Effects of *Bordetella pertussis* on neutrophil leukocytes.

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DECLARATION

This thesis is the original work of the author except where otherwise stated.

Frank F. Craig.

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LIST OF ABBREVIATIONS AND TERMS

ADP	Adenosine-5'-diphosphate
cAMP	3',5'-cyclic adenosine monophosphate
Bis	N'N'-methylenebisacrylamide
BSA	Bovine serum albumin
C-mode	Avirulent (cyanic) phenotype of *B.pertussis*
conc	concentration
DPT	Diphtheria-pertussis-tetanus triple vaccine
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FHA	Filamentous haemagglutinin
fMLP	N'-formyl-L-methionyl-L-leucyl-L-phenylalanine
G-protein	Guanine nucleotide binding protein
HBS	Hepes-buffered saline
HEPES	N-2-hydroxyethyl piperazine N'-2-ethane sulphonic acid
HSF	Histamine-sensitizing factor
IAP	Islets-activating protein
LPF	Leukocytosis- or lymphocytosis-promoting factor
LPF-HA	Leukocytosis-promoting factor haemagglutinin
LPS	Lipopolysaccharide endotoxin
MIC	Minimum inhibitory concentration
mV	milliVolts
PIF	Polymorphonuclear leukocyte-inhibitory factor
PT	Pertussis toxin
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SS	Stainer-Scholte
TEMED	NNNN'-tetra-methyl-1,2-diamino-ethane
X-mode	Virulent (xanthic) phenotype of *B.pertussis*

SUMMARY

This thesis describes and discusses the effects that various components of Bordetella pertussis produce on neutrophil leukocytes; particularly those of the rabbit. The work was done with crude fractions and purified components of the bacterium and also whole-cells of mutant strains and of antigenically-modulated variants of B.pertussis.

The neutrophil functions which were investigated in detail were

(1) adhesiveness, as assayed by aggregation.

(2) bactericidal response, as assayed by luminol-enhanced chemiluminescence, to a pro-inflammatory mediator (N'-formyl-L-methionyl-L-leucyl-L-phenylalanine; fMLP) or to intact X-mode B.pertussis Tohama.

The filamentous haemagglutinin (FHA) of B.pertussis had at least four noteworthy effects on the function of rabbit peritoneal neutrophils: it enhanced neutrophil aggregation; it directly induced neutrophil chemiluminescence; it enhanced the neutrophil response to fMLP; and it induced a faster rate of chemiluminescence production to intact B.pertussis. There were species differences in neutrophils and FHA-enhancement of aggregation with rabbit neutrophils was not found with human or pig cells.

Lipopolysaccharide endotoxin (LPS) of B.pertussis, unlike FHA, had no effect on the aggregation of rabbit peritoneal neutrophils. However, it resembled FHA in inducing neutrophil chemiluminescence directly and in enhancing neutrophil response to fMLP. LPS from Salmonella minnesota behaved similarly in each of the above. B.pertussis LPS also induced a

faster rate of neutrophil chemiluminescence to intact B.pertussis.

Pertussis toxin (PT) enhanced aggregation of rabbit peritoneal and peripheral neutrophils and human peripheral neutrophils. It inhibited neutrophil chemiluminescent response to fMLP although only after a lag period of 30-40 min. PT overrode the enhancing effects of FHA and LPS on neutrophil response to fMLP. The latter observation was applied to monitoring the toxoiding of an experimental acellular pertussis vaccine, consisting predominantly of PT and FHA. PT slightly suppressed the neutrophil chemiluminescent response to intact B.pertussis.

With intact B.pertussis there was a positive correlation between hydrophobicity and the induction of neutrophil chemiluminescence.

The work supports the view that a significant feature of infection with B.pertussis is the ability of the pathogen to impair the functional effectiveness of host phagocytes.

INTRODUCTION

Part 1: Bordetella pertussis

2.1.1. Taxonomy of the genus Bordetella

The genus Bordetella has four members: B.pertussis, B.parapertussis, B.bronchiseptica, and the recent addition B.avium. B.pertussis, B.parapertussis and B.bronchiseptica were first assigned to the separate genus Bordetella by Moreno-Lopez in 1952. Previously B.pertussis and B.parapertussis were classified as members of the genus Haemophilus. B.bronchiseptica was formerly placed successively in the genera Bacillus, Alcaligenes, Brucella then Haemophilus. B.avium was previously identified as a member of the Alcaligenes genus before its assignment to the Bordetella genus. Kersters et al. (1984) listed the major characteristics of B.avium; Pittman and Wardlaw (1981) described the characteristics of the other three members of the genus. Goodnow (1980) reviewed the biological properties and characteristics of B.bronchiseptica. Both B.bronchiseptica and B.avium are motile whereas B.pertussis and B.parapertussis are not.

Bordetella species are aerobic, gram-negative, minute, coccobacilli. All are pathogens of the respiratory tract; B.pertussis and B.parapertussis cause whooping cough in humans, B.bronchiseptica causes respiratory infections in many mammalian species but only occasionally in man, and B.avium is a pathogen of birds.

2.1.2. Pertussis - the disease

Whooping cough, or pertussis, is an epidemic respiratory disease that mainly affects infants and young children. The

disease was mentioned in 1540 by Moulton; DeBaillou (or Baillon, or Ballonius) first described an epidemic in 1578 (cited in Lapin, 1943). The World Health Organisation estimates that nearly one million children still die each year from the disease (Hinman, 1985). The incubation period for pertussis is considered to be 6 to 20 days, with an average of 7 days. After the incubation stage the infection progresses to the catarrhal stage. The onset of this stage is non-specific and symptoms resemble the ones associated with an ordinary upper respiratory tract infection such as coughing, sneezing, and oedema of the eyelids. In some cases slight fever may also occur. As the disease progresses the symptoms continue to worsen with coughing becoming more frequent and more violent. After one to two weeks the paroxysmal stage gradually supervenes. During this stage the patient suffers severe bouts of coughing or occasionally fits of sneezing. The characteristic whooping noise associated with pertussis occurs when, after a bout of paroxysmal coughing, inspiration ensues through the partially swollen pharynx into the lungs. At the end of a paroxysm a patient will often vomit, which together with the severe coughing bouts, interferes with the patient's nutrition and fluid intake. The physical exertions of coughing may also cause various damaging effects such as conjunctival haemorrhage, hernia, emphysema, and pneumothorax. Complications include damage to the central nervous system which may occur following cerebral anoxia and haemorrhage induced by the paroxysmal coughing (Linneman, 1979). Encephalopathic states may occasionally occur although the reasons have not yet been established (Joint Committee on Vaccination and Immunisation, 1981).

Superinfection of pertussis patients can occur and may lead

to primary or secondary bronchopneumonia. The bacteria most often involved are encapsulated organisms such as Streptococcus pneumoniae, Haemophilus influenzae, and pathogenic staphylococci (Jamieson, 1973). Olson (1975) and Manclark and Cowell (1984) reviewed pertussis and its sequelae.

The convalescent stage of pertussis is characterised by coughing episodes of diminished frequency and severity. Cases of reinfection with the redevelopment of pertussis have been reported (Wilson et al., 1965). The susceptibility of pertussis patients to secondary infections and the afebrile responses usually associated with the disease suggests that the host's normal inflammatory responses and immune system may be altered by B.pertussis infection.

2.1.3. Pertussis vaccine and vaccination

The preparation of a pertussis vaccine was first made possible in 1906 when the organism was isolated and grown on laboratory medium (Bordet and Gengou, 1906). Vaccines prepared in the 1920s and 1930s gave inconsistent results with some investigators finding a correlation with immunisation and protection against disease whereas others did not. Manclark and Cowell (1984) reviewed the early history of pertussis vaccine and vaccination. The reasons for these inconsistencies were probably due to differences in the methods used to inactivate the bacterium and to reduce its toxicity and the absence of a standard method for measuring vaccine potency or toxicity. The latter problem was solved when clinical trials in the 1940s and 1950s related efficiency of whole-cell pertussis vaccines with potency in the intracerebral mouse

protection test (Kendrick et al, 1947; Armitage et al., 1956).

Pertussis vaccines in current use are either of the whole-cell type or the acellular type. Whole-cell vaccines were first used in the 1920s while an acellular pertussis vaccine was first used in trials in the late 1950s (Armitage et al., 1959). The majority of the whole-cell vaccines in current use consist of B.pertussis cells that have been killed by treatment with formaldehyde, thiomersal, heating at 56°C, or by a mixture of heat and chemicals. Most of the recent studies concerned with the development of an acellular vaccine have involved the isolation and purification of the major haemagglutinins of B.pertussis. An acellular pertussis vaccine in current use in Japan consists of formalin-detoxified pertussis toxin and filamentous haemagglutinin, a trace amount of other B.pertussis proteins, reduced amounts of LPS, and aluminium hydroxide as an adjuvant (Sato et al., 1984). A noticeable omission from the Japanese vaccine is the heat-labile agglutinogens. Various studies have indicated that these agglutinogens may be important components of whole-cell vaccines (Armitage et al., 1956; Preston, 1963; Preston and TePunga, 1959) and their inclusion in an acellular vaccine for use in the U.K. is under investigation; field trials are currently being performed. Robinson et al. (1985) reviewed progress towards the development of improved acellular pertussis vaccines.

Pertussis vaccine can be given alone or in a triple vaccine (DPT) which also contains diphtheria and tetanus toxoids and the adjuvant aluminium hydroxide (Butler et al., 1969). The vaccination schedule in current use in the U.K. advocates a series of three DPT injections, with an interval of 6-8 weeks between each, to be given to children aged 3-6 months old

(Joint Committee on Vaccination and Immunisation, 1981). A booster DTP injection is then given once the child is five years old.

The antibiotic erythromycin can be used in the treatment of pertussis since B.pertussis is susceptible to this drug (Bass et al., 1969). Pertussis antiserum is also available although no evidence exists to prove that it is effective in preventing pertussis (Morris and McDonald, 1957).

The use of effective pertussis vaccines has contributed greatly towards the decline in incidence of the disease. The relatively low incidence of pertussis at present has given rise to the opinion held by some that the well-publicised side effects of the vaccine may constitute a greater risk than the disease (Dick, 1974; Stewart, 1985). Risks associated with pertussis vaccine include local reactions such as redness and swelling; systemic reactions such as fever, vomiting and diarrhoea; and rare but serious neurological reactions. A study in the U.K. estimated that the risk of neurological illness attributed to DTP vaccine was 1 in 110,000 vaccinations (Joint Committee on Vaccination and Immunisation, 1981). However, the report also reaffirmed that pertussis vaccine is effective against pertussis and suggested that the advantages of the vaccine in preventing pertussis outweighed the risks associated with its use.

2.1.4. Biologically-active components of B.pertussis

Bordetella pertussis produces a wide range of biologically-active components that may individually or in conjunction with other components be important in the

pathogenesis of disease and in immunity. These components are reviewed briefly. The articles of Munoz and Bergman (1977), Wardlaw and Parton (1983), Manclark and Cowell (1984), and Robinson et al. (1985) give a more detailed consideration of individual B.pertussis components.

(a) Adenylate cyclase

B.pertussis adenylate cyclase activity was first observed in the supernate of a whole-cell pertussis vaccine (Wolff and Cook, 1973). Adenylate cyclase was later purified from B.pertussis culture supernates and characterized by Hewlett and Wolff (1976). The purified enzyme was heat-labile (90% activity lost after 20 min at 56°C), required Mg^{2+} for maximal activity, and had a molecular mass of 70 kDal. Hewlett et al. (1976) also reported that the enzyme was extracytoplasmic. Paradoxically, Confer and Eaton (1982) have reported that urea-extracted adenylate cyclase from B.pertussis was heat-stable; the enzyme resisted heating at 100°C for 5 min. B.pertussis adenylate cyclase is stimulated by calmodulin; a Ca^{2+}-binding protein found in most, if not all, eukaryotic cells (Wolff et al., 1980; Goldhammer et al., 1981). Greenlee et al. (1982) estimated the molecular mass of adenylate cyclase to be 77 kDal and noted that calmodulin stimulated adenylate cyclase both in the presence and absence of free Ca^{2+}. The lower molecular mass adenylate cyclase, isolated by the technique of Hewlett and Wolff (1976), was not stimulated by calmodulin perhaps because of partial proteolytic degradation, which would account for its insensitivity to calmodulin stimulation (Greenlee et al., 1982). A second form of adenylate cyclase was reported by

Wolff et al. (1984). This enzyme was isolated from intact B.pertussis, had a molecular mass of 47 kDal and was stimulated by calmodulin even in the absence of free Ca^{2+}.

Speculations concerning the exact role of the B.pertussis adenylate cyclase have been made since its first isolation (Hewlett and Wolff, 1976). Brownlie et al. (1985) showed that B.pertussis adenylate cyclase is not involved in regulating the synthesis of virulence-associated proteins. Transposon-induced mutants of B.pertussis that were deficient in both adenylate cyclase and haemolysin were avirulent in a mouse respiratory infection model (Weiss et al., 1984). The interaction of B.pertussis adenylate cyclase with phagocytes is reviewed in section 2.3.2.

(b) Filamentous haemagglutinin

Haemagglutinating activity of B.pertussis was first reported by Keogh et al. (1947). Arai and Sato (1976) later demonstrated that B.pertussis actually produces two haemagglutinins: one designated fimbrial haemagglutinin and the other leukocytosis-promoting factor haemagglutinin. The former haemagglutinin had a filamentous structure (approximately 2 by 40 nm), had no histamine-sensitizing activity in mice, and was once thought to be associated with the fimbriae. However, other studies have indicated that this haemagglutinin is not associated with fimbriae (Ashworth et al., 1982b) and it is now usually referred to as filamentous haemagglutinin (FHA). Early work by Fisher (1949) indicated that cholesterol may be important in B.pertussis haemagglutinin interaction with erythrocytes and liposomes containing cholesterol were later used as a means of achieving

purification of FHA (Irons and MacLennan, 1979). FHA has been purified by various techniques and estimates of its molecular mass and subunit structure vary widely (Arai and Sato, 1976; Irons and MacLennan, 1979; Cowell et al., 1982). SDS-PAGE analysis of FHA shows multiple polypeptides with molecular mass from 220 to 58 kDal. Studies using monoclonal antibodies indicate that the lower molecular mass bands were probably fragments of the high molecular mass polypeptide (Irons et al., 1983). FHA production is favoured in static, poorly-aerated conditions (Arai and Munoz, 1979) or in shaken cultures containing heptakis (2,6-O-dimethyl)beta-cyclodextrin (Suzuki et al., 1985).

The importance of FHA in pathogenesis of B.pertussis has been shown in various studies. Antibodies against FHA protect mice from intranasal infection by B.pertussis, suggesting that immunity to FHA is important in immunity to the disease (Sato et al., 1981). Absence of FHA from the organism has been associated with loss of adherence to human ciliated respiratory epithelial cells in vitro (Tuomanen and Weiss, 1985). FHA may also function as an important adhesin in vivo. The interaction of FHA with phagocytes has not been reported.

(c) Haemolysin

Haemolysin production is characteristic of virulent B.pertussis and is readily lost on sub-culture in vitro (Lautrop, 1960). Genetic studies indicated that mutants deficient in both haemolysin and adenylate cyclase have reduced virulence in the mouse respiratory infection model (Weiss et al., 1984). The exact role of the haemolysin in infection is unknown.

(d) Heat-labile agglutinogens

The agglutinogens of B.pertussis can be defined as surface antigens which stimulate the production of antibodies which will in turn cause bacterial cell agglutination. The agglutinogens were first described by Andersen (1953) and later classified using the numbers 1 to 14 by Eldering et al. (1957). Eight agglutinogens were proposed for B.pertussis: 1-7 and 13. Agglutinogens 1 and 7 were found in all strains of B.pertussis with factor 7 being common to all Bordetella species. Some of the early methods of purification of agglutinogens are summarized by Munoz and Bergman (1977). Both agglutinogens 2 and 3 have recently been purified and reported to have a subunit molecular mass of 22 kDal (Zhang et al., 1985) and 20.8 kDal respectively (Fredriksen et al., 1985). Although the agglutinogens are generally thought to be envelope antigens, recent work has indicated that some are of fimbrial origin (Ashworth et al., 1982b; Carter and Preston, 1984).

Antibodies against agglutinogens may be important in immunity to pertussis (Preston and TePunga, 1959; Preston, 1963). Current WHO recommendations are that vaccines should include the three major agglutinogens (1, 2, and 3).

(e) Heat-labile toxin (Dermonecrotising toxin)

This was first reported by Bordet and Gengou (1909) who described a heat-labile substance of B.pertussis which was lethal to guinea-pigs and rabbits when given intraperitoneally or intravenously and dermonecrotising when given subcutaneously. A heat-labile product of B.pertussis also

induces splenic atrophy in mice (Iida and Okonigi, 1971) and is cytotoxic to cell cultures (Felton et al., 1954). The dermonecrotising activity of B.pertussis is destroyed by heating at 60°C for 10 min (Munoz et al., 1959). The instability of the toxin has been a major impediment to its isolation and subsequent purification. Properties of partially-purified toxin preparations have been reported (Onoue et al., 1963; Nakase et al., 1969). The preparation of Livey and Wardlaw (1984) had a molecular mass of approximately 90 kDal. Nakase and Endoh (1985) reported the isolation of purified heat-labile toxin which had a total molecular mass of 102 kDal and consisted of two polypeptides of molecular mass 30 kDal and 24 kDal. When administered intracutaneously into guinea-pigs and mice the purified toxin induced vasoconstriction which subsequently resulted in necrosis and haemorrhage. In addition, the toxin appeared to inhibit rat kidney Na+ - K+ ATPase in vitro. Inhibition of this enzyme may cause constriction of vascular smooth muscle and this action may explain the ability of the toxin to induce dermonecrosis and splenic atrophy in mice (Nakase and Endoh, 1985). Kume et al. (1986) reported the purification of heat-labile toxin from Bordetella bronchiseptica. This preparation had a molecular mass of about 190 kDal and dissociated into two components of molecular mass about 118 kDal and 75 kDal after treatment with dithiothreitol and urea. Heat-labile toxin is an intracellular protein although small amounts may be detected extracellularly (Banerjea and Munoz, 1962; Cowell et al., 1979). The exact role of heat-labile toxin in pathogenesis is at present unknown.

(f) Lipopolysaccharide endotoxin

B.pertussis is unusual in that it has two chemically distinct forms of lipopolysaccharide endotoxin (LPS), termed LPS-I and LPS-II, which differ in their polysaccharide components (Le Dur et al., 1980). Both LPS-I and LPS-II appear to have similar biological properties to those of the unfractionated LPS preparation. B.pertussis LPS has properties similar to the LPSs of other gram-negative bacteria (Nakase et al., 1970; Ayme et al., 1980). It is unusual, although not unique, in being mitogenic for murine spleen cells (Girard et al., 1981). Polyclonal activation of rabbit immunoglobulin-forming cells by B.pertussis LPS is macrophage-dependent (Haeffner-Cavaillon et al., 1982a) and B.pertussis LPS binds specifically to rabbit peritoneal macrophages (Haeffner-Cavaillon et al., 1982b). B.pertussis LPS may also have adjuvant activity (Morse 1976; Ayme et al., 1980). Aprile and Wardlaw (1973) reported that different strains of B.pertussis had different LPS antigenic determinants although other studies failed to confirm this (Le Dur et al., 1980).

(g) Pertussis toxin

Nomenclature

B.pertussis produces a toxin which appears to be the major virulence factor of the organism (Pittman, 1979). The diverse biological activities of this toxin are reflected in the different names assigned to it. The concept that these various activities may actually be attributable to the one substance

was first stated in the Unitarian Hypothesis of Levine and Pieroni (1966). The name pertussigen was later used by Munoz (1976) for a substance, which among other activities, sensitized mice to histamine and induced leukocytosis. Wardlaw and Parton (1983) reviewed the evidence for and against the pertussigen concept. A factor isolated from B.pertussis, termed islet-activating protein, had histamine-sensitizing activity and leukocytosis-promoting activity (Mizushima et al., 1979). Pittman (1979) first used the term pertussis toxin for a substance which had histamine-sensitizing, leukocytosis-promoting, and islets-activating activity. To avoid confusion, I propose to use the term pertussis toxin (PT) for a substance which has the biological activities listed below and the other properties attributed to the pertussigen molecule in Wardlaw and Parton (1983). Before a review of some of the more important biological activities of PT, the physicochemical properties and ADP-ribosylating activity of the molecule will be briefly discussed.

Physicochemical properties

PT has been purified by various investigators although, as mentioned previously, the product may have been called by another name. Estimates of the molecular mass of the toxin are summarised in Table 1.

The subunit structure of PT has also been variously estimated. The purified islets-activating protein (IAP) of Yajima et al. (1978a) consisted of four polypeptide chains of molecular mass 12, 20, 20, and 25 kDal. Irons and MacLennan (1979) purified the lymphocytosis-promoting factor hemagglutinin which consisted of four component polypeptides

Table 1

Estimates of the molecular mass of pertussis toxin.

Reference	Authors' designation of substance*	Molecular mass (kDal)
Lehrer et al. (1974)	HSF	90
Arai and Sato (1976)	HA-LPF	107, 103, 30 (by different methods)
Morse and Morse (1976)	LPF	67-74
Yajima et al. (1978a)	IAP	77
Cowell et al. (1982)	LPF-HA	170
Tamura et al. (1982)	IAP	117
Sekura et al. (1983)	Pertussis toxin	113
Nicosia et al. (1986)	Pertussis toxin	109

*HSF=Histamine-sensitizing factor, HA-LPF=Hemagglutinin-leukocytosis-promoting factor, LPF=Lymphocytosis- (or leukocytosis-) promoting factor, IAP=Islet(s)-activating protein, LPF-HA=Leukocytosis-promoting factor-hemagglutinin.

of molecular mass 12.6, 21.1, 22.4, and 27.2 kDal. The purified IAP of Tamura et al. (1982) consisted of six subunits, one copy of subunits 1, 2, 3, and 5 and two copies of subunit 4. The molecular mass of the subunits were 28, 23, 22, 9.3, and 11.7 kDal respectively. Sekura et al. (1983) proposed slightly different molecular masses for the subunits but agreed that PT consists of six subunits. Using genetic techniques the PT genes have now been cloned and sequenced (Locht and Keith, 1986; Nicosia et al., 1986). The cloned toxin has a deduced molecular mass of about 109 kDal.

PT is an A-B type toxin. The B-oligomer of the toxin comprises subunit 5, a dimer of subunit 2 and 4, and a dimer of subunit 3 and 4. Subunit 1 is the enzymically-active A component (Tamura et al., 1982).

ADP-ribosylating activity

The A-protomer of PT catalyzes the ADP-ribosylation of a membrane-bound protein of molecular mass 39-41 kDal in target cells (Tamura et al., 1982). The toxin substrates include the guanine nucleotide binding proteins (G-proteins) G_i, G_o, and transducin (Milligan et al., 1985). The major PT substrate of C6 glioma cells modulates adenylate cyclase activity causing receptor mediated stimulation of cAMP accumulation (Katada and Ui, 1982). PT also ADP-ribosylates a membrane protein in neutrophils causing an inhibition of normal response to chemotactic factors, however, the basal cAMP levels in these cells remains unaffected (Bokoch and Gilman, 1984; Okajima and Ui, 1984). There is now evidence to suggest that the PT substrates of both C6 glioma cells and neutrophils are G-proteins immunochemically distinct from G_i,

G_o, or transducin (Milligan et al., 1986; Gierschik et al., 1986).

Major biological activities

The major activities of PT are reviewed below. The effect of PT on phagocytes is covered in section 2.3.3. Munoz and Bergman (1977), Wardlaw and Parton (1983), and Sekura et al. (1985) reviewed the different biological properties of PT. These articles also cover activities of the toxin such as toxicity, adjuvancy, mitogenicity, and the ability to confer protection in the murine intracerebral challenge test. Due to the constraints of a review such as this, these activities are not covered below.

Haemagglutination

Arai and Sato (1976) first demonstrated that B.pertussis actually produced two haemagglutinins. One (FHA) was non-toxic in mice whereas the other was toxic, induced histamine sensitivity and leukocytosis in mice, and was termed the leukocytosis-promoting factor hemagglutinin (LPF-HA). Differentiation of the two haemagglutinin types can be done using the physical character of the erythrocyte agglutination they produce. LPF-HA produces firmly-bound, coherent sheets of agglutinated erythrocytes whereas FHA appears to influence only the settling of the erythrocytes. The most sensitive erythrocyte species to agglutination by LPF-HA are goose erythrocytes (Irons and MacLennan, 1979).

Histamine-sensitization

B.pertussis histamine-sensitizing activity was first described by Parfentjev and Goodline (1948). Pertussis vaccine injected intraperitoneally into mice made the animals hypersensitive to a subsequent injection of histamine. The term histamine-sensitizing factor (HSF) was first proposed by Maitland *et al.* (1955). HSF also increases susceptibility to the effects of serotonin, bradykinin, and a variety of other stress stimuli (Munoz and Bergman, 1968). The exact mechanism of histamine-sensitization in mice remains unknown. However, histamine-sensitization is an activity of the B-oligomer of PT (Nogimori *et al.*, 1984).

Islets-activation

A tendency towards hypoglycaemia during or after infections with *B.pertussis* has been observed both in children (Regan and Tolstoouhov, 1936) and experimental animals (Oddy and Evans, 1940; Pittman *et al.*, 1980). Pertussis vaccine causes hyperinsulinaemia in rats (Gulbenkian *et al.*, 1968). Studies have correlated the hyperinsulinaemia with the time-course of hypoglycaemia in the same animals suggesting that both effects are linked (Tabachnick and Gulbenkian, 1969). Similar effects were noted with the purified IAP of *B.pertussis* by Yajima *et al.* (1978 a, b). IAP exerts its effects by acting on the pancreatic islet B-cells, blocking the inhibition of insulin release. This suggests that the hypoglycaemia associated with *B.pertussis* infections is probably due to an IAP-induced alteration in the pancreatic B-cells leading to hypersecretion of insulin in response to various insulin-releasing stimuli

(Katada and Ui, 1979; Katada and Ui, 1980).

Leukocytosis promotion

The lymphocytosis frequently observed in whooping cough patients was first reported by Frolich in 1897 (cited by Wardlaw and Parton, 1983). Sauer (1933) noted the occurrence of lymphocytosis in children injected with pertussis vaccine. The factor responsible for these effects has been designated leukocytosis-promoting factor and lymphocytosis-promoting factor, both abbreviated to LPF. The blood counts of both lymphocytes and polymorphonuclear leukocytes are increased by pertussis or purified LPF-treated animals (Morse, 1965; Morse and Morse, 1976). Morse and Reister (1967) indicated that the lymphocytosis observed in pertussis-treated mice was not due to stimulation of cell division but rather blockade of lymphocyte recirculation from the bloodstream back to extravascular sites. Also the neutrophilia appeared to be due to active cell proliferation as well as neutrophil migration into the bloodstream from tissue reserves. Lymphocytosis may be induced by a change in lymphocyte rather than vascular endothelial cell properties (Morse and Barron, 1970). Spangrude et al. (1984) found that pertussigen-treated murine lymphocytes lost their ability to localize in lymph nodes or Peyer's patches. Adherence of the lymphocytes to endothelial cells in vitro was unaffected. Lymphocyte extravasation may involve a receptor-specific binding event followed by an adenylate cyclase-dependent activation of cell motility. PT may inhibit the second process thus explaining its capacity to block lymphocyte recirculation in vivo (Spangrude et al., 1984).

(h) Tracheal cytotoxin

B.pertussis has the ability to adhere to and destroy both hamster tracheal organ and epithelial cells in vitro (Collier et al., 1977; Goldman et al., 1982). Culture supernates from virulent B.pertussis cultures appear to inhibit DNA synthesis in uninfected tracheal cells in vitro. The substance responsible was designated tracheal cytotoxin and the purified product appeared to consist of a peptide (of molecular mass 1.5 kDa), muramic acid and glucosamine (Goldman et al., 1982). The exact structure of the toxin and its role in virulence remains unknown.

2.1.5. Phase variation and antigenic modulation

Bordetella species can undergo a form of variation termed phase variation (Leslie and Gardner, 1931). Freshly isolated and undegraded strains of B.pertussis are termed phase I organisms; completely degraded, antigenically changed, avirulent strains which appear on prolonged subculture are termed phase IV organisms. Phase II and phase III strains are intermediate forms between phase I and phase IV organisms. Factors lost during change from phase I to phase IV include HSF (Kind, 1953), agglutinogen 1 (Eldering et al., 1962), haemagglutinating activity (Keogh and North, 1948; Standfast, 1951), haemolysin (Peppler, 1982), and certain envelope proteins (Parton and Wardlaw, 1975; Ezzell et al., 1981). Increased tolerance to erythromycin was used by Weiss and Falkow (1984) as an avirulent-phase marker. They found that avirulent phase organisms failed to produce PT, FHA or haemolysin.

Although phase variation was thought to be essentially irreversible (Parker, 1979), recent studies have indicated that phase variation may be reversible and is possibly regulated by the production of a positive effector protein which switches on the virulent phase genes (Weiss and Falkow, 1984).

