the antibody responses in other children immunised with these vaccines, they suggest that the lack of correlation between the titres of IgG to PT and of IgG to FHA, and the subsequent incidence of pertussis in immunised infants was not due to the variable presence of an antibody to another *B. pertussis* antigen.

Data from the Swedish clinical efficacy trials of JNIH 6 and JNIH 7 vaccines suggests
that the protection they confer is no greater than and may be less than that provided by whole cell pertussis vaccine (2). We found that the antibody responses to PT in the two children immunised with JNIH 6 or JNIH 7 vaccine, and to FHA in the child immunised with JNIH 6 vaccine, were at least as strong as those to PT and FHA either in children immunised with whole cell pertussis vaccine or in patients with pertussis. This implies that antibody responses to PT, with or without antibody responses to FHA, are not sufficient to ensure complete immunity to pertussis. Instead complete immunity may depend on antibody responses to a number of antigens. While immunisation with acellular pertussis vaccines may consistently induce an optimal serological response to PT (and to FHA for JNIH 6 vaccine), if antibodies to other antigens are necessary to confer complete immunity to pertussis, then levels of antibody to PT may not correlate with protection against disease. Thus failure to induce antibody responses to an appropriate mix of antigens rather than failure to induce an adequate antibody response to a single (or two) antigen(s) may be the reason for the incomplete immunity to pertussis provided by acellular pertussis vaccines.

Immunisation with whole cell pertussis vaccine and *B. pertussis* infection in unimmunised children induces serological responses to many other antigens as well as to PT and FHA but the nature of these antigens, their roles in pathogenesis, and their importance in inducing protective immunity is largely uncertain. 69 kD protein is functionally related to extracytoplasmic adenylate cyclase, a virulence factor of *B. pertussis* (17). While serological responses to 69 kD protein can be detected following immunisation with whole cell pertussis vaccine and following *B. pertussis* infection (18), their importance in conferring immunity to disease is uncertain. *B. pertussis* infection and immunisation with whole cell pertussis vaccine induce an agglutinogen response which is directed against fimbrial antigens and which is correlated with subsequent immunity to pertussis (19-21). The efficacy of acellular pertussis vaccines may be enhanced by supplementing PT and FHA with a significant amount of agglutinogens. Correlation of antibody responses with protective efficacy for acellular pertussis vaccines of varying constitution will help to define the requirements for effective immunity to pertussis and thus guide the formulation of optimal vaccines.
REFERENCES


Figure 1.
Protein profile of PT demonstrated by gold staining in lane 1, and by immunoblotting in lanes 2 to 11. Sera used for immunoblotting were:
lanes 2 and 4 = Swedish infants before immunisation;
lane 3 = Swedish infant between two and four months after the second immunisation with JNIH 6 acellular pertussis vaccine;
lane 5 = Swedish infant between two and four months after the second immunisation with JNIH 7 acellular pertussis vaccine;
lanes 6-9 = English children aged less than two years old, between three and nine months after their third immunisation with whole cell pertussis vaccine;
lane 10 = three year old child 33 days after the onset of pertussis;
lane 11 = 21 year old nurse 13 days after the onset of pertussis.
Figure 2.
Protein profile of FHA demonstrated by gold staining in lane 1, and by immunoblotting in lanes 2 to 11. Sera used for immunoblotting were as described in the legend to Figure 1.
Figure 3.
Protein profile of OMPs demonstrated by gold staining in lane 1, and by immunoblotting in lanes 2 to 11. Sera used for immunoblotting were as described in the legend to Figure 1.
Figure 4.
Protein profile of 69 kD protein demonstrated by gold staining in lane 1, and by immunoblotting in lanes 2 to 11. Sera used for immunoblotting were as described in the legend to Figure 1.
Nasal Immunoglobulin A Responses To Pertussis Toxin, Filamentous Hemagglutinin and Agglutinogens 2 and 3 after Infection with *Bordetella pertussis* and Immunization with Whole-Cell Pertussis Vaccine

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*Bordetella pertussis* is a noninvasive human pathogen which exclusively infects ciliated respiratory epithelium (13). Adhesion by *B. pertussis* to the base of cilia prevents clearance by the mucociliary stream (9) and allows bacterial replication and release of extracellular toxins at the site of infection (4). Pertussis toxin (PT), filamentous hemagglutinin (FHA), and agglutinogens 2 and 3 (Agg 2/3) contribute to the adherence of *B. pertussis* to ciliated respiratory epithelial cells (6, 11, 14, 15). It has been suggested that secretory immunoglobulin A (IgA) to these or other *B. pertussis* antigens is responsible for inhibiting or eradicating infection of the ciliated respiratory epithelium, whereas circulating humoral antibodies provide immunity to the toxins responsible for the symptoms of disease (10).

In the present study, we have measured IgA to PT, FHA, and Agg 2/3 in nasal secretions from patients with pertussis and from healthy 11-month-old infants 6 weeks after their third DTP vaccination, to determine the magnitude of the secretory immune response to infection and vaccination. We also attempted to determine whether the nasal secretory immune response might be used as a diagnostic marker of recent *B. pertussis* infection.

**MATERIALS AND METHODS**

Patients with pertussis were recruited as previously described (12). Pertussis was diagnosed in the presence of a paroxysmal cough lasting more than 2 weeks, associated with a typical whoop, vomiting, or apneic episodes (2). Nasal swabs cultured on charcoal agar supplemented with 10% sheep blood and 40 μg of cephalaxin per ml were used to isolate *B. pertussis* from patients with pertussis.

Nasal mucus was collected from patients with pertussis by gently rotating a calcium-alginate-tipped swab on a flexible wire shaft (Transwab Medical Wire & Equipment Co.) against the nasal turbinates and septum approximately 1 to 2 cm proximal to the anterior nares. The nasal-mucus-bearing tip of the swab was then amputated into an airtight polyethylene tube containing 2 ml of transport medium and stored at 4°C until assayed. Serial samples from each subject were stored and assayed in parallel.

Nasal mucus was collected by a similar technique from infants attending vaccination clinics in Hertfordshire, England, who had no history of either paroxysmal cough or exposure to pertussis. A cotton-tipped swab was first moistened in sterile water and then rotated within the anterior nares. The tip of the swab was broken off into a tube containing transport medium, stored, and then assayed in a manner identical to that used for the swabs from the patients with pertussis. Samples were collected from 10-month-old infants 6 weeks after their third immunization with DTP vaccine (Wellcome Trivax, Beckenham, England).

Total IgA and specific IgA to FHA, PT, and Agg 2/3 in nasal mucus were determined by an enzyme-linked immunosorbent assay method as previously described (1). For nasal mucus samples with a total IgA concentration greater than 1 μg/ml, titers of specific IgA were normalized on the basis of total IgA concentration to correct for differences in the volume of nasal mucus collected. Re-
TABLE 1. Mean log₁₀ (SD) titers of IgA to FHA, PT, and Agg 2/3 in nasal mucus samples

<table>
<thead>
<tr>
<th>Antibody response</th>
<th>Days from onset of cough</th>
<th>IgA to FHA</th>
<th>IgA to PT</th>
<th>IgA to Agg 2/3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8-14 days</td>
<td>43-84 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(n = 13)</td>
<td>(n = 14)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgA to FHA</td>
<td>2.36 (0.57)</td>
<td>3.26 (0.90)</td>
<td>0.037</td>
<td></td>
</tr>
<tr>
<td>IgA to PT</td>
<td>2.10 (0.56)</td>
<td>2.64 (0.70)</td>
<td>0.133</td>
<td></td>
</tr>
<tr>
<td>IgA to Agg 2/3</td>
<td>2.00 (0.40)</td>
<td>2.19 (1.04)</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

Samples were collected from patients with culture-positive pertussis at the onset of symptoms and during convalescence. IgA titers were normalized based on total IgA concentration.

RESULTS

Nasal mucus samples (n = 258) were collected between July 1985 and July 1987 from 114 patients with pertussis; patient ages ranged between 1 month and 49 years. The characteristics of these subjects and their serum IgG, IgA, and IgM responses to FHA, PT, and Agg 2/3 have been described previously (12). In brief, episodes of whooping, vomiting, apnea, and cyanosis occurred in 63%, 82%, 66%, and 29% of patients, respectively. Further, 63% of the patients were admitted to the hospital because of their illness. B. pertussis was isolated from 30/91 (33%) patients who had a postnasal swab collected within 42 days of the onset of cough. B. pertussis was isolated from 7/23, 12/29, 6/18, 4/13, 1/5, and 0/3 subjects who had a postnasal swab collected in weeks 1 to 6 of illness, respectively.

Mean titers of IgA to FHA, PT, and Agg 2/3 in nasal secretions from the 30 patients with culture-positive pertussis rose from approximately log₁₀2 at the onset of symptoms to peak at approximately log₁₀3 between 6 and 12 weeks later (Table 1). Overall mean titers of IgA to FHA, PT, and Agg 2/3 in 84 patients from whom B. pertussis was not isolated (16 of whom did not have postnasal swabs collected) were almost identical to those in patients with culture-positive pertussis. However, in samples collected between 8 and 14 days after the onset of cough, mean titers of IgA to FHA, PT, and Agg 2/3 were significantly higher (P < 0.05, P < 0.05, P = 0.001, respectively) in 17 patients with culture-negative pertussis than in 13 patients with culture-positive pertussis. There were no significant differences in mean titers of IgA to FHA, PT, and Agg 2/3 between culture-positive and culture-negative patients for all other time intervals studied (0 to 7, 15 to 21, 22 to 28, 29 to 42, 43 to 84, and >84 days after the onset of cough).

Because the differences in titers of IgA between culture-positive and culture-negative patients for samples collected between 8 and 14 days after the onset of cough might have been due to differences in subject ages in these two groups, the comparison was repeated for subjects aged less than 11 years. Although mean titers of IgA to FHA in 13 patients with culture-positive pertussis (mean age = 1.7 yr, SD = 2.1 yr) and 16 patients with culture-negative pertussis (mean age = 1.4 yr, SD = 2.0 yr) were not significantly different, the mean titers of IgA to PT and to Agg 2/3 were significantly higher in the culture-negative patients (P < 0.05 and P < 0.01, respectively).

Titers of IgA to FHA, PT, and Agg 2/3 in nasal mucus collected between 28 and 84 days after the onset of cough from 20 patients with pertussis, aged less than 1 year old, were significantly higher (P < 0.001) than those in nasal mucus collected from 42 healthy 11-month-old infants 6 weeks after their third DTP vaccination (Fig. 1).

DISCUSSION

Mean titers of IgA antibodies to FHA, PT, and Agg 2/3 in nasal secretions from patients with pertussis were found to increase over the period to 12 weeks after the onset of cough. By this time most subjects had detectable antibodies, although the level varied considerably between subjects. This is the first study in which antibodies to the major fimbrial antigens of B. pertussis have been shown to be induced in the upper respiratory tract where they have the potential to inhibit fimbriae-mediated adherence. Although the nasal mucosal responses were modest, they were of the same order, showed the same overall progression, and persisted as long as the serum antibody responses (12). Other studies of nasopharyngeal antibody responses to B. pertussis antigens have not shown large rises in antibody titers with duration of illness (7, 8). The protective significance of these apparently modest secretory responses to B. pertussis antigens is unknown. Also, they may not be typical of the levels of response at other significant sites of mucosal immunity such as the trachea or bronchi.

We found that titers of antibodies in nasal mucus collected during the second week of cough were significantly lower in culture-positive patients than in culture-
negative patients, suggesting that secretory responses may contribute to the eradication of *B. pertussis* infection. This finding should be interpreted with caution as the rate of isolation of *B. pertussis* in the patients studied remained at about 30% throughout the first 4 weeks of cough.

Other workers (3, 5, 7) have suggested that detection of *B. pertussis*-specific IgA in respiratory secretions might be a useful adjunct to culture in the diagnosis of pertussis. We found that mean titers of specific IgA in nasal mucus from infants 4 to 12 weeks after the onset of cough were significantly higher than those in vaccinated infants with no history of pertussis. Although titers in these two groups of patients demonstrated considerable overlap, the results suggest that the finding, in an infant, of a log_{10} titer greater than 3.3 (for IgA to FHA, PT, or Agg 2/3) is strong retrospective evidence in favor of a diagnosis of pertussis.

**ACKNOWLEDGMENTS**

This work was supported in part by a grant from the Department of Health and Social Security (to M.G.T).
We thank the subjects and their families for their generous cooperation; the nursing and medical staffs of St. Georges Hospital, Kingston Hospital, Brocklebank Health Centre, and the vaccination clinics of Kent and Surrey Health Authority for valuable assistance; Andrew Robinson and Laurie Irons for providing the antigens used in the enzyme-linked immunosorbent assays; Andrew Day and Nicholas Hornigold for assisting with enzyme-linked immunosorbent assays measurements; and Valerie Dickinson and Andrew Tickle for data analysis.

LITERATURE CITED


THE CONTRIBUTION OF BREAST FEEDING TO IMMUNITY TO PERTUSSIS

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INTRODUCTION

Secretory IgA and other components of human breast milk, such as complement, lysozyme, lactoferrin, and lactoperoxidase inhibit the growth of bacterial, viral and protozoal pathogens in the infant gut and thus ameliorate infectious gastrointestinal disease (1-4). These anti-infective components of human milk may also contribute to a reduction in the severity of respiratory infections in breast fed infants (5). We have compared titres of IgA to three Bordetella pertussis antigens: filamentous haemagglutinin (FHA), pertussis toxin (PT), and agglutinogens 2 and 3 (Agg 2/3), in milk from mothers breast feeding infants with pertussis with those in milk from mothers breast feeding healthy infants. We have also compared titres of IgA to FHA, PT and Agg 2/3 in sera and nasal secretions from breast fed infants with pertussis with those in sera and nasal secretions from bottle fed infants with pertussis.

MATERIALS AND METHODS

Breast milk specimens were donated by the mothers of breast fed infants aged less than six months old admitted to hospital with pertussis. Pertussis in the infant was diagnosed in the presence of a prolonged paroxysmal cough associated with whoop, vomiting, or apnoea (6). Breast milk specimens were also donated by the mothers of healthy breast fed infants, aged three months old, attending a vaccination clinic. Breast milk was expressed by hand or breast pump into sterile polystyrene containers and stored at -20°C until tested.

Serum and nasal mucus specimens were collected from infants aged less than six months old admitted to hospital with pertussis, and from healthy infants attending a clinic for their first DTP vaccination. Serum samples collected by venepuncture and centrifugation of clotted blood were stored in polyethylene tubes at -20°C until tested. Nasal mucus was collected by gently rotating a calcium alginate or cotton wool tipped swab against the nasal septum and the inferior nasal turbinate approximately one centimetre proximal to the external nares. The swab tip was amputated into a polyethylene tube containing 0.5 ml of transport medium, and stored at 4°C until tested. Pernasal swabs for isolation of B. pertussis were collected from infants with pertussis and where possible from their mothers.
Specific IgA to FHA, PT, and Agg 2/3 in breast milk, nasal mucus, and sera, and total IgA in breast milk and nasal mucus were measured by an ELISA technique similar to that previously described (7). Antigens used in ELISA were purified by techniques used to make antigens for an acellular pertussis vaccine (8) and were of comparable purity to those in the acellular vaccine, but were not toxoided. Microplates (96-well Nunc Immunoplate 1) were coated with the appropriate antigen at 2 μg/ml. Horseradish peroxidase - conjugates of affinity-purified goat IgG antibodies to human IgG, IgA and IgM (Northeast Biomedical Labs, Ltd.) were used at concentrations determined by preliminary titration. Starting with a 36-fold dilution (for serum antibodies), and a 5-fold dilution (for nasal and breastmilk IgA antibodies), 3-fold dilution series of sera and breastmilk, and 2-fold dilution series of nasal mucus, were tested and titres expressed as the reciprocal of the dilution giving 50% maximum colour development with tetramethylbenzidene as substrate (9). Titres were standardised against a working standard assayed in duplicate on each plate. Titres of specific IgA in nasal mucus were divided by titres of total IgA in nasal mucus to derive corrected figure which compensated for differences in the volume of nasal mucus collected.

Differences between population means were tested by Student's T-test.

RESULTS

Ten breast milk samples were donated by eight mothers breast feeding infants with pertussis (Table 1). The infants were aged between one and five months old, and had been coughing for seven to fifty-nine days at the time of breast milk donation. *B. pertussis* was isolated from the infant and/or the mother in six of eight families. The mothers of all eight infants had a cough at the time of their infant’s illness and/or a twofold or greater rise in titre of IgG to at least two *B. pertussis* antigens (FHA, PT, Agg 2/3) in serum samples collected at least fourteen days apart (7). Sixty-nine breast milk samples were donated by the mothers of sixty-nine healthy three month old infants attending a vaccination clinic for their first DTP vaccination.