Bordetella species can also undergo another kind of variation termed antigenic modulation. The organism may be classified into three different modes: xanthic (X)-mode, cyanic (C)-mode, or intermediate-mode (Lacey 1951, 1960). When NaCl was replaced by $MgSO_4$ as the principal salt in a solid medium devised by Lacey, B.pertussis colonies lost agglutinogens, haemagglutinating and haemolytic activity, and had decreased virulence in mice. The NaCl-grown bacteria were designated X-mode and the $MgSO_4$-grown bacteria were designated C-mode. Lower temperatures favour C-mode growth and higher temperatures favour X-mode growth. Later studies showed that C-mode bacteria were deficient in protective antigen, HSF, and two major outer membrane polypeptides of molecular mass 28 and 30 kDal, (Parton and Wardlaw, 1975, Wardlaw et al., 1976). In addition, heat-labile toxin and adenylate cyclase are lost during X- to C-mode modulation (Livey et al., 1978; Parton and Durham, 1978). Pyridines and related compounds also affect antigenic modulation (Schneider and Parker, 1982). Idigbe et al. (1981) found that the loss of X-mode components during modulation occurred within 10 hours. Genetic studies have indicated that antigenic modulation may be governed by the same genetic locus as phase variation (Weiss and Falkow, 1984).

Part 2: Neutrophils: activities and functions

To appreciate the implications of the effects *B.pertussis* may have on neutrophils, their activities and functions must be understood. Murphy (1976) and Smith and Lumsden (1983) give a good coverage of neutrophil activities. Neutrophils are complex cells that have a wide range of activities which may be subdivided into four processes: adhesion, locomotion, phagocytosis and killing of microorganisms. Although these activities can be treated as separate they are all part of an ongoing process which usually culminates in destruction of foreign material in the host. Therefore, for example, an agent which inhibits adhesion may also prevent normal locomotion and an agent which inhibits phagocytosis may also inhibit killing of microorganisms.

2.2.1. Classification

The white cells of blood, leukocytes, can be divided into mononuclear and polymorphonuclear forms. Depending on the affinity of their cytoplasmic granules for certain acidic and basic dyes used in stains, the polymorphonuclear cells (or granulocytes) can be classified as neutrophils, basophils, or eosinophils. The mononuclear forms are further subdivided into monocytes and lymphocytes and can be distinguished by staining and nuclear shape; monocytes have characteristic kidney-shaped nuclei. Monocytes leave the bloodstream and enter the tissues where they undergo morphologic and biochemical modifications and become resident or wandering macrophages. The role of macrophages in the immune response was reviewed by Kende (1982) and Takemura and Werb (1984). Neutrophils are the most

common leukocytes in the blood (60-70%) with the lymphocytes being the second most common (20-30%). The most active phagocytic cells are the neutrophils, monocytes, and macrophages (Ryan and Majno, 1977; Van Furth et al., 1979). The eosinophil also has phagocytic activity although it does not appear to be as efficient as neutrophils at ingestion and killing of bacteria (Yazdanbakhsh et al., 1986). Mahmoud et al. (1979) reviewed the role of eosinophils in the immune process.

2.2.2. Adhesive properties

Neutrophils are found in the blood as circulating non-adherent cells, however, their role in many pathological processes is to leave the bloodstream and migrate into damaged or infected tissues (Van Furth and Willemze, 1979). During the early parts of the inflammatory response neutrophils begin to adhere to the endothelium of the post-capillary venules, a process called margination (Beesley et al., 1978; Hoover et al., 1978). The apparent predilection of neutrophil adhesion for postcapillary venules may be related primarily to shear forces; the postcapillary venule is the site of the first major decrease in vessel wall shear stress (Mayrovitz et al., 1977). However, changes in neutrophil adhesiveness may also be important in margination (Lackie, 1982). After margination, the cells leave the bloodstream and emigrate towards the damaged or infected tissue, a process called diapedesis. Cell movement during diapedesis involves the locomotory machinery of the cell and the continual making and breaking of adhesive contacts between the cell and its substratum (Snyderman and Goetzl, 1981). Under conditions of either excessive

adhesiveness or non-adhesiveness neutrophils fail to translocate (Keller et al., 1979; Smith et al., 1979). Several syndromes have been associated with abnormal neutrophil adherence; decreased granulocyte adherence has been linked with post-streptococcal glomerulonephritis (Ruley et al., 1976) and multiple myeloma (Spitler et al., 1975) while increased granulocyte adherence has been linked with acute inflammatory disease (Lentnek et al., 1976; Smith et al., 1981).

Secondary granule contents of neutrophils, such as the acidic proteins (Bockenstedt and Goetzl, 1980) and lactoferrin (Oseas et al., 1981), may play a role in mediating cell adherence. Wright and Gallin (1979) reported that exocytosis of secondary granules occurred during human neutrophil adherence in vitro and during exudation in vivo. However, other studies indicated that neutrophil adhesion may not be mediated by the contents of the secondary granules. For example, Gallin et al. (1982) reported that neutrophils from a patient with a congenital deficiency of secondary granule contents marginated normally in vivo in response to endotoxin infusion and aggregated normally in response to the chemotactic agent N'-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP). In addition, Boxer et al. (1982) found that fMLP induced normal aggregation of lactoferrin-deficient neutrophils. However, small amounts of lactoferrin (4-8% of normal) were still present in both neutrophils from the patient with the secondary granule content-deficiency and from the patient with the lactoferrin-deficiency. These low lactoferrin levels may still have been enough to promote adhesion and aggregation.

English and Gabig (1986) reported that cellular metabolism

may only be required for the initial promotion of neutrophil adhesion while other factors may be more important in the maintenance of adhesion. They found that the stimulation of human neutrophil adherence by phorbol myristate acetate could be functionally separated into an induction phase, which required cellular metabolism, and a Mg^{2+}-dependent maintenance phase, that was independent of cellular metabolism. Neutrophil membrane glycoproteins may also play an important role in neutrophil adhesion (Kobayashi et al., 1984). Congenital defects in neutrophil adhesion have been correlated with the absence of one or more high molecular mass glycoproteins, such as glycoprotein gp150 (Arnaout et al., 1982) or glycoprotein gp183 (Anderson et al., 1984).

2.2.3. Locomotion and chemotaxis

Neutrophil movement towards an inflammatory focus is an essential part of the acute inflammatory response and any defects, either inherent or induced, are likely to have serious effects on the host defence system. An extensive list of clinical conditions associated with defective neutrophil locomotion reflects this importance (Gallin et al., 1980; Quie and Cates, 1977). Neutrophils move over surfaces by the use of traction gained from adhesive contacts (Lackie, 1982). In situations where adhesion is poor, such as in three-dimensional matrices of collagen, it has been suggested that neutrophils may use pseudopodia to link round tissue fibres and then use leverage to pull the cell in a specific direction (Brown, 1982).

Neutrophil movement is of obvious importance in neutrophil chemotaxis where the cell reacts to a chemical concentration

gradient by directed locomotion along the axis of the gradient (Keller et al., 1977). The metabolic processes which occur to transfer the chemotactic signal into directed movement are complicated (Becker, 1980; Snyderman and Goetzl, 1981). The nature of chemotactic factors and the regulation of specific chemotactic factor receptors were discussed by Wilkinson (1981) and Zigmond and Sullivan (1981). Wilkinson (1982) reviewed the chemotaxis of leukocytes with emphasis on the inflammatory process.

Chemotactic factors which have been studied include the complement factors (C3b, C5a), leukotrienes, the peptide fMLP and IgG immune complexes. In general, most chemotactic factors can modulate other functions as well as chemotaxis (Romeo, 1982) and these factors are perhaps better termed pro-inflammatory mediators. Table 2 lists the neutrophil functions induced by fMLP and other formyl-methionyl peptides, C5a, and certain other pro-inflammatory mediators. Wilkinson (1980) reviewed the effects of certain microorganisms on phagocyte locomotion. Bacterial virulence factors which inhibit neutrophil chemotaxis include cholera toxin (Roch-Arveiller et al., 1979) and LPS (Hartiala et al., 1985).

2.2.4. Phagocytosis

The microbicidal activity of neutrophils is dependent upon two important processes which precede intracellular killing; opsonization and phagocytosis. Opsonins were first described by Wright and Douglas in 1903 as factors which convert bacteria into "ready prey" for phagocytes. B.pertussis has low susceptibility to surface phagocytosis by human neutrophils. However, after the addition of IgG antibodies

Table 2

The effects of fMLP, C5a and certain other pro-inflammatory mediators on neutrophil function*.

1. Stimulation of locomotion
a. Chemotaxis
b. Chemokinesis
2. Stimulation of secretion of lysosomal enzymes
3. Stimulation of "respiratory burst"
a. Superoxide generation
b. Hexose-monophosphate shunt activity
c. Increased O_2 consumption
d. Increased H_2O_2 formation
e. Increased luminescence
4. Induced aggregation

*Modified from Becker (1980).

specific for the bacterium, opsonization and subsequent increased phagocytosis occurred (Utsumi et al., 1978). Currently the immunoglobulins and the complement system are regarded as the most important serum opsonins for promoting phagocytosis of microorganisms.

Although phagocytosis may be divided into attachment and ingestion phases, attachment of a bacterium does not necessarily lead to subsequent ingestion (Densen and Mandell, 1978; Horwitz and Silverstein, 1980). Ingestion of particles may be by a "zipper mechanism" (Griffin et al., 1975) which appears to involve both complement and Fc receptors on the neutrophil cell membrane (Horwitz, 1982). Van Oss et al. (1975) and Stendahl (1983) indicated that the size, shape, charge, hydrophobicity, and chemical composition of a particle influence its susceptibility to phagocytosis. Horwitz (1982) reviewed the congenital and acquired defects in phagocytosis and some of the bacterial substances which interfere with the phagocytic process.

2.2.5. Microbicidal activity

When microorganisms bind to the surface of neutrophils and ingestion begins, several mechanisms that result in destruction of the ingested microorganism are activated. These mechanisms involve two major events: (i) the fusion of the cytoplasmic granules of the neutrophil with the plasma membrane and the subsequent delivery of the granule contents into the phagosome, a process termed degranulation (Hirsch and Cohn, 1960; Zucker-Franklin and Hirsch, 1964), and (ii) the generation of highly toxic products of oxygen reduction in a process termed the "respiratory burst" (Babior, 1978). The

respiratory burst does not require either phagocytosis or degranulation but simply a contact between the phagocyte and stimulating agent (Curnutte et al., 1979). It is the action of both the granule contents and the products of the respiratory burst that causes the actual killing and digestion of microorganisms.

Bainton and Farquhar (1966) first reported that neutrophils contain two forms of granule: the primary or azurophil granule and the secondary or specific granule. Azurophil granules contain myeloperoxidase and various hydrolytic enzymes that break down organic material. In addition, they contain some non-enzymic materials, such as the cationic proteins (Odeberg and Olsson, 1975), which may have important anti-bacterial activities. Lysozyme is found in both the azurophil and specific granules. Murphy (1976) reviewed the contents of neutrophil granules. The specific granules of human neutrophils contain the high affinity iron-binding protein lactoferrin (Masson et al., 1969). Lactoferrin is thought to act by reducing the free-iron concentration far below that required for bacteria to grow thus inhibiting bacterial growth. However, it has also been reported to be a significant promoter of neutrophil adhesion. Therefore, it may also act by enhancing the localization of the neutrophils at the site of infection, thereby aiding the clearance of bacteria by recruited neutrophils (Oseas et al., 1981).

The increase in O_2 consumption which occurs during leukocyte phagocytosis was first observed in 1933 by Baldridge and Gerard. This was later correlated with the production of toxic oxygen products in the "respiratory burst". Changes which occur in neutrophils during the "respiratory burst" are shown in Table 2. The toxic oxygen species produced by phagocytes

were reviewed by Badwey and Karnovsky (1980). The general microbicidal responses of phagocytes are summarised in Table 3.

Another important anti-bacterial property of neutrophils is the increased hydrogen ion concentration that is generated in the phagosome (Rous, 1925a, b). Estimates of the pH within phagosomes range from 3.0 to 6.0 depending on the species studied and the time of sampling (Jensen and Bainton, 1973; Mandell, 1970).

B.pertussis has been reported to live for a time as an intracellular parasite of macrophage (Gray and Cheers, 1967a). The bacterium appeared to survive intracellularly for periods of up to one month in murine macrophage, albeit at levels of less than 1% of the original numbers. However, no studies on whether B.pertussis could outlast the macrophage, multiply and possibly reinitiate infection have been reported. This B.pertussis intracellular phase was termed the "complaisant phase" and occurred also with Mycobacterium tuberculosis (Gray and Cheers, 1967b). Mycobacterium tuberculosis survives intracellularly by preventing phagosome-lysosome fusion (Goren et al., 1976). The mechanism by which B.pertussis may survive in macrophage is unknown.

Inherited disorders of neutrophil microbicidal activity include myeloperoxidase deficiency (Lehrer et al., 1969) and chronic granulomatous disease (Holmes et al., 1967), the latter normally being fatal in the first decade of life. Neutrophils from chronic granulomatous disease patients lack a NADPH oxidase which, when activated, triggers the respiratory burst (Babior et al., 1975; Curnutte et al., 1975). Bacterial products which interfere with microbicidal activity of neutrophils include LPS (Weiss et al., 1980; Henricks et al.,

Table 3

Microbicidal responses of phagocytes*.

A. Degranulation (O_2-independent)

1. Cationic proteins
2. Proteases
3. Lactoferrin
4. Lysozyme
5. Acid hydrolases
6. Myeloperoxidase

B. Oxidative respiratory burst (O_2-dependent)

1. Myeloperoxidase-associated
   a. Hydrogen peroxide
   b. Halogenation
   c. Luminol-enhanced chemiluminescence
2. Superoxide, singlet oxygen, and other reactive oxygen radicals

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*Modified from Quie (1983).

1983) and enzymes such as catalase (Mandell, 1975). In vitro methods of measuring neutrophil microbicidal activity involve either direct (Quie et al., 1967) or indirect methods such as nitroblue tetrazolium reduction (Baehner et al., 1976) or chemiluminescence (Allen et al., 1972). Since a major portion of the work contained in this thesis involves the use of chemiluminescence a review of the technique is given in 2.2.6.

2.2.6. The use of chemiluminescence as an assay of microbicidal activity

Chemiluminescence was first described by Allen et al. (1972) who found that opsonized bacteria elicited a chemiluminescent response from human neutrophils upon phagocytosis. They correlated chemiluminescence with hexose-monophosphate shunt activity and suggested that chemiluminescence reflected the generation of singlet oxygen. Cheson et al. (1976) later found that superoxide dismutase and catalase both inhibited chemiluminescence and proposed that light production was due to reactions between ingested particles and some or all of the active oxidising agents produced during the neutrophil "respiratory burst". Allen and Loose (1976) described the use of the compound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) to enhance chemiluminescence. They demonstrated that when luminol was added to a rabbit macrophage suspension chemiluminescence was observed following phagocytosis of bacteria. Previously, in the absence of luminol no chemiluminescence had been detected. The compound lucigenin (bis-N-methylacridinium nitrate) has also been used to amplify the chemiluminescent response produced by phagocytes (Allen, 1981). Luminol is oxidised by

products of the myeloperoxidase system (DeChatelet et al., 1982; Dahlgren and Stendahl, 1983) whereas lucigenin-dependent chemiluminescence is almost entirely dependent upon superoxide production (Allen, 1981).

Hatch et al. (1978) used luminol-enhanced chemiluminescence to study the anti-bacterial activity of neutrophils stimulated by N-formyl-methionyl peptides. They found that there appeared to be a definite relationship between peptide structure and chemiluminescence activity, with fMLP being the most active peptide tested. Neutrophils stimulated by fMLP give a unique bimodal chemiluminescent response in the presence of luminol. The first peak is extracellular in origin while the second peak is cell-associated (Bender and Van Epps, 1983). In the presence of lucigenin, fMLP-stimulated neutrophils give only a single chemiluminescent peak which appears to be extracellular in origin (Dahlgren et al., 1985).

Other studies using luminol-enhanced chemiluminescence have correlated neutrophil bactericidal ability directly with chemiluminescence production (Horan et al., 1982). Chemiluminescence has also been used to evaluate the effect of serum opsonins on the anti-bacterial activity of phagocytes. (Easmon et al., 1980; Robinson et al., 1984). Opsonization of microorganisms causes an increased chemiluminescent response and this can be used as a probe for the presence of specific antibodies against a certain organism (Welch, 1980). Lilius et al. (1985) reported that chemiluminescence can be be used to detect the presence of opsonins against B.pertussis; higher levels of opsonins against B.pertussis were found in whooping cough patients than in vaccinated controls. In addition, they reported that sera from unvaccinated infants contained no opsonins against

B.pertussis.

Boeckx (1984) reviewed the use of chemiluminescence as a tool for studying phagocyte function. Harber (1984) reviewed the use of bioluminescence, which mainly involves the use of firefly luciferase; an enzyme which reacts with ATP to give off light. The luminescence produced during the ATP-firefly luciferase reaction can be used to check for the presence of viable bacteria. This is useful as a probe for bacteraemia and bacteriuria, anti-microbial susceptibility testing, and antibiotic and vitamin assays.

Part 3: Effect of B.pertussis components on phagocytes

2.3.1. Vaccine

A pertussis vaccine was reported to inhibit macrophage outpouring into a zone of thermal coagulation necrosis in the brains of experimental animals (Levine and Sowinski, 1972). In addition, pertussis vaccine was also reported to inhibit murine macrophage elongation, migration, and peritoneal cellular exudate formation (Benjamin et al., 1981). The component responsible for all activities was labile to heating at 80°C for 30 min.

2.3.2. Adenylate cyclase

Confer and Eaton (1982) found that extracts or culture supernates of B.pertussis containing high levels of heat-stable adenylate cyclase activity had a wide-range of anti-phagocyte activities. These included inhibition of superoxide production by human neutrophils and alveolar macrophages, a reduction of the direct bactericidal capacity of neutrophils, and inhibition of neutrophil chemotaxis and particle ingestion. Symes et al. (1983) reported that B.pertussis adenylate cyclase suppressed the oxidative burst of human monocytes although it had no effect on phagocytosis. Confer et al. (1984) suggested that the adenylate cyclase entered the phagocytes and induced unregulated cyclic AMP formation which impaired cell function. Hewlett et al. (1985) observed the considerable similarity between the adenylate cyclase of B.pertussis and the oedema factor of Bacillus anthracis, which is also a heat-stable, calmodulin-dependent

adenylate cyclase that inhibits neutrophil function (Leppla, 1982; O'Brien et al., 1985). The oedema factor requires the Bacillus anthracis protective antigen before it can enter mammalian cells and induce an increase in cAMP. Leppla (1982) suggested that the protective antigen binds to mammalian cells and provides a receptor for the oedema factor. A similar receptor-mediated mode of entry may exist for the B.pertussis adenylate cyclase (Hewlett et al., 1985).

2.3.3. Pertussis toxin

The polymorphonuclear leukocyte-inhibitory factor (PIF) of B.pertussis was first described by Utsumi et al. (1978). This factor inhibited the chemotactic and phagocytic capacity of neutrophils. In a later publication Imagawa et al. (1980) suggested that PIF may be identical to the lymphocytosis-promoting factor of B.pertussis. Granule enzyme secretion by rabbit neutrophils induced by the pro-inflammatory mediators fMLP, C5a, and leukotriene B4 was suppressed by purified PT (Becker et al., 1985). PT also inhibited the rise in intracellular calcium concentrations induced by fMLP in both rabbit (Molski et al., 1984) and guinea-pig neutrophils (Okajima and Ui, 1984). Both fMLP- and leukotriene B4-induced increases in actin association with the cytoskeleton in rabbit neutrophils is also inhibited by PT-treatment (Shefcyk et al., 1985). The increase in the cytoskeletal actin produced by phorbol myristate acetate, on the other hand, was not affected by the toxin. PT has now become useful as a tool for the study of signal transduction during neutrophil activation by pro-inflammatory mediators (Becker et al., 1986).

PT inhibited murine macrophage migration and chemotaxis *in vitro* as well as altering their spreading capacity on tissue culture dishes (Meade *et al.*, 1984). The toxin also inhibited the chemotaxis of human neutrophils *in vitro* and partially inhibited neutrophil adherence to protein-coated plastic surfaces, most notably after augmentation of adherence with fMLP (Spangrude *et al.*, 1985). Lad *et al.* (1986) reported that PT does not influence phagocytosis by human neutrophils. Meade *et al.* (1984), however, have reported that PT inhibited F_c-mediated phagocytosis by murine macrophage. This discrepancy can perhaps be explained by differences between the cells and in the methods used to assay phagocytosis.

OBJECT OF RESEARCH

Many successful pathogenic microorganisms have mechanisms for interfering with the anti-microbial activities of host phagocytes. However, the extent to which this generalization applies to Bordetella pertussis infection has only recently been considered. The aim of the present investigation was to assay the effects of B.pertussis components on phagocytes. This study focused on neutrophils exclusively and explored the effect on neutrophil function of the following components of B.pertussis: PT, FHA, and LPS. The neutrophil functions of primary interest were adhesiveness and bactericidal response to a pro-inflammatory mediator (fMLP) and whole-cells of B.pertussis. As the project developed it became apparent that the neutrophil response to fMLP had applications for monitoring the preparation of acellular pertussis vaccines.

MATERIALS AND METHODS

4.1. Growth of the bacteria

The strains used are listed in Table 4. The *B.pertussis* Tohama and 18334 strains were obtained from stock cultures kept at Dept. of Microbiology, Glasgow University, Glasgow, U.K. The other four strains were kindly supplied by Dr. A. A. Weiss (Department of Microbiology and Immunology, Box 678, Medical College of Virginia, Richmond, Virginia 23298-0001). Weiss *et al.* (1983) have described the isolation of the avirulent mutants noted in Table 4.

A loopful of growth from a 72 h culture grown at 37°C on Bordet-Gengou medium (Gibco Biocult Diagnostics Ltd., Paisley, Scotland), containing 20% (v/v) sterile, defibrinated horse blood (Gibco), was used to inoculate each litre of Stainer-Scholte (SS) medium modified such that the level of Tris present was 1.25 g l^{-1} (Stainer and Scholte, 1971). The organisms were either grown in shaken culture in X-mode (NaCl-containing) medium or in C-mode medium containing $MgSO_4$ instead of NaCl (Brownlie *et al.*, 1985). The intact bacteria used for the chemiluminescence and hydrophilicity assays were from 48 h shaken cultures grown at 37°C. The bacteria were then stored frozen at -70°C in phosphate-buffered saline (PBS; section 8.1) containing 10% (v/v) glycerol until used.

4.2. Processing of bacterial culture supernates

Cultures of bacteria grown for 48 h at 37°C in modified SS medium were centrifuged at 6000 g for 30 min at 4°C. The supernates were then filtered through a glass fibre paper filter (Whatman) then a Millipore filter (0.45 µm pore size)

Table 4

A summary of the *B.pertussis* strains used in the chemiluminescence studies.

Organism	Virulence factors absent†
B.pertussis 18334	NONE
B.pertussis Tohama	NONE
B.pertussis 347	PT, AC, HLY, FHA, HLT
B.pertussis 348	AC, HLY
B.pertussis 353	FHA
B.pertussis 357	PT‡

‡Some cell-associated PT present.
(Weiss *et al.*, 1983)

†AC=adenylate cyclase, HLY=haemolysin, FHA=filamentous haemagglutinin, HLT=heat-labile toxin.

to remove residual cells. Culture supernates were added to an Amicon DC2 Hollow Fibre Dialyzer/Concentrator with a molecular mass cut-off of 1000kDal. The resulting filtrates were then re-filtered using a filter with a molecular mass cut-off of 100 kDal and concentrated 15-30 fold.

4.3. Preparation of filamentous haemagglutinin

Filamentous haemagglutinin was kindly given by Dr. L. A. E. Ashworth (Public Health Laboratory Service, Centre of Applied Microbiology and Research, Porton Down, Salisbury, U.K.) and was stored at -20°C until use. It was diluted in HEPES-buffered saline (HBS) prior to use.

4.4. Preparation of lipopolysaccharide endotoxins

B.pertussis 165 (phase I) LPS was obtained from LIST Biological Laboratories Ltd (501-B Vandell Way, Campbell, California 95008, USA). Salmonella minnesota (smooth) and Salmonella minnesota Re595 (rough) LPS were obtained from Sigma. All were reconstituted according to manufacturer's instructions except that HBS was used in place of distilled water. Any further dilutions were also made in HBS.

4.5. Preparation of pertussis toxin

PT was obtained from two sources. One sample was obtained from LIST Biological Laboratories Ltd and was reconstituted in 2 ml of sterile, distilled water and stored at 4°C until use. Further dilutions were made using HBS. The adenylate cyclase activity of this preparation was less than 1.0

picomoles/min/μg in the presence of 1.0 μM calmodulin, according to the supplier. In addition, the lowest concentration of the toxin which caused haemagglutination of goose red blood cells was 195 ng ml^{-1}, according to the supplier. The other PT preparation used was kindly donated by Dr. V. Y. Perera (Dept. of Microbiology, Glasgow University). This toxin preparation was purified using the method of Perera et al. (1985). Briefly, the toxin was isolated by Fetuin-Sepharose affinity chromatography of cytoplasmic fractions of cultures of B.pertussis 18334, phase I, grown for 48 h at 37°C in cyclodextrin liquid medium (Imaizumi et al., 1983).

4.6. Pertussis vaccine preparation

An acellular pertussis vaccine was kindly supplied by Mr. M. Christodoulides (Dept. of Microbiology, Glasgow University). The vaccine, which consisted predominantly of PT and FHA, had been prepared from a culture supernate extract of B.pertussis 77/18319 grown statically for 5 days at 37°C in SS X-mode medium. The culture supernate extract had been detoxified by the carbodiimide-toxoiding technique of Christodoulides et al., (1987).

4.7. Protein estimation

Protein content of samples were determined by the method of Herbert et al. (1971). Bovine serum albumin (BSA) was used as a standard.

4.8. Treatment of samples for electrophoresis

Samples were adjusted to 100-200 µg of protein ml^{-1}. Each sample (0.1 ml) was added to 0.1 ml of solubilising buffer (0.1 M Tris-HCl buffer, pH 6.8, containing 3% (w/v) sodium dodecyl sulphate (SDS), 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01% (w/v) of bromophenol blue) and the mixtures heated at 100°C for 5 min prior to loading on the gel.

4.9. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

A modification of the method of Laemmli (1970) was used. Stacking gels contained 4.5% (w/v) acrylamide and 0.12% (w/v) N'N'-methylenebisacrylamide (Bis). Separating gels contained 15% (w/v) acrylamide and 0.4% (w/v) Bis. Both stacking and separating gels contained 0.2% (w/v) SDS.

Samples (10-30 µl) were layered directly onto the gels and electrophoresis was performed at 30 mA per gel until the dye-front reached the separating gel when the current was increased to 45 mA per gel. The gels were then stained either by the silver stain technique of Oakley et al. (1980) or by the periodate LPS stain technique of Tsai and Frasch (1982).

For molecular mass determinations, some of the samples were run side-by-side with marker proteins. These were bovine serum albumin (BSA; molecular mass=66 kDal), egg albumin (molecular mass=45 kDal), glyceraldehyde-3-phosphate dehydrogenase (molecular mass=36 kDal) carbonic anhydrase (molecular mass=29 kDal), trypsinogen (molecular mass=24 kDal), trypsin inhibitor (molecular mass=20.1 kDal) and alpha-lactalbumin (molecular

mass=14.2 kDal), all obtained from Sigma.

4.10. Heat-labile toxin assay

Samples (0.05 ml) containing 100-200 µg protein ml^{-1} were injected subcutaneously into groups of 3-week old HaM/ICR female mice. Skin reactions were scored after 24 h by measuring the zone of haemorrhage at the site of injection (Parton, 1986).

4.11. Haemagglutination assay

Defibrinated horse blood was obtained from Gibco. Fresh rabbit blood was collected in 3.8% (w/v) sodium citrate. Both preparations were washed three times in normal saline. A wash comprised centrifugation at 400 g for 5 min with removal of the supernates between washes. After the final wash the erythrocyte pellets were resuspended to give a 0.5% (v/v) suspension in normal saline. Aliquots (50 µl) of erythrocyte suspension were added and mixed to the contents of separate wells containing 50 µl of dilutions of test sample. All samples were tested in duplicate and the experiment was performed in round-bottomed Cooke microtitre plates. Haemagglutination was assayed after overnight incubation at 4°C. The haemagglutinin titre was recorded as the reciprocal of the highest dilution giving complete haemagglutination.

4.12. Adenylate cyclase assay

The assay was performed as described by Brownlie et al. (1985) except that calmodulin (bovine brain; Sigma) was added

to the reaction mixture to give a final concentration of 0.3 µM. Cyclic AMP was assayed by the cAMP-binding protein method of Tovey et al. (1974) using a cyclic AMP assay kit (Amersham. Kit Code no. TRK.482). The assay could detect a minimum of 0.2 pmol cAMP per assay tube.