Log₁₀ titres of total IgA and of IgA to FHA,PT and Agg 2/3 in breast milk from mothers of infants with pertussis did not appear to be influenced by the duration of cough in the infant with pertussis (Figure 1). Mean titres of total IgA and of IgA to
FHA and IgA to PT were not significantly raised in breast milk from mothers of infants with pertussis, compared with breast milk from mothers of vaccinees. However the mean titre of IgA to Agg 2/3 was significantly raised in breast milk from mothers of infants with pertussis, compared with breast milk from mothers of vaccinees (p=0.001).

Serum and nasal mucus samples were collected from 35 infants with pertussis aged less than six months old (Table 2). Infants had been coughing for two to fifty-seven days at the time of collection of first specimens. B. pertussis was isolated from 13/31 (42%) infants who had pernasal swabs collected. Only four infants had received one or more pertussis vaccination.

Titres of IgA to FHA, PT and Agg 2/3 in sera from infants with pertussis rose from low levels in the first ten days after the onset of cough, to higher levels later in the illness (Figure 2). Mean titres of IgG to PT and of IgA to PT in sera collected less than 31 days after the onset of cough, were significantly higher in breast fed infants than in bottle fed infants regardless of whether all infants with pertussis or only infants with culture positive pertussis were considered (Table 3). There were no significant differences in titres of antibodies in sera collected more than thirty days after the onset of cough.

Titres of IgA to FHA, PT and Agg 2/3 in nasal mucus from infants with pertussis showed a modest upward trend with increasing duration of illness (Figure 3). There were no significant differences in mean titres of specific antibody or in mean titres of total IgA in nasal mucus, between breast fed infants and bottle fed infants, regardless of whether all infants or only infants with culture positive pertussis were considered.

**DISCUSSION**

We found that breast milk contains IgA to FHA, PT and Agg 2/3. These antigens are thought to be important in the pathogenesis of pertussis and antibodies to them appear to be associated with immunity to disease. Breastfeeding may enhance immunity to these antigens and hence to pertussis. When suckled by mice previously immunised with FHA and PT, infant mice were protected against the effects of B. pertussis.
respiratory infection (10). Oda et al found that antibodies to FHA, PT and agglutinogens were commonly present in colostrum samples from Indonesian mothers and that intraperitoneal administration of these antibodies to suckling mice protected them from the effects of *B. pertussis* respiratory infection (11). Oda et al (11) and other authors (12) have suggested that colostrum inhaled during feeding might localise in the respiratory tract and thereby protect the infant. Our results, which show that titres of IgA to FHA, PT and Agg 2/3 in nasal mucus are not significantly higher in breast fed infants than in bottle fed infants, suggest that inhalation of breast milk does not provide a major contribution to infant immunity to pertussis.

We found that titres of IgA to FHA and IgA to PT in breast milk from mothers of infants with pertussis were similar to titres of the same antibodies in breast milk from mothers of vaccinees. In contrast we found that titres of IgA to Agg 2/3 were significantly higher in breast milk from mothers of infants with pertussis. Evidence for a common mucosal immune system implies that the IgA response in breast milk is likely to be similar to that in the bronchial mucosa (13). Our results suggest that *B. pertussis* infection, for which there was strong clinical, microbiological, or serological evidence in all eight mothers of infants with pertussis, induces a modest secretory immune response to these *B. pertussis* antigens. This conclusion is consistent with our finding (14) that the levels of IgA to FHA, PT and Agg 2/3 in nasal mucus from infants with pertussis are not greatly elevated above the levels found in nasal mucus from healthy infants.

We have shown that titres of IgA to PT and IgG to PT in serum collected in the first thirty days after the onset of pertussis are significantly higher in breastfed infants than in bottle fed infants with pertussis. This is a surprising finding and we are uncertain of the mechanism(s) responsible. Ogra et al found that IgA could be absorbed from colostrum and be detected in infant sera (13), and our findings may provide a clinically important reflection of this process. However our findings that serum immunoglobulins to FHA and Agg 2/3 were not significantly raised in breastfed infants with pertussis, and that titres of serum immunoglobulins to FHA, PT and Agg 2/3 were no higher in healthy breastfed infants (compared with healthy bottlefed infants) at the time of their first DTP vaccination (data not shown) suggests that immunoglobulins are not readily absorbed from breast milk into infant sera. An alternative explanation for the elevated titres of IgA and IgG to PT in breastfed infants with pertussis is that cytokines and/or lymphocytes are absorbed from breast milk and stimulate antibody production in the infant. This explanation is consistent with the
finding that breast feeding enhances the serum antibody response to conjugate
Haemophilus influenzae type B vaccine (16). Another possible explanation for our
finding is that maternal B. pertussis infection allows PT to enter the circulation from
where it is transported via breast milk to the infant and then augments the infant's
immune response to PT. FHA and Agg 2/3 are not thought to enter the circulation
during B. pertussis infection, while the lymphocytosis seen in patients with pertussis is
strong evidence that PT is a systemically active toxin. Our results may provide evidence
for the immunisation of breastfed infants by antigens derived from maternal infection
via ingested breast milk.

Breastfeeding obviously does not provide complete protection against pertussis. It
may however reduce the frequency of disease or ameliorate disease when present. Such
an effect has been shown with infections due to respiratory syncytial virus (RSV).
Breast milk contains IgA to RSV and RSV neutralising activity (17-19), and
breastfeeding provides partial protection against severe RSV infection (12,20). The
mechanism of this protection is unclear. It appears to be present even following
cessation of breast feeding (20) and in some studies (21) was associated with elevated
titres of antibody to RSV in nasal mucus. Like RSV, B. pertussis is an important
cause of infant respiratory morbidity and mortality. Current immunisation practice does
not confer protection against pertussis until approximately six months age and
antibacterial agents are largely ineffective in treatment of the disease. Our results suggest
that breastfeeding may confer a small contribution to immunity to pertussis and
possibly ameliorate this disease in infants.

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TABLE 1

Clinical details of the infants of mothers who donated breast milk samples.

<table>
<thead>
<tr>
<th>Infant</th>
<th>Age (mths)</th>
<th>Infant culture positive</th>
<th>Mother culture positive</th>
<th>Duration of cough in infant (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>yes</td>
<td>yes</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>no</td>
<td>not tested</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>yes</td>
<td>no</td>
<td>34</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>yes</td>
<td>no</td>
<td>12</td>
</tr>
<tr>
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<td>6</td>
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<td>17</td>
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<td></td>
<td>23</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>no</td>
<td>no</td>
<td>7</td>
</tr>
</tbody>
</table>
TABLE 2

Clinical details of infants aged less than six months old with pertussis.

<table>
<thead>
<tr>
<th></th>
<th>Breast fed</th>
<th>Bottle fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>Number who had received &gt;1 pertussis vaccination</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Mean age (days)</td>
<td>76</td>
<td>90</td>
</tr>
<tr>
<td>Mean duration of cough at time of first specimen collection (days)</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Culture positive</td>
<td>5/7</td>
<td>8/24</td>
</tr>
<tr>
<td>Number of serum collections</td>
<td>12</td>
<td>41</td>
</tr>
<tr>
<td>Number of nasal mucus collections</td>
<td>13</td>
<td>46</td>
</tr>
</tbody>
</table>
TABLE 3

Mean log\textsubscript{10} titres of antibodies to FHA, PT and Agg 2/3 in serum specimens, collected less than 31 days after the onset of cough, from infants less than six months old with pertussis.

<table>
<thead>
<tr>
<th></th>
<th>All infants with pertussis</th>
<th>Infants with culture-positive pertussis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Breast-fed</td>
<td>Bottle-fed</td>
</tr>
<tr>
<td>Number of infants</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Mean age at onset of pertussis (weeks)</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>Mean duration of cough at time of serum collection (days)</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>IgG to FHA</td>
<td>1,400</td>
<td>886</td>
</tr>
<tr>
<td>IgA to FHA</td>
<td>118</td>
<td>14</td>
</tr>
<tr>
<td>IgM to FHA</td>
<td>102</td>
<td>78</td>
</tr>
<tr>
<td>IgG to PT</td>
<td>8,467</td>
<td>562*</td>
</tr>
<tr>
<td>IgA to PT</td>
<td>445</td>
<td>11*</td>
</tr>
<tr>
<td>IgM to PT</td>
<td>654</td>
<td>175</td>
</tr>
<tr>
<td>IgG to Agg 2/3</td>
<td>169</td>
<td>345</td>
</tr>
<tr>
<td>IgA to Agg 2/3</td>
<td>21</td>
<td>6</td>
</tr>
<tr>
<td>IgM to Agg 2/3</td>
<td>22</td>
<td>77</td>
</tr>
</tbody>
</table>

* p < 0.005
** p < 0.001
Figure 1.

Log$_{10}$ titres of total IgA and of IgA to FHA, PT and Agg 2/3 in breast milk from mothers of infants with pertussis, in relation to the duration of cough in the infant. The broken line shows mean log$_{10}$ titres (+/- 1 SD) of total IgA and of IgA to FHA, PT and Agg 2/3 in 69 samples of breast milk from mothers of healthy 3 month old infants attending a vaccination clinic.
Figure 2.

Log$_{10}$ titres of IgA to FHA, PT and Agg 2/3 in sera from infants with pertussis, in relation to duration of cough. The solid circles represent breast fed infants and the open circles represent bottle fed infants.
Figure 3.

Log$_{10}$ titres of IgA to FHA, PT and Agg 2/3 in nasal mucus from infants with pertussis, in relation to duration of cough. The solid circles represent breast fed infants and the open circles represent bottle fed infants.
BORDETELLA PERTUSSIS INFECTION DOES NOT SUPPRESS HUMAN SERUM IMMUNOGLOBULIN RESPONSES.

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SUMMARY

Concentrations of IgG, IgA and IgM and titres of IgG, IgA and IgM to *Escherichia coli* were measured in serum samples from 46 infants with pertussis, aged less than one year old at the onset of cough. There was no evidence of a consistent suppression either of immunoglobin concentrations or of titres of functional antibodies following *Bordetella pertussis* infection. These results suggest that B lymphocyte function is not significantly impaired by *B. pertussis* infection.

INTRODUCTION

It has been suggested that infection with *Bordetella pertussis* results in a suppression of immune responsiveness which may inhibit clearance of infection and predispose the patient to other infections (1-3). Antigens postulated to contribute to this effect include adenylate cyclase (AC), which has been shown to suppress chemotaxis, superoxide production, and bacterial killing by neutrophils in vitro (3), and pertussis toxin (PT), which has been shown to inhibit the function of a wide range of immune effector cells in vitro (1,2). Evidence that secondary infections are more common in patients with pertussis is, however, largely anecdotal (4) and may reflect local damage to the respiratory epithelium rather than a systemic effect of *B. pertussis* toxins on immune defenses. We have measured the total concentrations of IgG, IgA and IgM and the titres of IgG, IgA and IgM to *Escherichia coli* in the serum of children less than one year old with pertussis in an attempt to determine whether natural *B. pertussis* infection is a cause of a significant impairment in B lymphocyte function.

SUBJECTS AND METHODS

Patients with pertussis were prospectively recruited between July 1985 and July 1987 from patients admitted to the paediatric wards of two South West London hospitals, and from patients referred by local general practitioners. All patients for whom consent
was available were included in the study. Pertussis was diagnosed in the presence of a paroxysmal cough lasting more than two weeks and associated with a typical whoop, vomiting or apnoeic episodes (5). A postnasal swab was collected at presentation and cultured on charcoal agar supplemented with 10% sheep blood and 40µg of cephalexin per ml as previously described (6). Blood samples were obtained by venepuncture, allowed to clot at room temperature for one hour, centrifuged at 2,000 G for ten minutes and the serum collected and stored at -20 C. All samples were assayed in parallel. Another venous blood sample was used to measure absolute lymphocyte count at presentation.

Concentrations of IgG, IgA and IgM were determined by immunoturbidimetry using a Cobas Bio centrifugal analyser (Roche Diagnostics, Welwyn Garden City, Herts, UK). Samples with IgA and IgM levels below the detection limit for turbidimetry were re-assayed by an ELISA technique using polyclonal goat anti-human IgA or IgM (Kallestad UK Ltd, Brill, Bucks, UK) as the capture antibody and alkaline phosphatase conjugated anti-human IgA and IgM (Sigma, Poole, Dorset, UK) as detection antibody. Functional antibodies were similarly measured by ELISA using an \textit{E. coli} preparation as capture antigen. The antigen was prepared from six random isolates from normal stools. The isolates were grown agar slopes for 24 hours and solubilised in 0.25\% (w/v) sodium dodecyl sulphate in phosphate-buffered saline. Titres of functional antibodies were compared with a gamma globulin preparation (Pentaglobin, Biotest Pharma GMBH, Frankfurt/Main, W. Germany) which was arbitrarily assigned a value of 10,000 units for each of IgG, IgA and IgM.

RESULTS

Eighty-two serum samples were collected from 46 subjects (24 female, 22 male) aged between two weeks and 50 weeks at the onset of cough. Subjects were recruited from a larger study of serum immunoglobulin responses to \textit{B. pertussis} antigens in patients with pertussis and their family contacts (6). \textit{B. pertussis} was isolated from 17/42 (40\%) subjects who had a postnasal swab collected. 13/41 (32\%) subjects had an absolute lymphocyte count greater than \(10 \times 10^9\)/L. A single serum sample was collected from 20 subjects, two serum samples were collected from 16 subjects, and three serum samples were collected from 10 subjects. Serum samples were collected between one and 244 days after the onset of cough.
Figure 1 shows concentrations of IgG, IgA and IgM in sera in relation to time from onset of cough. There was no evidence of a consistent temporary suppression of concentrations of IgG, IgA or IgM at any time after the onset of cough. Concentrations of IgG and IgA were not significantly correlated with time from onset of cough, but concentrations of IgM were positively correlated with time from onset of cough (correlation coefficient = .306, p<0.01).

Figure 2 shows titres of IgG, IgA and IgM to E. coli in sera in relation to time from onset of cough. There was no evidence of a consistent temporary suppression of titres of functional antibodies at any time after the onset of cough. Titres of IgG to E. coli were not significantly correlated with time from onset of cough, but titres of IgA and IgM to E. coli were positively correlated with time from onset of cough (correlation coefficient = .226, p<0.5; correlation coefficient = .327, p<0.005 respectively).

DISCUSSION

While several B. pertussis virulence factors have been shown to have effects on cells in culture and on experimental animals (2,7), the relative importance of these virulence factors in the pathogenesis of pertussis in humans is unclear. Pertussis toxin produces a range of effects on lymphocytes including impaired migration (8), enhanced proliferation (9), enhanced gamma interferon release (10), enhanced interleukin 2 release (11), inhibition of B cell responses to bacterial lipopolysaccharide (12), and suppression of the IgM response of mouse splenocytes to sheep erythrocytes (1). An increase in the numbers of circulating lymphocytes is a common feature of B. pertussis infection in humans (4) but there is no evidence that this or other abnormalities in lymphocyte function cause any significant impairment of immune responsiveness.

The finding that PT suppresses in vitro IgM responses of mouse splenocytes to sheep erythrocytes (1), prompted us to study total and specific immunoglobulin concentrations in humans with pertussis. We attempted to control for the effects of age on immunoglobulin concentrations by studying only children less than one year old. We found a significantly raised lymphocyte count in 32% of our subjects but did not find a reduction either in concentrations of serum IgG, IgA or IgM, or in titres of serum.
antibodies to *E. coli*. We consider the significant correlation between time of onset of cough and concentrations of total IgM, specific IgM and specific IgA are almost certainly a result of increasing immunologic maturity with age rather an effect of *B. pertussis* infection. These results imply that the severity of any immune suppression induced by *B. pertussis* infection may be less than has previously been suggested. Further studies of polymorphonuclear leukocyte, monocyte and T lymphocyte function in patients with pertussis should be done to determine whether *B. pertussis* infection causes a significant impairment of the function of these cell types.

REFERENCES


Figure 1. Total concentrations of IgG, IgA and IgM (g/L) in serum samples from subjects, less than one year old with pertussis, in relation to days from the onset of cough.
Figure 2. Titres of IgG, IgA and IgM to *E. coli* in serum samples from subjects, aged less than one year old with pertussis, in relation to days from the onset of cough.
Human cellular immune responses to *Bordetella pertussis* infection

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Key words: *Bordetella pertussis*; Cellular immunity; Filamentous haemagglutinin; Pertussis toxin

1. SUMMARY

We have compared the responses of peripheral blood leucocytes from three groups (i) patients suffering from pertussis (whooping cough), (ii) clinical staff caring for those patients and laboratory staff working with *Bordetella pertussis*, and (iii) staff with no known recent contact with *B. pertussis*. In vitro stimulation with filamentous haemagglutinin (FHA) caused significant increases in proliferation of only the patient group’s lymphocytes. In vitro stimulation with pertussis toxin (PT) caused a large increase in proliferation of lymphocytes from all three groups and in the patient group the increase in proliferation was related to the dose of PT. Interleukin 2 (IL-2) production by leucocytes from all three groups was significantly increased following challenge with FHA or PT. The increases in IL-2 production were greatest in lymphocytes from patients with pertussis. Challenge with toxoided pertussis toxin had no effect on either proliferation or IL-2 production in any of the groups.

2. INTRODUCTION

Information on the immune response in man to *B. pertussis* has been limited to measurement of antibody levels in the serum of infected or vaccinated individuals. Specific antibodies directed against several *B. pertussis* antigens including filamentous haemagglutinin (FHA), pertussis toxin (PT), lipopolysaccharide (LPS) and various outer membrane proteins [1,2] can be assayed. Several of these, particularly FHA and PT, have been selected as components of acellular vaccines developed with the intention of replacing the current whole cell vaccine [3] and have recently been tested in clinical trials in Sweden. We have previously demonstrated that rodents immunized with whole cell vaccine develop strong T cell immunity to *B. pertussis* antigens [4]. T cells can release a range of soluble mediators (lymphokines), including interleukin 2 (IL-2), granulocyte macrophage colony stimulating factor (GMCSF) and gamma-interferon (IFNγ), which have been shown to stimulate anti-bacterial responses in vitro and in vivo [5–7]. It is therefore of importance to determine whether or not T cell responses, which may be protective are generated in humans following contact with *B. pertussis*. We have studied three groups: patients diagnosed as suffering from...
whooping cough, a contact group comprising clinical staff who have contact with these patients or laboratory staff working with *B. pertussis* cultures, and a control group with no known recent contact. Serum antibody titres to purified FHA and PT were measured. Proliferation and IL-2 production in cultures of peripheral blood leucocytes stimulated with a range of *B. pertussis* antigens were also determined.

3. MATERIALS AND METHODS

3.1. Patients

All patients diagnosed as having pertussis had a prolonged paroxysmal cough with whoop, vomiting or apnoea. Patients 1 and 2 were parents of an infant with culture positive pertussis. Patients 3 and 4 were brothers aged 13 yrs and 15 yrs with pertussis. Patient 5 was the mother of an infant with pertussis and was the only subject from whom *B. pertussis* could be isolated from nasopharyngeal swabs. Blood was collected 7, 10, 5, 4 and 1 weeks after the onset of cough in patients 1, 2, 3, 4 and 5 respectively.

The contact group comprised clinical staff caring for patients with whooping cough and laboratory personnel working with the bacterium. The control group comprised clinical and laboratory staff with no known recent exposure to *B. pertussis*. The vaccination history of these control and contact groups is not recorded.

3.2. Antigens

Bacterial vaccine and purified components. The second British Reference Preparation for pertussis vaccine (NIBSC 66/84) reconstituted in phosphate buffered saline (PBS) was used as the source of killed whole *B. pertussis* cells. The material was washed once in PBS, before use.

Late exponential cultures of *B. bronchiseptica* NCTC8344 grown in Stainer and Scholte medium [8] and *E. coli* 0111 strain J5 grown in brain-heart infusion broth (Difco) at 37 °C under agitated conditions were harvested by centrifugation, resuspended in PBS and killed by heating to 56 °C for 30 min in a water bath. The cells were washed once in PBS before use.

Purified active PT was isolated from 3-day suspension cultures of *B. pertussis* strain Welcome 28 (W28) by affinity chromatography as described by Irons [9]. Purified PT was treated with glutaraldehyde as described by Munoz [10] to yield a PT toxoid (PTd). Purified FHA was kindly supplied by Dr Y Sato (National Institute of Health, Japan). Purity of PT and FHA was confirmed by SDS polyacrylamide gel electrophoresis and silver staining. LPS was purified from *B. pertussis* W28 by the method of Staub [11]. An endotoxin reference preparation (Mallinckrodt Inc, St. Louis, MO, U.S.A.) was used as a source of *E. coli* LPS.

When required the protein content of the preparations was determined by the method of Schacterle and Pollock [12] using bovine serum albumin as a reference.

3.3. Preparation of peripheral blood leucocytes and serum

Blood was collected by venepuncture into heparinized collecting vessels, and diluted with an equal volume of RPMI 1640 culture medium. The leucocytes were separated from erythrocytes on a Ficoll hypaque cushion as previously described [13]. A sample of the diluted cell-free plasma above the cell layer was collected for antibody measurement.

3.4. Antibody measurement using ELISA

The antibody levels in the sera were estimated by an enzyme-linked immunosorbent assay (ELISA) described by Melville-Smith [14]. Pertussis toxin and FHA were used to coat the plates at concentrations of 2 µg/ml and 6 µg/ml respectively. Heparinised plasma samples were further diluted in saline prior to assay. A serum pool from uninfected DTP-vaccinated children was used as a reference. Antibodies were detected using alkaline phosphatase-conjugated anti-human polyvalent immunoglobulin (Sigma Chemical Company, Poole, Dorset). Antibody levels to each antigen preparation were estimated by assigning a nominal potency of 100 units/ml to the reference serum pool. The absorbances were plotted against the log10 of the concentration for each serum and the antibody levels, expressed in units/ml of undi-
luted serum determined from the reference using a parallel line assay.

3.5. Interleukin 2 production

The capacity of lymphoid cells to produce IL-2 in vitro in response to the panel of antigens described above was studied by culturing $5 \times 10^6$ cells/ml at $37^\circ$C in the presence of 5% (v/v) CO$_2$. After 36 h the cells were centrifuged and the cell-free supernatant frozen at $-20^\circ$C prior to assay of IL-2.

3.6. Interleukin 2-dependent cell line assay

Interleukin 2 activity in culture supernatants was determined by its effect on the proliferation of an IL-2 dependent T cell line, CTLL. CTLL cells ($5 \times 10^3$) were incubated in a total volume of 100 μl of RPMI 1640 containing 5% v/v foetal calf serum and dilutions of test supernatants. Proliferation was measured by the incorporation of [$^3$H]thymidine (0.5 μCi, specific activity 20 μCi/mM Amersham) during the last 4 h of a 24 h incubation period [15].

3.7. Cell proliferation assay

Leucocytes were distributed into 96-well microtitre plates (Flow) at $5 \times 10^4$ cells/well in RPMI 1640 containing 3% v/v foetal calf serum. Antigens were added to wells at the concentrations indicated above. Each individual's cells were assayed in duplicate. After 18 h in culture, 0.5 μCi of [$^3$H]thymidine was added and the cells were harvested 4 h later on to fibreglass filters (Whatman). Incorporated radioactivity was determined by scintillation counting as described previously [4].

4. RESULTS

4.1. Antibody titres

Infection with B. pertussis stimulated the production of antibodies directed against PT and FHA (Table 1). Antibody titres in the patient group were significantly elevated above control group titres for each antigen. Patient 5 had lower titres of antibody to FHA and PT than the other four patients. Her immune responses to B. pertus-

Table 1

| Antibody titres to B. pertussis antigens in serum of patients with whooping cough, contact and control groups |
|----------------------------------|---|---|
| Antibody levels units/ml         | PT  | FHA |
| Disease group                    |     |     |
| 1                               | 379 | 180 |
| 2                               | 624 | 410 |
| 3                               | 1110| 440 |
| 4                               | 239 | 185 |
| 5                               | 68  | 32  |
| mean                            | 441.0| 248.6|
| Contact group                   |     |     |
| 8                               | 21  | 51  |
| 9                               | 115 | 86  |
| 10                              | 7   | 6   |
| 11                              | 11  | 21  |
| 12                              | 32  | 11  |
| 13                              | 42  | 50  |
| 14                              | 277 | 589 |
| 15                              | 85  | 230 |
| mean                            | 73.8| 130.5|
| Control group                   |     |     |
| 16                              | 6   | 9   |
| 17                              | ND  | ND  |
| 18                              | 4   | 16  |
| 19                              | 10  | 12  |
| 20                              | 67  | 67  |
| 21                              | ND  | ND  |
| 22                              | ND  | ND  |
| 23                              | ND  | ND  |
| 24                              | 3   | 41  |
| 25                              | 84  | 113 |
| 26                              | 20  | 98  |
| 27                              | 10  | 28  |
| mean                            | 25.5| 48.0|

Antibody levels in serum were determined by ELISA using a serum pool from DTP-vaccinated children as a standard of 100 units/ml. Levels in the patient group were significantly elevated above the other groups. $P = 0.016$ (FHA) and $P = 0.018$ (PT). ND = Not Done.
Table 2

Proliferation of leucocytes following antigenic challenge

<table>
<thead>
<tr>
<th>Subject</th>
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<th>Bb</th>
<th>FHA</th>
<th>PT</th>
</tr>
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<td>25</td>
<td>50</td>
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</tr>
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</tr>
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<td>0.8</td>
</tr>
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<td>1.6</td>
<td>2.2</td>
</tr>
<tr>
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<td>0.9</td>
<td>2.7</td>
<td>1.2</td>
<td>1.2</td>
</tr>
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<td>5.3</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>mean</td>
<td>2.8</td>
<td>3.3</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Leucocyte proliferation expressed as a stimulation index: proliferation in presence of antigen / proliferation in culture medium.
Proliferation in the patient group was significantly elevated above the other groups only following challenge with FHA at 50 μg/ml, P = 0.016. Bp = Bordetella pertussis, Bb = Bordetella bronchiseptica.

found nine months after finishing a full primary immunization course of DTP vaccine (unpublished observations).

4.2. Proliferative responses of leucocytes

The proliferative responses of leucocytes following in vitro challenge with a range of antigens are shown in Table 2. Each value is a stimulation index (SI) expressed as cpm in wells containing antigen, divided by cpm in wells containing culture medium alone. Tritiated thymidine incorporation in control cultures ranged from 400–8000 cpm.

No increase in proliferation was seen in response to challenge with E. coli or PTd (results not shown). An increase in proliferation was caused by LPS, B. pertussis and B. bronchiseptica, in all three subject groups. Responses to FHA at three doses were significantly elevated in the patient group only. Subjects 8, 9 and 10 in the contact group and subject 25 in the control group showed elevated responses to FHA, however overall the contact and control groups were not significantly different.

Challenge with PT at three doses caused a large
Table 3  
IL-2 production by leucocytes following antigen challenge

<table>
<thead>
<tr>
<th>Subject</th>
<th>Bp</th>
<th>Bb</th>
<th>FHA 12 µg/ml</th>
<th>FHA 25 µg/ml</th>
<th>FHA 50 µg/ml</th>
<th>PT 12 µg/ml</th>
<th>PT 25 µg/ml</th>
<th>PT 50 µg/ml</th>
</tr>
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<td>0.6</td>
<td>0.7</td>
<td>2.6</td>
<td>2.6</td>
<td>3.6</td>
<td>2.6</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>1.1</td>
<td>1.5</td>
<td>3.2</td>
<td>2.7</td>
<td>3.0</td>
<td>10.5</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>18.3</td>
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<tr>
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<td>1.4</td>
<td>1.5</td>
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<td>32.0</td>
<td>1.0</td>
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<td></td>
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<td>2.0</td>
<td>13.0</td>
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<td>0.2</td>
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<tr>
<td>mean</td>
<td>4.6</td>
<td>2.5</td>
<td>8.7</td>
<td>14.3</td>
<td>19.3</td>
<td>3.2</td>
<td>3.5</td>
<td>8.5</td>
</tr>
</tbody>
</table>

| Contact Group | 8 | 1.0 | 1.5 | 2.1 | 3.6 | 3.1 | 1.3 | 3.7 | 7.1 |
| | 9 | 0.8 | 0.6 | 2.0 | 2.0 | 2.0 | 0.5 | 1.0 | 2.2 |
| | 10 | 1.0 | 1.6 | 2.7 | 2.5 | 2.8 | 2.2 | 4.2 | 5.4 |
| | 11 | 1.8 | 2.1 | 1.0 | 1.4 | 1.6 | 6.5 | 7.8 | 7.4 |
| | 12 | 2.1 | 2.4 | 2.1 | 2.4 | 2.2 | 4.8 | 5.2 | 6.8 |
| | 13 | 1.1 | 0.8 | 1.7 | 1.2 | 2.2 | 1.2 | 1.1 | 1.4 |
| | 14 | 0.4 | 1.2 | 1.1 | 1.2 | 1.5 | 0.7 | 0.7 | 2.5 |
| | 15 | 0.6 | 1.2 | 2.2 | 1.3 | 2.0 | 0.8 | 0.8 | 1.2 |
| mean | 1.1 | 1.4 | 1.9 | 2.0 | 2.2 | 2.3 | 3.1 | 4.3 |

| Control Group | 16 | 0.7 | 2.0 | 1.8 | 4.0 | 4.0 | 1.8 | 1.1 | 3.8 |
| | 17 | 0.7 | 1.2 | 1.8 | 1.4 | 1.9 | 0.7 | 0.8 | 2.3 |
| | 18 | 0.8 | 1.4 | 0.9 | 0.8 | 1.3 | 0.7 | 1.6 | 2.8 |
| | 19 | 1.6 | 2.1 | 1.9 | 1.8 | 1.7 | 1.0 | 1.2 | 4.2 |
| | 20 | 0.6 | 0.6 | 1.4 | 2.1 | 1.9 | 1.6 | 3.0 | 2.1 |
| | 21 | 1.1 | 1.9 | 1.4 | 1.6 | 1.6 | 1.5 | 0.8 | 1.0 |
| | 22 | 1.2 | 1.9 | 1.1 | 1.6 | 2.1 | 6.4 | 6.4 | 7.4 |
| | 23 | ND | ND | ND | ND | ND | ND | ND | ND |
| | 24 | 0.6 | 0.7 | 0.7 | 1.3 | 1.5 | 2.0 | 2.2 | 2.4 |
| | 25 | 0.5 | 2.0 | 3.5 | 2.0 | 4.0 | 0.2 | 1.0 | 3.5 |
| | 26 | 0.5 | 1.0 | 6.0 | 13.0 | 9.0 | 0.3 | 0.6 | 1.5 |
| | 27 | 0.5 | 0.9 | 0.6 | 1.2 | 1.2 | 0.7 | 1.5 | 4.8 |
| mean | 0.8 | 1.4 | 1.9 | 2.8 | 2.8 | 1.5 | 1.8 | 3.2 |

IL-2 production expressed as a stimulation index: proliferation of CTLL cells in antigen-induced supernatant/ proliferation of CTLL cells in unstimulated supernatant. IL-2 production was significantly increased in the patient group following challenge with FHA at 50 µg/ml, P = 0.058. If patient 5 is excluded from the patient group, then PT at 50 µg/ml also causes a significant increase in IL-2 production. P = 0.104. ND = not done. Bp = Bordetella pertussis Bb = Bordetella bronchiseptica.

increase in proliferation in all three subject groups. In the contact and control group, proliferation did not significantly increase with an increase in PT dose. However, in the disease group, proliferation increased significantly with an increase in PT dose.

4.3. IL-2 production by leucocytes

IL-2 levels in the supernatant of leucocytes cultured with different antigens are given in Table 3. Results are given as an SI expressed as cpm of CTLL cells cultured with supernatant from leucocytes incubated with antigen divided by cpm of CTLL cells cultured with supernatant from leucocytes incubated in culture medium. Each supernatant was assayed at 25% final concentration in a single CTLL assay.

Overall, no significant increase in IL-2 levels were seen in cultures containing E. coli, PTd or LPS (results not shown). However, patient 3 did show marked IL-2 production in response to both B. pertussis and B. bronchiseptica. Patient 3 also gave the highest levels of antibody to FHA and
PT. Stimulation with FHA and PT caused an elevation in IL-2 titres in control and contact groups, but a significantly higher elevation in IL-2 titres in the disease group, particularly if subject 5 is excluded from the disease group.

5. DISCUSSION

We have demonstrated that following infection with *B. pertussis* both systemic antibody production and T cell immunity are induced. It is known that both infection with *B. pertussis* and vaccination with whole-cell vaccine, which contains some active PT, cause the production of antibodies directed against a range of *B. pertussis* antigens, including PT and FHA, and confer a high degree of immunity against further episodes of whooping cough [16,17]. However, the Swedish field trial of two acellular pertussis vaccines, comprising toxoided PT only and toxoided PT plus FHA, has shown levels of efficacy significantly lower than the rates reported for whole-cell vaccines [18]. This was in spite of the acellular vaccines inducing antibody responses as high as those produced by whole-cell vaccines. One possible area of difference between these two types of vaccine, which was not examined, was the cellular immune responses they engendered.