4.13. Enzyme-linked immunosorbent assay (ELISA) for filamentous haemagglutinin

The monoclonal antibody preparations and reference filamentous haemagglutinin preparation used for this assay were kindly supplied by Dr. L. A. E. Ashworth (Public Health Laboratory Service, Centre of Applied Microbiology and Research, Salisbury, Porton Down, U.K.). Both the monoclonal and FHA preparations were isolated as described by Irons et al. (1983). All incubations were done in a humidified box. Buffer recipes used are given in section 8.2. NUNC-immunoplate 1-96F ELISA plates were coated with 200 µl of a 1 µg ml^{-1} solution of the monoclonal antibody F2 in 0.05 M carbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were then washed with PBS containing 0.05% (v/v) Tween-20 (PBST). Serial dilutions of the unknowns and standard FHA preparation were prepared in PBST and 200 µl of each dilution added to each consecutive well. All samples were assayed in duplicate. The plate was then incubated at 37°C for 2 h, washed with PBST, and 200 µl of monoclonal antibody F3-horse radish peroxidase conjugate (0.5 µg ml^{-1}) in PBST then added to each well. After a further incubation at 37°C for 2 h and subsequent washing of the plate in PBST, 200 µl of the substrate was added to each well. The substrate, H_2O_2 and phenylenediamine, was prepared by the addition of 20 µl of a

20 volumes solution of H_2O_2 and 34 mg of O-phenylenediamine (Sigma) to 100 ml of citrate/phosphate buffer (pH 5.0). The plate was then incubated for 30 min in the dark at room temperature and the reaction stopped by the addition of 50 µl of 12.5% (v/v) H_2SO_4. The A_{492} of each well was then determined using a Titertek Multiskan MC photometer (Flow Laboratories). A graph of absorbance at 492 nm versus log_{10}FHA conc (ng/well) was drawn and the FHA content of the unknowns calculated by comparison with the reference FHA preparation using a parallel line assay.

4.14. Hydrophilicity assay

A modification of the method of Robinson et al. (1983) was used. The bacteria used were from frozen cultures as noted in section 4.1. After thawing, the bacteria were washed twice in PBS by centrifugation. Each wash comprised centrifugation at 9,000 g for 60 sec. After washing the bacteria were resuspended in PBS and the E_{550} set at 1.00. N-octane (300 µl) was then added to 4 ml of the bacterial suspension and the suspension mixed for 1 min. After standing for 20 min at room temperature the upper, hydrophobic layer was removed and the E_{550} of the remaining aqueous layer determined. This value, after correction for a PBS control, was multiplied by 100 and expressed as the % Hydrophilicity of the organism.

4.15. Viable count of bacteria

A nalidixic acid-resistant B.pertussis Tohama strain (Minimum inhibitory concentration (MIC) of 60 µg nalidixic acid ml^{-1} of Bordet-Gengou medium) was kindly supplied by

Dr C. J. Smith (Dept. of Microbiology, Glasgow University). This organism was grown for 48 h on Bordet-Gengou medium at 37°C, harvested in sterile PBS and then plated onto fresh Bordet-Gengou medium for overnight incubation at 37°C. This overnight culture was harvested in HBS and washed twice by centrifugation at 9000 g for 30 sec. The bacterial suspension was then standardised so that it had an E_{600} of 0.50. Dilutions of the preparation made in sterile PBS were plated onto selective Bordet-Gengou plates containing 40 μg cephalexin ml^{-1} (Sigma) and 30 μg nalidixic acid ml^{-1} (Sigma). Duplicate plates were used for each dilution (0.1 ml) and the viable counts obtained from each plate after incubation for 5 days at 37°C. The viable counts obtained were expressed as colony-forming units (C.F.U.) ml^{-1} of the original suspension.

4.16. Neutrophil preparation

Human peripheral blood from normal healthy volunteers was collected in heparin (10 units ml^{-1} of blood) and the neutrophils obtained after dextran sedimentation and density gradient centrifugation on Ficoll-Paque (Pharmacia) as described by Boyum (1968). Rabbit peripheral neutrophils were obtained by the method of Henson (1971). Briefly, rabbit peripheral blood was collected in acid citrate dextrose solution, centrifuged at 550 g for 20 min and the plasma and buffy coat layers discarded. Neutrophils were then isolated from the erythrocyte/neutrophil suspension by sedimentation of erythrocytes by addition of an equivalent volume of saline containing 2.5% (w/v) gelatin (Difco). Neutrophils were isolated from peripheral pig blood by the same technique except that saline containing 3% (w/v) gelatin was used. All

peripheral neutrophil preparations were washed twice in HBS prior to use.

Rabbit peritoneal neutrophils were obtained from female New Zealand White albino rabbits. Briefly, 500ml of normal saline containing 0.1% (w/v) oyster glycogen (Sigma) was injected intraperitoneally and the peritoneal exudate collected 4 h later (Lackie, 1977). The exudate was stored at 4°C and used within 2 days of isolation. Before use the cells were washed once in divalent-cation-free HBS-EDTA then in HBS. Contaminating erythrocytes were removed from all neutrophil preparations after the first wash, using hypotonic lysis. Washed cells were used within 2 h of preparation. To obtain a monodisperse population of cells for the aggregation and chemiluminescence assays the cells were passed through a 10 µm Nitex filter (Plastok Associates, Birkenhead). Cell suspensions contained >95% neutrophils and cell viability was >95% as determined by Trypan Blue exclusion. Cell numbers were standardized by counting with a haemocytometer.

4.17. Aggregation assay

The method of Lackie (1977) was used. HBS was used for suspension of the neutrophils and as diluent in all aggregation experiments. Cell suspensions were placed in polystyrene vials (41x15 mm) to which appropriate samples were added, the final volume in each vial being 1 ml. The number of cells in each vial was approximately 10^6 ml^{-1}. Vials were shaken at 140 reciprocations min^{-1} in a water bath for 1 h at 37°C. The total particle count was determined by removing an aliquot of 0.5 ml using a micropipette and a tip with an aperture enlarged to approximately 2 mm in diameter. This

sample was then diluted in 10 ml of cold, filtered 0.9% (w/v) NaCl solution containing 0.01% (w/v) sodium azide and counted on a Model Z_B Coulter Counter (Coulter Electronics Ltd. Dunstable, Bedfordshire, England). The counter was fitted with a 200 µm aperture tube; the amplification^{-1} setting was 0.5 and the aperture current^{-1} setting was 0.177. Only particles greater than 5 µm in diameter were therefore registered by the counter.

For convenience the results shown in tabular form were presented as the "Effect" (E) which was calculated as

$$E = 10.\ \log_{10}\left[\frac{\text{(count in control)}}{\text{(count in experimental)}}\right]$$

Thus inhibitors of aggregation will give a negative value of E and agents which increase aggregation will give a positive value. An E value of zero indicates no effect (Lackie, 1977). The results shown in the figures were presented as % Aggregation. This value was calculated as

$$\%\ \text{Aggregation} = \left[\frac{\text{(control count)} - \text{(experimental count)}}{\text{(count in control)}}\right] \times 100\%$$

4.18. Chemiluminescence assay

This assay was performed using an automated luminometer (Wallac LKB 1251 Luminometer) connected to a Acorn BBC 'B microcomputer. Chemiluminescence emission was measured in millivolts (mV) at 37°C. The number of neutrophils per assay tube was 10^6 and the final volume per tube was 700 µl. Luminol (Sigma) was added at the start of each assay by automatic

dispenser to a final conc of 10^{-5} M. Luminol was first prepared as a 10^{-2} M solution in dimethyl sulphoxide, diluted 100-fold in HBS, then stored at -20°C until use. The stimulus used was either the soluble synthetic peptide fMLP (Sigma) or suspensions of intact bacteria. The final concentration of fMLP per assay tube was 10^{-7} M. The bacterial stimulus was prepared from frozen (-70°C) suspensions as noted in section 4.1. The bacteria were washed twice in HBS and resuspended so that the bacterial suspension had an E_{600} of 0.50. Each wash comprised centrifugation at 9000 g for 60 sec. This suspension (50 µl) was used as the intact bacterial stimulus. If fMLP was used as the stimulus then it was added by automatic dispenser in a luminol/fMLP mixture. In the chemiluminescence assays in which intact bacteria were used as the stimulus, the bacteria were added prior to automatic dispensing of luminol.

Before addition of a stimulus, the neutrophils were always pre-heated for 20 min at 37°C. If neutrophils were pre-treated with toxins or test agents the pre-incubation times were always lenghtened to 60 min unless otherwise specified. When either fMLP or intact bacteria were used as the stimulus, samples were usually assayed in triplicate or duplicate respectively and the chemiluminescence results shown are mean values. The count time per tube was varied according to the stimulus used: 0.5 sec for fMLP and 12 sec for the bacterial stimulus. Although shown as continuous traces, the chemiluminescence curves shown in the results section are actually derived from a finite number of observations. The frequency of sampling was varied for the different stimuli: approximately every 90 sec for fMLP and every 225 sec for the bacterial stimulus. The peak values and total

chemiluminescence counts of certain of the chemiluminescence results were tabulated for easier comparison and chemiluminescence curves are usually not shown for these results.

4.19. Statistical tests

The Student t-test as described in Campbell (1981) was used to evaluate the significance of differences between the two population means in various experiments. This test was used only if the data had been obtained by random sampling from a normal, or approximately normal, distribution and the variances of the two populations did not differ significantly when tested using the F-test. If the variances of the two groups differed significantly the Fisher-Behrens test was then used to evaluate any differences between the population means (Campbell, 1981). The degree of difference between the means of the control and experimental groups were either not significant ($P>0.05$), marked with no asterisk, or significant at the levels $P<0.05$, $P<0.01$, or $P<0.001$ which was denoted with *, **, or *** respectively.

RESULTS

Part 1: The influence of B.pertussis fractions and purified components on neutrophil aggregation

5.1.1. Effect of cytoplasmic and supernatant fractions

Preliminary investigations showed the presence of neutrophil aggregating activity in cytoplasmic fractions of B.pertussis (table 5). However, cytoplasmic fractions contain a relatively large number of different components and therefore B.pertussis culture supernates were prepared since they contain fewer components, which would make it easier to characterise any aggregating factors. Identification of the aggregating factor(s) was also made easier by the separation of B.pertussis culture supernates into three fractions by pressure dialysis as follows: molecular mass > 1000 kDal (High molecular mass fraction), molecular mass < 1000 kDal but > 100 kDal (Medium molecular mass fraction), and molecular mass < 100 kDal (Low molecular mass fraction). The Low molecular mass fraction had no detectable aggregating activity while both the other two fractions were more active than the cytoplasmic fraction, with the Medium molecular mass fraction being the most active (table 5). The Medium molecular mass fraction was then used for further aggregation studies. The activity of both X-mode (virulent) and C-mode (avirulent) supernatant fractions were compared. The results indicated that the X-mode supernatant fraction had approximately four times more aggregating activity than the corresponding C-mode fraction (figure 1). B.parapertussis culture supernates were also separated into three fractions, identical to the molecular mass ranges used for the B.pertussis culture supernates. B.parapertussis X-mode Medium molecular mass fractions did

Table 5

Influence of various crude B.pertussis X-mode fractions on the aggregation of rabbit peritoneal neutrophils.

Sample	Conc (ng/ml)	Mean effect* ± SEM
Cytoplasmic	7680	+3.47*** ± 0.33
fraction	1536	+0.81*** ± 0.15
	307	+0.10*** ± 0.06
High molecular	6667	+4.18*** ± 0.48
mass supernatant	2222	+2.27*** ± 0.16
fraction	741	+0.78*** ± 0.05
Medium molecular	1000	+5.16*** ± 0.39
mass supernatant	200	+4.93*** ± 0.32
fraction	40	+3.01*** ± 0.19
Low molecular	6667	+0.16 ± 0.18
mass supernatant	2222	-0.01 ± 0.10
fraction	741	-0.10 ± 0.06

*Derived using Effect = $10.\log_{10}\left[\frac{\text{(count in control)}}{\text{(count in experimental)}}\right]$; n>4.

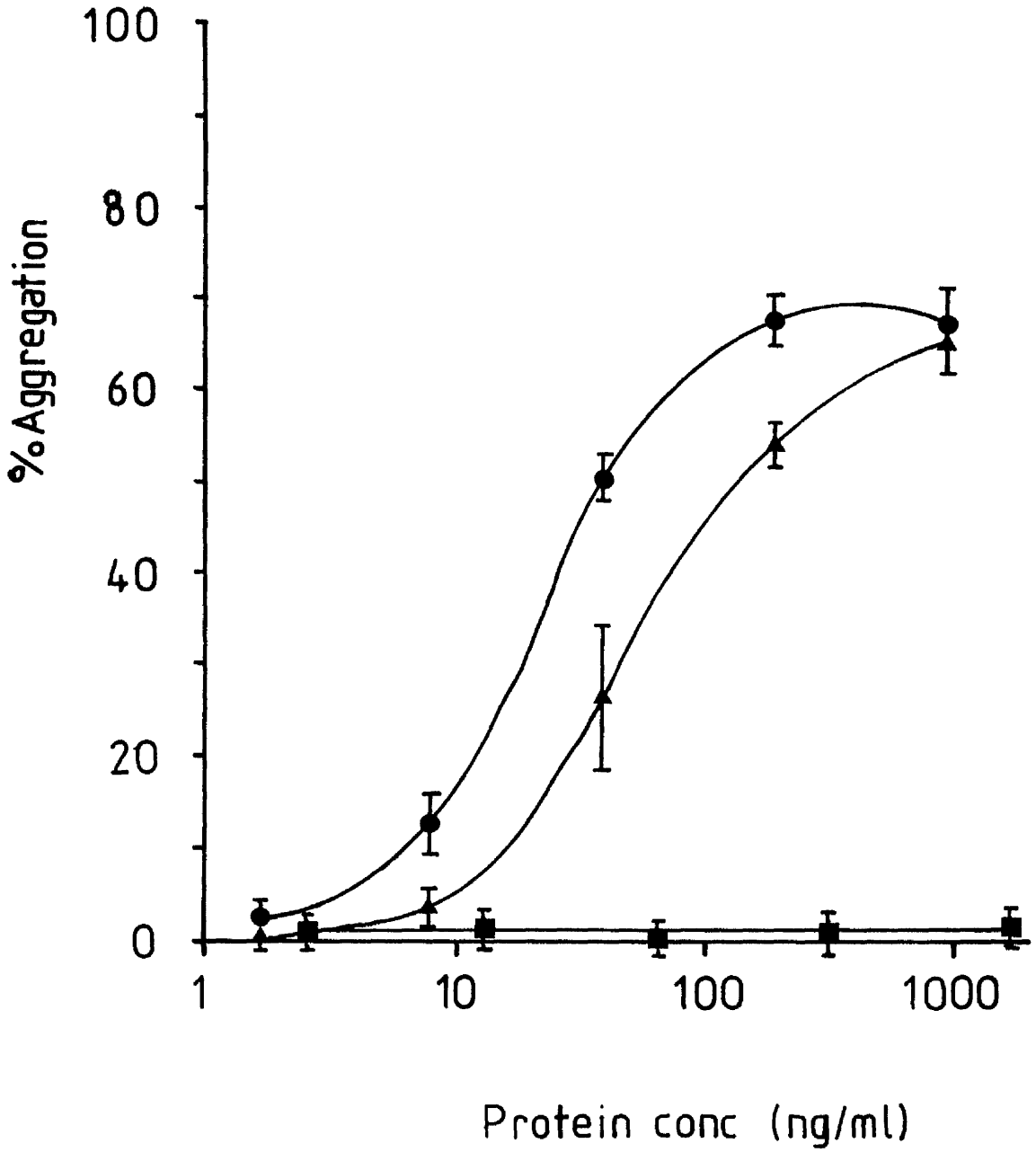
A positive value for the effect indicates an enhancement of aggregation and a negative value indicates inhibition. ***=P≤0.001, **=P≤0.01, *P=≤0.05, otherwise not significantly different from control suspension (P>0.05).

Figure 1. The aggregation of rabbit peritoneal neutrophils by B.pertussis X- and C-mode and B.parapertussis X-mode supernatant fractions. Each point shown is a mean and SEM of 8 observations.

● = B.pertussis X-mode.

▲ = B.pertussis C-mode.

■ = B.parapertussis X-mode.


100
80
60
40
20
0
% Aggregation
1
10
100
1000
Protein conc (ng/ml)

not enhance aggregation (figure 1). In addition, neither of the other two (High molecular mass or Low molecular mass) B.parapertussis supernatant fractions were active (table 6).

A Medium molecular mass fraction from uninoculated Stainer-Scholte X-mode (SS-X) culture medium had no significant effect on neutrophil aggregation (table 7). This suggested that the component(s) responsible for aggregation was a bacterial product rather than some component of the medium. In addition, the neutrophils showed the expected response to known inhibitors, theophylline and caffeine (Lackie, 1974), and a known inducer of aggregation, fMLP (O'Flaherty et al., 1977), indicating that the assay was capable of showing either inhibition or enhancement of aggregation (table 7). To confirm that the neutrophil aggregating activity observed in the B.pertussis supernatant fractions was not a general response to added protein, of any nature, BSA and fibronectin were assayed for their effects on neutrophil aggregation. These proteins were chosen since their molecular mass, BSA (66 kDal) and fibronectin (approximately 400 kDal), approximated to the lower molecular mass (70 kDal for adenylate cyclase) and higher molecular mass (220 kDal for FHA) range of the protein-containing virulence factors produced by B.pertussis. Neither BSA nor fibronectin significantly influenced neutrophil aggregation at the doses tested (table 7).

The B.pertussis supernatant fractions were also assayed on rabbit peripheral blood neutrophils (table 8). The aggregation of the rabbit peripheral cells was enhanced by both X- and C-mode fractions with the X-mode fraction being the most active. The enhancement of aggregation of rabbit peripheral cells, by either X- or C-mode fraction, was not as marked and

Table 6

Influence of *B.parapertussis* X-mode supernatant fractions on the aggregation of rabbit peritoneal neutrophils.

Supernatant fraction[1]	Conc (ng/ml)	Mean effect[*] ± SEM
High molecular mass	8000	-0.04 ± 0.16
	1600	+0.09 ± 0.16
	320	-0.04 ± 0.07
Medium molecular mass	1600	+0.04 ± 0.09
	320	+0.01 ± 0.09
	64	+0.02 ± 0.05
Low molecular mass	8000	-0.15 ± 0.17
	1600	-0.06 ± 0.12
	320	+0.01 ± 0.12

[1]Supernatant fractions derived as shown in section 4.2.

[*]Derived as in table 5; n>4. No effect was significantly different from control suspension (P>0.05).

Table 7

Influence of inhibitors, an inducer and other agents on the aggregation of rabbit peritoneal neutrophils.

Agent	Conc	Mean effect* ± SEM
Theophylline	5.0 mM	-0.45*** ± 0.04
Caffeine	5.0 mM	-0.50*** ± 0.02
FMLP	2×10^{-6} M	+11.6*** ± 0.30
BSA	12.0 µg ml^{-1}	-0.17 ± 0.06
	4.0 µg ml^{-1}	-0.10 ± 0.05
Fibronectin	3.8 µg ml^{-1}	+0.18 ± 0.09
	0.9 µg ml^{-1}	-0.11 ± 0.10
SS-X medium[1]	1 in 25 (v/v)	+0.09 ± 0.08

[1]A Medium molecular mass fraction, which had been concentrated 26-fold, obtained from uninoculated X-mode medium.

*Derived as in table 5; n>4.

Table 8

Influence of the *B.pertussis* X- and C-mode supernatant fractions[1] on the aggregation of peripheral neutrophils.

Mode	Protein conc (ng/ml)	Mean effect[*] ± SEM Human	Pig	Rabbit
X	2222	+0.03 ± 0.18	-0.13 ± 0.08	+0.79*** ± 0.16
	741	-0.01 ± 0.04	-0.05 ± 0.09	+0.51** ± 0.03
	247	+0.07 ± 0.06	-0.09 ± 0.07	+0.44** ± 0.05
C	2222	-0.03 ± 0.18	+0.02 ± 0.07	+0.29** ± 0.11
	741	-0.04 ± 0.21	-0.05 ± 0.07	+0.26* ± 0.08
	247	+0.03 ± 0.17	-0.02 ± 0.07	+0.17 ± 0.10

[1]Medium molecular mass fractions.

[*]Derived as in table 5; n>4.

did not occur at such low doses as with rabbit peritoneal neutrophils. When similar doses of either X- or C-mode fraction were assayed on human or pig peripheral blood neutrophils, no significant effects were found.

The data for the influence of the B.pertussis supernatant fractions on neutrophils from different species, although obtained using the same assay techniques, had been obtained at different times. Further measurements were then made using human peripheral and rabbit peritoneal neutrophils in the same experiment. The cells were placed in separate vials and the influence of the B.pertussis X-mode supernatant fraction on neutrophil aggregation was assayed. The results confirmed that the B.pertussis X-mode supernatant fraction enhanced the aggregation of rabbit peritoneal but not that of human peripheral neutrophils (table 9).

An additional experiment was performed to assay whether trace erythrocyte contamination of the peripheral neutrophil preparations was perhaps affecting neutrophil aggregation. Trace amounts of erythrocytes may have preferentially bound any active component(s) in the supernatant fractions and therefore have stopped binding of the active component(s) to the peripheral neutrophil preparations. This would perhaps have explained why the aggregation of human or pig peripheral neutrophils was not influenced by X- or C-mode B.pertussis supernatant fractions. Lysed human erythrocytes were added to the most responsive cells, rabbit peritoneal neutrophils, and the aggregation pattern compared with that of an erythrocyte-free preparation of the same neutrophil suspension. A study showed that the B.pertussis X-mode supernatant fraction caused aggregation of rabbit peritoneal neutrophils even in the presence of erythrocytes (table 10).

Table 9

The species-specificity of the neutrophil aggregating activity in the *B.pertussis* X-mode supernatant fraction[1].

Protein conc (ng/ml)	Mean effect[†] ± SEM	
	Rabbit peritoneal	Human peripheral
1000	+5.27* ± 0.59	+0.05 ± 0.05
200	+4.04* ± 0.33	+0.12 ± 0.09
40	+1.35* ± 0.05	+0.11 ± 0.09

[1]Medium molecular mass fraction.

[†]Derived as in table 5; n=2.

Table 10

The influence of human erythrocyte contamination on the rabbit peritoneal neutrophil aggregating activity in the *B.pertussis* X-mode supernatant fraction[1].

Protein conc (ng/ml)	Mean effect[†] ± SEM minus erythrocytes	plus erythrocytes[2]
1000	+5.31* ± 0.04	+5.98* ± 0.24
200	+3.29* ± 0.11	+3.84* ± 0.32
40	+0.68 ± 0.07	+0.85 ± 0.02

[1]Medium molecular mass fraction.

[2]Peripheral human blood erythrocytes had been obtained by dextran sedimentation after 30 min at 37°C. After two washes in HBS, 10 µl of the packed human erythrocyte pellet was lysed in 1 ml of distilled water. 25 ml of HBS was then added to the lysed cell suspension and 0.15 ml of this preparation was added to each vial.

[†]Derived as in table 5; n=2.

These results (tables 9 and 10) appeared to confirm that the species-specificity of the neutrophil aggregating activity in the supernatant fractions was not artefactual.

5.1.2. Influence of heating on supernatant fraction activity

A known characteristic of PT is that it loses biological activity after heating at 80°C for 30 min (Wardlaw and Parton, 1983). No aggregation-promoting activity was found in the X- or C-mode B.pertussis supernatant fractions after such heat-treatment (table 11). This indicated that the component(s) responsible for aggregation was not heat-stable, thereby ruling out LPS, and that the neutrophil aggregating activity in the supernatant fractions may have been due to PT. The effect of similar heat-treatment on FHA activity is unknown (but see section 5.1.7.).

5.1.3. Enhancement by pertussis toxin

To find whether the neutrophil aggregating activity detected in the B.pertussis supernatant fractions was due in part or full to PT or to some other component, purified PT (LIST Biological Laboratories Ltd) was assayed on various neutrophil types (table 12). Purified PT enhanced aggregation of all three neutrophil types tested, with rabbit peritoneal neutrophils being the most responsive. Unlike the B.pertussis supernatant fractions, PT enhanced aggregation of human peripheral neutrophils. PT was not assayed on pig peripheral neutrophils. The X- and C-mode B.pertussis supernatant fractions both enhanced the aggregation of rabbit peritoneal neutrophils at lower doses than PT. The differences

Table 11

Influence of heat-treatment of the *B.pertussis* supernatant fractions[1] on the aggregation of rabbit peritoneal neutrophils.

Mode	Protein conc (ng/ml)	Mean effect* ± SEM Unheated	Heated[2]
X	1000	+5.16*** ± 0.39	+0.03 ± 0.12
	200	+4.93*** ± 0.32	-0.13 ± 0.09
	40	+3.01*** ± 0.19	-0.12 ± 0.06
C	1000	+4.82*** ± 0.39	+0.03 ± 0.13
	200	+3.43*** ± 0.20	-0.16 ± 0.03
	40	+1.56*** ± 0.40	-0.09 ± 0.07

[1]Medium molecular mass fractions.

[2]80°C for 30 min.

*Derived as in table 5; n=8 for unheated and 4 for heated.

Table 12

Influence of purified pertussis toxin on neutrophil aggregation.

Dose (ng/ml)	Mean effect* ± SEM Rabbit peritoneal[a]	Rabbit peripheral	Human peripheral[a]
1000	+6.54*** ± 0.32	+2.32*** ± 0.08	+2.34*** ± 0.33
200	+1.32** ± 0.21	+0.15* ± 0.02	+0.51 ± 0.15
40	+0.20 ± 0.08	+0.18* ± 0.03	+0.26 ± 0.08

*Derived as in table 5; n=4 or [a]8 observations.

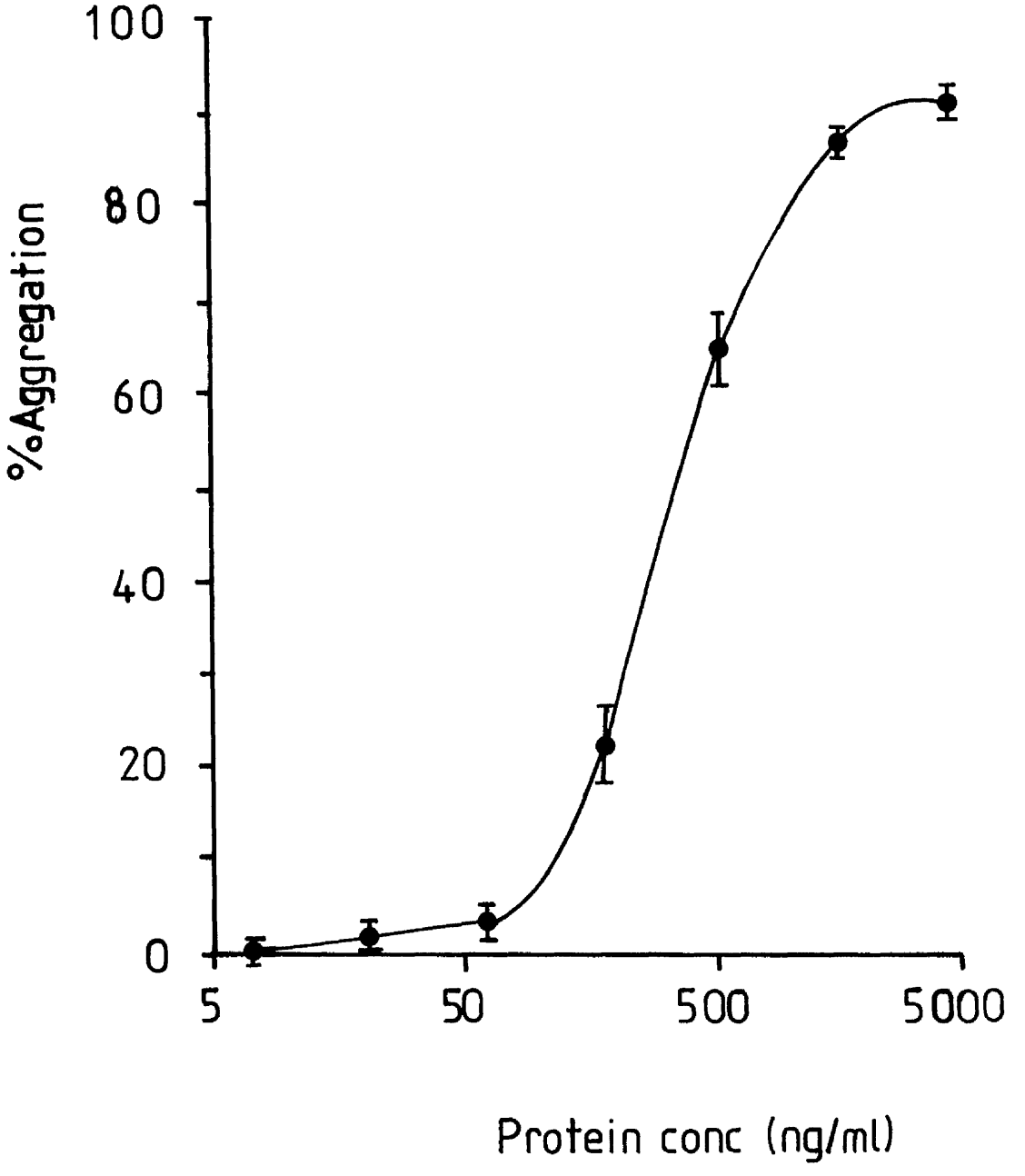
observed between the aggregating activities of PT and the supernatant fractions suggested that other factors may be involved in influencing the aggregation patterns obtained with the supernatant fractions. A dose-response curve (figure 2) was obtained using PT prepared by the method of Perera et al. (1985). This toxin sample appeared to have similar aggregating activity to the preparation from LIST Biological Laboratories Ltd (table 12). The PT preparation of Perera et al. (1985) was used for all further studies involving this toxin.