The development of cell mediated immunity following disease or immunization with whole-cell pertussis vaccine has not been studied previously in man. We and others have shown that T cell responses are stimulated in rodents following immunization with *B. pertussis* whole-cell vaccine, and some of its component antigens [4,19]. After immunization of mouse footpads with a whole-cell vaccine, cell mediated responses in the draining popliteal lymph nodes were measured. Strong proliferative responses were seen on secondary challenge with whole *B. pertussis*, and PT but not with PTd. Responses to FHA were less marked. IL-2 production was elevated in response to the same antigens. In this study of human responses to *B. pertussis*, leucocyte proliferation and IL-2 production while raised to PT and FHA were not significantly elevated following challenge with PTd or whole bacteria.

This difference in the pattern of responses may reflect the differing methods of presenting these antigens (injection of killed *B. pertussis* into mouse footpads, and natural infection of human respiratory epithelium with live *B. pertussis*) or the differing origins of the leucocyte populations investigated (mouse lymph nodes and human peripheral blood).

The occurrence of T cell responses to *B. pertussis* antigens in vitro following natural infection may be a reflection of similar responses in vivo. It is important to consider the role such T cells could play in combating whooping cough within the respiratory tract. *B. pertussis* is a non-invasive pathogen of the ciliated respiratory epithelium [17]. While antibodies to FHA or to PT could inhibit the adherence of *B. pertussis* to human ciliated respiratory epithelial cells [17], T cells normally cannot be detected on the surface of lung epithelium, and are probably restricted to the interstitial tissues [20] and tracheobronchial lymph nodes [21]. T cell action is therefore probably mediated by cytokines which can influence the behaviour of other more mobile cell types such as alveolar macrophages and neutrophils which can reach the epithelial surface. IL-2 has recently been shown to activate macrophages directly in vitro [6], and to protect mice from a lethal challenge with *E. coli* [22]. Cheers and Gray [23] have shown that in mice given an aerosol challenge with *B. pertussis*, viable bacteria can be found inside alveolar macrophages, these bacteria remain until neutrophils are observed to infiltrate the lung. It is not known whether this happens in the human respiratory tract, but obviously activation of macrophages could contribute to clearing this source of bacteria.

Antibodies are thought to be important both in preventing attachment of *B. pertussis* to cilia and hence in limiting the initial colonization phase of infection, and also in preventing the systemic effects of PT and other toxins. T cell responses, particularly lymphokine production, should also be considered as potentially protective. It will therefore be important to investigate the T cell immunity produced following vaccination with either the currently used whole-cell vaccines, in which active PT is present, or with the new acellu-
lar component vaccines containing PT only in the toxoided form.

Toxoiding of PT does not seem to affect antibody production but the results of this study suggest that it does affect T cell stimulation. Therefore failure of toxoided acellular pertussis vaccines to stimulate a sufficiently strong cellular immune response could account for their reduced efficacy compared to whole-cell vaccines and infection itself. We are currently investigating these possibilities.

ACKNOWLEDGEMENTS

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REFERENCES


Severe hypoxaemia in pertussis

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*Department of Paediatrics, Cardiothoracic Institute, Brompton Hospital, London and †Communicable Disease Unit, St George's Hospital Medical School, London

SUMMARY Overnight tape recordings of breathing movements, airflow, and arterial oxygen saturation from six infants aged 3 weeks to 7 months, who had cyanotic episodes associated with pertussis, were compared with recordings from 12 age matched healthy controls. In all patients clinically apparent apnoeic episodes were associated with the rapid onset and progression of central cyanosis. When overnight recordings were compared, patients with pertussis had a greater frequency of apnoeic pauses (particularly those ≥12-0 seconds duration) and a greater frequency of episodes of hypoxaemia (oxygen saturation ≤80% for ≥0-5 seconds) associated with apnoeic pauses. In addition to episodes of hypoxaemia associated with a prolonged absence of breathing movements, patients with pertussis had frequent dips in oxygen saturation in association with continued breathing movements with and without continued inspiratory airflow. These episodes of hypoxaemia during continued breathing movements were more common in patients with pertussis.

These findings suggest that episodes of abnormal apnoea accompanied by evidence of a mismatch between ventilation and perfusion of the lungs may produce the rapid onset of severe hypoxaemia in infants with pertussis.

Apnoeic and cyanotic episodes often occur during pertussis particularly when it occurs in early infancy.1 2 The underlying mechanisms responsible for this dangerous complication are, however, unknown. In this paper we describe the results of an investigation by non-invasive techniques of infants with pertussis experiencing frequent and severe cyanotic episodes and compare these results with those from 12 healthy, age matched infants.

Patients and methods

Details of the six patients are given in the table. Pertussis was diagnosed by the presence of characteristic paroxysms of repetitive coughing that persisted for longer than one week and were associated with retching, vomiting, cyanosis, or convulsions in an infant who otherwise appeared to be in good health.3 Three patients had a history of recent exposure to pertussis. Nasopharyngeal swabs ('Transwab', Medical Wire and Equipment Co) from all six patients were cultured on cephalixin supplemented charcoal agar and Bordetella pertussis was isolated from two patients. None of the patients had suffered episodes of cyanosis before the onset of the present illness.

All patients had a paroxysmal cough and a history of apnoea associated with the rapid onset of
profound cyanosis. These apnoeic cyanotic episodes began at least one week after the onset of cough in all patients except patient 1 who suffered apnoic episodes from the outset of the illness. In all patients the cyanotic episodes had a sudden unpredictable onset. They occurred when awake and when asleep and were often not immediately preceded by cough. In many cases coughing followed the onset of the cyanosis (see below). Patients 1, 4, 5, and 6 had a history of convulsions with loss of consciousness during their cyanotic episodes. Patients 1 and 6 were cyanosed and convulsing at the time of admission to hospital. (The twin sister of patient 6, with an identical history of pertussis, died during a cyanotic convulsion while the infants were being brought to hospital.) Apart from patient 2 who had a birthweight of 680 g at 25 weeks' gestation all the patients were delivered at term with birth weights of at least 2000 g.

All patients were nursed under close continuous supervision and were treated by positioning, oropharyngeal suctioning, and administration of oxygen via a face mask at the onset of any clinically apparent episodes of severe apnoea or cyanosis. Patients 1, 3, 5, and 6 had been treated with erythromycin, patients 3 and 5 were treated with phenobarbital and salbutamol, and patients 4 and 5 were treated with aminophylline before and during investigation. Chest radiography showed normal results in all patients at the time of investigation. The recordings described in this paper were measured by non-invasive sensors, did not influence the clinical care of the patients, and were approved by the St George's Hospital ethical committee.

The case history of patient 1 shows the clinical features seen in these six infants. This 3 week old girl was brought to hospital with a two week history of paroxysmal cough and repeated episodes of apnoea with cyanosis, loss of consciousness, and generalised convulsions. Cyanosis typically occurred within 10 seconds of the onset of apnoea and loss of consciousness and convulsions followed within a further 20 seconds. On the day of admission she had twice lost consciousness. On admission she was apnoic, cyanotic, and convulsing but quickly recovered spontaneously. After admission she had further repeated episodes of apnoea, cyanosis, and convulsions requiring resuscitation by nursing and medical staff.

Healthy term infants being studied as part of a large survey of normal breathing patterns were used as control subjects. Two age matched controls were randomly selected for comparison with each patient with pertussis.

Investigations centered around overnight tape recordings (Racal Store 4 FM with a 1 channel multiplexer) of beat to beat arterial oxygen saturation (from a probe placed on the big toe and a modified Nellcor pulse oximeter Respox 2), airflow measured at the external nares by a thermistor (time constant 0.5 seconds, Yellow Springs Instrument Co) or by expired carbon dioxide and a scavenger tube (sampling at 100 ml/minute, with an electronic response time of 100 milliseconds, Engstrom Eliza), and breathing movements from inductance plethysmography (Studley Data Systems) or a pressure capsule (Graseby Dynamics). In order to verify the accuracy of the oxygen saturation measurements the oximeter was used in a beat to beat mode and every plethysmographic waveform representing the arterial pulsation used to derive oxygen saturation was recorded onto the tape recordings along with the oxygen saturation signal. Only oxygen saturation measurements accompanied by pulse waveforms of adequate quality were regarded as accurate. In three infants (patients 1, 3, 5) oesophageal tube and pressure transducer (Gaeltec). In patient 1 an electroencephalogram was recorded from a centrotemporal configuration during cyanotic episodes. Tape recordings were printed onto an ink jet chart recorder (Siemens 34T) or an electrostatic chart recorder (Gould ES 1000).

Parents of patients and controls gave informed consent for the investigations.

Recordings were analysed by a technician who was unaware of the clinical details of each subject. Periods during which signals were uninterpretable owing to movement artefact were documented and excluded from analysis. Breathing patterns were classified as regular or non-regular breathing according to previously published criteria. Regular breathing was defined as episodes of \( \geq 1 \) minute where the breathing pattern was relatively regular in amplitude and rate, interrupted by sighs but without movement artefact. Non-regular breathing was all the remainder of the recording, and included intermittent brief periods of movement artefact. All apnoeic pauses (absent inspiratory efforts for \( \geq 4 \) seconds) were counted and their durations measured and the frequency of these pauses was determined by dividing the total number of pauses by the total duration of interpretable recording. All episodes of hypoxaemia, defined for this study as a situation where oxygen saturation fell to \( \leq 80\% \) for \( \geq 0.5 \) seconds were identified. This measurement was chosen after our experience of analysing similar recordings from large numbers of healthy infants wherein a cut off of 80% was helpful in defining normality. The duration of this hypoxaemia was...
measured for each episode and the breathing pattern during the preceding 12 seconds and during the period of hypoxaemia recorded. Breathing movements or airflow, or both, were regarded as absent if there was complete cessation for a period of ≥4-0 seconds. The frequency of episodes of hypoxaemia was determined by dividing the total number of episodes of hypoxaemia by the total duration of interpretable recording. The duration of hypoxaemia was determined by totalling the lengths of each episode and dividing this by the total duration of interpretable recording.

The frequency of apnoeic pauses of different durations, and the frequency of episodes of hypoxaemia associated with apnoeic pauses of different durations, in patients with pertussis and control subjects were compared by the Mann-Whitney U test.

Results

Clinical observations showed that although most of the overt cyanotic episodes began in association with coughing or crying, about one third, especially those occurring during sleep, began with an absence of inspiratory efforts (apnoeic pause). In all the observed cyanotic episodes the onset of cyanosis was rapid, usually occurring between four and eight seconds after the onset of the cough, crying, or apnoeic pause. Recovery from a cyanotic episode was frequently associated with a large inspiratory effort (a gasp).

All subjects had at least 4-9 hours of interpretable recordings (patients with pertussis 5-7-9-8 hours, normal subjects 4-9-12-5 hours.) Regular breathing and non-regular breathing alternated at intervals of about 20-100 minutes throughout the recordings. Both in the pertussis patients and in the normal subjects apnoeic pauses and episodes of hypoxaemia occurred almost exclusively during the periods of non-regular breathing.

Patients with pertussis had a greater mean frequency of apnoeic pauses than control subjects (fig 1). This difference was greatest for apnoeic pauses with a duration of ≥12-0 seconds (p<0-005). The longest apnoeic pauses for patients 1-6 were 15-0, 28-5, 11-5, 15-0, 53-8, and 17-0 seconds, respectively. The longest apnoeic pause in a control subject was 14-1 seconds. Thus five of the six patients had abnormally prolonged apnoeic pauses.

![Fig 1](image1.png)

**Fig 1** Frequency of apnoeic pauses (duration 4-0-7-9, 8-0-11-9, ≥12-0 seconds) for patients with pertussis (solid circles), and control subjects (open circles). Note logarithmic scale on the vertical axis.

![Fig 2](image2.png)

**Fig 2** Frequency of episodes of hypoxaemia associated with either continued breathing movements, or apnoeic pauses with durations of 4-0-7-9, 8-0-11-9, and ≥12-0 seconds, for patients with pertussis (solid circles), and control subjects (open circles). Note logarithmic scale on the vertical axis.
The frequency of episodes of hypoxaemia related to the duration of associated apnoeic pauses for patients with pertussis and control subjects is shown in fig 2. Patients with pertussis had a greater frequency of episodes of hypoxaemia associated with apnoeic pauses with a duration of 4.0—7.9 seconds (p<0.005), 8.0—11.9 (p<0.05), and ≥12.0 seconds (p<0.05). The greatest difference between patients with pertussis and control subjects, however, was in the frequency of episodes of hypoxaemia during continued breathing movements (p<0.001).

An example of a hypoxaemic episode associated with a prolonged absence of breathing movements and airflow is shown in fig 3. These episodes usually began at end expiration and were associated with a raised oesophageal pressure. Prolonged apnoeic pauses often included a period of breathing movements without airflow before normal breathing was resumed ('mixed' apnoea) (fig 4). Some hypoxaemic episodes terminated with a period of sharply positive oesophageal pressure excursions suggesting coughing (fig 4). Patients with pertussis also had episodes of hypoxaemia associated with absent airflow despite continued breathing movements (fig 5). Patient 2 had prolonged periods of hypoxaemia despite continued breathing movements and continued airflow (fig 6) and patients 3–5 had similar but briefer episodes. Electroencephalographic recordings during prolonged pauses in inspiratory efforts with hypoxaemia in patient 1 did not show a seizure preceding the apnoeic pauses.

**Discussion**

All six patients in this study presented under 6 months of age and had cyanotic episodes characterised by the extremely rapid onset and progression...
Fig 4  Recording of an episode of hypoxaemia associated with 'mixed' apnoea in patient 5. A prolonged period of absent airflow (A-B) is initially associated with absent inspiratory efforts but a raised oesophageal pressure (C). Ineffective breathing efforts then occur (D). Towards the end of the episode there are sharply positive excursions in oesophageal pressure (E), suggestive of coughing. I: Arterial saturated oxygen. III: Oesophageal pressure from a balloon catheter; scale is to the left of the signal. Positive pressure changes are represented by upward deflections. IVb: Airflow at the nose from a thermistor; inspiratory flows are represented by upward deflections.

Fig 5  Recording of an episode of hypoxaemia associated with continued breathing movements but absent airflow (A-B) in patient 4. I: Arterial saturated oxygen. IIa: Abdominal breathing movements from a pressure capsule transducer: expansion is an upward deflection. III: Arterial pulse waveform. IVb: Airflow at the nose from a thermistor; inspiratory flows are represented by upward deflections.
of severe hypoxaemia similar to that occurring during a condition previously termed prolonged expiratory apnoea and suggesting the acute onset of a mismatch between ventilation and perfusion of the lungs. Between clinically overt cyanotic events infants with pertussis showed repeated abnormal dips in arterial oxygen saturation in association with four breathing patterns: prolonged (>15 seconds) pauses in inspiratory efforts (fig 3), an initial absence of effort and airflow followed by continued efforts but absent airflow (fig 4), continued breathing efforts but absent inspiratory airflow (fig 5), and continued inspiratory efforts and continued airflow (fig 6). Hypoxaemia was not seen in association with these patterns of breathing in control subjects. As with the rapidity of onset and severity of the clinically apparent cyanotic episodes, the occurrence of hypoxaemia during continued breathing movements is also characteristic of prolonged expiratory apnoea and suggests that both mismatch between ventilation and perfusion of the lungs, and apnoea (absent ventilation of the lungs) contribute to the pathogenesis of hypoxaemia in infants with pertussis.

We have previously suggested that alveolar atelectasis may be responsible for these disturbances in ventilatory perfusion relations and that the accompanying prolonged pauses in breathing movements may result from the effects of atelectasis on lung reflexes. This hypothesis is supported by the recordings shown in fig 6, which shows that hypoxaemia may develop despite continued airflow into the lungs. If alveolar atelectasis is the cause of the hypoxaemic episodes, a defect in lung surfactant may represent the primary pathology. In infants with pertussis this may be due to a direct effect of B pertussis toxins on surfactant synthesis, secretion or function.

Although a proportion of the cyanotic events began during coughing, some followed crying, and others, especially during sleep, followed a pause in inspiratory efforts. In this last case the infant would characteristically stop breathing, become cyanosed, arouse, and then begin coughing.
Cyanotic convulsions have been reported to precede death in pertussis, and changes suggesting anoxia are a consistent finding in the histology of the brains of children dying of pertussis complicated by convulsions. Our recordings suggest that the convulsions are secondary to severe cerebral hypoxaemia.