5.1.4. Biological, enzymic and SDS-PAGE analysis of supernatant fractions

In an attempt to identify which component of B.pertussis was responsible for the rabbit neutrophil aggregating activity in the supernatant fractions, the fractions were examined for the presence of known biologically-active components of B.pertussis. The heat-labile toxin of B.pertussis can be assayed by its ability to induce a haemorrhagic response in mouse skin. Parton (1986) reported that a dose of 1.2 µg of crude heat-labile toxin (B.pertussis cytoplasmic fraction) caused a 17-20 mm haemorrhagic zone when injected subcutaneously into 3-5 week old mice. Graded doses (containing 5-10 µg protein) of the supernatant fractions were injected subcutaneously into groups of mice but no zones of reaction were observed after 24 h, indicating that no detectable heat-labile toxin was present.

The B.pertussis supernatant fractions were examined for the presence of haemagglutinating activity since both PT and FHA are haemagglutinins (Irons and MacLennan, 1979). No detectable agglutination of horse or rabbit erythrocytes was found in

Figure 2. The influence of pertussis toxin on the aggregation of rabbit peritoneal neutrophils. Each point shown is a mean and SEM of 5 observations.


100
80
60
40
20
0
%Aggregation
5
50
500
5000
Protein conc (ng/ml)

either the B.pertussis X- or C-mode supernatant fraction at the doses tested (up to 5 µg protein per well). These erythrocyte species are not the most sensitive for assaying B.pertussis haemagglutinins (Irons and MacLennan, 1979). Gorringe et al. (1986) reported that the haemagglutinating activity of FHA is lost when FHA is incubated under shaken conditions. The B.pertussis supernate fractions used were isolated from a shaken culture and any FHA present may have lost, or have reduced, haemagglutinating activity. Although active PT might have been present, low levels may not have been detected using horse or rabbit erythrocytes. A more sensitive assay for PT would have been a PT ELISA. An FHA ELISA of the B.pertussis supernatant fractions was done and the results are shown in section 5.1.9.

The adenylate cyclase of B.pertussis can be measured in an enzymic assay which involves two steps: incubation of the sample with ATP, the substrate from which the adenylate cyclase generates cAMP, and then measurement of the cAMP generated by a competitive binding assay involving radiolabelled cAMP (section 4.12.). The cAMP assay used would detect 0.2 pmol per assay tube. Using this assay, no detectable adenylate cyclase activity was found in doses (5 µg protein per assay tube) of B.pertussis supernatant fraction (table 13). However, a low level of adenylate cyclase activity was detected in the more concentrated B.parapertussis supernatant fraction (14 µg protein per assay tube).

The B.pertussis supernatant fractions were also examined using SDS-PAGE to assay whether any of the bands correlated with the known molecular mass of any of the B.pertussis virulence factors. SDS-PAGE of the samples (containing 1.5 µg protein) showed mainly the presence of low molecular mass,

Table 13

Adenylate cyclase activity of the supernatant fractions[1].

Supernatant fraction	Protein conc (µg/tube)	Cyclic AMP* produced (pmol cAMP min^{-1})	Adenylate cyclase (pmol cAMP min^{-1} mg protein^{-1})
B.pertussis (X-mode)	5	none detected	-
B.pertussis (C-mode)	5	none detected	-
B.parapertussis (X-mode)	14	4	286

[1]Medium molecular mass fractions.

*Standard curve shown in section 8.4. (figure 19).

diffuse bands which were of similar molecular mass to purified B.pertussis LPS and stained using a LPS-specific stain (plate 1). These bands were resistant to protease suggesting that the bands were non-proteinaceous in nature (plate 2). Coomassie blue stain of the gels was not done since there was not enough protein present in the samples to be detected by this staining method.

5.1.5. Effect of LPS from B.pertussis and other bacteria

B.pertussis LPS had been detected in those B.pertussis supernatant fractions which contained potent neutrophil aggregating activity. A visual comparison of SDS-PAGE patterns (plate 1) indicated that the probable levels of LPS in the X- or C-mode B.pertussis supernatant fraction loaded onto the gel was less than the concentration of the purified reference B.pertussis LPS i.e. less than 1.5 µg LPS. Since the samples of supernatant fractions loaded onto the gels contained 1.5 µg protein, and the protein dose-range of the supernatant fractions which contained active neutrophil aggregating activity had previously been determined (figure 1), the approximate concentrations of LPS over this active dose-range could be calculated. The highest degree of neutrophil aggregation observed with a supernatant fraction occurred with a dose of 1.0 µg protein ml^{-1} and therefore doses of LPS around the figure of 1.0 µg LPS ml^{-1} were assayed. No significant enhancement of the aggregation of rabbit peritoneal neutrophils was found using purified B.pertussis LPS over the range tested (table 14). (The limulus amoebocyte lysate assay (Sigma Chemical Company, 1973) would have given a more exact estimation of the LPS

Plate 1. SDS-PAGE of B.pertussis Medium molecular mass supernatant fractions. The final concentration of each component loaded per well was: 1.5 µg protein of the B.pertussis X- or C-mode supernatant fraction, and 1.5 µg of B.pertussis LPS. The gel was stained using the periodate LPS staining technique of Tsai and Frasch (1982).

1 = C-mode supernatant fraction.

2 = X-mode supernatant fraction.

3 = B.pertussis LPS.


1 2 3

Plate 2. Effect of protease-treatment on the SDS-PAGE profiles of the B.pertussis Medium molecular mass supernatant fractions. B.pertussis X- and C-mode supernatant fractions samples (containing 10 µg protein) and 10 µg B.pertussis LPS were treated with 1 µg of proteolytic enzyme (protease (type VII) or ∝-chymotrypsin (type VII); Sigma) for 30 min at 25°C. Solubilizing buffer was then added to each sample and the mixtures heated at 100°C for 5 min prior to loading on the gel. The final concentration of each component loaded per well was: 1.5 µg protein of the X- and C-mode supernatant fraction, 1.5 µg of B.pertussis LPS, and 0.15 µg of protease or ∝-chymotrypsin. The gel was stained using the silver stain technique of Oakley et al. (1980).

1 = Molecular mass standards.

2 = Protease.

3 = ∝-chymotrypsin.

4 = B.pertussis LPS and protease.

5 = B.pertussis LPS and ∝-chymotrypsin.

6 = B.pertussis LPS.

7 = Molecular mass standards.

8 = C-mode supernatant fraction and protease.

9 = C-mode supernatant fraction and ∝-chymotrypsin.

10 = C-mode supernatant fraction.

11 = X-mode supernatant fraction and protease.

12 = X-mode supernatant fraction and ∝-chymotrypsin.

13 = X-mode supernatant fraction.

14 = B.pertussis LPS.

Molecular mass (kDal)

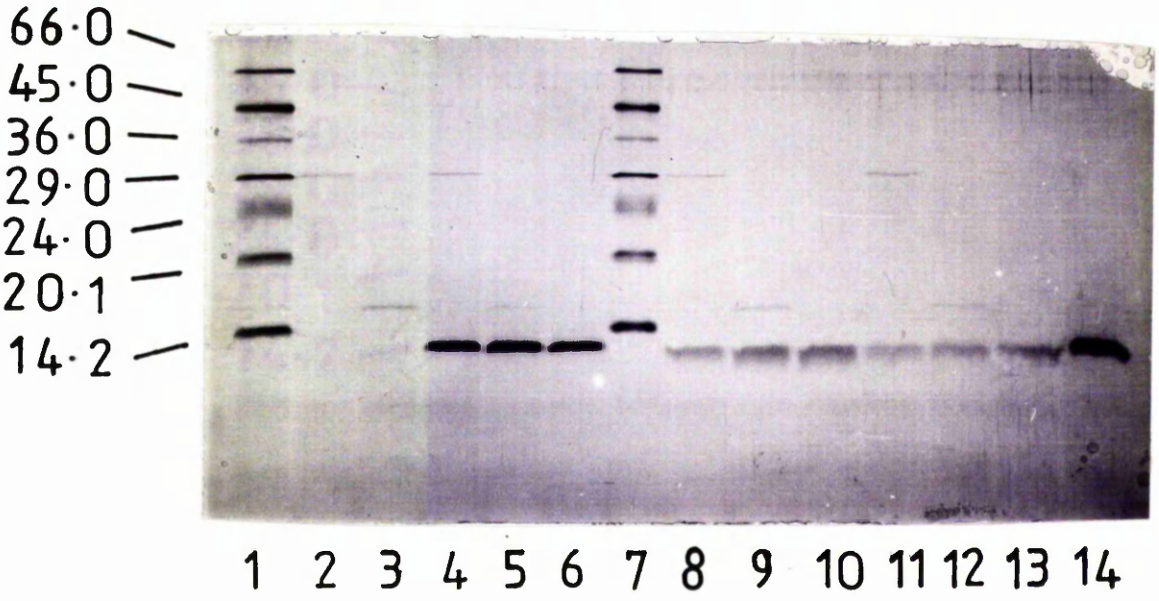


Table 14

Influence of different types of LPS on the aggregation of rabbit peritoneal neutrophils.

Dose (µg/ml)	Mean effect* ± SEM, with LPS from *B.pertussis*	*S.minnesota* Re595	*S.minnesota*
10.0	−0.01 ± 0.16	−0.01 ± 0.24	−0.01 ± 0.04
1.0	+0.05 ± 0.16	+0.16 ± 0.19	−0.13 ± 0.06
0.1	+0.08 ± 0.07	+0.20 ± 0.12	−0.11 ± 0.01

*Derived as in table 5; n>4. No effect was significantly different from control suspension (P>0.05).

concentration of the B.pertussis supernatant fractions.)

A characteristic of smooth LPS is that it produces a laddering effect, with multiple bands, on SDS-PAGE (Tsai and Frasch, 1982). The results indicated that B.pertussis LPS is a a rough LPS, since no laddering effect was observed after SDS-PAGE (plates 1 and 2). To assess whether a smooth LPS may influence the aggregation of rabbit peritoneal neutrophils smooth LPS from Salmonella minnesota was assayed. This was compared with the rough LPS of Salmonella minnesota Re595 and the rough LPS of B.pertussis. No LPS had any significant effect over the range tested (table 14). Doses of LPS over 10 µg diminished neutrophil viability.

5.1.6. Influence of filamentous haemagglutinin

FHA is a potent haemagglutinin, especially active against goose erythrocytes, although erythrocytes from other species are also agglutinated. The effect of this haemagglutinating factor on neutrophil aggregation was investigated. FHA enhanced the aggregation of rabbit peritoneal neutrophils and appeared to be the most active bacterial component tested (table 15). FHA also enhanced the aggregation of rabbit peripheral neutrophils though the effect was not as marked and did not occur at such low doses as with rabbit peritoneal cells. Human peripheral neutrophil aggregation was not influenced by FHA. The effect of FHA on the aggregation of pig peripheral neutrophils was not investigated. There appears to be a similarity between the neutrophil aggregating activity of both purified FHA and the B.pertussis supernatant fractions since both enhanced the aggregation of rabbit but not human neutrophils. A dose-response curve of FHA-enhancement of

Table 15

Influence of purified FHA on neutrophil aggregation.

Dose (ng/ml)	Mean effect* ± SEM Rabbit peritoneal	Rabbit peripheral	Human peripheral
344	+5.80*** ± 0.32	+1.33** ± 0.29	+0.14 ± 0.37
115	+4.82*** ± 0.31	+0.52** ± 0.14	-0.15 ± 0.24
38	+4.22*** ± 0.18	-0.07 ± 0.15	-0.24 ± 0.11

*Derived as in table 5; n=4.

aggregation of rabbit peritoneal neutrophils was obtained (figure 3). These results suggested that FHA may be the major neutrophil aggregating factor present in the B.pertussis supernatant fractions.

5.1.7. Effect of heating on aggregating activity of filamentous haemagglutinin

Heat-treatment (80°C for 30 min) had destroyed the neutrophil aggregating activity present in the B.pertussis supernatant fractions (table 11). If FHA was the major neutrophil aggregating factor present in the supernatant fractions then similar heat-treatment should destroy the neutrophil aggregating activity of FHA. The neutrophil aggregating activity of FHA was indeed destroyed by such heat-treatment (table 16). This again suggested that FHA may have been the active neutrophil aggregating factor present in the B.pertussis supernatant fractions.

5.1.8. Enzymic, immunological and SDS-PAGE analysis of pertussis toxin preparations

The results had so far suggested that FHA and possibly PT were both capable of enhancing neutrophil aggregation, with FHA being the most active. Although PT appeared to have a different species-specificity from FHA, it was possible that minor contaminants of the PT preparations used were contributing to the neutrophil aggregation patterns obtained for the toxin. The PT preparations of both LIST Biological Laboratories Ltd and that obtained by the method of Perera et al. (1985) were therefore analysed for contamination by

Figure 3. The influence of purified FHA on the aggregation of rabbit peritoneal neutrophils. Each point shown is a mean and SEM of 4 observations.

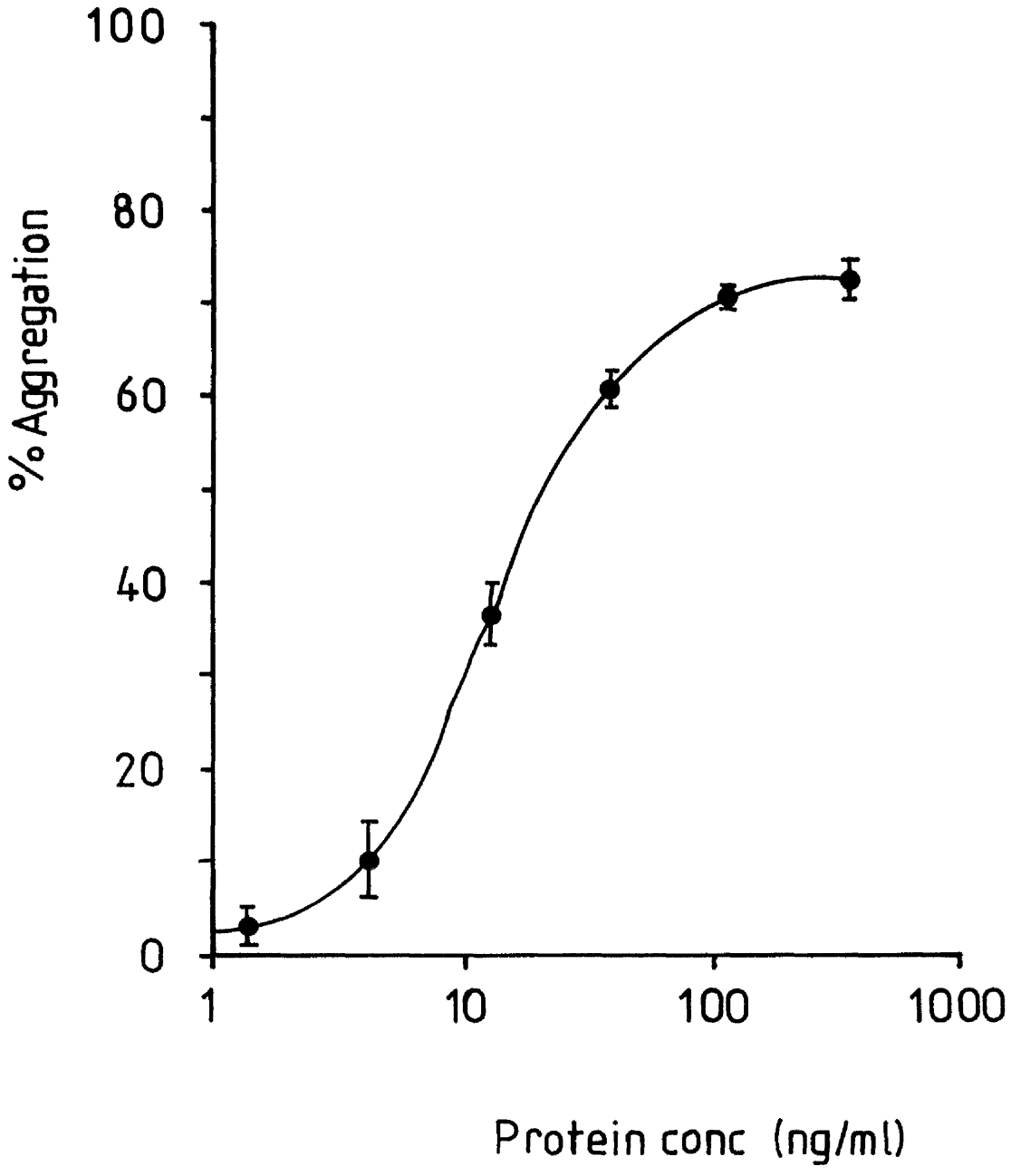

100
80
60
40
20
0
% Aggregation
1
10
100
1000
Protein conc (ng/ml)

Table 16

Influence of heat-treatment of FHA on its aggregating activity for rabbit peritoneal neutrophils.

Dose (ng/ml)	Mean effect† ± SEM Unheated	Heated‡
344	+5.80*** ± 0.32	+0.14 ± 0.11
115	+4.82*** ± 0.31	+0.06 ± 0.14
38	+4.22*** ± 0.18	-0.03 ± 0.12

‡80°C for 30 min.

†Derived as in table 5; n=4.

other B.pertussis products.

No detectable adenylate cyclase activity (i.e. < 0.2 pmol/min/µg protein) was found in a sample (13 µg per assay tube) of the PT preparation of Perera et al. (1985). A representative standard curve is shown in section 8.4. (figure 19). The adenylate cyclase activity of the LIST Biological Laboratories Ltd preparation was reported to contain less than 1.0 pmol/min/µg of toxin by the manufacturer.

The LIST Biological Laboratories Ltd preparation was assayed in an FHA ELISA. No detectable levels of FHA were found (table 17). The FHA ELISA could detect a minimum of between 3-5 ng FHA per well. The PT preparation of Perera et al. (1985) was not analysed by FHA ELISA due to an insufficient concentration of toxin.

Examination of both PT preparations by SDS-PAGE showed the expected pattern of five subunits (Tamura et al., 1982). However, a contaminating band was also observed in both preparations (plate 3). An additional three contaminating bands were observed in the toxin preparation of Perera et al. (1985). SDS-PAGE of FHA shows multiple polypeptides, ranging from molecular mass 58,000 to 220,000 kDal (Irons et al. 1983), and it is possible that some of the contaminating bands observed in the PT preparations were actually FHA fragments. The low molecular mass, diffuse band observed only in the toxin preparation of Perera et al. (1985) has been confirmed as being LPS, which comprised about 4% (w/w) of this PT preparation (Dr. U. Y. Perera: personal communication).

Table 17

An FHA ELISA of the supernatant fractions[1] and the pertussis toxin preparation supplied by LIST Biological Laboratories Ltd.

Sample	Protein conc (ng/well)	A_{492}	FHA[†] conc (ng/well)
B.pertussis X-mode	2212	0.029	< 3
supernatant fraction	1106	0.025	< 3
B.pertussis C-mode	2225	0.034	< 3
supernatant fraction	1112	0.030	< 3
B.parapertussis X-mode	5571	0.025	< 3
supernatant fraction	2786	0.030	< 3
Purified PT	2500	0.026	< 3
preparation (LIST)	1250	0.026	< 3

[1]Medium molecular mass fractions.

[†]Standard curve shown in section 8.4. (figure 20).

Plate 3. SDS-PAGE of the different pertussis toxin preparations. The plate was supplied by Dr. V. Y. Perera (Dept. of Microbiology, Glasgow University). The source of the toxin sample is shown in brackets. The gel was comprised of a discontinuous gradient of 13.5-20 % Acrylamide. The solubilizing buffer contained 4 M urea. The gel was stained using the silver stain technique of Oakley et al. (1980). The contaminating bands, possibly FHA fragments, are labelled "a". The LPS band present in the toxin preparation of Dr. V. Y. Perera is labelled with a "b".

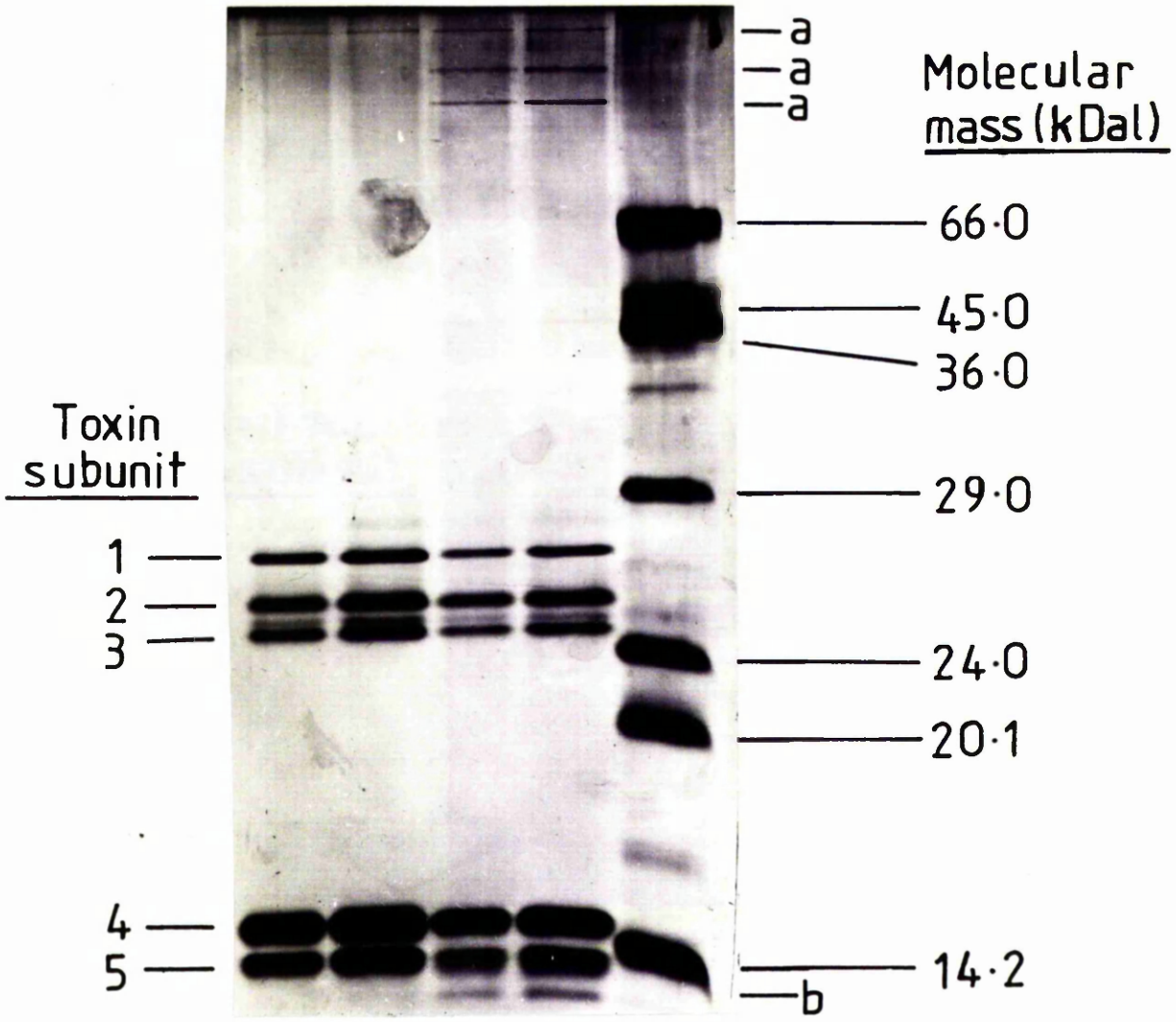
1 = 1.0 µg pertussis toxin (LIST Biological Laboratories Ltd).

2 = 2.0 µg pertussis toxin (LIST Biological Laboratories Ltd).

3 = 1.0 µg pertussis toxin (Dr. V. Y. Perera).

4 = 2.0 µg pertussis toxin (Dr. V. Y. Perera).

5 = Molecular mass standards.


a
a
a
Molecular mass (kDal)
66·0
45·0
36·0
29·0
24·0
20·1
14·2
b
Toxin subunit
1
2
3
4
5

5.1.9. Immunological analysis of the B.pertussis supernatant fractions

Studies had indicated that the neutrophil aggregating factor present in the B.pertussis supernatant fractions was possibly FHA. However, the B.pertussis culture supernate fractions used had been isolated from shaken cultures, in the absence of cyclodextrin, and would be expected to contain little, if any, FHA activity (Arai and Munoz, 1979). An FHA ELISA of both the X- and C-mode supernatant fractions found no detectable FHA (table 17). The FHA ELISA used could detect a minimum of 3-5 ng FHA per well. A possible explanation of this apparent contradiction is discussed in section 6.1.1.

Part 2: The influence of purified bacterial components on rabbit peritoneal neutrophil chemiluminescence

5.2.1. Enhancement by filamentous haemagglutinin and LPS

FHA enhances rabbit peritoneal neutrophil aggregation (figure 3); since enhancement of aggregation is often associated with metabolic effects, the influence of FHA on the chemiluminescent response of rabbit peritoneal neutrophils was assayed. *B.pertussis* LPS had no influence on neutrophil aggregation (table 14) and was assayed to determine whether any correlation could be made between the ability of a bacterial component to influence neutrophil aggregation and chemiluminescence. Both FHA and LPS induced neutrophil chemiluminescence directly (table 18). A longer count time gave higher chemiluminescence counts, as expected, and was more sensitive in detecting any differences between the experimental and control mixtures. The smooth LPS of *Salmonella minnesota* and the rough LPS of *Salmonella minnesota* Re595 were also assayed for their effects on neutrophil chemiluminescence. The LPS of *Salmonella minnesota* Re595 induced higher levels of neutrophil chemiluminescence than either the rough LPS of *B.pertussis* or the smooth LPS of *Salmonella minnesota* at the 0.5 sec count time. The LPS of *Salmonella minnesota* Re595 was the roughest LPS assayed and there may be a correlation between degree of roughness of an LPS and its ability to stimulate neutrophil chemiluminescence directly. The results suggested that bacterial components which induce neutrophil chemiluminescence do not necessarily also influence neutrophil aggregation.

The chemiluminescence counts obtained at each individual

Table 18

Direct stimulation of rabbit peritoneal neutrophil chemiluminescence by various purified bacterial products.

Count time (sec)	Test agent conc per vial	mV chemiluminescence ± SEM[1] Experimental	Control
0.5[2]	50 ng FHA	20.61** ± 0.28	18.34 ± 0.16
	1 µg *B.pertussis* LPS	25.65** ± 1.32	19.02 ± 0.67
	1 µg *S.minnesota* smooth LPS	23.61** ± 1.00	19.02 ± 0.67
	1 µg *S.minnesota* Re595 LPS	61.73*** ± 1.09	19.02 ± 0.67
12.0[3]	50 ng FHA	285.75*** ± 13.28	170.50 ± 4.56
	1 µg *B.pertussis* LPS	472.67*** ± 23.02	242.00 ± 14.5

[1]Individual chemiluminescence counts were summed over the total assay time and the mean value obtained for each sample. ($n>4$ observations). For experimental values: ***=$P\leq 0.001$, **=$P\leq 0.01$, otherwise not significantly different from control value ($P>0.05$).

[2]The assay was done for 30 min with each vial being counted approximately every 90 sec.

[3]The assay was done for 60 min with each vial being counted approximately every 225 sec.

sampling time were not much higher than background levels. A more sensitive technique of assaying the influence of bacterial components on neutrophil chemiluminescence is to assay the effects of the components on neutrophils stimulated by a different, more active stimulus. Both fMLP and intact bacteria stimulate a strong, characteristic chemiluminescence response when incubated with neutrophils. (Bender and Van Epps, 1983; Horan et al., 1982). The effects of purified bacterial components and B.pertussis fractions on the chemiluminescent response of rabbit peritoneal neutrophils induced by either fMLP or intact B.pertussis were therefore studied. The results are shown in Part 3 and Part 4 of the Results section.

Part 3: The influence of purified bacterial components and B.pertussis fractions on the chemiluminescent response of rabbit peritoneal neutrophils to fMLP

5.3.1. Effect of B.pertussis supernatant fractions

The B.pertussis X- and C-mode Medium molecular mass supernatant fractions, used for the aggregation studies, were also assayed for their influence on the chemiluminescent response of rabbit peritoneal neutrophils to the pro-inflammatory mediator fMLP. The neutrophils were pre-incubated in the presence of the fraction before addition of the stimulus and the light-enhancing reagent luminol. FMLP-induced stimulation of neutrophils causes a bimodal chemiluminescent response in the presence of luminol. The first peak is extracellular in origin while the second peak is cell-associated (Bender and Van Epps, 1983). A typical bimodal chemiluminescent response of rabbit peritoneal neutrophils induced by fMLP is shown in figure 4. To simplify the presentation of the chemiluminescence results, the individual points were joined and the chemiluminescence curves given as shown are continuous traces through the points and without the SEM bars.

A B.pertussis X-mode supernatant fraction enhanced chemiluminescence (figure 5). The enhancing effect was observed even after pre-incubation for 15 min (figure 5, curve c) and longer pre-incubation times led to increased enhancement. Maximal enhancement appeared to occur after pre-incubation for approximately 60 min (figure 5, curve a). A longer pre-incubation period of 90 min gave no further enhancement (data not shown). The peak values (highest count

Figure 4. Neutrophil chemiluminescent response to fMLP. Rabbit peritoneal neutrophils were pre-incubated for 60 min before addition of the stimulus. Each point shown is a mean and SEM of 3 observations. The data used was from figure 10 (curve d).

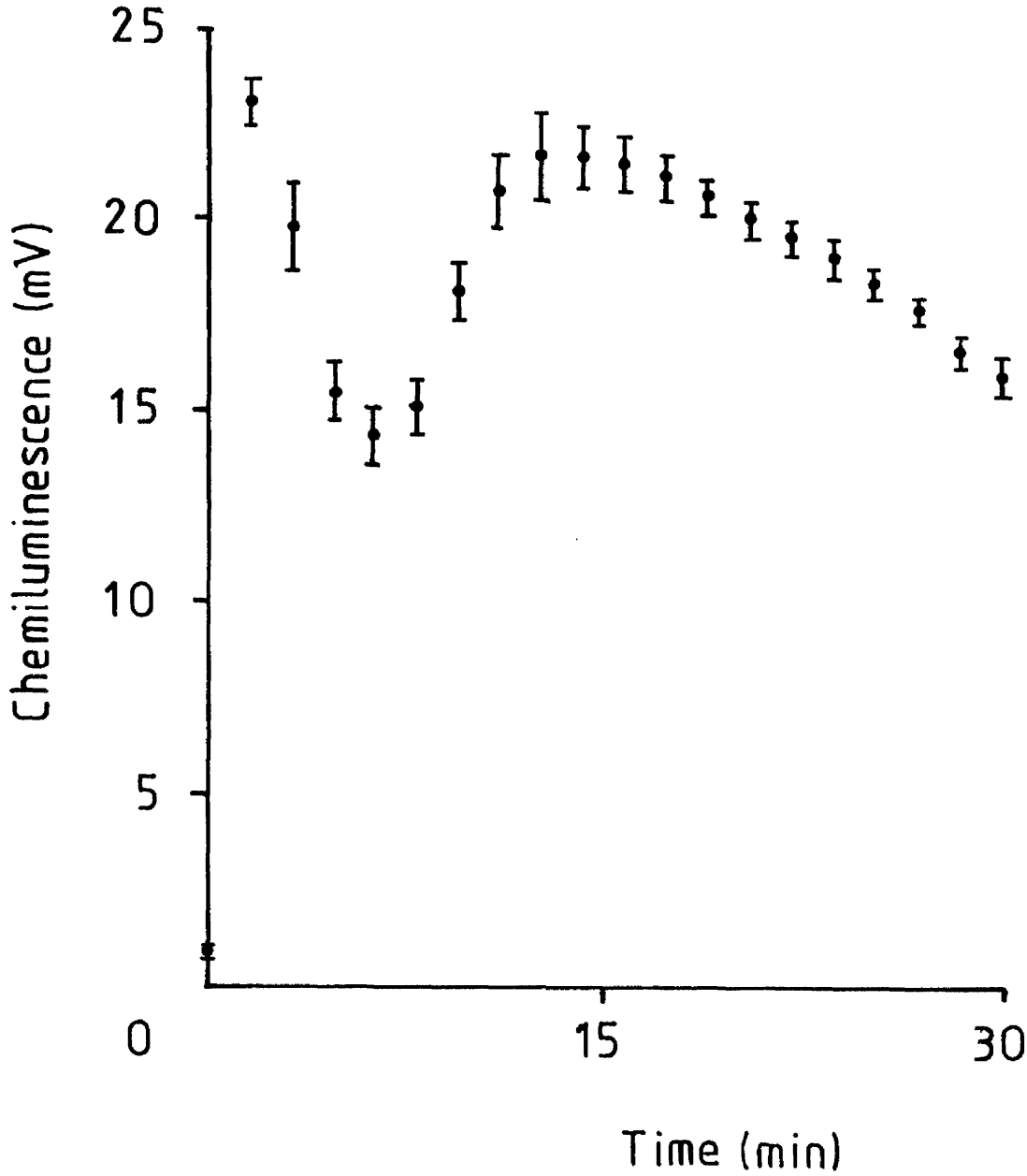

25
20
15
10
5
0
15
30
Chemiluminescence (mV)
Time (min)

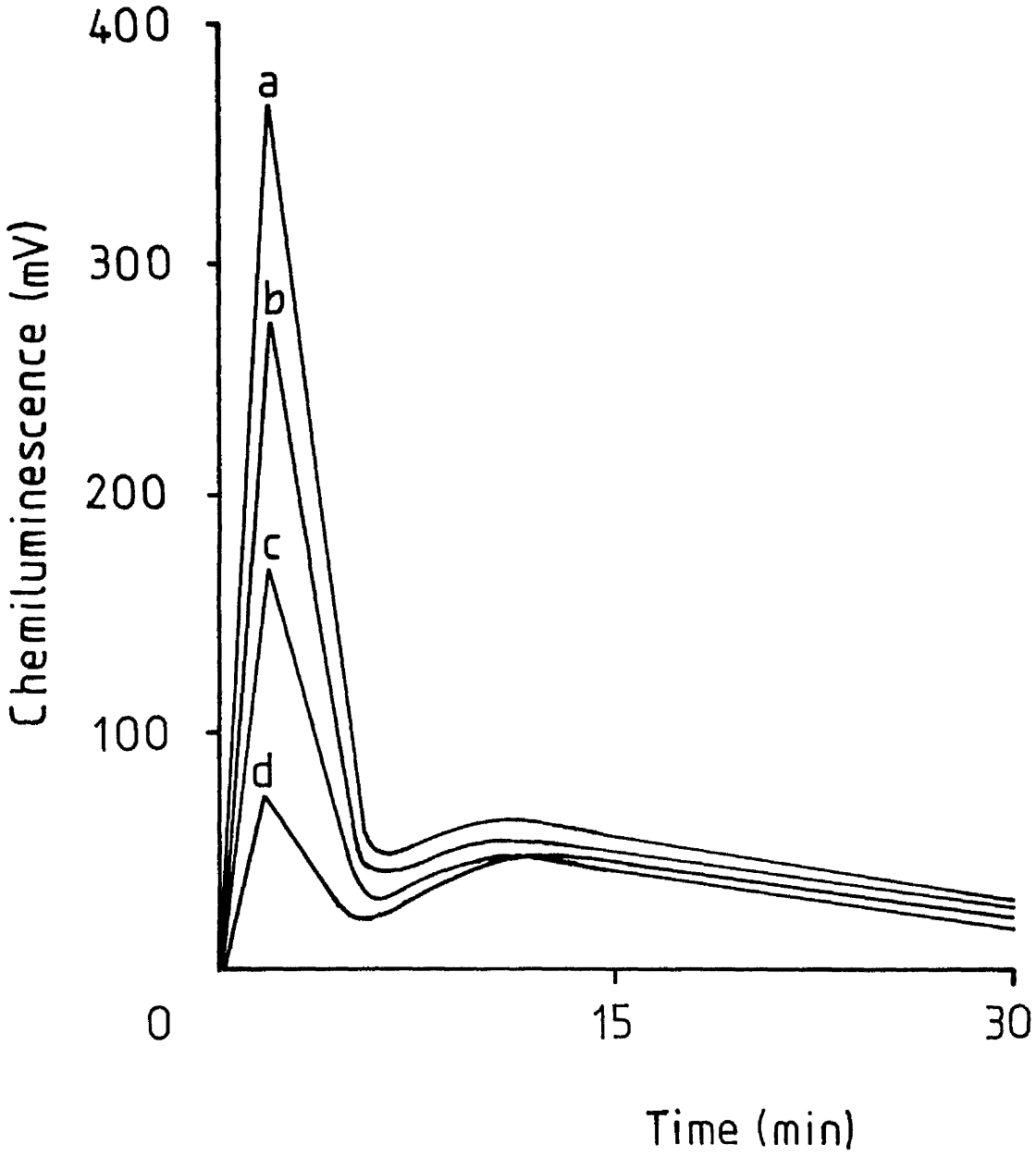
Figure 5. The influence of pre-incubation time on the effect of a B.pertussis X-mode supernatant fraction on fMLP-induced chemiluminescence of neutrophils. Rabbit peritoneal neutrophils were pre-incubated in the presence of of the supernatant fraction (containing 2100 ng protein per vial) for various times before addition of the stimulus. Supernatant fraction was not added to the control. The figure shown is representative of 2 separate experiments.

a = 60 min.

b = 30 min.

c = 15 min.

d = Control (buffer).


400
300
200
100
0
15
30
Chemiluminescence (mV)
Time (min)
a
b
c
d

over the time of assay) and total chemiluminescence counts of the data shown in figure 5 were tabulated and there was a positive correlation between chemiluminescence peak value and the total chemiluminescence produced (table 19).

An uninoculated culture medium fraction, as used in table 7, was assayed to determine its effect on the chemiluminescent response of rabbit peritoneal neutrophils to fMLP. This was done to assess whether some component of the medium was perhaps responsible for the enhancement of the neutrophil chemiluminescent response to fMLP shown in figure 5. The 1 in 50 and 1 in 100 dilutions caused a slight enhancement of the peak values but did not effect the overall chemiluminescence produced (table 20). The 1 in 10 dilution had no significant effect on either the peak chemiluminescence value or on the overall chemiluminescence produced. In comparison, the B.pertussis X-mode supernatant fraction dramatically enhanced both the chemiluminescence peak value and total chemiluminescence produced (table 19). An approximately equivalent concentration of the culture medium fraction had no effect confirming that a component produced by B.pertussis, and not some component of the medium, was responsible for the effects noted. A B.pertussis C-mode supernatant fraction also enhanced neutrophil chemiluminescence (table 21). At both doses tested the B.pertussis X-mode supernatant fraction induced significantly higher chemiluminescence peak values ($P<0.01$) and total chemiluminescence production ($P<0.001$) than similar doses of the C-mode supernatant fraction.

5.3.2. Enhancement by filamentous haemagglutinin

FHA induced the chemiluminescence of rabbit peritoneal

Table 19

The influence of pre-incubation time on the effect of a *B.pertussis* X-mode supernatant fraction[1] on fMLP-induced chemiluminescence of neutrophils: peak values and total chemiluminescence.

Sample	Pre-incubation time (min)	mV chemiluminescence ± SEM[†] Peak value		Total	
Buffer	30	70.77	± 7.81	722.58	± 29.13
Supernatant	15	167.53**	± 9.31	889.28*	± 44.04
Supernatant	30	274.17***	± 6.18	1130.62**	± 66.91
Supernatant	60	364.53***	± 32.91	1336.57***	± 16.28

[1]The Medium molecular mass supernatant fraction; dose=2100 ng protein per vial.

[†]Peak value was the highest chemiluminescence value observed over 30 min while total chemiluminescence was the sum of the individual chemiluminescence counts over 30 min. Values given are a mean of 3 observations. For experimental values: ***=$P \leq 0.001$, **=$P \leq 0.01$, or *=$P \leq 0.05$, otherwise not significantly different from control value ($P > 0.05$).

Table 20

The influence of uninoculated culture medium[1] on fMLP-induced chemiluminescence of neutrophils.

Sample	Dilution	mV chemiluminescence ± SEM[†]	
		Peak value	Total
Buffer	-	24.40 ± 0.77	346.92 ± 35.56
Medium	1 in 10	24.75 ± 0.49	267.62 ± 11.88
Medium	1 in 50	27.21* ± 0.64	337.30 ± 38.61
Medium	1 in 100	27.84* ± 1.26	354.28 ± 36.16

[1]A Medium molecular mass fraction which had been concentrated 26-fold.

[†]Derived as in table 19; n=6.

Table 21

The influence of B.pertussis X- and C-mode supernatant fractions[1] on fMLP-induced chemiluminescence of neutrophils.

Sample	Protein conc (ng/vial)	mV chemiluminescence ± SEM[†] Peak value	Total
Buffer	-	55.72 ± 4.69	555.76 ± 37.11
C-mode	420	123.26*** ± 5.67	778.19*** ± 40.58
	2100	213.41** ± 11.51	981.96*** ± 23.52
X-mode	420	248.82** ± 18.94	1187.53*** ± 76.26
	2100	321.67** ± 25.07	1301.35*** ± 32.21

[1]Medium molecular mass fractions.

[†]Derived as in table 19; n=6.

neutrophils directly (table 18), therefore FHA was assayed on the chemiluminescent response of these cells to fMLP. FHA enhanced neutrophil chemiluminescence at the relatively low doses of 10-50 ng (figure 6). A dose of 250 ng (figure 6, curve a) had approximately the same enhancing effect as a dose of 50 ng (figure 6, curve b) for about the first 10 min, although a slight inhibitory effect was observed with the 250 ng dose after about 20 min. A study of the response with time (figure 7) indicated that maximal enhancement occurred after a pre-incubation time of about 60 min. An enhancing effect was first observed after a pre-incubation time of about 15 min. These results implicated FHA as the factor in the B.pertussis supernatant fractions responsible for the enhancement of the neutrophil chemiluminescent response to fMLP (section 5.3.1.).

To determine whether the enhancement of the neutrophil chemiluminescent response to fMLP by FHA was non-specifically caused by the addition of extracellular protein, 100 ng BSA (molecular mass=66 kDal) and 100 ng fibronectin (molecular mass approximately 400 kDal) were assayed for their effect on the neutrophil chemiluminescent response to fMLP (table 22). Of the different proteins tested, only FHA had any significant influence on the neutrophil chemiluminescent response. Thus, FHA-enhancement of the neutrophil chemiluminescent response to fMLP appears to be due to the specific structure of FHA, rather than the fact that it is was simply a protein. Although, a dose of 50 ng FHA had a slight stimulatory effect even in the absence of fMLP (table 18), this stimulation is not enough to account for the FHA-enhancement of neutrophil chemiluminescence in the presence of fMLP. Thus, the enhancement of neutrophil chemiluminescent response to fMLP by

Figure 6. The effect of purified FHA on fMLP-induced chemiluminescence of neutrophils. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of FHA for 60 min before addition of the stimulus. No FHA was added to the control. The figure shown is representative of 3 separate experiments.

a = 250 ng.

b = 50 ng.

c = 10 ng.

d = Control (buffer).

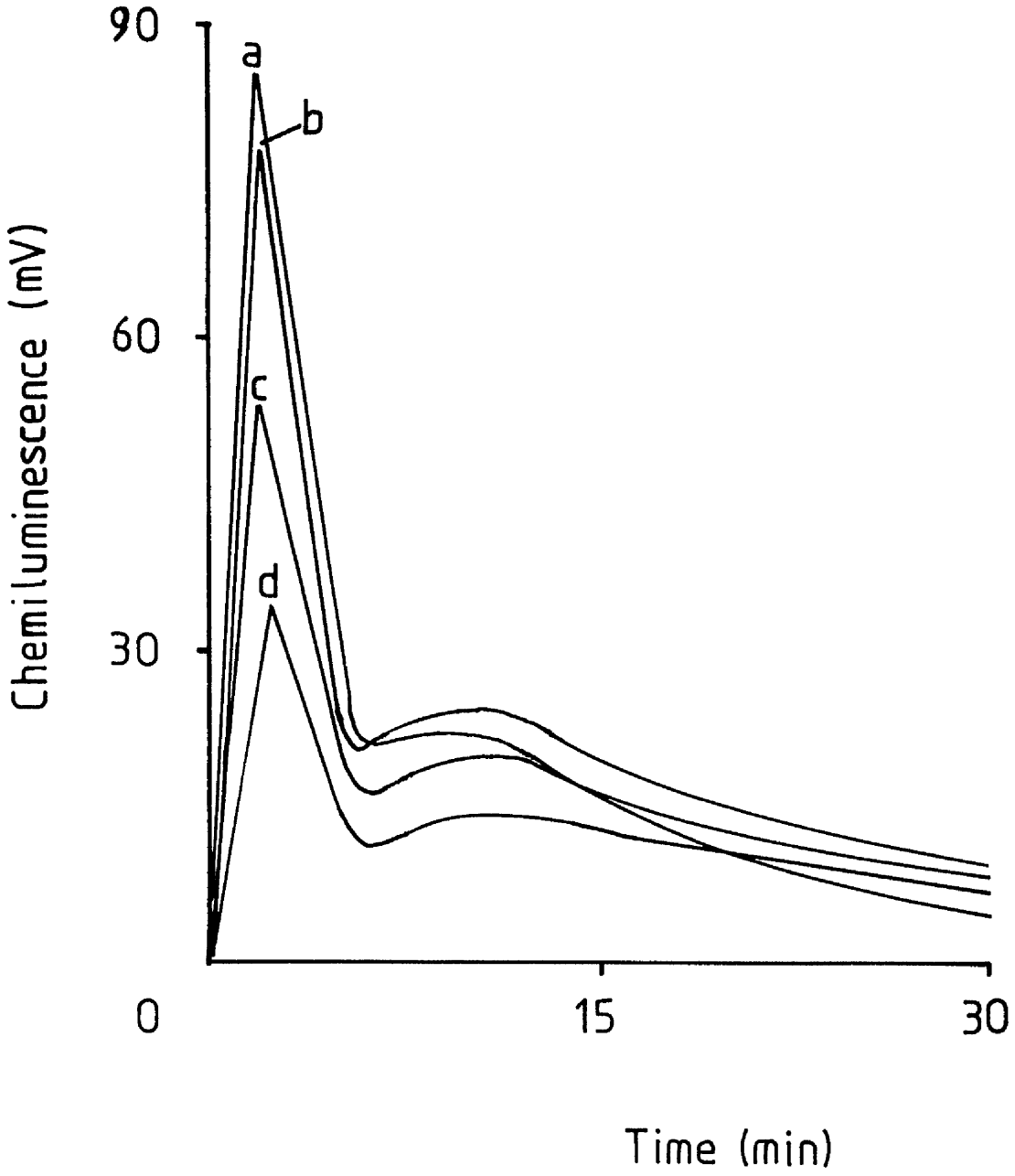

90
60
30
0
15
30
a
b
c
d
Chemiluminescence (mV)
Time (min)

Figure 7. The effect of pre-incubation time on the influence of purified FHA on neutrophil chemiluminescent response to FMLP. Rabbit peritoneal neutrophils were pre-incubated in the presence of 50 ng FHA per vial for various times before addition of the stimulus. No FHA was added to the control. The individual chemiluminescence counts were summed over 30 min and expressed as % Chemiluminescence compared to the control. The control was given a value of 100%. Each point shown is a mean and SEM of 6 observations.

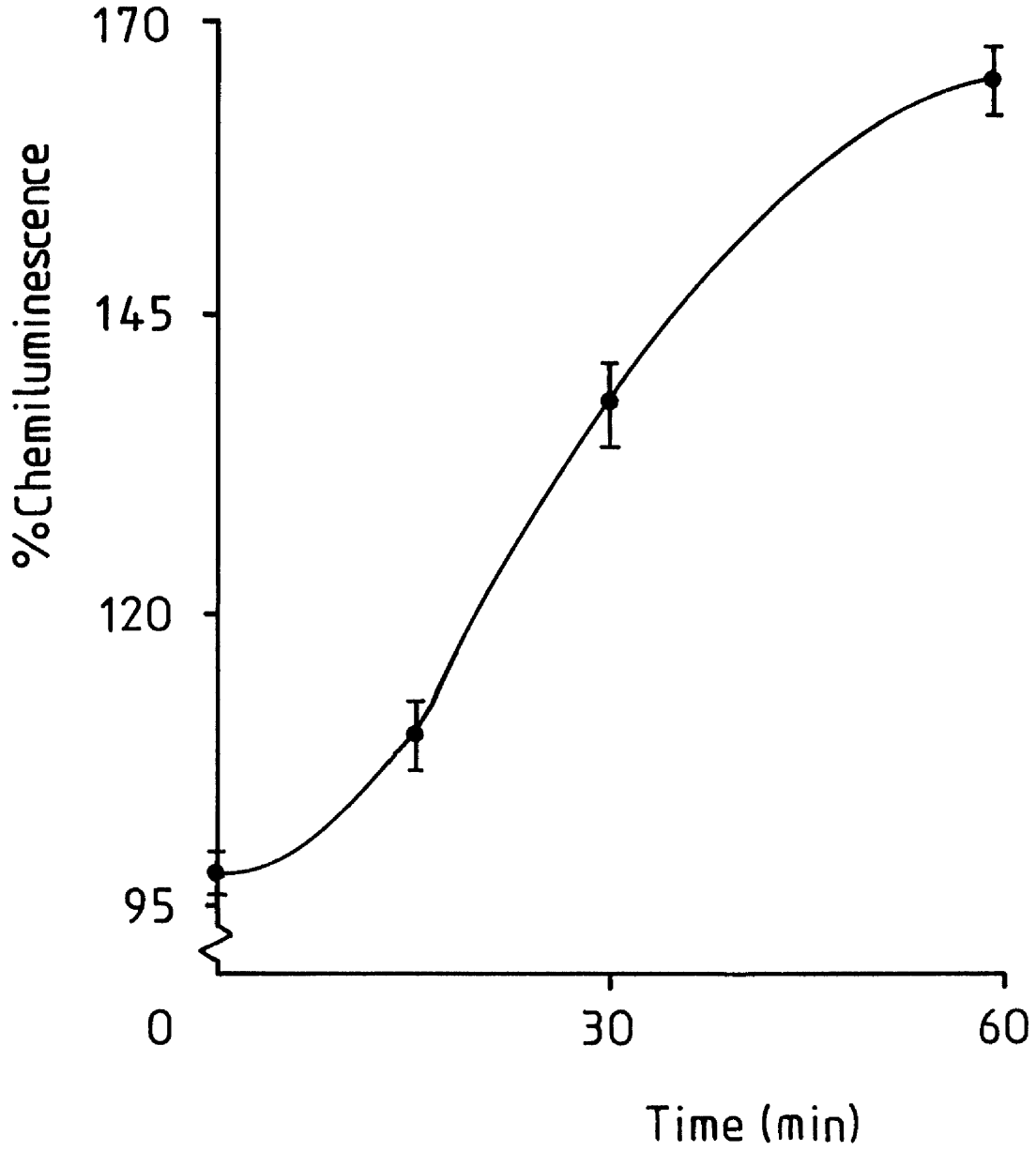

170
145
120
95
0
30
60
%Chemiluminescence
Time (min)

Table 22

The effect of various proteins on neutrophil chemiluminescent response to fMLP.

Sample	Conc per vial (ng)	mV chemiluminescence ± SEM† Peak value		Total	
Buffer	-	46.32	± 6.69	420.00	± 25.98
FHA	50	173.42***	± 11.66	809.94***	± 4.96
BSA	100	41.05	± 9.64	389.71	± 42.95
Fibronectin	100	43.95	± 7.27	387.36	± 20.66

†Derived as in table 19; n=6.

FHA appears to be a synergistic effect of the two agents.

5.3.3. Enhancement by B.pertussis LPS

LPS appeared to be a major component of the B.pertussis X- and C-mode supernatant fractions (plates 1 and 2). Since B.pertussis LPS may have influenced the chemiluminescence results obtained for the supernatant fractions LPS was therefore assayed for its possible effects on neutrophil chemiluminescence (figure 8). B.pertussis LPS enhanced neutrophil chemiluminescence at doses as low as 1 µg (figure 8, curve b) with maximal enhancement occurring with a dose of 10 µg (figure 8, curve a). A 0.1 µg dose of LPS had no significant effect on the neutrophil chemiluminescent response to fMLP (figure 8, curve c). Although increased neutrophil chemiluminescence was obtained with doses of LPS exceeding 10 µg, neutrophil viability was diminished by such treatment.

B.pertussis LPS required a pre-incubation time of about 15 min before any enhancement effect was observed (figure 9). Maximal enhancement occurred with a pre-incubation time of about 60 min. The results (table 21) indicated that doses of the B.pertussis supernatant fractions, down to 420 ng protein per vial, significantly enhanced neutrophil chemiluminescence. Using the same calculations as in 5.1.5., the levels of LPS likely to be present in the lowest dose of the B.pertussis supernatant fractions assayed (420 ng protein per vial) would be less than 420 ng LPS per vial. LPS enhanced neutrophil chemiluminescence only at doses of about 1000 ng and above. This indicated that some factor, other than LPS, was responsible for enhancement of the neutrophil chemiluminescent response to fMLP observed with the X- or

Figure 8. The influence of B.pertussis LPS on fMLP-induced chemiluminescence of neutrophils. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of LPS for 60 min before addition of the stimulus. No LPS was added to the control. The figure shown is representative of 8 separate experiments.

a = 10 µg LPS.

b = 1 µg LPS.

c = 0.1 µg LPS

d = Control (buffer).