The patterns of breathing and hypoxaemia identified above have been described in apnoea of prematurity suggesting that the pathogenesis of these two conditions might be similar. Moreover, similar cyanotic episodes, sometimes with evidence of prolonged pauses in inspiratory efforts, have been reported in other respiratory tract infections during infancy. In two retrospective studies respiratory syncytial virus infection was associated with prolonged apnoea or cyanotic episodes in 10% and 20% of cases respectively. In the prospective study of infection by respiratory syncytial virus reported by Anas et al, 25% of infants showed prolonged apnoeic episodes. These often began early in the disease before other symptoms of infection had developed and were more frequent in infants less than 3 months of age and in previously preterm infants especially those who had suffered from apnoea of prematurity. Yolken and Murphy described cyanotic episodes in five infants with rotavirus infection, three of whom were temporarily resuscitated but subsequently died.

Despite a distinctive clinical picture, doctors are often slow to diagnose pertussis, and notifications greatly underestimate the true incidence of the disease. Even when pertussis is diagnosed, however, clinical observation alone is likely to greatly underestimate the frequency and severity of hypoxaemia as shown by our overnight recordings. Nicoll and Gardner and Cherry have suggested, on the basis of epidemiologic studies, that pertussis may be responsible for some cases of sudden infant death. Williams and Jones have produced evidence that the convulsions and apnoea complicating pertussis may be accompanied by subsequent intellectual impairment. These data and our findings suggest that infants with a history of cough with apnoea or cyanosis, even if apparently well at initial examination, should be admitted to hospital for a period of meticulous observation. Our results also underline the need to prevent this serious and as yet untreatable infectious disease and clearly this can be best achieved by a more complete programme of immunisation.

In conclusion episodes of abnormal apnoea accompanied by evidence of a mismatch between ventilation and perfusion of the lungs may cause severe arterial hypoxaemia in infants with pertussis. This hypoxaemia may be life threatening, may result in seizures, and may be one cause of the brain damage reported in some infants with pertussis.

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Severe hypoxaemia in pertussis

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**Effects of *Bordetella pertussis* Infection on Human Respiratory Epithelium In Vivo and In Vitro**

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*Bordetella pertussis* infection probably involves attachment to and destruction of ciliated epithelial cells, but most previous studies have used animal tissue. During an epidemic, nasal epithelial biopsy specimens of 15 children (aged 1 month to 3 1/2 years) with whooping cough were examined for ciliary beat frequency, percent ciliation of the epithelium, and ciliary and epithelial cell ultrastructure. In addition, the in vitro effects of filtrates from a 24-h broth culture and of tracheal cytotoxin derived from *B. pertussis* on human nasal tissue organ culture were measured. *B. pertussis* was cultured from nasal swabs of 12 children. The mean ciliary beat frequency of their nasal biopsy specimens, 11.3 Hz (range, 10.4 to 13.0 Hz) was similar to that found in biopsy specimens from 10 normal children (mean, 12.5 Hz; range, 11.8 to 13.5 Hz). The abnormalities of the epithelium observed in 14 of 15 patients were a reduction in the number of ciliated cells, an increase in the number of cells with sparse ciliation, an increase in the number of dead cells, and extrusion of cells from the epithelial surface. In vitro, neither culture filtrate nor tracheal cytotoxin had any acute effect on ciliary function, but culture filtrate and tracheal cytotoxin (1 and 5 μM, respectively) caused extrusion of cells from the epithelial surface of turbinate tissue, loss of ciliated cells, an increased frequency of sparsely ciliated cells, and toxic changes in some cells. These changes were dose dependent and progressive, and between 36 and 90 h ciliary beating ceased. The observations made with patient tissue confirm that *B. pertussis* infection damages ciliated epithelium, and the in vitro experiments suggest that tracheal cytotoxin may be responsible for the abnormalities observed in vivo.

*Bordetella pertussis* is a bacterial respiratory tract pathogen and is the major cause of whooping cough (pertussis) in children. Earlier this century whooping cough was common, but use of the current inactivated whole-cell vaccine significantly reduced its incidence (5). More recently, in the United Kingdom, however, concern about adverse effects associated with the administration of this vaccine led to loss of public confidence in it, and reduced use of the vaccine was associated with several large epidemics. Although the clinical features of pertussis are well recognized (paroxysmal coughing and choking often culminating in vomiting or retching and cyanosis, apneic attacks, and long duration of illness), the pathogenesis of the disease is still understood. Almost 80 years ago, Mallory and Hornor (12) histologically characterised pertussis as a noninvasive bacterial colonization of ciliated cells in the respiratory epithelium. *B. pertussis* has been shown to adhere to epithelial cells, and filamentous hemagglutinin (a cell surface protein) has been proposed as the bacterial adhesin (17, 23, 24). However, the precise interaction between *B. pertussis* and the respiratory mucosa has not been described. For example, how the bacterium interacts with mucus, beating cilia (26), and epithelial cells is poorly understood. Organ culture studies using animal tissue suggest that late in the infection epithelial cell injury occurs, resulting in cessation of ciliary beating (2, 14, 15); at this stage the requirements for bacterial colonization may change (11).

*B. pertussis* produces a number of biologically diverse toxins, including pertussis toxin (also known as lymphocytosis-promoting factor), extracellular adenylate cyclase, and heat-labile toxin (25). Until recently, none of the toxins described above produced the characteristic changes induced by *B. pertussis* infection in respiratory epithelium (2, 12, 14, 15). Tracheal cytotoxin was first characterized by Goldman and coworkers and is a 1,6-anhydromuramic acid-containing monomeric disaccharide-tetrapeptide of peptidoglycan (3, 6-9, 18) which can be purified from culture supernatants of *B. pertussis*. It causes progressive cytotoxic changes in ciliated cells of hamster tracheal organ cultures.

The aims of the present study were to examine the function and ultrastructure of respiratory epithelial tissue obtained from the upper respiratory tract of children with whooping cough and to investigate for the first time the effects of tracheal cytotoxin on normal human respiratory epithelial tissue in vitro.

**MATERIALS AND METHODS**

Patients. In 1985 and 1986, during a pertussis epidemic, 15 children (nine boys and six girls aged 1 to 42 months) with whooping cough were investigated. All patients had a prolonged paroxysmal cough associated with whooping, vomiting, and/or apnea. Pertussis was confirmed by isolation of *B. pertussis* from a postnasal swab cultured on charcoal agar supplemented with 10% sheep blood and 40 μg of cephalaxin per ml (16) and/or by an increase in antibody titers to pertussis antigens (22). Ten normal children without recent history of respiratory infection (five boys and five girls, aged 1 to 28 months) acted as controls for ciliary beat frequency.
dom) supplemented with penicillin (50 U/ml), streptomycin essential medium (MEM) (GIBCO. Uxbridge, United Kingdom) for short experiments or minimal concentration in either Ringer's solution (Na+ [147 mmol/liter], Cl- [150 mmol/liter]) or MEM-ATB (50 µg/ml), and gentamicin (50 µg/ml) (MEM-ATB) for longer experiments.

Normal human respiratory epithelium. For shorter experiments (4 h), strips of nasal turbinate epithelium obtained from normal volunteers by the brushing technique described above. The tissue was used in longer experiments (up to 90 h) derived after nasal turbinate. These were resected from patients undergoing operations for relief of nonallergic nasal obstruction (by V. Lund). Tissue was transported to the laboratory for MEM-ATB. The turbinate were checked by light microscopy, and those that had a smooth, fully ciliated surface with normal ciliary beating were then transferred to fresh MEM-ATB and cut aseptically into adjacent 3-mm-thick cross sections.

**Effect of broth culture filtrates and tracheal cytotoxin on human respiratory epithelium in vitro.** The effect of culture filtrates (in a 1:1 mixture with medium 199 plus epithelium) was assayed on strips of epithelium obtained by the brushing technique for 4 h. The effect of culture filtrates (in a 1:1 mixture with MEM-ATB plus tissue) was assayed with epithelium obtained from nasal turbinate and incubated for 60 h. The effect of tracheal cytotoxin (20 and 40 µM) in Ringer's solution plus epithelium from nasal brushing was assayed for 4 h, and the effect of tracheal cytotoxin (1, and 5 µM) in MEM-ATB plus epithelium from nasal turbinate was assayed for 90 and 36 h, respectively. CBF was measured at hourly intervals from each of 10 strips of nasal epithelium in a sealed microscope coverslip-slide preparation. CBF was measured from the cell's luminal surface, and mitochondrial damage. The last two features were scored separately for ciliated and unciliated cells.

**Examination of ultrastructure by transmission electron microscopy.** The effect of culture filtrates and tracheal cytotoxin on the ultrastructure of epithelial cells and cilia of epithelium in a sealed microscope coverslip-slide preparation. CBF was measured from the cell's luminal surface, and mitochondrial damage. The last two features were scored separately for ciliated and unciliated cells.

**Statistical analysis.** Unpaired Student's t test was used to compare the CBF of children with pertussis with the CBF of normal children. By comparing the control mean CBF with the test mean CBF at each time point during an experiment, maximum ciliary slowing was detected. The 10 control readings of CBF were compared with the 10 test readings at the time point of maximum ciliary slowing by the unpaired Student's t test.

**RESULTS**

Biopsy specimens were taken from the nasal epithelium of 15 children with pertussis infection (Table 1). B. pertussis...
with respect to cell cytopathic effects and the number of cilia on the cell surface. Controls. A large sample of epithelium was obtained, probably because the epithelium was relatively normal. Nasal biopsies from 10 nonsmoking normal adults acted as controls.

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<th>Patient</th>
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was isolated from 12 of 15 children, and significant rises in antibody titers to two or more B. pertussis antigens were present in all 15. All patients had nasal symptoms, including blockage, snuffles, and anterior rhinorrhea. The nasal epithelium was usually reddened, but in some patients it was pale and edematous. Excess tenacious mucoid secretions were always seen. Good samples of strips of nasal epithelium were obtained from 11 of the patients, but only cellular debris, mucus, and single epithelial cells were obtained from the other 4 patients. The failure to obtain a good sample is unusual in our experience unless there is an abnormality of the epithelium, and the biopsy was not repeated because of the patients' condition. It is likely that these children had extensive damage to their epithelium in the area that was biopsied. The mean CBF on epithelial strips of the children with pertussis was within the normal range (113 Hz; range, 10.4 to 13.0 Hz; standard deviation, 0.78 Hz). Although it was a little lower, it did not differ significantly from the mean CBF of 10 normal children used as controls (12.5 Hz; range, 11.8 to 13.5 Hz; standard deviation, 0.58 Hz). In all 10 of the normal children, >95% of the epithelial strips were ciliated, but in 10 of 11 of the children with pertussis the percentage of strips of epithelium that were ciliated was reduced, in some cases markedly (Table 1). For one patient (patient 15), the biopsy specimen obtained appeared completely normal by light microscopy.

The results of ultrastructural studies of patients' nasal epithelial samples are shown in Table 2, and a representative example is shown in Fig. 1. Patient 15 was analyzed separately, as a large sample of epithelium was obtained, probably because the epithelium was relatively normal, which would otherwise bias the results. In the nine specimens examined in which sufficient tissue was available for electron microscopy, there was an increase in the percentage of cells extruding from the epithelial surface, an increase in the number of dead cells, and a decrease in the number of cilia on ciliated cells. However, the most noticeable change was the increased frequency of cells with no cilia. The nasal epithelium of patient 15 was also abnormal when examined by electron microscopy. There was an increased number of dead cells and an increased frequency of poorly ciliated cells, but the percentage of cells with no cilia was similar to that of control subjects. Examination of the ultrastructure of cilia from children infected with pertussis (excluding patient 15) showed no increase in abnormal forms, either of the dynein arms (patients, 304 cilia examined, 98.6% normal; controls, 560 cilia examined, 98.9% normal) or the microtubular pattern (patients, 790 cilia examined, 97.8% normal; controls, 990 cilia examined, 92.6% normal). Bacteria were not seen associated with the epithelial biopsy specimens taken from B. pertussis-infected children, except in one sample. In this sample, from patient 4 (Table 1), rod forms were seen closely associated with cilia (Fig. 2).

The effect of a Stainer-Scholte broth B. pertussis culture filtrate on human respiratory epithelial tissue in vitro was assessed by light microscopy measurement of CBF and electron microscopy of epithelial cell and ciliary ultrastructural

**TABLE 1.** Examination of nasal biopsy specimens taken from 15 children with B. pertussis infection

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<td>0</td>
</tr>
<tr>
<td>13</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
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<td>0</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>11.0</td>
<td>95</td>
</tr>
</tbody>
</table>

**TABLE 2.** Results of transmission electron microscopy of nasal biopsy specimens taken from children with B. pertussis infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Normal cells</th>
<th>Cells extruding from epithelial surface</th>
<th>Single cells</th>
<th>Dead cells</th>
<th>Cells with normal complement of cilia</th>
<th>Cells showing loss of cilia</th>
<th>Cells showing gross loss of cilia</th>
<th>Cells showing no cilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>2</td>
<td>1</td>
<td>0</td>
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<td>10</td>
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<tr>
<td>2</td>
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<td>13</td>
<td>8</td>
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<td>0</td>
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<td>0</td>
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<td>3</td>
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<tr>
<td>7</td>
<td>36</td>
<td>5</td>
<td>4</td>
<td>7</td>
<td>24</td>
<td>33</td>
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<td>8</td>
<td>56</td>
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<td>4</td>
<td>7</td>
<td>24</td>
<td>33</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>9</td>
<td>15</td>
<td>2</td>
<td>14</td>
<td>8</td>
<td>10</td>
<td>19</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Total*</td>
<td>336 (79.2)</td>
<td>37 (8.7)</td>
<td>33 (7.8)</td>
<td>18 (4.2)</td>
<td>38 (12.6)</td>
<td>82 (19.3)</td>
<td>17 (4.0)</td>
<td>268 (63.1)</td>
</tr>
<tr>
<td>15*</td>
<td>174 (81.3)</td>
<td>9 (4.2)</td>
<td>6 (2.8)</td>
<td>25 (11.7)</td>
<td>136 (63.6)</td>
<td>50 (23.4)</td>
<td>4 (1.9)</td>
<td>24 (11.2)</td>
</tr>
</tbody>
</table>

* Nine nasal biopsies of patients provided sufficient tissue for examination by electron microscopy. The results for patient 15 are shown separately because a large sample of epithelium was obtained, probably because the epithelium was relatively normal. Nasal biopsies from 10 nonsmoking normal adults acted as controls.

**Values in parentheses are the percentages of the total number of cells examined that showed the indicated characteristics. The results are analyzed separately with respect to cell cytopathic effects and the number of cilia on the cell surface.**
The culture filtrate had no effect on CBF over 4 h. Two experiments were performed. In the first, the mean CBF of the control sample after 4 h was 14.0 Hz and that of the test sample was 13.9 Hz; in the second, the mean CBFs were 12.7 and 13.4 Hz, respectively, after 4 h. Similarly, the mean CBF measurements at each of the hourly time points up to 4 h were equal. However, over a longer period (Fig. 3), the mean CBF was affected by the culture filtrate. The mean CBF began to decrease after 22 h, and by 60 h there was no ciliary beating present in the organ culture. However, the effect of the culture filtrate did not appear to be a direct effect on ciliary beating but rather a cytotoxic effect, i.e., disruption of the epithelial surface. For example, in an experiment done after incubating samples with culture filtrate for 36 h, 5 of 10 CBF readings yielded values of 12.5, 12.1, 12.5, 10.9, and 11.3 Hz, and 5 other readings, taken from areas where ciliary beating had previously occurred but had ceased, yielded values of 0 Hz. In addition, mucus and cell debris accumulated in the areas in which ciliary beating did not occur, making it difficult to distinguish the surface of the organ culture.

The effect of the B. pertussis culture filtrate on the ultrastructure of respiratory epithelial tissue is shown in Table 3. In the presence of culture filtrate for 36 h, there was an increase in the number of cells extruding from the epithelial surface and an increase in the frequency of poorly ciliated cells, and a greater proportion of the epithelial cells became unciliated. An increased number of the epithelial cells showed toxic changes, with increased cytoplasmic blebbing from their luminal surface and mitochondrial damage. All of these changes were more pronounced after 60 h. There seemed to be no difference between ciliated and unciliated cells with respect to their susceptibility to the toxic effects of culture filtrate. No changes were detected in the ultrastructure of cilia after 60 h of exposure to culture filtrate.