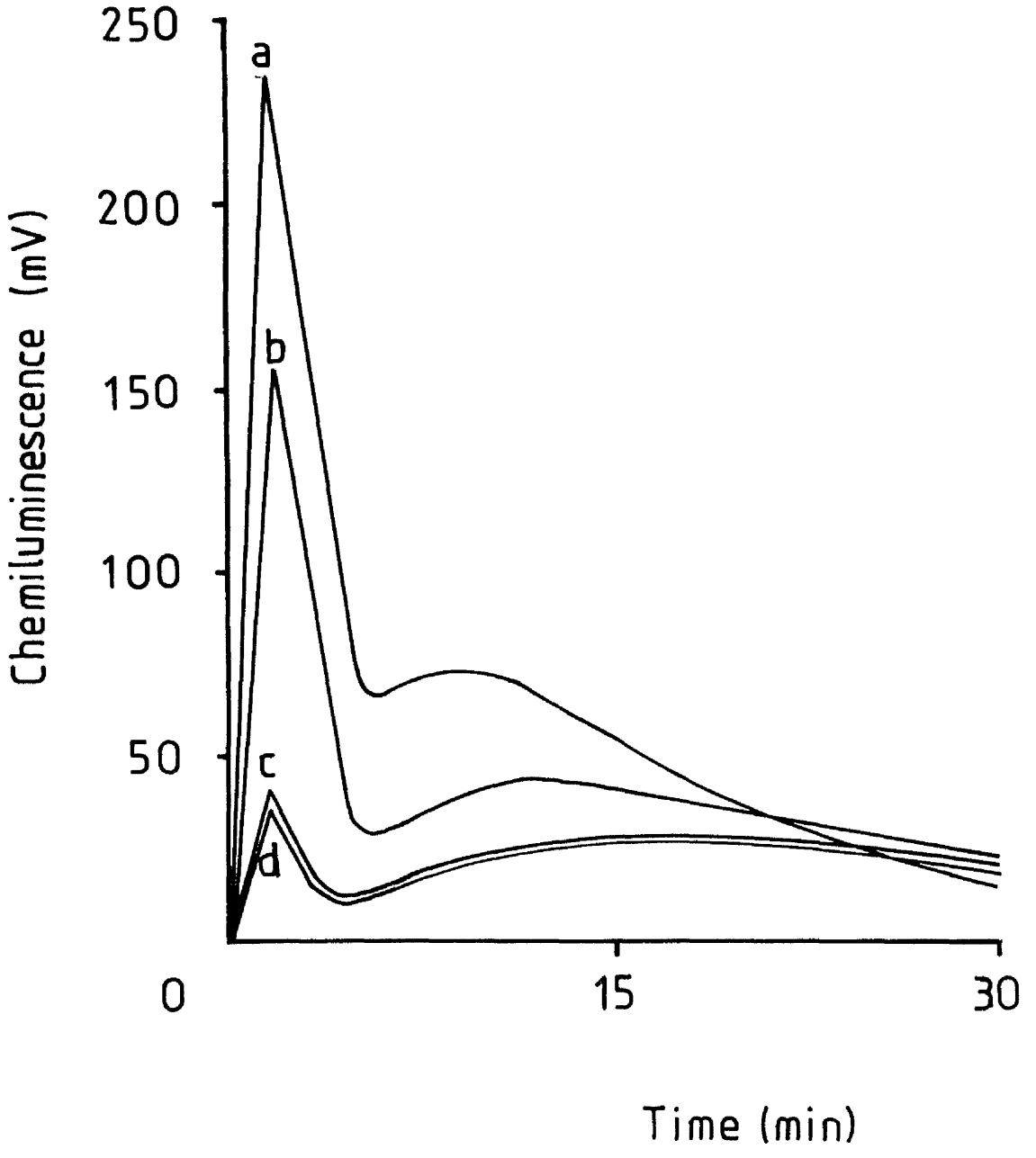
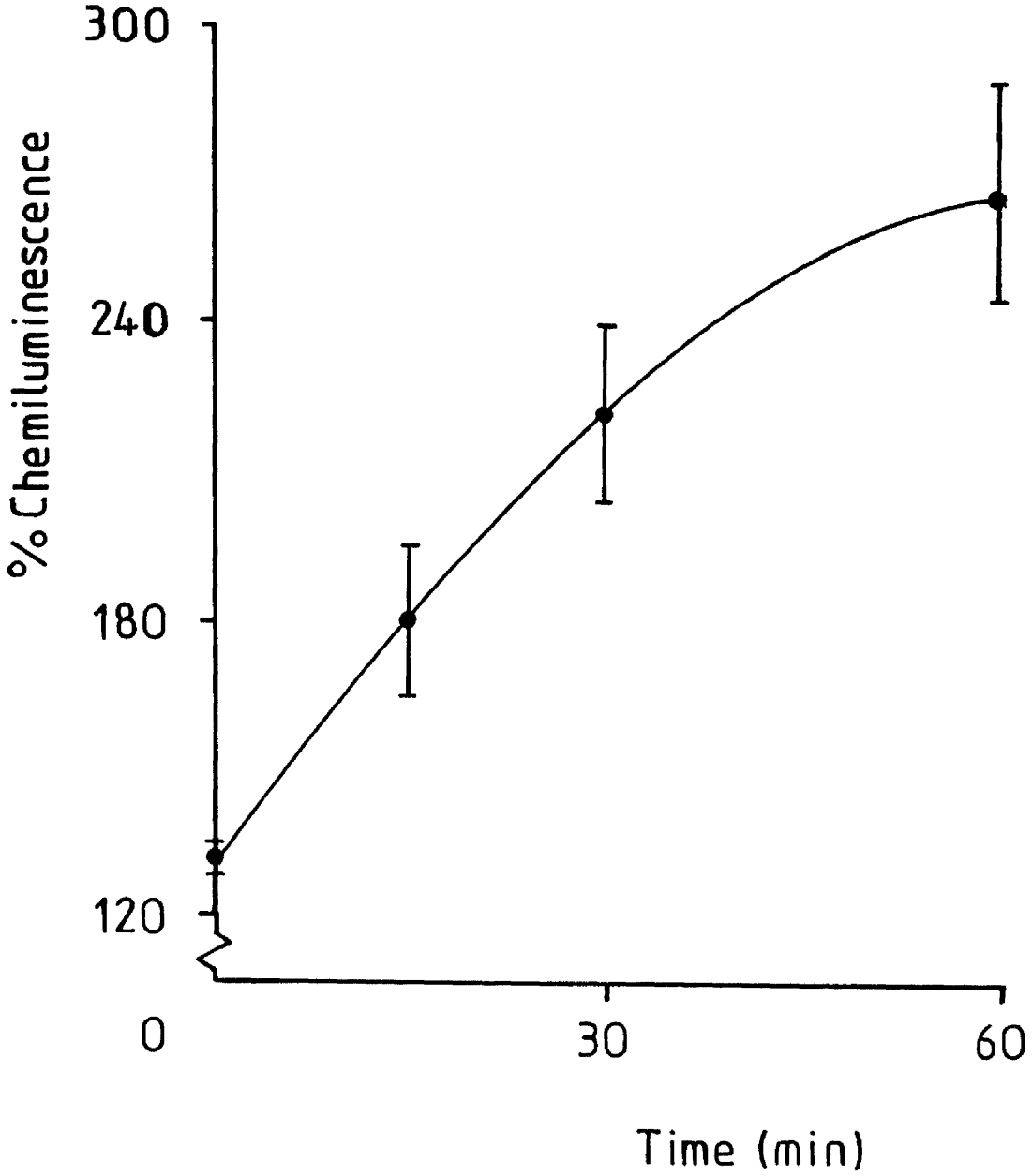

250
200
150
100
50
0
15
30
Chemiluminescence (mV)
Time (min)
a
b
c
d

Figure 9. The effect of pre-incubation time on the influence of B.pertussis LPS on fMLP-induced chemiluminescence of neutrophils. Rabbit peritoneal neutrophils were pre-incubated in the presence of 10 µg LPS per vial for various times before addition of the stimulus. No LPS was added to the control. The individual chemiluminescence counts were summed over 30 min and expressed as % Chemiluminescence relative to the control. The control was given a value of 100%. Each point shown is a mean and SEM of 6 observations.


300
240
180
120
0
% Chemiluminescence
30
60
Time (min)

C-mode B.pertussis supernatant fraction. In addition, the C-mode supernatant fraction contained slightly more LPS, as judged from SDS-PAGE (plate 1), than the X-mode supernatant fraction but was not as active at enhancing the neutrophil chemiluminescent response to fMLP. This also suggests that some other factor, possibly FHA, was the dominant factor responsible for the enhancement of the neutrophil chemiluminescent response to fMLP noted with the B.pertussis X- or C-mode supernatant fraction. Although B.pertussis LPS caused a slight enhancement of neutrophil chemiluminescence, even in the absence of fMLP (table 18), this stimulation was not enough to account for B.pertussis LPS-enhancement of neutrophil chemiluminescent response seen in the presence of fMLP. Thus, like FHA, LPS-enhancement of neutrophil chemiluminescent response to fMLP appears to be due to a synergistic effect of the two agents.

5.3.4. A comparison of B.pertussis LPS with LPS from Salmonella minnesota

B.pertussis LPS (rough) was compared to the LPS from Salmonella minnesota (smooth) and Salmonella minnesota Re595 (rough) to find whether B.pertussis LPS enhancement of the neutrophil chemiluminescent response to fMLP was specific to B.pertussis LPS, or typical of LPS in general (table 23). Salmonella minnesota Re595 (rough) LPS was the most active, B.pertussis LPS the second most active, while Salmonella minnesota (smooth) LPS was the least active enhancer of neutrophil chemiluminescence. The higher dose (10 µg) of Salmonella minnesota (smooth) LPS was not as active as the lower dose (1 µg), suggesting that neutrophil responsiveness

Table 23

A comparison of the influence of B.pertussis and Salmonella minnesota LPS on fMLP-induced chemiluminescence of neutrophils.

Sample	Conc per vial (μg)	mV chemiluminescence ± SEM* Peak value	Total
Buffer	-	26.35 ± 2.62	346.62 ± 11.17
B.pertussis LPS	1	90.36*** ± 13.20	667.34*** ± 21.84
	10	151.00*** ± 4.37	962.81** ± 39.29
S.minnesota Re595	1	192.92*** ± 2.38	847.31*** ± 21.65
LPS (rough)	10	176.01*** ± 6.07	912.29** ± 29.39
S.minnesota LPS	1	74.96** ± 10.12	578.55*** ± 21.96
(smooth)	10	50.86*** ± 3.13	361.86 ± 25.97

*Derived as in table 19; n=6.

was diminished by treatment with the higher but not the lower dose. Thus, the effect of B.pertussis LPS on fMLP-induced chemiluminescence of neutrophils was not unique to that LPS. All three LPS types stimulated neutrophil chemiluminescence in the absence of fMLP (table 18). This stimulatory effect may have contributed to LPS-enhancement of the neutrophil chemiluminescent response observed in the presence of fMLP (table 23). There was a correlation between degree of stimulus by LPS in the absence of fMLP and enhancement of chemiluminescence by LPS in its presence. For example, Salmonella minnesota Re595 (rough) LPS induced the highest levels of chemiluminescence both in the absence and presence of fMLP.

5.3.5. Inhibition by pertussis toxin

PT inhibits rabbit peritoneal neutrophil secretory responses and chemotaxis induced by fMLP (Becker et al., 1985). The effect of PT on fMLP-induced chemiluminescence of rabbit peritoneal neutrophils was investigated. The PT preparation of Perera et al. (1985) was inhibitory (figure 10), and the degree of inhibition increased with dose of toxin: 6.4 ng caused a slight suppression (figure 10, curve c) with 160 ng causing almost complete suppression of chemiluminescence (figure 10, curve a). Pre-incubation of the neutrophils for 15 min in the presence of the toxin had no significant effect ($P>0.05$) on total chemiluminescence production (figure 11(i)). Significant inhibition of chemiluminescence ($P<0.01$) was first observed after a pre-incubation time of 30 min with maximal suppression occurring after a pre-incubation time of 60 min. The results suggest that 50% inhibition of total

Figure 10. The inhibitory effect of pertussis toxin on fMLP-induced chemiluminescence of neutrophils. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of the toxin for 60 min before addition of the stimulus. No toxin was added to the control. The figure shown is representative of 7 separate experiments.

a = 160 ng.

b = 32 ng.

c = 6.4 ng.

d = Control (buffer).

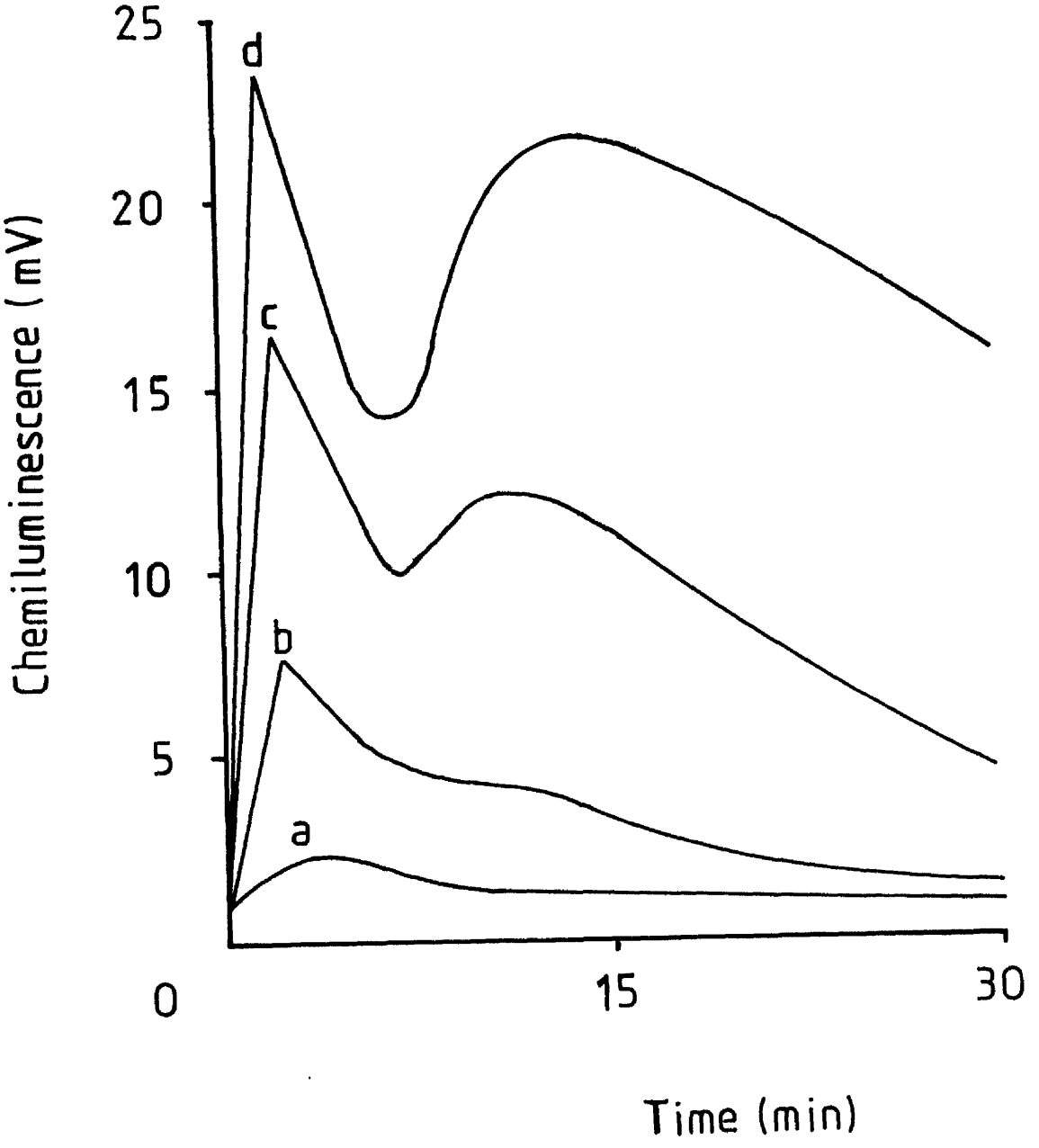

25
20
15
10
5
0
15
30
d
c
b
a
Chemiluminescence (mV)
Time (min)

Figure 11. The influence of pre-incubation time on the effect of pertussis toxin on neutrophil chemiluminescent response to fMLP. Rabbit peritoneal neutrophils were pre-incubated for various times in the presence of 160 ng of toxin per vial before addition of the stimulus. No toxin was added to the control.

(i) Total measured chemiluminescence.

The individual chemiluminescence counts were summed over 30 min and expressed as % Chemiluminescence relative to the control. The control was given a value of 100%. Each point shown is a mean and SEM of 6 observations.

(ii) A chemiluminescence curve.

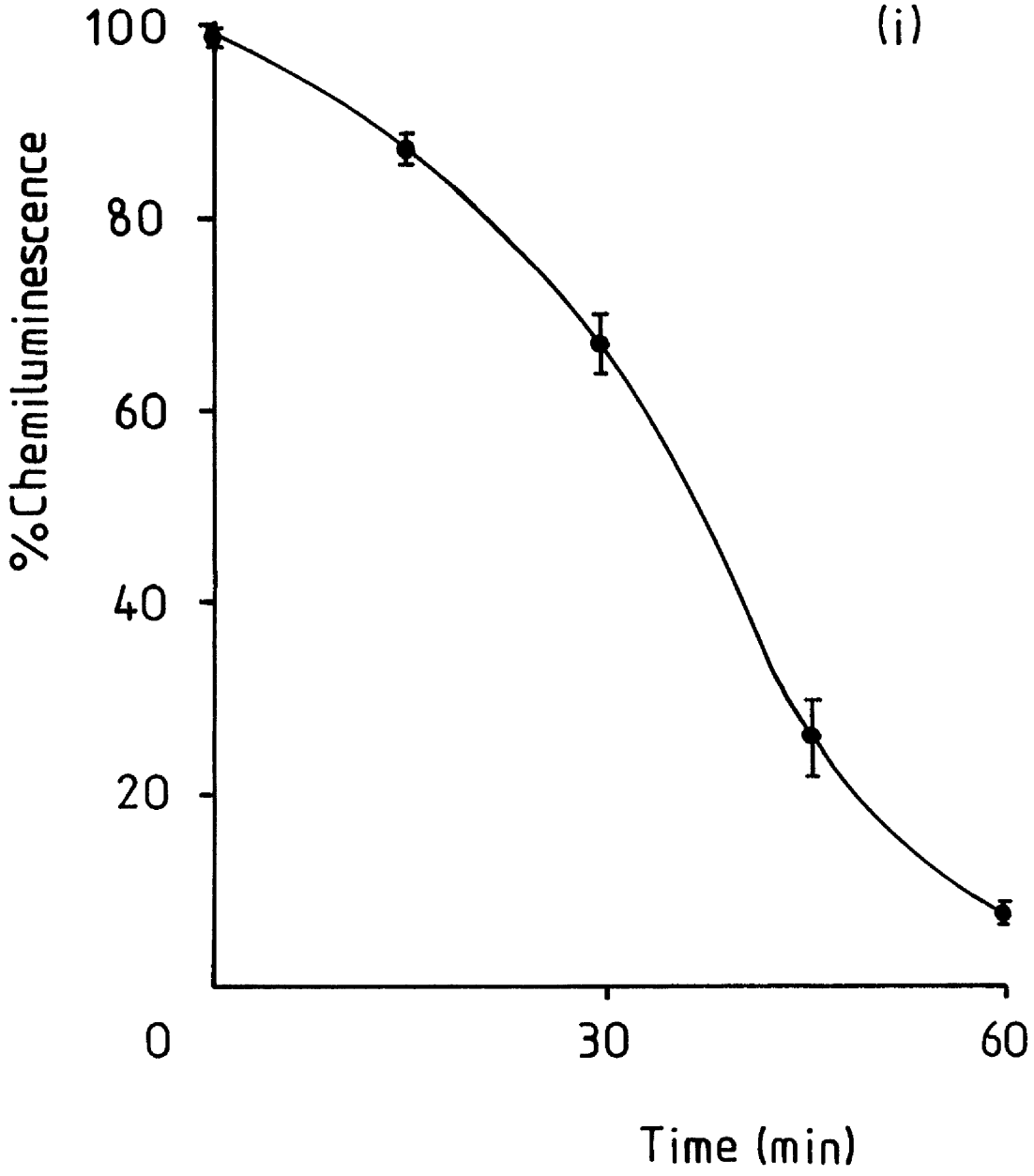
The figure shown is representative of 2 separate experiments.

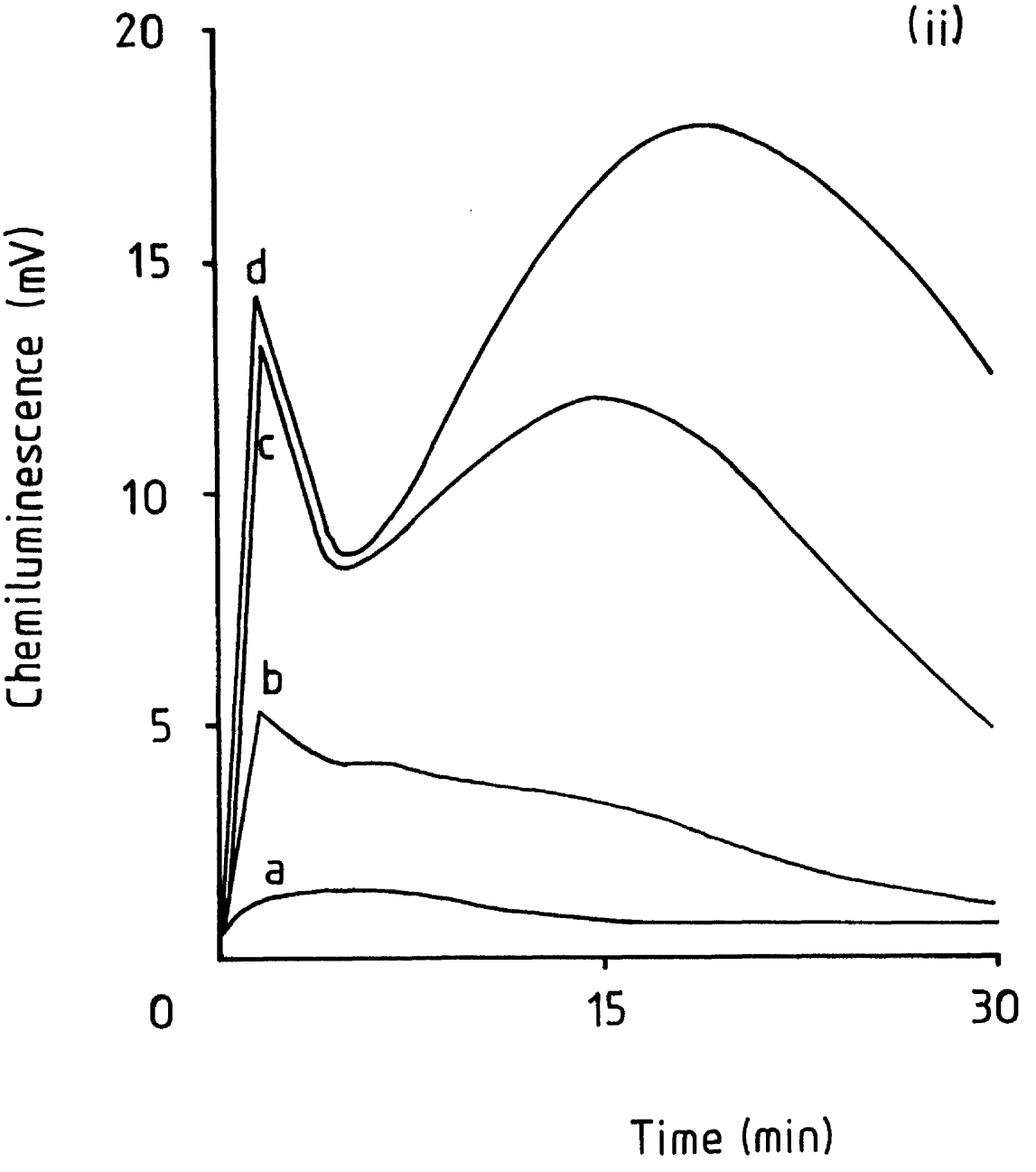
a = 60 min

b = 45 min

c = 30 min

d = Control (buffer).


(i)
100
80
60
40
20
0
30
60
%Chemiluminescence
Time (min)


(ii)
20
15
10
5
0
Chemiluminescence (mV)
d
c
b
a
15
30
Time (min)

chemiluminescence would occur if the neutrophils were pre-incubated in the presence of 160 ng of toxin for about 35-40 min. Analysis of a chemiluminescence curve suggested that there was a lag period of between 30-40 min before any inhibitory effects of PT was evident (figure 11(ii)).

Earlier studies indicated that the PT preparation of Perera et al. (1985) was contaminated by trace amounts of other components; LPS and possibly FHA (section 5.1.8). Both FHA and PT activity are heat-labile whereas LPS activity is heat-stable. About 1 µg of B.pertussis LPS is required to enhance the fMLP-induced chemiluminescent response of rabbit peritoneal neutrophils significantly (figure 8). Levels of LPS as high as this were unlikely to be present in the relatively small amounts of PT used in the present chemiluminescence studies (6.4-160 ng per vial). However, to verify that LPS was not modulating the inhibitory effect of PT, a PT preparation was heat-treated at 80°C for 30 min to destroy PT activity but leave any LPS activity intact. The inhibitory effect of PT was partially abolished by heating for 10 min and completely destroyed by heating for 30 min (table 24). No enhancement of chemiluminescence was observed with the PT which had been heat-treated at 80°C for 30 min confirming that there was insufficient LPS present to influence the results obtained.

5.3.6. Agents which elevate neutrophil intracellular cAMP levels

It has been well documented that cAMP and agents which elevate intracellular cAMP levels diminish both degranulation and other cellular responses of neutrophils (Cox and Karnovsky, 1973; Weissman et al., 1972). As a control

Table 24

The effect of heating on the inhibitory activity of pertussis toxin on fMLP-induced chemiluminescence of neutrophils.

Sample	Conc per vial (ng)	mV chemiluminescence ± SEM* Peak value		Total	
Buffer	-	31.21	± 3.74	234.46	± 11.63
Toxin (unheated)	160	2.97**	± 0.82	27.71**	± 3.49
Toxin (80°C for 10 min)	160	22.59	± 3.31	122.34***	± 17.71
Toxin (80°C for 30 min)	160	26.80	± 2.37	219.97	± 9.08

*Derived as in table 19; n=6.

experiment, neutrophils were pre-incubated in the presence of various agents (caffeine, theophylline, and dibutyryl cAMP) known to increase intracellular cAMP levels. As might have been expected, the fMLP-induced chemiluminescent response of rabbit peritoneal neutrophils was inhibited by all three agents tested (table 25).

5.3.7. Influence of a pertussis toxin and filamentous haemagglutinin mixture

FHA enhanced the fMLP-induced chemiluminescent response of rabbit peritoneal neutrophils to fMLP (section 5.3.2.) whereas PT inhibited it (section 5.3.5.). Since both factors are secreted during bacterial growth, it was of interest to determine which effect would predominate. After pre-incubation with equal doses (50 ng) of both factors, the suppressive effect of PT overrode the enhancing effect of FHA. Pre-incubation of the neutrophils with 50 ng FHA and a higher dose of PT (160 ng) caused a further reduction in chemiluminescence (table 26).

5.3.8. Influence of a pertussis toxin and B.pertussis LPS mixture

B.pertussis LPS enhanced the neutrophil chemiluminescent response to fMLP (section 5.3.3.) whereas PT inhibited it (section 5.3.5.). Rabbit peritoneal neutrophils were pre-incubated in the presence of a PT and B.pertussis LPS mixture to find which effect would predominate. Using a maximum enhancing dose of LPS (10 µg) and a maximum inhibitory dose of PT (160 ng) the inhibitory activity of PT

Table 25

The effects of agents which elevate intracellular cAMP levels on fMLP-induced chemiluminescence of neutrophils.

Sample	Conc per vial (mM)	mV chemiluminescence ± SEM* Peak value	Total
Buffer	-	129.25 ± 4.88	691.58 ± 8.27
Caffeine	5.0	2.31** ± 0.09	35.30** ± 1.02
Theophylline	5.0	3.49** ± 0.19	50.61** ± 1.82
Dibutyryl cAMP	0.2	51.48** ± 1.26	275.42** ± 4.56

*Derived as in table 19; n=7.

Table 26

The influence of a mixture of pertussis toxin and FHA on fMLP-induced chemiluminescence of neutrophils.

PT conc per vial	FHA conc per vial	mV chemiluminescence ± SEM* Peak value		Total	
zero	zero	16.87	± 0.45	250.32	± 3.52
50 ng	zero	6.71*	± 1.88	82.72***	± 22.90
160 ng	zero	3.26**	± 0.78	40.14**	± 8.65
zero	50 ng	117.00**	± 16.18	867.97**	± 73.03
50 ng	50 ng	14.09	± 3.74	141.60*	± 42.00
160 ng	50 ng	6.80***	± 1.25	65.35***	± 12.40

*Derived as in table 19; n=9.

almost completely suppressed the enhancing effect of LPS (table 27).

5.3.9. Monitoring of a toxoiding procedure

An acellular pertussis vaccine, which was a culture supernate extract consisting predominantly of PT and FHA, was assayed for its effects on neutrophil chemiluminescent response to fMLP (table 28). An untoxoided vaccine preparation inhibited, whereas a toxoided vaccine preparation enhanced, neutrophil chemiluminescent response to fMLP. The enhancement of neutrophil chemiluminescent response to fMLP by the toxoided vaccine sample was abolished by heat-treatment of the vaccine at 80°C for 30 min. This suggests that the factor(s) responsible for enhancement of chemiluminescence was not heat-stable, thereby ruling out LPS. It also indicated that the enhancement of chemiluminescence observed with the unheated vaccine sample was not caused non-specifically by the addition of extracellular protein. Since PT inhibits fMLP-induced chemiluminescence of neutrophils (figure 10), this indicated that the toxoiding procedure used had abolished most, if not all, of the PT inhibitory activity present in the untoxoided vaccine preparation. This correlated with observations that the toxoiding procedure used had abolished the leukocytosis-promoting and histamine-sensitizing activity of the untoxoided vaccine preparation (Mr. M. Christodoulides: personal communication).

The enhancement of neutrophil chemiluminescence observed with the pertussis vaccine (table 28) may have been due to FHA. A toxoided FHA preparation (kindly supplied by Mr. M. Christodoulides, Dept. of Microbiology, University of Glasgow)

Table 27

The influence of a mixture of pertussis toxin and *B.pertussis* LPS on fMLP-induced chemiluminescence of neutrophils.

PT conc per vial	LPS conc per vial	mV chemiluminescence ± SEM† Peak value		Total	
zero	zero	20.21	± 1.23	292.78	± 16.13
160 ng	zero	1.43**	± 0.02	19.87**	± 0.20
zero	10 µg	107.55**	± 2.99	898.42***	± 13.87
160 ng	10 µg	5.14***	± 0.18	51.34***	± 1.53

†Derived as in table 19; n=6.

Table 28

The effect of an acellular pertussis vaccine on fMLP-induced chemiluminescence of neutrophils.

Sample	Protein conc per vial (µg)	mV chemiluminescence ± SEM† Peak value	Total
Buffer	-	22.03 ± 2.85	198.11 ± 14.18
Untoxoided vaccine	0.1	3.89** ± 1.07	26.12** ± 2.43
Toxoided vaccine	10.0	52.00** ± 1.14	354.62** ± 4.67
	1.0	34.58** ± 1.43	290.27* ± 28.37
Toxoided vaccine[1]	1.0	24.43 ± 1.99	219.72 ± 25.93

[1]Heated at 80°C for 30 min.

†Derived as in table 19; n=6.

was assayed for its effects on neutrophil chemiluminescence (table 29). This FHA preparation had been toxoided using the same procedure as the one applied to the B.pertussis culture supernate extract. A 50 ng dose of toxoided FHA caused a slight enhancement of neutrophil chemiluminescence, however, the degree of enhancement of both peak value and total chemiluminescence was not as great as that induced by a similar dose of untoxoided FHA ($P<0.001$). The peak neutrophil chemiluminescence value induced by a dose of 2500 ng toxoided FHA was not significantly different from that induced by a dose of 50 ng untoxoided FHA ($P>0.05$). However, the untoxoided preparation induced a greater level of total neutrophil chemiluminescence ($P<0.01$) than neutrophils treated with the highest dose of toxoided FHA. The results suggest that the enhancement of chemiluminescence observed with the toxoided vaccine was probably due to residual FHA activity. Thus, the toxoiding procedure used reduces the biological activity, as assayed using chemiluminescence, of both PT and FHA.

Table 29

The effect of a toxoided FHA preparation on fMLP-induced chemiluminescence of neutrophils.

Sample	Dose per vial (ng)	mV chemiluminescence ± SEM† Peak value		Total	
Buffer	-	14.83	± 1.04	232.03	± 11.78
Untoxoided FHA	50	45.41**	± 4.00	458.50***	± 7.59
Toxoided FHA	50	21.04*	± 1.33	297.26**	± 8.84
	250	40.56***	± 1.47	349.99***	± 21.92
	2500	45.40**	± 4.53	377.98***	± 22.38

†Derived as in table 19; n=6.

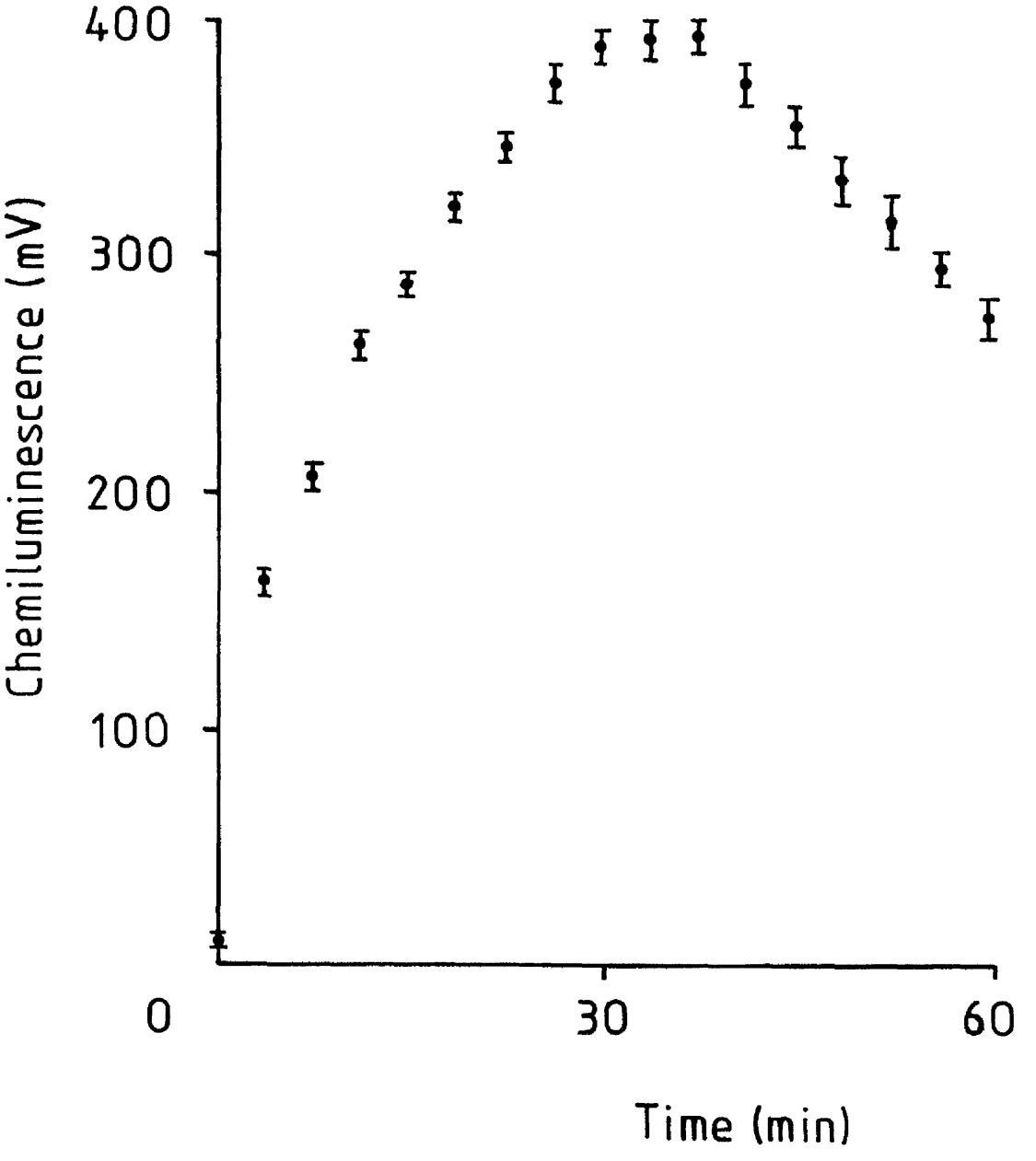
Part 4: The chemiluminescent response of rabbit peritoneal neutrophils to different strains and mutants of Bordetella pertussis

5.4.1. Effect of antigenic modulation

Chemiluminescence studies using whole-cells of B.pertussis as a stimulus were performed to assess the effects of B.pertussis virulence factors on the response of rabbit peritoneal neutrophils to intact bacteria. A noticeable difference between the chemiluminescence patterns obtained when using either fMLP or an intact bacterial stimulus is that fMLP induces a bimodal response (figure 4) whereas intact bacteria induce only a single peak (figure 12). The first, rapid chemiluminescence peak seen after neutrophil stimulation by fMLP is an extracellular peak (Bender and Van Epps, 1983). This rapid peak is absent from the slower chemiluminescent response induced by whole-cells of bacteria.

During antigenic modulation, B.pertussis loses a wide range of virulence-associated factors and undergoes changes in outer membrane proteins (section 2.1.5). Two B.pertussis strains, 18334 and Tohama, were assayed for differences in chemiluminescence patterns to find whether antigenic modulation significantly influenced the ability of these bacteria to avoid killing by neutrophils. The chemiluminescence peaks took up to 2 h for the full rise and subsequent decline to background levels although the chemiluminescence peak values occurred within the first 60 min of assaying. The X-mode organisms induced chemiluminescence patterns with peak values at about 30-40 min which declined to background levels at about 75 min. The C-mode organisms

Figure 12. Neutrophil chemiluminescent response to intact X-mode B.pertussis Tohama. Rabbit peritoneal neutrophils were pre-incubated for 60 min before addition of the stimulus. Each point shown is a mean and SEM of 2 observations. The data used was from figure 16 (curve d).


400
300
200
100
0
30
60
Chemiluminescence (mV)
Time (min)

induced chemiluminescence patterns with peak values between 40-50 min which persisted for about a further 40 min before declining to background chemiluminescence levels. Since the first 60 min of the chemiluminescence peaks induced by intact B.pertussis gave the most interesting information, most experiments were run for 60 min only and all data shown here is from this period only. To simplify the chemiluminescence results individual time points were linked and the chemiluminescence curves shown are continuous traces through individual time points and without the SEM bars.

B.pertussis Tohama and 18334 C-mode organisms induced significantly lower chemiluminescence peaks and total levels of chemiluminescence than their respective X-mode counterparts (figure 13). A transposon-induced mutant, B.pertussis 347 grown in X-mode culture medium (figure 13, curve c), induced a similar chemiluminescence pattern to the C-mode organisms (figure 13, curves b and d). The B.pertussis Tohama X-mode strain (figure 13, curve f) induced a significantly higher chemiluminescent peak and total chemiluminescence than the B.pertussis 18334 X-mode strain (figure 13, curve e). The susceptibility of an organism to phagocytosis decreases as its hydrophilicity increases (Stendahl, 1983). A lower rate of phagocytosis would correspond with a lower rate of intracellular, but perhaps not extracellular, bacterial killing. B.pertussis 347 and the B.pertussis C-mode organisms were significantly more hydrophilic ($P<0.001$) compared to the B.pertussis X-mode organisms (figure 15(i)). Thus there was a positive correlation between the chemiluminescence levels generated and the hydrophobicity of the organisms.

Figure 13. Effect of B.pertussis antigenic modulation on the induction of neutrophil chemiluminescence. The bacteria were grown in either X- or C-mode SS medium in shaken culture; the mode of the organism is given in brackets. Rabbit peritoneal neutrophils were pre-incubated at 37°C for 20 min before addition of the stimulus. The figure shown is a mean of 4 observations and is representative of 8 separate experiments.

a = No stimulus.

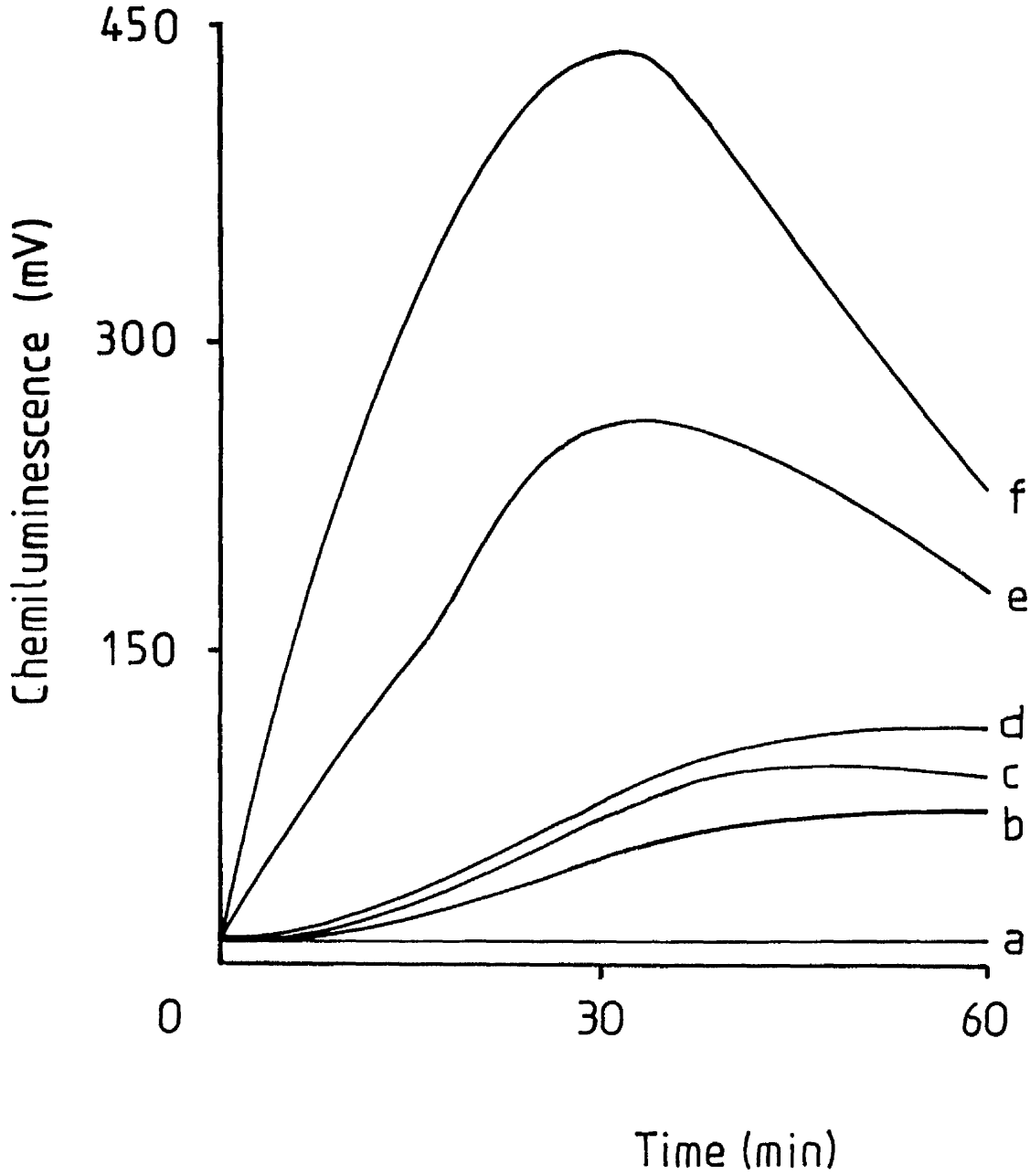
b = 18334 (C).

c = 347 (X).

d = Tohama (C).

e = 18334 (X).

f = Tohama (X).


450
300
150
Chemiluminescence (mV)
f
e
d
c
b
a
0
30
60
Time (min)

5.4.2. Study of mutants

A study of the effect of individual components on neutrophil chemiluminescence generation to intact B.pertussis was done using transposon-induced mutants deficient in one or more virulence factors (table 4). The mutants were derived from a B.pertussis Tohama X-mode parental strain so this strain was included as a reference (figure 14). B.pertussis Tohama X-mode induced the highest chemiluminescence peak value and the highest level of total chemiluminescence (figure 14, curve f) which were significantly different from all other strains tested ($P<0.05$). The total amount of chemiluminescence and the chemiluminescence peak values produced by the adenylate cyclase- and haemolysin-deficient strain B.pertussis 348 (figure 14, curve e), the FHA-deficient strain B.pertussis 353 (figure 14, curve c), and the PT-deficient strain B.pertussis 357 (figure 14, curve d) were not significantly different ($P>0.05$). The virulence factor-deficient strain B.pertussis 347 (figure 14, curve b) induced the lowest chemiluminescence peak value. It also induced the lowest levels of total chemiluminescence over the time tested and was the only strain to differ significantly ($P<0.001$) in its degree of hydrophilicity when compared to the other mutants or parental strain (figure 15(ii)). The extreme hydrophilicity of B.pertussis 347 may explain the ability of the organism to stimulate a low level of neutrophil chemiluminescence only.

5.4.3. Effect of pertussis toxin on neutrophil chemiluminescent response to whole-cells of B.pertussis

PT (160 ng) almost completely suppressed the neutrophil

Figure 14. A study of neutrophil chemiluminescence generation against mutants of B.pertussis. The mutants were grown in X-mode SS medium in shaken culture. Rabbit peritoneal neutrophils were pre-incubated at 37°C for 20 min before addition of the stimulus. The figure shown is a mean of 4 observations and is representative of 8 separate experiments.

a = No stimulus.

b = 347 (X).

c = 353 (X).

d = 357 (X).

e = 348 (X).

f = Tohama (X).

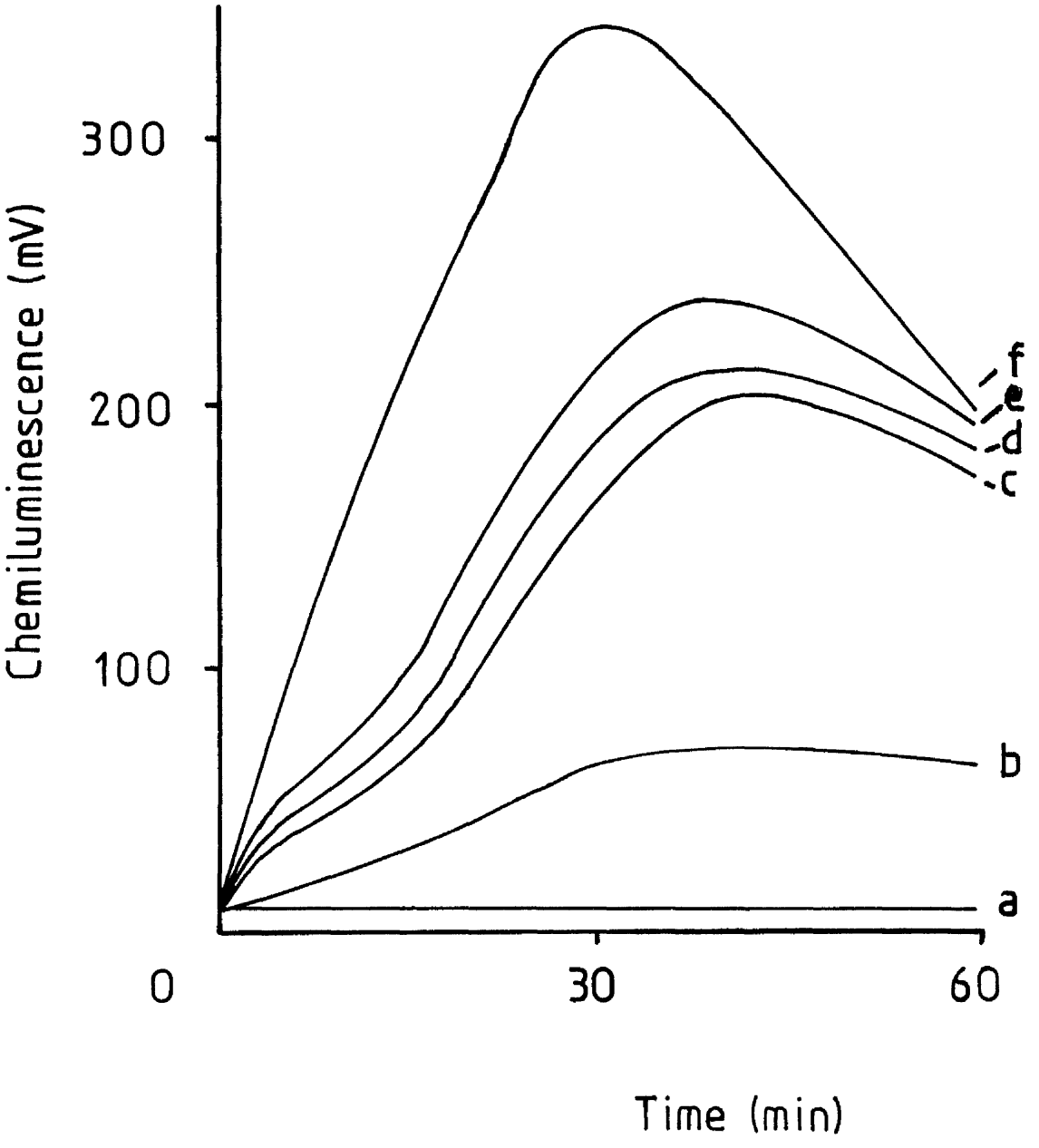

Chemiluminescence (mV)
300
200
100
0
30
60
Time (min)
f
e
d
c
b
a

Figure 15. A correlation of whole-cell B.pertussis chemiluminescence pattern with bacterial hydrophilicity.

(i) Antigenically-modulated strains.

(A) The individual chemiluminescence counts obtained for each B.pertussis strain over 60 min were summed. The mean total chemiluminescence count was then plotted. Conditions were as for figure 11. (n=8 observations).

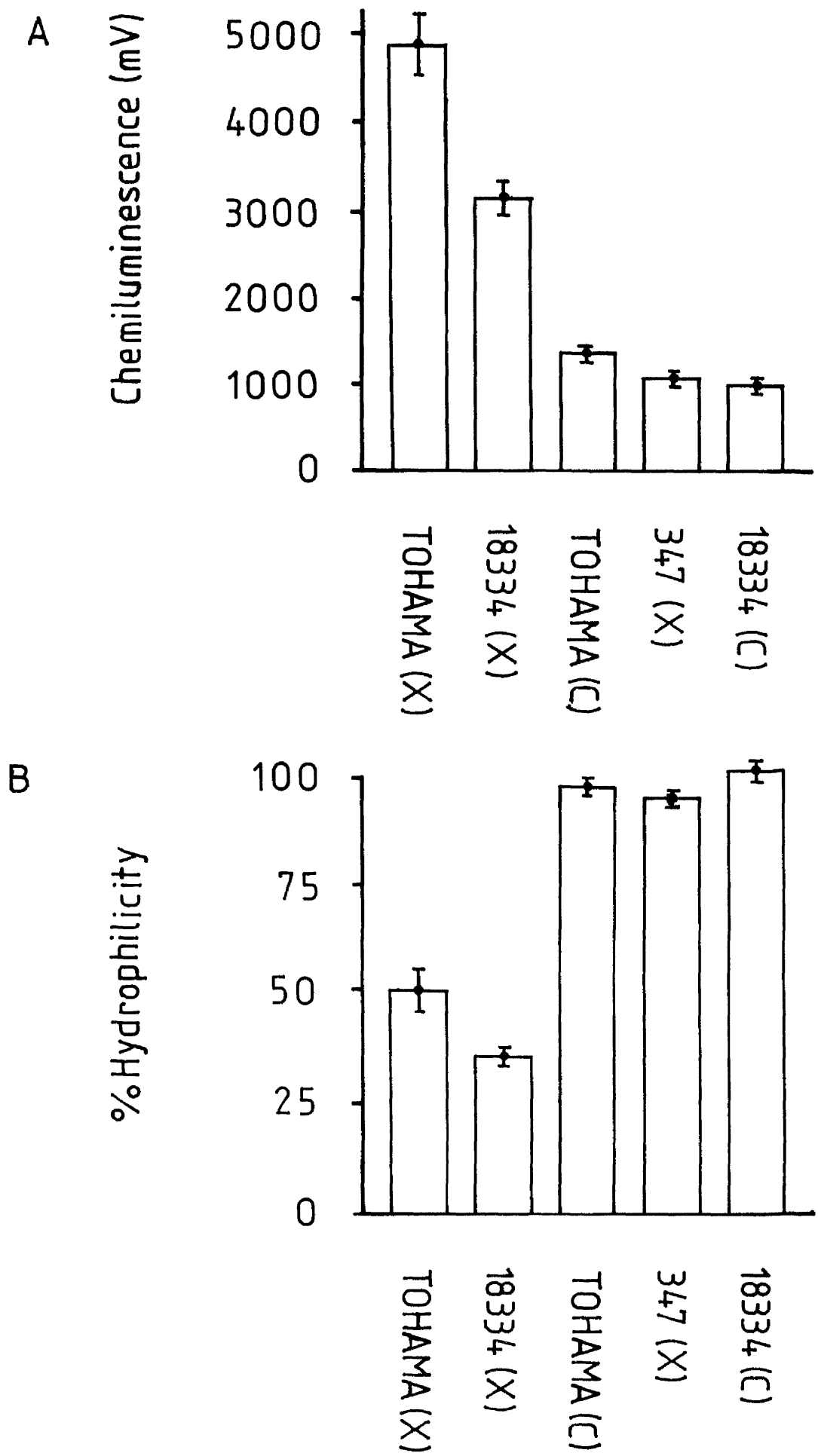
(B) Hydrophilicity of the different B.pertussis strains. (n=8 observations).

(ii) B.pertussis mutants grown in X-mode.

(A) The individual chemiluminescence counts obtained for each B.pertussis strain over 60 min were summed. The mean total chemiluminescence count was then plotted. Conditions were as for figure 12. (n=8 observations).

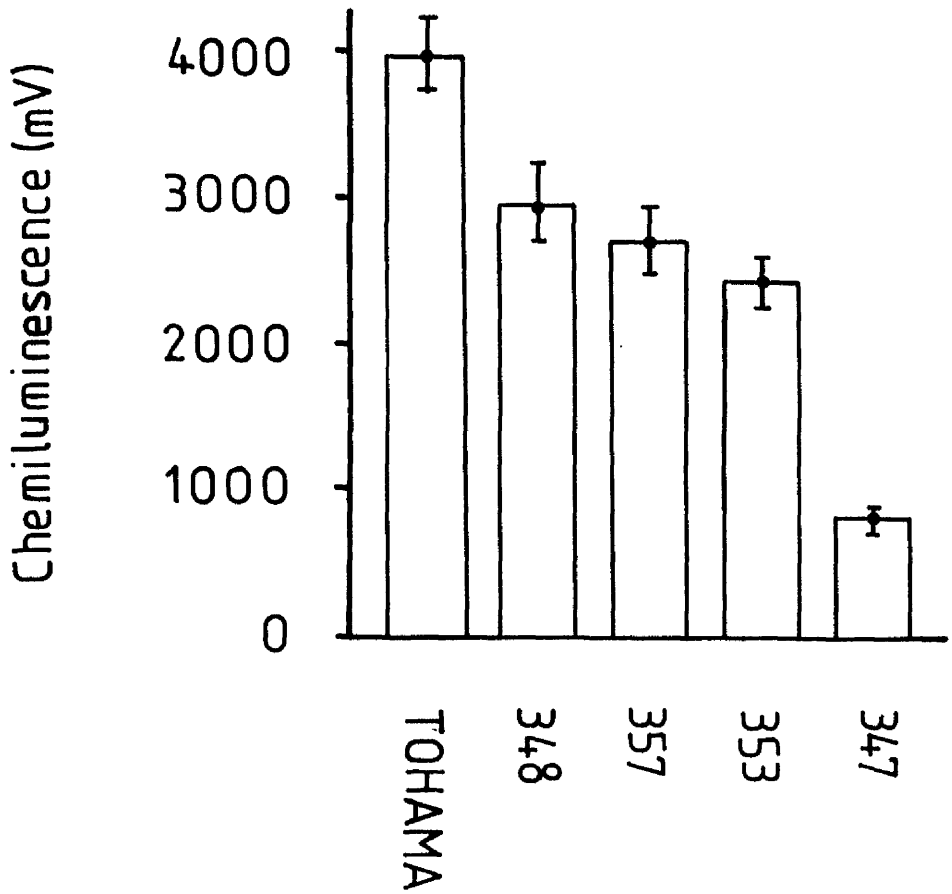
(B) Hydrophilicity of the different B.pertussis mutants. (n=8 observations).

(i)

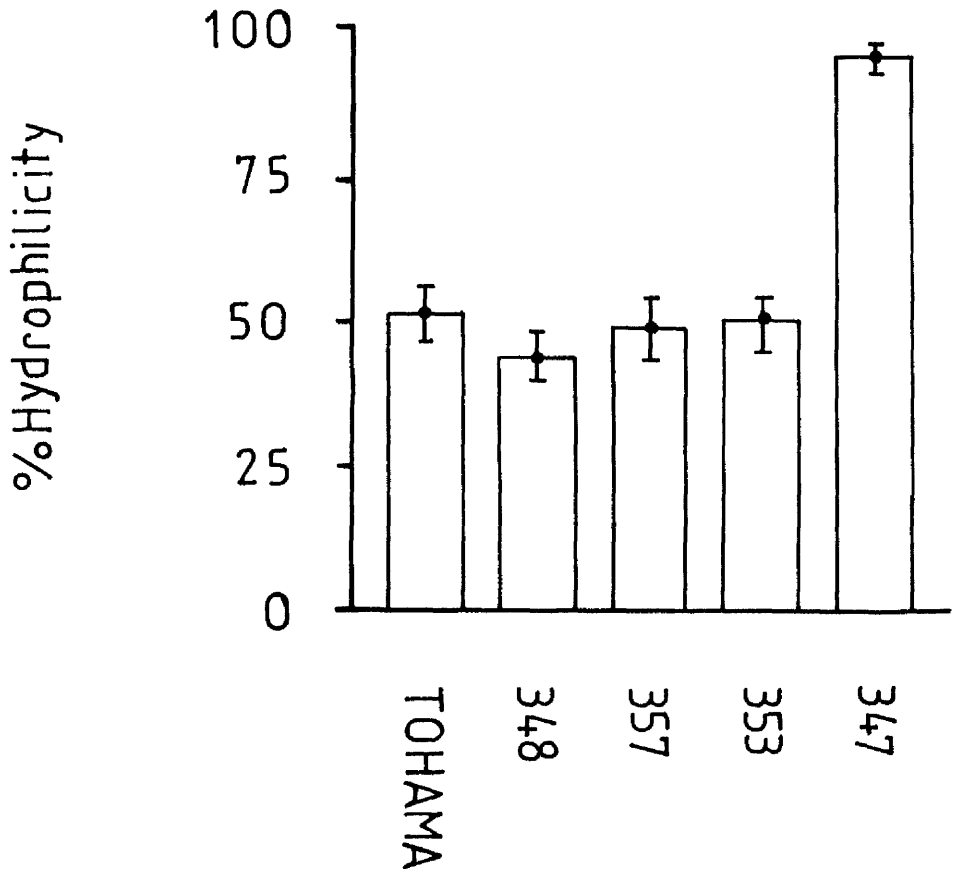

A
Chemiluminescence (mV)
5000
4000
3000
2000
1000
0
TOHAMA (X)
18334 (X)
TOHAMA (C)
347 (X)
18334 (C)
B
% Hydrophilicity
100
75
50
25
0
TOHAMA (X)
18334 (X)
TOHAMA (C)
347 (X)
18334 (C)

(ii)

A


Chemiluminescence (mV)
4000
3000
2000
1000
0
TOHAMA
348
357
353
347

B


%Hydrophilicity
100
75
50
25
0
TOHAMA
348
357
353
347

chemiluminescent response to fMLP (figure 10), but a similar dose of the toxin caused only a slight inhibition ($P<0.05$), about 30%, of the peak value and total chemiluminescence induced by intact B.pertussis (figure 16, curve b). The lower dose of PT (6.4 ng) caused a slight inhibition of the peak value ($P<0.05$) but had no significant effect ($P>0.05$) on total chemiluminescence production (figure 16, curve c). The sample of toxin used almost completely inhibited the neutrophil chemiluminescent response to fMLP, confirming that the slight inhibitory effect of PT on neutrophil chemiluminescent response to the intact bacteria was genuine, and not due to a loss in activity of the toxin (data not shown). This suggested that although PT inhibited the neutrophil chemiluminescent response to fMLP and to intact B.pertussis, the response of neutrophils to fMLP was more inhibited than the response to intact B.pertussis.