The effects of tracheal cytotoxin were examined in a similar way, and the effects of culture filtrate and tracheal cytotoxin on the ultrastructure of epithelial tissue are shown in Fig. 4. Tracheal cytotoxin did not have an acute effect on the CBF, even when large concentrations were used. After 4 h in one experiment, the mean control CBF was 11.5 Hz and the mean CBF in the presence of 20 μM tracheal cytotoxin was 12.3 Hz; after 4 h in another experiment, the mean control CBF was 12.1 Hz and the mean CBF in the presence of 40 μM tracheal cytotoxin was 13.2 Hz. The experimental CBFs at the other time points were similarly not significantly different from the control CBFs. However, as with culture filtrate, over a prolonged period tracheal cytotoxin caused loss of ciliary beating (Fig. 3). Tracheal cytotoxin at a 1 μM concentration caused a complete loss of ciliary beating in the organ culture after 90 h, and at a 5 μM concentration it caused a complete loss of ciliary beating after 36 h. Again the effects were predominantly cytotoxic, with disruption of the epithelial surface occurring after 36 h at 1 μM and 22 h at 5 μM, but the CBF in those areas where cilia continued to beat was normal.

The effects of tracheal cytotoxin on the ultrastructure of ciliated epithelial cells are shown in Table 4. The effects were very similar to those of the culture filtrate, with tracheal cytotoxin causing extrusion of cells from the epithelial surface, loss of ciliated cells, an increased frequency of sparsely ciliated cells, and toxic changes in some cells. The changes were dose dependent, and ciliated and unciliated cells showed toxic changes with equal frequency. No changes were detected in the ultrastructure of cilia after 24 h of exposure to tracheal cytotoxin (5 μM). Although no effect of tracheal cytotoxin on CBF was detected at 4 h, transmission electron microscopy did show abnormal changes in the epithelium even after this short exposure to tracheal cytotoxin.

DISCUSSION

The characteristic pathological phenomenon which has been described as following B. pertussis infection is specific colonization of ciliated epithelial cells and destruction of ciliated cells (12). The appearances of the nasal biopsy
samples taken from children with pertussis infection during our study were in keeping with this description, in that there was a marked reduction in the number of ciliated cells. The loss of ciliated cells appeared to be due to extrusion of cells from the epithelial surface and cell death and also due to loss of cilia from cells. However, the beat frequency of those cilia that remained was normal.

Incubation of respiratory epithelial tissue with B. pertussis culture filtrate showed that bacterial products did not affect ciliary function directly but that they did have cytotoxic effects on the epithelial cells, producing mitochondrial swelling and cell surface cytoplasmic blebbing. Cells were seen to extrude from the epithelium and were probably replaced by unciliated cells from below the epithelial surface, leading to a largely unciliated epithelium. In addition, the tight junctions between epithelial cells were broken and poorly ciliated cells were seen, suggesting that cilia were lost. These changes would cause a profound disturbance of the mucociliary clearance system. We previously determined that the concentration of tracheal cytotoxin in a 30-h shaking culture of B. pertussis in Stainer-Scholte medium is approximately 1.5 μM. Further experiments showed that the effects of the crude bacterial culture filtrate were reproduced by similar concentrations of purified tracheal cytotoxin from B. pertussis.

Tracheal cytotoxin is a single molecule produced by B. pertussis and is a 1,6-anhydromuramic acid tetrapeptide of peptidoglycan containing monomeric disaccharide (3, 4).
The production of tracheal cytotoxin is preserved among other *Bordetella* species (7), and the release of a peptidoglycan fragment which is toxic for ciliated epithelial tissue in *Neisseria gonorrhoeae* has also been described (13). The effects of tracheal cytotoxin have previously been studied with hamster respiratory epithelial cells (8, 9), but this is the first time that its effects on human epithelial cells have been described.

*B. pertussis* infection of animal organ culture causes ciliostasis due to extrusion of ciliated cells (2, 14, 15). Tracheal cytotoxin alone causes loss of ciliated cells and ciliostasis in hamster tracheal rings and cultured hamster tracheal cells, while adenylate cyclase, heat-labile toxin, and pertussis toxin have no effect on respiratory epithelial cells (9). Tracheal cytotoxin (1 to 5 μM) destroyed the ciliated cell population in 60 to 96 h, and nonciliated cells migrated to fill the gaps left by extruded cells. In the presence of tracheal cytotoxin, ciliated cells became more round, and loss of cilia, mitochondrial swelling, and loss of intercellular junctions occurred (8).

The effects of tracheal cytotoxin on human tissue were very similar to the effects on hamster tissue and had a similar time course. The effects of tracheal cytotoxin were seen earlier at a 5 μM concentration than at a 1 μM concentration, but even at high concentrations (40 μM) there was no immediate effect of tracheal cytotoxin on ciliary beating. Changes in human epithelial cell ultrastructure were, however, demonstrated after 4 h of incubation with tracheal cytotoxin.

The cytotoxic effect of tracheal cytotoxin on hamster tracheal epithelial cells was specific for ciliated cells, and the ultrastructure of unciliated cells remained normal (9). However, in the experiments with human nasal tissue described in this paper, mitochondrial swelling and cytoplasmic blebbing were seen equally in unciliated and ciliated cells. This most likely represents a species difference, or possibly the nonciliated epithelial cells in the upper respiratory tract are more responsive to tracheal cytotoxin than are those from the trachea. However, there are two further considerations. Firstly, we have shown that after exposure to tracheal cytotoxin, epithelial cells with poor ciliation are seen. If this process was complete, it would be impossible to assess whether an unciliated cell was originally unciliated or had become so during an experiment. Secondly, unciliated cells from below the epithelial surface probably replace the damaged cells that are shed. We examined the effect of tracheal cytotoxin on epithelial cell ultrastructure after 24 h, i.e., 12 h before complete ciliostasis occurred, by light microscopy.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Ciliary beat frequency [μb]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>30</td>
<td>8</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
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<tr>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
</tr>
<tr>
<td>70</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>90</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIG. 3.** Effect of *B. pertussis* culture filtrate and tracheal cytotoxin on CBF in vitro. Human nasal epithelial tissue was incubated with broth (○) and *B. pertussis* culture filtrate (●) in a 1:1 mixture with medium 199, with MEM alone (△) and MEM containing 5 μM tracheal cytotoxin (◆). +, Time at which disruption of the integrity of the epithelial surface was first observed.

The effects of tracheal cytotoxin on hamster tracheal epithelial cells were specific for ciliated cells, and the ultrastructure of unciliated cells remained normal (9). However, in the experiments with human nasal tissue described in this paper, mitochondrial swelling and cytoplasmic blebbing were seen equally in unciliated and ciliated cells. This most likely represents a species difference, or possibly the nonciliated epithelial cells in the upper respiratory tract are more responsive to tracheal cytotoxin than are those from the trachea. However, there are two further considerations. Firstly, we have shown that after exposure to tracheal cytotoxin, epithelial cells with poor ciliation are seen. If this process was complete, it would be impossible to assess whether an unciliated cell was originally unciliated or had become so during an experiment. Secondly, unciliated cells from below the epithelial surface probably replace the damaged cells that are shed. We examined the effect of tracheal cytotoxin on epithelial cell ultrastructure after 24 h, i.e., 12 h before complete ciliostasis occurred, by light microscopy.

If the response of the unciliated cells to tracheal cytotoxin had been examined later, then the effect of tracheal cytotoxin might have been underestimated because the unciliated cells appearing fresh on the epithelial surface from below would have been exposed to tracheal cytotoxin for less time. Since *B. pertussis* infection is believed to be limited to the ciliated epithelium of the respiratory tract, an understanding of bacterial interactions with the respiratory mucosa is crucial if the pathogenesis of whooping cough is to be explained. The production of tracheal cytotoxin alone is not

**TABLE 3.** Results of transmission electron microscopy of human respiratory tissue treated with *B. pertussis* culture filtrates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ciliated cells (%)</th>
<th>Unciliated cells (%)</th>
<th>Loss of cilia</th>
<th>Blebbing on:</th>
<th>Mitochondrial damage in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (60 h; n = 371)</td>
<td>88.9 + + +</td>
<td>11.1 0 0</td>
<td>60.9 14.8 0.8 23.5</td>
<td>76.5 0 0 21.0 2.2 0.3</td>
<td>50.7 0.8 0.2</td>
</tr>
<tr>
<td>Culture filtrate (60 h; n = 304)</td>
<td>72.4 0</td>
<td>25.7 0</td>
<td>33.9 17.1 1.6 46.4</td>
<td>51.0 0 0 42.8 2.6 0.9</td>
<td>48.8 0.9 0.2</td>
</tr>
<tr>
<td>Culture filtrate (60 h; n = 207)</td>
<td>70.5 0</td>
<td>29.5 0</td>
<td>33.3 15.5 1.4 34.9</td>
<td>49.3 0 0 47.3 1.9 0.5</td>
<td>49.3 1.0 0.2</td>
</tr>
<tr>
<td>Culture filtrate (60 h; n = 171)</td>
<td>57.3 0</td>
<td>29.8 10.5</td>
<td>23.2 12.3 17.5 22.5 56.7</td>
<td>26.9 10.5 7.6 37.2 2.4 0.6</td>
<td>32.1 10.5 9.7 0.2</td>
</tr>
</tbody>
</table>

* Each cell in a tissue section was examined for extrusion from the epithelial surface (a score of 0 indicates normal positioning in the epithelium), the presence of cilia (0 indicates a full complement of cilia on the cell surface), cell blebbing (cytoplasmic projections from luminal cell surfaces), and mitochondrial damage.
Filamentous hemagglutinin is a cell surface protein with a rodlike structure which appears to be involved in the adherence of *B. pertussis* to ciliated respiratory cells and unciliated cells (17, 23, 24). However, it may not be the sole determinant of adherence, as pertussis toxin may influence *B. pertussis* attachment to rabbit ciliated respiratory cells (17). Immunization with filamentous hemagglutinin causes a decrease in mouse pulmonary and tracheal colonization by *B. pertussis* (11). Using a filamentous hemagglutinin-negative strain of *B. pertussis*, Kimura et al. (11) showed that filamentous hemagglutinin was important during colonization of the trachea early in the infective process in the presence of a normal mucociliary barrier, but later in infection, when the epithelium had been damaged, the filamentous hemagglutinin-negative strain was able to colonize the trachea. These results suggest the *B. pertussis* adherence is a critical event during colonization in the presence of normal mucociliary clearance.

Pertussis is a disease in which a wide variety of toxic molecules act in concert. The initial event in the pathogenic process appears to be bacterial adherence to the epithelial
surface, possibly by multiple mechanisms and/or adhesins (17). Tracheal cytotoxin probably does not have a role at this stage, as it does not directly affect ciliary beating, unlike the toxins of some other bacterial species which colonize the human respiratory epithelium (21, 26, 28). Once the bacterium has become attached, the production of tracheal cytotoxin could facilitate contiguous spread by damaging the epithelium and compromising mucociliary clearance. This would result in B. pertussis proliferation and a buildup of mucus which would depend upon coughing for clearance. The local delivery of other toxins may then contribute to the disease process. It seems that colonization of the respiratory tract probably depends on adherence to respiratory epithelial tissue, which would permit local release of tracheal cytotoxin. The damage that this toxin causes to the epithelial cells and the loss of ciliary activity could in turn facilitate contiguous spread of the bacterium, while the accumulation of mucus is likely to contribute to the characteristic coughing episodes of pertussis.

**ACKNOWLEDGMENTS**

This work was supported in part by a grant from the National Fund for Research into Crippling Diseases (Action Research for the Crippled Child) and in part by the Public Health Service grant AI22243 from the National Institutes of Health.

**REFERENCES**


**TABLE 4. Results of transmission electron microscopy of human respiratory tissue treated with TCT**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cells extruding from epithelial surface</th>
<th>Loss of cilia</th>
<th>Blebbing on:</th>
<th>Mitochondrial damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 + + + + + + + + + +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (4 h; n = 225)</td>
<td>81.8 18.2 0 0 79.1 10.7 0 10.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCT (1 μM; 4 h; n = 263)</td>
<td>73.0 26.3 0.8 0 55.9 23.2 0.8 20.2</td>
<td>74.5 3.8 1.5 17.1 2.3 0.8</td>
<td>70.0 9.9 15.6 4.6</td>
<td></td>
</tr>
<tr>
<td>TCT (5 μM; 4 h; n = 165)</td>
<td>52.7 46.7 0.6 0 63.0 17.0 0 20.0</td>
<td>67.9 11.5 0.6 15.8 4.2 0</td>
<td>60.0 20.0 18.2 1.8</td>
<td></td>
</tr>
<tr>
<td>Control (24 h; n = 223)</td>
<td>63.5 33.9 1.3 1.3 57.5 17.2 0 25.3</td>
<td>72.5 1.3 0.9 24.0 0.9 0.4</td>
<td>70.0 4.7 21.0 4.3</td>
<td></td>
</tr>
<tr>
<td>TCT (1 μM; 24 h; n = 215)</td>
<td>27.9 43.7 14.0 14.4 34.9 15.3 7.2 42.6</td>
<td>55.3 5.3 1.4 28.8 1.0 1.0</td>
<td>51.9 10.1 29.8 8.2</td>
<td></td>
</tr>
<tr>
<td>TCT (5 μM; 24 h; n = 218)</td>
<td>51.4 46.8 1.8 0 42.7 17.9 2.8 36.7</td>
<td>57.8 4.6 0.9 29.8 6.9 0</td>
<td>37.6 25.7 25.2 11.5</td>
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* TCT, Tracheal cytotoxin.

Each cell in a tissue section was examined for extrusion from the epithelial surface (a score of 0 indicates normal positioning in the epithelium), the presence of cilia (0 indicates a full complement of cilia on the cell surface), cell blebbing (cytoplasmic projections from luminal cell surfaces), and mitochondrial damage.


The epidemiology of pertussis may be approximately represented by an equation that relates the rate of increase in incidence of pertussis to the contagiousness of pertussis, the number of contacts per case, and the proportion of the population susceptible. By use of this equation it is possible to estimate the proportion of the population that is susceptible to pertussis at any stage of a pertussis epidemic. Data from pertussis epidemics in England and Wales in the last 10 years suggest that the level of immunity to pertussis has fluctuated between 92% and 94% of the total population. During this period pertussis vaccine uptake in England and Wales has risen from a low of 30% to the present level of ~80%. Comparison with other countries suggests that a further increase in vaccine uptake to levels above 90% would reduce pertussis incidence to less than one-tenth of its present level. These aspects of pertussis epidemiology are likely to be of relevance in the planning and interpretation of future pertussis vaccine trials, such as are expected to start in England shortly.

Pertussis is an important childhood disease with a worldwide distribution. It causes a typical clinical syndrome that is followed by a high degree of immunity. An effective vaccine is available, but in many countries the levels of vaccine uptake have been insufficient to prevent regular epidemics. The epidemiology of pertussis is determined by the factors that influence the transmission of the disease within the community. The most important of these are the infectivity of the organism, the number of contacts exposed to infected individuals (and the degree of exposure), and the level of immunity to pertussis in contacts exposed to the infection. The relation among these factors may be expressed by the equation

\[ \text{Pertussis}_2 = \text{Pertussis}_1 \times \text{Contacts} \times \text{Susceptible} \times \text{Infectivity}, \]

when \( \text{Pertussis}_2 \) represents the incidence of pertussis at time 2, \( \text{Pertussis}_1 \) represents the incidence of pertussis at time 1, \( \text{Contacts} \) represents the number of contacts per case, \( \text{Susceptible} \) represents the proportion of contacts susceptible to infection, and \( \text{Infectivity} \) represents the contagiousness or ease of transmission of pertussis infection. The interval between time 1 and time 2 is the average time from acquisition of infection by one individual to transmission of infection to another susceptible individual (a slightly longer interval than the incubation period). This relation is illustrated by figure 1, which shows the spread of disease to some of the susceptible contacts of a patient with pertussis and the subsequent transmission from these patients with secondary cases to some of their susceptible contacts.

The interval between acquisition of infection and subsequent transmission to another susceptible individual is usually thought to be between 1 and 4 weeks [1–3]. Stocks [4] studied the time of onset of symptoms in siblings of patients with pertussis in a large-scale investigation of the epidemiology of pertussis in families living in London in the 1920s. He found that symptoms most commonly started ~7 days after the onset of symptoms in the first family member to become ill. Lawson [5] studied the transmission of pertussis within families in Boston during the same period. He found that the period from acquisition of infection to transmission to a sibling was ~14 days. These results, which suggest that patients with pertussis are most contagious during the first week of illness (~1–2 weeks after the acquisition of infection), are consistent with the finding that recovery of \textit{Bordetella pertussis} from cough plates is highest during the first week of illness and rapidly declines during the second and third weeks.
It therefore seems reasonable to estimate that the average interval from acquisition of infection to transmission to another individual is between 1 and 2 weeks.

Infection with *B. pertussis* is highly contagious. The peak reported prevalence in England and Wales and in the United States before the introduction of pertussis vaccination was in children 2-6 years of age [7-9]. Approximately 50% of children over 5 years of age had had pertussis [8], and only 1% of reported cases occurred in persons ≥15 years old [7-9]. It is thought that by the age of 15 years almost 100% of children had suffered an episode of pertussis but that ~20% of episodes went unrecognized [9]. In small isolated communities, such as Iceland and the Faeroe Islands, epidemics of pertussis affected almost 100% of children born since the previous epidemic [3, 10].

There is considerable evidence that the transmission of *B. pertussis* infection is influenced by the closeness of contact with the index case. In London, before the introduction of vaccination, the relative risk of pertussis for children living in the same house as a child with pertussis was 36 times greater than that for children living on a distant street. The relative risk for children living in a house next door to a child with pertussis was five times greater than that for children living on a distant street and was two times greater than that for children living between two and 10 houses away [4]. In Wales, during a recent pertussis epidemic, the prevalence of pertussis among unvaccinated children <5 years of age was 64% with exposure to pertussis by a family member but only 36% without such exposure [11].

It is possible to estimate the number of secondary cases produced by an infectious individual in a population where all individuals are susceptible [12]. This intrinsic reproductive rate (*Ro*) is determined partly by the infectivity or contagiousness of pertussis and partly by the number of contacts exposed to the index case during the course of illness. While it is usually impossible to directly determine *Ro*, it may be estimated from the equation

\[
Ro = 1 + \frac{\text{average life expectancy}}{\text{average age at infection}}
\]  

The intrinsic reproductive rate of pertussis in England and Wales and in the United States is estimated to be ~15 (1 + 65/4.5) [12].

The relation between the incidence of pertussis at time 1 (*Pertussis 1*) and the incidence of pertussis at time 2 (*Pertussis 2*) may then be simplified as

\[
Pertussis 2 = Pertussis 1 \times \text{Susceptible} \times Ro.
\]  

The proportion of the population susceptible to pertussis is determined by the birthrate, the uptake of pertussis vaccine, the efficacy of the vaccine used, the incidence of disease, the rate of decline in immunity following disease or vaccination, and the frequency of unsuspected infection boosting immunity. Both natural disease and vaccination confer immunity to disease; however, natural disease provides almost absolute protection against pertussis during childhood, whereas the degree of protection provided by vaccination is less complete, with an incidence of pertussis in fully vaccinated children that is ~10%-20% of that in unvaccinated children [13, 14]. The degree of protection provided by vaccination is further reduced in those children exposed to pertussis within the home, where incidence rates in fully vaccinated children are ~50% of those in unvaccinated children [11, 14, 15].

The immunity provided by vaccination declines with time. Lambert studied 89 families during the course of a pertussis epidemic in 1962 and found that the incidence of pertussis among vaccinated individuals increased from 20% for those vaccinated in the preceding 3 years to 95% for those vaccinated ≥12
Epidemiology of Pertussis

years earlier [16]. A recent English study found that pertussis vaccine efficacy decreased from 100% in the first year after vaccination to 52% in the fifth year after vaccination [17]. Neither does childhood pertussis provide lifelong immunity to pertussis. Lambert reported pertussis in eight of 156 persons with a history of previous pertussis [16]. Trollfors and Rabo [18] found that 41 of 174 adults with culture-positive pertussis had had pertussis as children, and Maclean [19] reported a similar finding in 19 of 80 adults with serologically confirmed pertussis. When older children or adults with declining immunity are exposed to pertussis, they may develop typical pertussis or a brief atypical illness, or they may remain asymptomatic. It is likely that brief atypical illness and asymptomatic infection in these individuals occur frequently and are responsible for boosting immunity and preventing episodes of typical disease. This suggestion is supported by the finding that IgG and IgA antibodies to B. pertussis may be present in the sera of adults with no recognized recent episode of pertussis [20, 21].

The wide use of pertussis vaccination has had a major effect on the epidemiology of pertussis. In England and Wales and in the United States during the prevaccine era, pertussis was endemic, with an estimated incidence of >100 cases per 100,000 population per year [22]. Since the introduction of pertussis vaccination in the 1950s, the endemic pattern of disease has been abolished, and a cyclic pattern of epidemics has been the dominant feature of pertussis epidemiology. It has been suggested [23] that the recurrent pattern of epidemic cycles is determined by the rate of influx of susceptible individuals into the population (birthrate × proportion unvaccinated) and the intrinsic reproductive rate of the disease. This hypothesis asserts that when the birthrate has raised the proportion of susceptibles in the population to the level where Susceptible × Ro > 1, an epidemic will occur and continue until the proportion of susceptibles has been reduced to the level where Susceptible × Ro < 1. This cycle will repeat itself when the birthrate again replenishes the proportion of susceptibles within the population. While this formulation may be appropriate for the dynamics of maintenance and termination of a single epidemic, it does not appear to adequately explain the regular initiation of pertussis epidemics at approximately 4-yearly intervals in many countries during recent years.

Figure 2 illustrates pertussis epidemiology for several countries between 1950 and 1986. In all of these countries [24-29] and in New Zealand [30, 31] and Kenya [32], epidemics have occurred at approximately 4-yearly intervals. Furthermore, pertussis epidemics occurred at approximately 3- to 4-yearly intervals in England and Wales [22] and the United States [22] in the prevaccine era (despite continued endemic disease) and continued to occur in Finland [26], the United States [24, 25], and Fiji [33] at approximately the same frequency despite a progressive rise in vaccine uptake to levels that have almost abolished pertussis from these countries. Reduction of the influx of susceptibles into the population in these countries through an increase in the rate of vaccine uptake has resulted in a reduction in epidemic size but has not significantly altered the interval between epidemics. Similarly, different rates of vaccine uptake in different regions of England and Wales have affected epidemic size, but not epidemic frequency, in the last 10 years [34].

Fine and Clarkson [23] attempted to explain the constancy of the interepidemic interval in England and Wales during a period of significant changes in
vaccine uptake. They suggested that changes in the rate of vaccine uptake had not affected the frequency of pertussis epidemics because “pertussis vaccines are more effective in protecting against disease than in protecting against infection.” They cited the incomplete protection against disease afforded by vaccination, the reduced severity of pertussis in vaccinated individuals, and the occasional isolations of B. pertussis from asymptomatic, fully vaccinated contacts of persons with pertussis as evidence in support of their hypothesis.

To explain the unchanged periodicity of pertussis epidemics, this hypothesis requires not only that infection occur frequently in vaccinated individuals but also that infection in these individuals be frequently transmitted to others. There is little evidence that this is so. While serologic evidence suggests that B. pertussis infection is common in the close contacts of patients with pertussis [35], B. pertussis is rarely isolated from such contacts unless they have typical symptoms of pertussis [35–40]. If culture positivity is a reliable correlate of infectivity, it would seem that asymptomatic infection or atypical disease, although perhaps common, is an unusual source of infection for other susceptible individuals. A number of reports have described the spread of pertussis from adults with unrecognized infection [41–43], but failure to consider the diagnosis rather than absence of symptoms was the common feature of these cases. It therefore seems unlikely that failure of vaccination to protect against infection and infectivity is an adequate explanation for the regular recurrence of pertussis epidemics.

While the initiation of pertussis epidemics may be relatively insensitive to changes induced in the rate of influx of susceptible individuals into the population by changes in the level of vaccine uptake, it is likely that equation 3 does approximately represent the dynamics of pertussis spread within a community during an epidemic. From this equation it is possible to estimate the level of immunity to pertussis in a community at different phases of an epidemic.

If the proportion of the population susceptible to pertussis at any point of an epidemic is approximated by the equation

\[ \frac{\text{Susceptible}}{\text{Pertussis 1} \times Ro} \]  

then substitution of the numbers of notified cases from two points on the exponential growth phase of the epidemic should allow estimation of the level of immunity to pertussis at the outset of the epidemic.

Figure 3 shows weekly pertussis notifications in England and Wales for the years 1977–1987. In the early stages of each epidemic (A–B, C–D, and E–F), the rate of increase in notifications followed an exponential curve of approximately similar slope. Substitution of pertussis notifications from two points separated by an interval of 1½ weeks for Pertussis 1 and Pertussis 2 in equation 4 suggests that the proportion of the population susceptible to pertussis at the outset of the 1978, 1982, and 1986 epidemics was 7.7%, 7.2%, and 7.5%, respectively. A further assessment of the level of immunity to pertussis in the population may be made at the peak of the epidemics when Pertussis 2 = Pertussis 1, and, therefore, the proportion of the population susceptible to pertussis = 1/Ro = 1/15 = 7.1%. Finally, the level of immunity to pertussis following the epidemic peak may be estimated from the curves P-Q, R-S, and T-U. Substitution in equation 4 of notifications from points separated by an interval of 1½ weeks suggests that following the peaks of the 1978, 1982, and 1986 epidemics, the proportion of the population susceptible was 6.1%, 6.1%, and 6.4%, respectively. These estimates of the level of immunity to pertussis in England and Wales during the last 10 years are very similar to those obtained by Fine and Clarkson [44]; the latter authors used a similar theoretical consideration of the transmission dynamics of pertussis plus extrapolation from data in the prevaccine era and a detailed cohort analysis of available notification and vaccine data to obtain their estimates.

If the level of immunity to pertussis in England and Wales has not changed significantly but has fluctuated between 92% and 94% during the last 10 years, then vaccination or natural disease must have induced immunity in ~93% of the annual birth cohort. The annual birthrate for England and Wales in the last 10 years has approximated 600,000 live births [45]. If one allows for an average vaccine uptake of 60% [34] and an overall vaccine efficacy of 80% [14], it is possible to estimate the average number of children who acquire immunity to pertussis following infection in England and Wales each year from the equation

\[ \text{Immune I} = \text{Immune V} - \text{Immune T}, \]  

where Immune I represents the number of children who acquire immunity to pertussis following infec-
Figure 3. Weekly pertussis notifications between 1977 and 1987 for England and Wales. Evident are an exponential increase in notifications at the onset of epidemics in 1977 (A–B), 1981 (C–D), and 1985 (E–F) and a less steep exponential decrease in notifications at the end of epidemics in 1979 (P–Q), 1983 (R–S), and 1987 (T–U).
tion each year, Immune T represents the total number of children who acquire immunity to pertussis each year, and Immune V represents the number of children who acquire immunity to pertussis following vaccination each year. Thus,

\[
\text{Immune I} = 0.93 \times 600,000 - 0.60 \times 0.80 \times 600,000 = 558,000 - 288,000 = 270,000.
\]

This estimate of the number of children who acquire immunity to pertussis following infection each year is likely to approximate the annual average incidence of pertussis, given that asymptomatic infection is unusual in children. Actual pertussis notifications for the 4-year periods 1977-1980 and 1981-1984 were 135,378 cases and 110,062 cases, respectively. Thus, the average notification rate during this period was ~31,000 cases per year. Comparison of this figure with the estimated annual average incidence suggests that only 11% of cases of pertussis are reported, an estimate similar to those of 5%-25% made by other workers [11, 46-48].

While pertussis notifications in England and Wales and in other countries [22, 29] greatly underestimate the true incidence of the disease, estimates of the effect of different levels of vaccine uptake on national pertussis incidence can be derived through comparison of pertussis vaccine uptake and pertussis incidence data. Figure 4 shows notified pertussis incidence and rates of pertussis vaccine uptake from several countries at different times during this century. These data are remarkably consistent despite differences between countries in the level of undernotification of pertussis and in the vaccination schedules used. Thus, in England and Wales, Sweden, and Canada, vaccine uptake rates of 80%-90% reduced pertussis notifications to a level approximately one-tenth of that before pertussis vaccination was introduced, whereas in Finland and the United States, vaccine uptake rates of ~95% reduced pertussis notifications to a level approximately one-hundredth of that before pertussis vaccination was introduced.

These results suggest that progressive increments in pertussis vaccine uptake above a level of 80% bring about disproportionately greater reductions in pertussis incidence and that eradication of pertussis might be possible with levels of pertussis vaccine uptake >95%. However, theoretical considerations [49-51] predict that a high (>90%) level of vaccine uptake will not result in the eradication of pertussis but will merely change the age-related incidence, and that although children and adolescents will be protected from pertussis by vaccination in infancy, declining immunity with age will result in an increasing number of susceptible adults and an increased incidence in this group. An increase in the incidence of pertussis in adults and in infants too young to have been vaccinated has been reported in the United States [43-52], but it is difficult to determine whether this trend represents a genuine increase in the incidence of disease in infants and adults or only an increased awareness and notification of disease in these patients.

In some countries, notably England and Wales, public anxiety about a possible relation between whole-cell pertussis vaccination and subsequent severe neurologic deficit has been a major obstacle to achieving high levels of vaccine uptake. This public concern has persisted despite considerable scientific doubt that whole-cell pertussis vaccine is a cause of
severe adverse effects [52]. It has been hoped that acellular pertussis vaccines might acquire a public reputation for safety and be acceptable to a larger proportion of the population and that inclusion of increased amounts of purified antigens in these vaccines would improve their protective efficacy. While controlled trials will allow comparisons of vaccine efficacy and of the rates of common minor adverse effects, comparison of the rates of severe adverse effects following vaccination with either whole-cell or acellular pertussis vaccine is almost impossible because of the rarity of these events. The results of a recent Swedish trial comparing two acellular pertussis vaccines with placebo [53] suggest that acellular pertussis vaccines are associated with a low rate of minor adverse effects but are no more effective in preventing pertussis than are whole-cell pertussis vaccines. Although trials comparing acellular and whole-cell pertussis vaccines are necessary to estimate relative risks and benefits, administrative changes that increase vaccine uptake are more likely to reduce the incidence of pertussis than are changes in vaccine composition.

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From whom do children catch pertussis?

The importance of asymptomatic infection and atypical illness in the transmission of pertussis is disputed. Fine and Clarkson suggested that such infection in vaccinated children and adults is commonly transmitted to infants and young children.¹ We studied the transmission of pertussis within 26 families during an epidemic to determine the incidence of asymptomatic infection and atypical illness in children and adults and how commonly such infection is a source of illness for other family members.

Patients, methods, and results

During a large survey of serological responses to infection with *Bordetella pertussis* in patients with pertussis and their close contacts we recruited 26 families (42 parents and 48 children) in which we were able to obtain clinical data on all family members. The families were seen in hospital and general practice in south west London from July 1985 to January 1987, and all of them included at least one member with typical pertussis. Families were followed up for from four to 44 weeks (mean 13 weeks) after presentation.

Forty three children and 11 parents had typical pertussis, defined as prolonged paroxysmal cough with whoop, vomiting, or apnoea. Five children and 12 parents had atypical pertussis, defined as brief non-paroxysmal cough without whoop, vomiting, or apnoea, which occurred within 20 days after the onset of typical symptoms of pertussis in another family member (table). Four children with typical pertussis and three children with atypical pertussis had received at least two pertussis vaccinations. Nineteen parents but no children remained with typical pertussis and three children with atypical pertussis had received at least two pertussis vaccinations. Nineteen parents but no children remained with typical pertussis and three children with atypical pertussis had received at least two pertussis vaccinations.

In four families a parent was the first member with symptoms and presumably responsible for transmitting pertussis to the children. Of these four parents, three had typical and one atypical pertussis. In the 22 other families the first family member with symptoms was a child with typical pertussis. Of the subjects exposed to pertussis by a family member, all children less than 4 years old developed typical pertussis; in contrast, five of 10 older children and 11 of 38 parents developed atypical pertussis. *B pertussis* was isolated from 14 of 39 children and from three of 32 parents from whom nasopharyngeal swabs were taken. The table shows the numbers of swabs that were positive on culture. Positive cultures were more common in unvaccinated children (12/29) than in children who had been vaccinated at least once (2/10).

Comment

In this study children were more commonly exposed to pertussis by a sibling (22/26) than a parent, and 25 of the 26 children and adults responsible for transmitting the infection had typical disease. Atypical disease occurred in older children and parents but was almost exclusively associated with secondary rather than primary infection. Furthermore, isolation of *B pertussis* (which may reflect a person’s infectiousness) was more common in those with typical pertussis (16 of 46 who had swabs taken) than in those who were asymptomatic or had atypical pertussis (one of 25 who had swabs taken). These results could not have been influenced by erythromycin treatment as the only family members treated in this way were children with typical pertussis (17/43).

We suggest that pertussis is most commonly transmitted by subjects with typical disease and that atypical disease and asymptomatic infection are not an important source of pertussis in this community. Thus a greater uptake of pertussis vaccination would probably affect transmission of the disease sufficiently to reduce its incidence.

We thank the doctors who referred families for investigation; the hospital microbiology laboratory for culturing the nasopharyngeal swabs; and Dr N Noah, consultant epidemiologist, Communicable Disease Surveillance Centre, Colindale, for valuable advice. MGT is supported by a grant from the Department of Health and Social Security.

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ANTIBIOTICS IN WHOOPING COUGH

Although whooping cough no longer carries a high mortality, symptoms may persist for several months so that both the child and its family suffer weeks of anxiety and disturbed sleep. The prevalence and severity of the disease have declined since the turn of the century. Publicity about the adverse effects of whooping cough vaccination steeply reduced the uptake of immunisation in England and Wales - from 78% in 1971 to 37% in 1974. The subsequent epidemics of whooping cough in 1977-79 and 1982-83 were the largest since vaccination began. The epidemic which started in September 1985 could well be of similar magnitude. The uptake of immunisation has now somewhat improved and is currently about 65% (information from Communicable Disease Surveillance Centre). This article discusses whether antibiotics can help in whooping cough, either in treatment of the illness or preventing its spread.

Vaccination is the most effective prophylaxis. Genuine contraindications are few and children up to the age of 6 years can be vaccinated if they have missed earlier immunisation.

Natural history - A prodromal illness develops 7 to 10 days after infection of the respiratory tract with Bordetella pertussis. During the initial catarrhal stage, the cough is rarely paroxysmal but after 1 to 2 weeks the typical cough and inspiratory "whoop" appear. However, the "whoop" is often absent, particularly in young infants. Vomiting, cyanosis and apnoea may accompany bouts of coughing. The symptoms, which may be caused by bacterial toxins, persist much longer than the bacteria and can take many weeks to resolve. The typical syndrome may recur in subsequent months during any upper respiratory infection. The disease tends to be more severe and the mortality is highest in infants.

Secondary spread - Patients with whooping cough are infectious during the prodromal and early paroxysmal stages. About 20% of vaccinated and 70% of unvaccinated family contacts develop the disease. The protection given by vaccination appears to decline gradually over 5 years, but illness in vaccinated individuals tends to be shorter and less severe.

Diagnosis - A cough lasting longer than one week, associated with cyanosis, vomiting or whoop is almost certainly whooping cough. The diagnosis may be confirmed by isolation of B. pertussis from nasopharyngeal swabs, but positive cultures are obtained only in about 30% of cases. The finding of an absolute lymphocytosis supports the diagnosis. A doctor who ignores the mother's account of the illness will miss the diagnosis.

Drug treatment - None of the antibacterial drugs evaluated in whooping cough has shown major clinical benefit. In an early placebo-controlled trial in young children chloramphenicol or chlorotetracycline started within a week of onset of symptoms slightly reduced the frequency and severity of paroxysms. These drugs are no longer recommended for children. Erythromycin is now widely used because it is safer, more active in vitro against B. pertussis, and penetrates sputum well. In one series of 50 children with proven whooping cough, nasopharyngeal swabs took a mean of 12 days to become negative without treatment, but did so after 1 day on erythromycin. Swabs took 24 days to
become negative on ampicillin in this study. Co-trimoxazole can also eradicate nasopharyngeal infection but none of these drugs alters the course of the established illness or alleviates symptoms. Small uncontrolled studies suggest that erythromycin may abort clinical illness in asymptomatic recently infected patients.

Does chemotherapy reduce spread? - Many authorities recommend treatment of children with whooping cough to reduce infectivity although this has not been shown to prevent spread of infection. Close contacts probably become infected before the index case is recognised and treatment started. Treatment for less than 14 days risks bacteriological relapse. Spread occurred in a paediatric ward after treatment of the index case with erythromycin for 7 days.

Prophylactic use - Two small placebo-controlled trials of erythromycin prophylaxis for family contacts have shown no benefit. Co-trimoxazole has also been suggested for prophylaxis, but no controlled trials exist.

COMPLICATIONS - Secondary bacterial infection, most commonly otitis media or pneumonia, may require treatment with an appropriate antibiotic.

CONCLUSION - Antimicrobial drugs appear to give no direct benefit in established whooping cough or in the prophylaxis of close contacts. Erythromycin may eradicate early nasopharyngeal infection and prevent progression to clinical disease, but the evidence is inconclusive.

It seems reasonable (though of unproven benefit) to prescribe erythromycin for patients likely to expose young children to the disease, and to infants who are diagnosed in the prodromal phase as they may become severely ill. Erythromycin should be given for 14 days. Doctors should use the increased community awareness of the disease during epidemics to encourage vaccination of susceptible children.

DISCUSSION

The major component of the research presented in this thesis was an investigation of the serological responses to *B. pertussis* antigens in patients with pertussis, their family contacts and infants immunised with whole cell pertussis vaccine. We demonstrated that:

(i) pertussis produces a prompt serum IgG, IgA and IgM response to PT, FHA, Agg 2/3 and other *B. pertussis* antigens

(ii) the magnitude of the class antibody responses to PT, FHA and Agg 2/3 is broadly similar for patients with pertussis and vaccinated infants

(iii) initial titres of serum antibodies are lower in patients with pertussis than in their family contacts who remain well

(iv) serum antibody to PT is primarily to the enzymatic subunit and not to the binding subunits.

However despite the results of the research presented in this thesis, and similar investigations conducted by others(1,2), the serologic determinants of immunity have not been clearly and consistently defined. Similarly the large Swedish trial of immunisation with acellular pertussis vaccines containing PT or PT plus FHA also failed to demonstrate that postvaccination titres of antibodies to these two antigens were correlated with protection against disease(3). The results of further trials of primary immunisation with acellular pertussis vaccines may help to resolve whether serologic responses to one or more *B. pertussis* antigens can be used as determinants of immunity. It seems likely however that serologic determinants of immunity will remain elusive and that investigation of other components of the immune response will be needed to determine what confers immunity following infection or vaccination.

Until recently it was thought that the secretory immune response was likely to be a major determinant of immunity to pertussis. Our results however demonstrate that there is only a modest IgA response in nasal mucus following either disease or vaccination. While this result is at odds with previous expectations, it is supported by our finding of minimal responses to *B. pertussis* antigens in breast milk from mothers of infants with pertussis. Zackrisson et al investigated the IgA response to PT and FHA in saliva from patients with pertussis(4). They found a fourfold rise in titres (for samples collected at intervals of 2-5 weeks) occurred in only 19/62 (31%) patients for IgA to FHA and in only 14/62 (23%) patients for IgA to PT. Shahin et al found that mucosal immunisation of mice with FHA provided protection against *B. pertussis* infection despite the fact that such immunisation induced no detectable IgA response in nasal wash fluid and only a modest IgA response in bronchoalveolar.
These results indicate a weak secretory immune response to *B. pertussis* infection and imply that some other component of the immune response may be more important in clearing infection and conferring protection against future disease.

Our demonstration of a cell mediated response in patients with pertussis provided the first evidence that a T lymphocyte response to *B. pertussis* antigens is present in humans. Subsequently investigations of the T cell response to experimental *B. pertussis* respiratory tract infection in the mouse have demonstrated that this response is crucial to the eradication of infection and the establishment of immunity(6,7). Further results from human studies are necessary to determine if T cell responses are of similar importance in man. If such studies do indicate that T cell responses to one or more *B. pertussis* antigens are strongly correlated with immunity to pertussis they may allow a more accurate identification of the determinants of immunity to pertussis and a more informed decision on the optimal composition of an acellular pertussis vaccine.

We found that the serum titre of IgA to PT was higher in patients with pertussis than in recently vaccinated infants. Other investigators have described similar serologic criteria for the diagnosis of *B. pertussis* infection but in general these criteria have been somewhat imprecise and have often required consideration of more than one assay for a useful sensitivity and specificity(8-10). If acellular pertussis vaccines completely replace whole cell pertussis vaccines in the future then detection of a serological response to a *B. pertussis* antigen not present in the acellular vaccine may prove to be a more simple and reliable test for recent infection.

Our results and those of Goldman (11) suggest that tracheal cytotoxin (TCT) is important in the pathogenesis of pertussis. Unfortunately TCT does not stimulate antibody production even when conjugated to a variety of protein carriers. Therefore unless TCT stimulates an effective T cell response it may not be useful as a component of a vaccine against pertussis. If agents which can inactivate TCT can be synthesised they may provide some benefit in the treatment of pertussis. However delayed diagnosis will commonly mean that treatment cannot be started early in the course of the disease. Furthermore treatment may fail if the toxin binds irreversibly to its receptor, or if brief exposure to the toxin initiates a very prolonged effect.

The immediate purpose of much of the research described in this thesis was to provide information to assist with the planning, execution and assessment of a large clinical trial of acellular pertussis vaccines anticipated to begin in England in 1990. It
was expected that determinations of the serum and nasal antibody responses to purified *B. pertussis* antigens following natural infection would provide information which might assist with:

(i) the selection of the vaccines to be trialled
(ii) the evaluation of immune responses induced by the various vaccines
(iii) the diagnosis of *B. pertussis* infection in trial subjects.

At the time the research was being performed candidate vaccines were being evaluated for inclusion in this trial. (Indeed the efforts of an English group to produce an acellular pertussis vaccine had provided the pure *B. pertussis* antigens used in the ELISA tests described in Papers 2, 4 and 5) The vaccines being considered contained PT, FHA and Agg 2/3 in varying proportions and were to have been compared with the killed whole-cell pertussis vaccine used in England. A major stimulus to the performance of this trial was the resurgence of pertussis in England following a reduction in pertussis vaccine uptake in the 1970's. A further stimulus was the decision by Wellcome, the manufacturers of the whole-cell pertussis vaccine used in England, to cease vaccine production. The vaccine trial was expected to identify vaccines with equal or greater efficacy than the Wellcome whole-cell vaccine. Furthermore it was hoped that acellular pertussis vaccines would be found to have a low incidence of adverse effects, and hence be regarded more favorably by the public and their doctors and consequently be administered to a higher proportion of children.

For a variety of reasons an efficacy trial of acellular pertussis vaccines was not performed. While the high cost of the trial and delays in the availability of a suitable English acellular vaccine contributed to the failure to perform this trial the most important reason was the decline in the incidence of pertussis in England which followed successful efforts to increase the uptake of childhood vaccines (12). The declining incidence of pertussis made the need for new vaccines less pressing, and reduced the chances of a trial conducted in England showing a significant difference in efficacy between vaccines.

The persistent recurrence of epidemics of pertussis, albeit of a much lesser magnitude than in the past, has led to reviews of the pertussis immunisation schedule in a number of countries (13). A central issue of these reviews has been consideration of whether booster doses of pertussis vaccine are necessary for effective control of pertussis. While the need for a primary immunising course of three doses of vaccine, given early in infancy, is widely accepted, uncertainty persists about the need for further booster doses given in the second year of life, at school entry, or in
adolescence or adulthood. In some countries (eg England and Wales, New Zealand) no further doses of pertussis vaccine are given after the initial three doses in infancy, while in other countries (eg Australia) a single booster is recommended at 15 or 18 months, and in the United States two boosters at 15 months and five years are recommended. The advent of new acellular pertussis vaccines which induce greater serological responses to *B. pertussis* antigens and less severe local reactions in the vaccinated infant(14,15), has led to the recommendation that they replace whole cell pertussis vaccine for the two boosting doses (given at 15-18 months and 4-6 years of age) in the United States (16), and has prompted other nations to consider adding a further booster dose to their current vaccine schedules.

A commonly stated reason for booster doses of pertussis vaccine following the primary immunising course is concern that the incidence of pertussis in infants, in whom it is most severe, may increase as a result of transmission from adolescents and adults with pertussis (17). The administration of booster doses of pertussis vaccine is intended to prevent the emergence of a cohort of young adults susceptible to pertussis because waning post-vaccination immunity has not been stimulated by natural infection due to the success of the primary vaccine programme in reducing the incidence of disease.

A number of studies in the United States have suggested that unrecognised *B. pertussis* infection is common in adolescents and adults (17,18). However these studies have usually relied on serological criteria for diagnosis of infection and have almost invariably failed to isolate *B. pertussis* from nasopharyngeal samples. The epidemiological significance of these asymptomatic or mildly symptomatic infections therefore remains uncertain. It seems unlikely that adults with minimal or no cough and negative nasopharyngeal cultures pose a significant risk of transmitting pertussis to others. Instead it seems likely that most adolescents and adults, having acquired infection from a person with typical pertussis, are briefly colonised, with a prompt immune response ensuring rapid clearance of infection, prevention of typical pertussis symptoms, and little or no transmission of infection to others.

Typical culture positive pertussis does however occur in adults (19,20) and is evidence that immunity does wane following either immunisation or disease. Vaccine induced immunity declines from approximately 100% at one year after immunisation to approximately 60% at five years after immunisation, and has been estimated to be virtually zero at twelve years after immunisation (21-23). The duration and solidity of protection following childhood pertussis cannot be estimated with similar accuracy but
is widely thought to be greater than that provided by vaccination.

Nelson raised concern about transmission of pertussis from adults to infants when he reported that a large proportion of infants aged less than three months admitted to hospital with pertussis in Dallas between 1966 and 1977 had acquired the infection from their parents (24). Pertussis in the infant was more commonly acquired from a parent in the second six year period (12/22; 55%) than it was in the first six year period (3/24; 13%) of his study. While Nelson's study and other reports of small series of infants with pertussis (25,26) might suggest that parent to infant transmission of pertussis is common, larger family studies have usually found that the first person to develop pertussis in most families is a child rather than a parent (27-34) (Table 1). It therefore seems unlikely that prevention of pertussis in adults will prevent the entry of pertussis into most families.

An increase in the incidence of pertussis in adults in the United States in the 1980s has been cited as evidence of waning immunity in adults and of the need for booster doses of pertussis vaccine after childhood (17,18,35). Approximately 12% of reported pertussis in the United States is in persons aged at least 15 years and the incidence in this group appears to have increased approximately five-fold during the 1980s (36). However the incidence of reported pertussis in the United States during this period was approximately 0.5/10^5 in adolescents and adults, approximately 5/10^5 in children aged 1-4 years old, approximately 20/10^5 in children aged 3-12 months old, and approximately 60/10^5 in infants aged less than 3 months old(36).

A major increase in pertussis vaccine uptake in England and Wales in the last 20 years has been followed by a dramatic decline in pertussis incidence to levels which approximate those in the United States. However these changes have not led to a significant increase in the number of notified cases in infants, and adults continue to comprise less than 5% of all pertussis notifications (37). These data from England and Wales and the United States demonstrate that it is children rather than adults who remain the most significant reservoir of disease in the community. In consequence any intervention administered to adolescents and adults in an attempt to reduce disease incidence in this group (or as a secondary effect in infants), is likely to be less cost effective than the same intervention administered to children who have a very much higher burden of disease.

In summary there is conflicting evidence about the importance of transmission of
pertussis from adults to infants. A combination of organisational change, improved health education, and increased professional commitment to childhood immunisation is likely to increase childhood vaccine uptake significantly(12) and may be expected to have a greater impact on the epidemiology of pertussis than would the administration of booster doses of vaccine to children or adults.
### TABLE 1.

Source of *Bordetella pertussis* infection within families

<table>
<thead>
<tr>
<th>Reference (year country)</th>
<th>Number of families</th>
<th>Number of family members</th>
<th>Total number of cases</th>
<th>Number (%) of primary cases in children (age cutoff)</th>
</tr>
</thead>
<tbody>
<tr>
<td>27 (1976-77 Finland)</td>
<td>49</td>
<td>209</td>
<td>113</td>
<td>47 (96%) (&quot;children&quot;)</td>
</tr>
<tr>
<td>28 (1977 USA)</td>
<td>78</td>
<td>416</td>
<td>153</td>
<td>64 (82%) (&lt;11 years)</td>
</tr>
<tr>
<td>29 (1979-81 Finland)</td>
<td>21</td>
<td>97</td>
<td>55</td>
<td>18 (86%) (&lt;16 years)</td>
</tr>
<tr>
<td>30 (1984-90 Japan)</td>
<td>89</td>
<td>NS</td>
<td>NS</td>
<td>79 (89%) (&quot;children&quot;)</td>
</tr>
<tr>
<td>31 (1985-87 England)</td>
<td>26</td>
<td>90</td>
<td>54</td>
<td>22 (85%) (&lt;12 years)</td>
</tr>
<tr>
<td>32 (1985 USA)</td>
<td>61</td>
<td>265</td>
<td>94</td>
<td>15 (20%) (&lt;12 years)</td>
</tr>
<tr>
<td>33 (1988 USA)</td>
<td>37</td>
<td>184</td>
<td>66</td>
<td>29 (78%) (&lt;15 years)</td>
</tr>
<tr>
<td>34 (1989 USA)</td>
<td>14</td>
<td>116</td>
<td>31</td>
<td>5 (24%) (&lt;14 years)</td>
</tr>
</tbody>
</table>
REFERENCES