5.4.4. Effect of filamentous haemagglutinin on neutrophil chemiluminescent response to intact B.pertussis

FHA stimulated neutrophil chemiluminescence directly (section 5.2.1.) and enhanced neutrophil chemiluminescent response to fMLP (section 5.3.2.) so FHA was assayed for its effects on the chemiluminescence of neutrophils stimulated by whole-cells of B.pertussis. FHA induced a greater rate of neutrophil chemiluminescence production while the peak value and total chemiluminescence production were slightly reduced ($P<0.05$) by treatment with a dose of 50 ng of FHA (figure 17, curve b). The decrease in peak value and total chemiluminescence observed after pre-treatment of the neutrophils with FHA may have been artefactual. Since

Figure 16. The effect of pertussis toxin on neutrophil chemiluminescent response to intact X-mode B.pertussis Tohama. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of the toxin for 60 min before addition of the stimulus. No toxin was added to the control. The figure shown is representative of 6 separate experiments.

a = No stimulus.

b = 160 ng.

c = 6.4 ng.

d = Control (buffer).

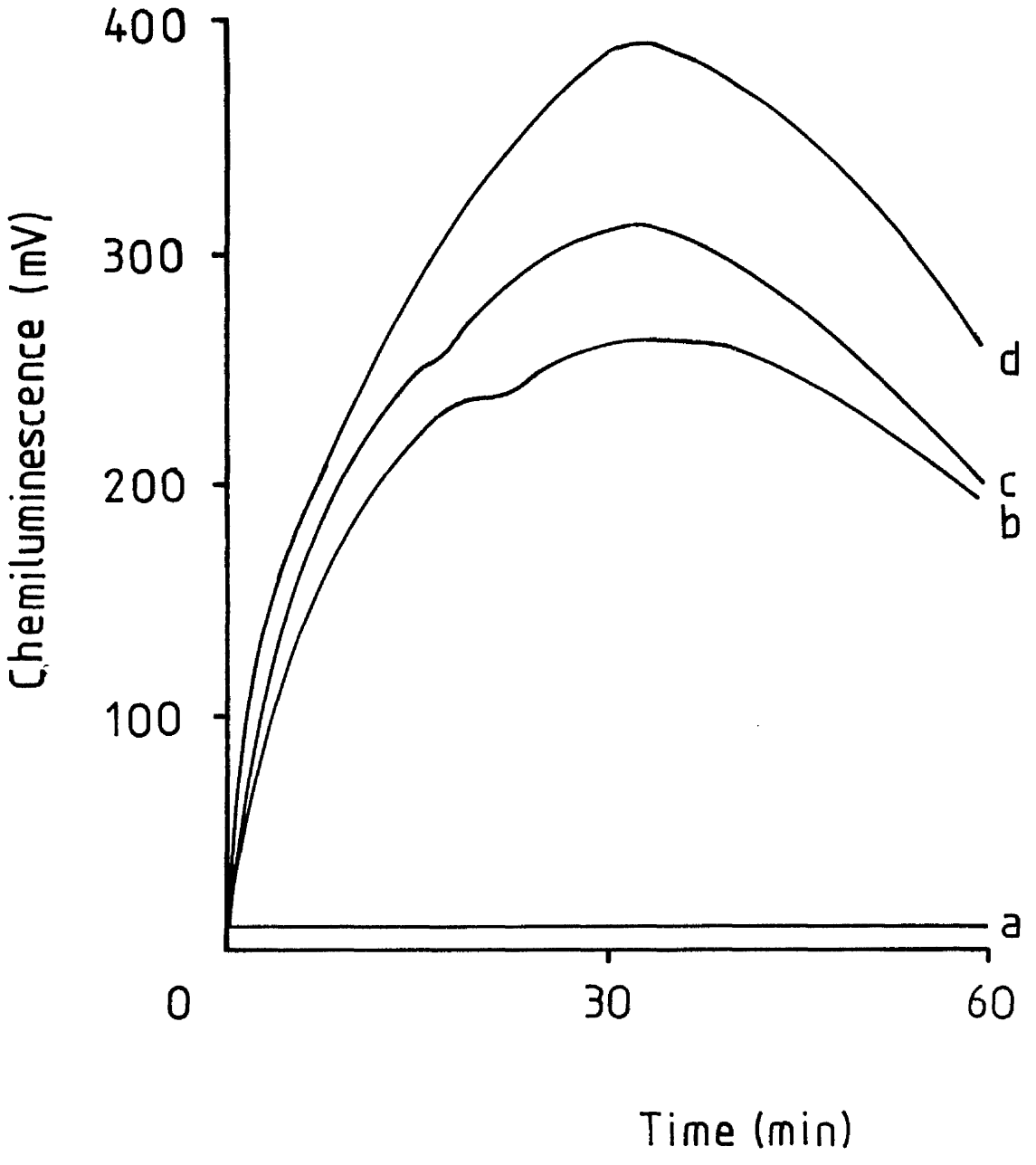

400
300
200
100
Chemiluminescence (mV)
d
c
b
a
0
30
60
Time (min)

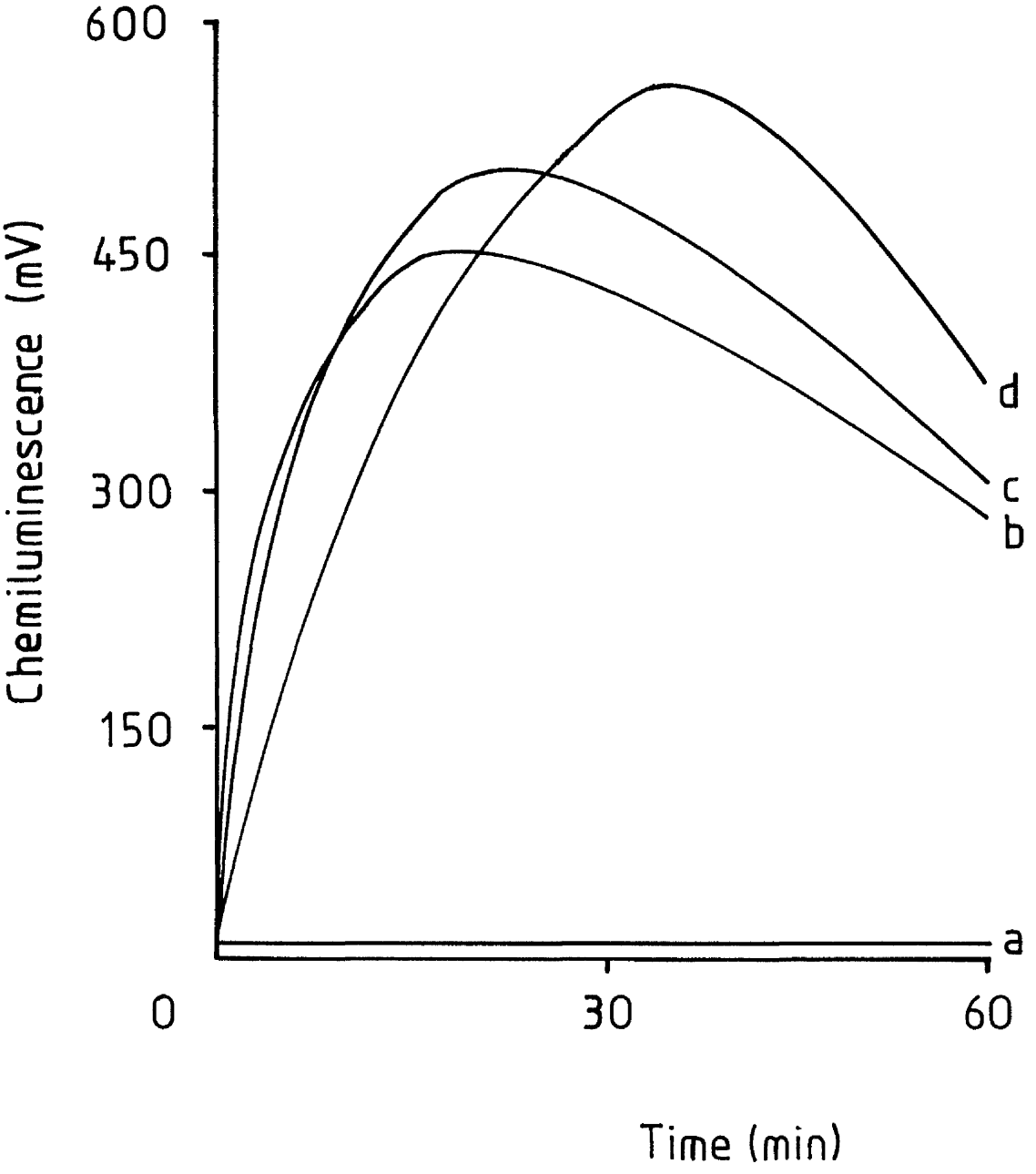
Figure 17. The effect of purified FHA on neutrophil chemiluminescent response to intact X-mode B.pertussis Tohama. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of FHA for 60 min before addition of the stimulus. No toxin was added to the control. The figure shown is representative of 8 separate experiments.

a = No stimulus.

b = 50 ng.

c = 10 ng.

d = Control (buffer).


600
450
300
150
0
30
60
Chemiluminescence (mV)
Time (min)
a
b
c
d

neutrophil pre-incubation with FHA induced a faster rate of chemiluminescence production, the sampling regime used in which each sample was counted approximately every 225 sec, may miss the chemiluminescence peak values of the FHA-treated neutrophils. Also this may explain the apparent depression in total chemiluminescence measured. A reduction in the time between counting of each sample may have indicated whether the decrease in chemiluminescence peak value and total chemiluminescence of FHA-treated neutrophils was artefactual. Neutrophil chemiluminescence was enhanced significantly by FHA even in the absence of any other stimulus (table 18). This may have contributed to the chemiluminescent pattern obtained in the presence of FHA and whole-cells of B.pertussis.

5.4.5. Effect of a mixture of pertussis toxin and filamentous haemagglutinin on neutrophil chemiluminescent response to whole-cells of B.pertussis

A mixture of PT and FHA (50 ng dose of each) was assayed to see which factor, if any, would predominate in modulating neutrophil chemiluminescent response to intact B.pertussis (table 30). The chemiluminescence peak value and total chemiluminescence produced by neutrophils was slightly decreased after pre-incubation with either PT or FHA alone. Pre-incubation of neutrophils with a mixture of both PT and FHA reduced both chemiluminescence peak value and total chemiluminescence produced. Neutrophils pre-incubated with both PT and FHA had a similar rate of chemiluminescence production as neutrophils pre-treated with FHA alone (data not shown). The results (table 30) showed that the chemiluminescence peak value and total chemiluminescence

Table 30

The influence of a mixture of pertussis toxin and FHA on neutrophil chemiluminescent response to X-mode *B.pertussis* Tohama.

PT conc per vial	FHA conc per vial	mV chemiluminescence ± SEM* Peak value		Total	
zero	zero	332.83	± 14.40	4338.00	± 154.37
50 ng	zero	276.34*	± 13.50	3654.43**	± 165.71
zero	50 ng	291.78*	± 7.49	3851.14*	± 92.68
50 ng	50 ng	240.97***	± 9.30	3084.85***	± 103.74

*Derived as in table 19 except values were obtained over 60 min; n=9.

produced by the PT and FHA-treated neutrophils was, however, significantly reduced compared to the FHA-treated neutrophils ($P<0.001$). The effects of a mixture of both PT and FHA on the neutrophil chemiluminescent response to intact B.pertussis were approximately similar to the cumulative effects of PT and FHA alone.

5.4.6. Effect of B.pertussis LPS on neutrophil chemiluminescent response to intact B.pertussis

B.pertussis LPS induces neutrophil chemiluminescence directly (table 18) and enhances the neutrophil chemiluminescent response to fMLP (figure 8), so the effects of LPS on the neutrophil chemiluminescent response to intact B.pertussis were investigated (figure 18). A dose of 10 µg of B.pertussis LPS induced a greater rate of chemiluminescence production accompanied with an apparent decrease in the chemiluminescence peak value and total chemiluminescence production ($P<0.05$). The effects noted were similar to those observed after neutrophil pre-treatment with FHA (figure 17). Thus, the decreased chemiluminescence peak value and total chemiluminescence production of the LPS-treated neutrophils may also have been due to the sampling regime missing out the highest peak values of these cells. LPS induced neutrophil chemiluminescence even in the absence of any other stimulus (table 18). This may have influenced the results obtained with LPS in the presence of intact B.pertussis.

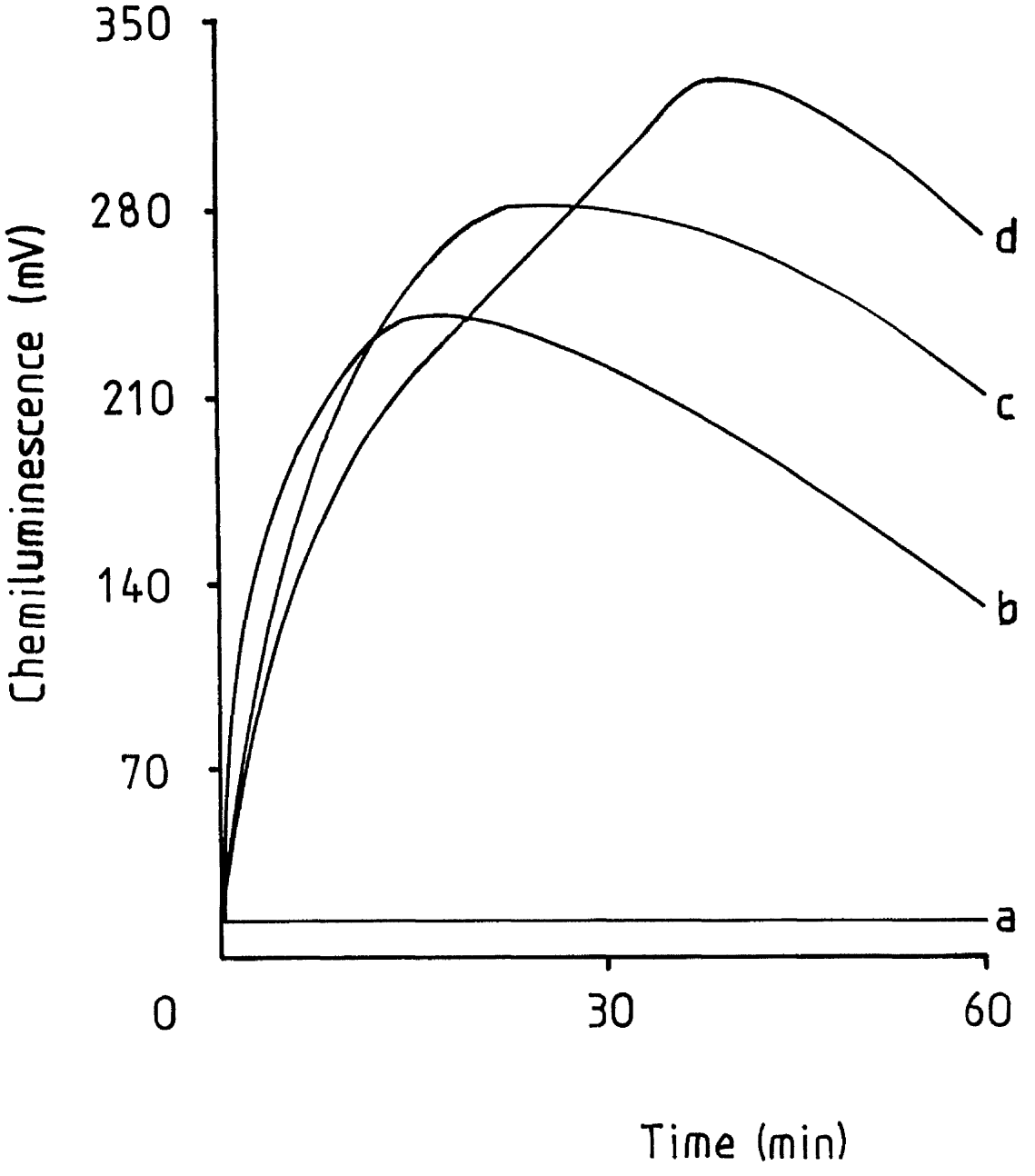
Figure 18. The effect of B.pertussis LPS on neutrophil chemiluminescent response to intact X-mode B.pertussis Tohama. Rabbit peritoneal neutrophils were pre-incubated in the presence of various doses of the toxin for 60 min before addition of the stimulus. No toxin was added to the control. The figure shown is representative of 4 separate experiments.

a = No stimulus.

b = 10 μg LPS.

c = 1 μg LPS.

d = Control (buffer).


350
280
210
140
70
Chemiluminescence (mV)
d
c
b
a
0
30
60
Time (min)

5.4.7. Ratio of bacteria to neutrophil in the chemiluminescence assay

The number of intact B.pertussis per neutrophil in the neutrophil chemiluminescence assay was unknown, so the number of bacteria per assay tube was determined. In preliminary experiments with B.pertussis Tohama X-mode as the test bacterium contamination of viable counts was frequently encountered because of the number of manipulations involved. To overcome this, a B.pertussis Tohama X-mode organism, resistant to nalidixic acid (MIC of 60 µg nalidixic acid ml^{-1} of Bordet-Gengou medium), was used in the bacterial viable count assay. Nalidixic acid and one other antibiotic, cephalexin, were incorporated into the growth medium to minimise the risk of contamination. The results obtained indicated that the B.pertussis suspension used in the chemiluminescence assays contained between $2.6-3.7 \times 10^8$ viable organisms ml^{-1} (table 31). Since only 50 µl of a similarly standardised suspension was used in the chemiluminescence assay, the ratio of neutrophils to viable bacteria used in the chemiluminescence assay could be calculated. The ratio was calculated to be between 13-18 bacteria per neutrophil.

Table 31

Estimation of the numbers of B.pertussis organisms in a suspension using the viable count technique.

Dilution	C.F.U.*/0.1 ml of dilution					C.F.U. ml^{-1}
	1	2	3	4	Mean	of original
1 in $2.5x10^{3}$	s-con†	s-con	s-con	s-con	s-con	-
1 in $2.5x10^{4}$	1052	945	1103	987	1022	$2.6x10^{8}$
1 in $2.5x10^{5}$	149	127	153	168	149	$3.7x10^{8}$
1 in $2.5x10^{6}$	13	14	13	18	15	$3.6x10^{8}$

*Colony-forming units.

†Semi-confluent growth.

DISCUSSION

The underlying theme of this work was the belief that B.pertussis infections may alter the behaviour of neutrophils and thereby contribute to the pathogenesis of whooping cough. In particular, an impairment of neutrophil function may cause the host to become susceptible to secondary infections.

Neutrophils from various species were studied but those from the rabbit received most attention because of their ease of procurement. The aspects of neutrophil function which received attention were adhesion and the bactericidal response to fMLP and intact B.pertussis. The neutrophil aggregation assay measured changes in cell adhesion. The chemiluminescence assay measured potential bactericidal capacity of the neutrophils.

Both culture supernate fractions and purified B.pertussis components were assayed for their effects on neutrophil aggregation and chemiluminescent response to fMLP. Both antigenically-modulated strains of and mutants of B.pertussis were assayed to find the role of virulence factors on neutrophil chemiluminescent response to intact B.pertussis. Purified virulence factors of B.pertussis were also assayed on neutrophil response to intact B.pertussis.

Part 1: The effect of B.pertussis products on neutrophil aggregation

Aggregation involves changes in the adhesive properties of cells. Neutrophil adhesive changes are important during margination, locomotion, and for phagocytosis.

6.1.1. Identification of active factor(s)

B.pertussis cytoplasmic and culture supernate fractions

enhanced aggregation of rabbit peritoneal neutrophils and certain observations implicated PT as the causative factor. For example, supernatant fractions of the virulent B.pertussis X-mode strain, which produces PT, had greater neutrophil aggregating activity than those of the avirulent B.pertussis C-mode strain, which is PT-deficient. In addition, both B.pertussis X- and C-mode supernatant fractions lost neutrophil aggregating activity after heating at 80°C for 30 min, a treatment which destroys the activity of PT. Supernatant fractions of B.parapertussis, a species which does not produce PT, contained no detectable neutrophil aggregating activity. However, on more detailed investigation purified PT was less active at enhancing neutrophil aggregation than the B.pertussis X- or C-mode supernatant fraction, suggesting that PT was not the major factor in the B.pertussis supernatant fractions which enhanced neutrophil aggregation.

The B.pertussis X- and C-mode supernatant fractions were therefore examined for the presence of other factors which may have influenced neutrophil aggregation. The major candidates were adenylate cyclase, heat-labile toxin, LPS, and FHA. The heat-stable adenylate cyclase of B.pertussis has important anti-phagocytic properties (section 2.3.2.), and it has been suggested that the enzyme acts by inducing unregulated cAMP formation in phagocytes (Confer et al., 1984). The agents theophylline and caffeine raise neutrophil intracellular cAMP levels and both inhibited aggregation of rabbit peritoneal neutrophils. This suggested that B.pertussis adenylate cyclase would also inhibit neutrophil aggregation. The heat-stable adenylate cyclase of B.pertussis is resistant to heating at 100°C for 5 min (Confer and Eaton, 1982), therefore the enzyme may be resistant to heating at 80°C for 30 min.

Weiss and Hewlett (1986) recently reported that B.pertussis produces two forms of adenylate cyclase; adenylate cyclase enzyme and adenylate cyclase toxin. The adenylate cyclase enzyme was originally isolated from culture supernates of B.pertussis, has enzymic activity, and has no effect on eukaryotic cells. The adenylate cyclase toxin also has enzymic activity, but is mainly cell-associated and has the ability to enter eukaryotic cells and catalyze the formation of cAMP. If sufficient of the latter form of adenylate cyclase was present in the B.pertussis X- or C-mode supernatant fractions then inhibition of neutrophil aggregation would have been observed after heat-treatment (80°C for 30 min) of the fractions. Heat-treated B.pertussis X- and C-mode supernatant fractions did not inhibit neutrophil aggregation suggesting that there was little or no active, heat-stable adenylate cyclase present in the B.pertussis supernatant fractions. This was confirmed by direct enzymic assays: there was no adenylate cyclase activity detectable in either the B.pertussis X- or C-mode supernatant fraction. Low levels of adenylate cyclase activity were, however, found in a B.parapertussis X-mode supernatant fraction. The B.parapertussis X-mode supernatant fraction had no significant effect on neutrophil aggregation suggesting that there was insufficient levels of adenylate cyclase toxin present to influence aggregation.

B.pertussis adenylate cyclase enzyme is extracellular and it is perhaps surprising that little or no cyclase activity was found in the supernatant fractions isolated. This is explicable: since the supernatant fractions used in the experiments were of molecular mass > 100 kDal whereas the molecular mass of the cyclase found in culture supernates is 70 kDal, one would expect to find very little adenylate

cyclase activity in the supernatant fractions used. In addition, the fractionation procedures destroy adenylate cyclase activity (Hewlett and Wolff, 1976). Although adenylate cyclase activity was not detected in the B.pertussis supernatant fractions, low levels were found in a more concentrated sample of the B.parapertussis X-mode supernatant fraction. B.parapertussis produces greater levels of extracellular adenylate cyclase than B.pertussis (Endoh et al., 1980) and this may explain why adenylate cyclase activity was found in supernatant fractions of the former but not the latter organism.

Purified adenylate cyclase was not assayed for its effects on neutrophil aggregation since no purified enzyme was available. The purification procedures used for isolation of B.pertussis adenylate cyclase are complex and time-consuming. In addition, the adenylate cyclase isolated from Bordetella culture supernates has no effect on eukaryotic cells (Weiss and Hewlett, 1986).

The supernatant fractions were also assayed for heat-labile toxin, but no toxin activity was found. It is probable that the toxin was not detected because it is an intracellular protein (Cowell et al., 1979) and is not likely to be present in significant levels in 48 h culture fluids. If heat-labile toxin was the factor responsible for inducing aggregation then B.parapertussis supernatant fractions should also have had aggregating activity.

Another toxin, LPS, was found as a major component in both the X- and C-mode B.pertussis supernatant fractions using SDS-PAGE. Significantly, the C-mode fraction contained more LPS than the X-mode fraction, as estimated by SDS-PAGE, but had lower neutrophil aggregating activity. Purified

B.pertussis LPS did not influence aggregation. In addition, smooth and rough LPS from Salmonella minnesota and Salmonella minnesota Re595 respectively did not influence aggregation. This suggested that LPS had no influence on aggregation under the conditions used. This agrees with the findings of Dahinden and Fehr (1983) who reported that Salmonella LPS did not induce granulocyte aggregation, as measured by an aggregometer. The above results differ from the findings of Thorne et al. (1977) who, using a similar aggregation assay to that used in the present investigation, suggested that LPS from Acinetobacter species enhanced rabbit peritoneal neutrophil aggregation. The difference in results may be explained by the different species of LPS used.

FHA was the most active bacterial component tested in the neutrophil aggregation assay, suggesting that it was responsible for the neutrophil aggregating activity present in the B.pertussis supernatant fractions. In addition, like the activity in the supernatant fractions, the neutrophil aggregating activity of purified FHA was destroyed by heat-treatment at 80°C for 30 min. Surprisingly, no neutrophil aggregating activity was found in any of the B.parapertussis supernatant fractions. B.parapertussis has a faster growth rate than B.pertussis and FHA production by B.parapertussis may have been suppressed at an earlier stage during the 48 h incubation period than FHA production by B.pertussis. Any FHA produced by B.parapertussis would have been subjected to prolonged mechanical, and possibly chemical, degradative processes which may have destroyed the neutrophil aggregating activity.

The results suggested that B.pertussis produces two neutrophil aggregating factors, FHA and PT. To investigate

whether the PT preparations were contaminated by a trace amount of FHA, the toxin preparations were examined by SDS-PAGE and FHA ELISA. SDS-PAGE showed one or more contaminating bands in the PT preparations used. It is possible that the contaminating bands (except the LPS band) were breakdown products of FHA and that they may have been responsible for the neutrophil aggregating activity credited to PT. This is not implausible since it would only require about 4-5% contamination of the PT preparations by FHA. However, an FHA ELISA of both PT preparations indicated that no FHA was present in either sample. The FHA ELISA utilised the monoclonal antibody F3. Irons et al. (1983) reported that this monoclonal fails to detect SDS-dissociated FHA and suggested that F3 may be directed against a conformational determinant of FHA. If some of the contaminating bands present in the PT preparations were FHA fragments, then the conformation of the FHA molecule, and possibly the F3 receptor, may have been altered. This would explain the apparent absence of immunologically-reactive FHA in the PT sample assayed. It may also explain why the FHA ELISA results indicated that there was no detectable FHA present in either the B.pertussis X- or C-mode supernatant fraction. No bands, except LPS, were observed in SDS-PAGE profiles of either B.pertussis X- or C-mode supernatant fraction. However, the protein concentration of each supernatant fraction sample loaded onto the gel was very low (1.5 µg) and a small amount of FHA may have been below the level of detection. Concentration of the B.pertussis supernatant fractions by membrane dialysis may have allowed visualisation of any proteins present by SDS-PAGE. The question of FHA contamination might have been resolved had

other monoclonal antibodies, which would react with denatured FHA, been available. These could have been used as a FHA probe in either an ELISA or in an immunoblotting technique. One indication that FHA and PT may both enhance neutrophil aggregation is the difference in neutrophil species-specificity observed for each B.pertussis component (see section 6.1.2.).

The various activities of PT depends on at least two mechanisms: the ADP-ribosyltransferase activity of the 'A' moiety or the binding of the 'B' moiety to the cell surface (Nogimori et al., 1984). The ADP-ribosylating activity of the toxin was reviewed in section 2.1.4.; the 'B' moiety interacts with lymphocytes inducing lymphocytosis-promotion and a mitogenic response (Nogimori et al., 1984). If the 'B' moiety of the toxin was responsible for enhancing neutrophil aggregation then two possible processes may be visualised. The neutrophils may be passively agglutinated by cross-linking of the cells by toxin. Alternatively, the binding of PT to neutrophils may stimulate neutrophil adhesiveness and thus agglutination, possibly by the induction of neutrophil secretion. A mixture of both passive and stimulated agglutination may even be involved. The enhancement of neutrophil aggregation by FHA may also be mediated by similar processes.

The chemiluminescence results showed that PT inhibited, whereas FHA enhanced, neutrophil bactericidal responses. The bactericidal responses of neutrophils normally involves degranulation. Possibly FHA-enhancement of neutrophil aggregation was due to induction of neutrophil secretion by FHA. Since PT actually inhibits neutrophil secretory responses (Becker et al., 1986) this suggests that any PT-enhancement

of neutrophil aggregation is due to passive cross-linking of the cells by toxin. A similar aggregation response, the clumping of Chinese hamster ovary cells, also occurs in the presence of PT and has been used as an *in vitro* assay for the toxin. The clumping of these cells is not due to passive agglutination, nor alteration of the basal cyclic AMP levels of the cells, and it has been suggested that a toxin-mediated alteration of the cell surface occurs (Hewlett *et al.*, 1983).

Although both FHA and PT enhanced neutrophil aggregation, FHA was more active than PT. FHA is also a more active haemagglutinin than PT. A third haemagglutinin of *B.pertussis* has been reported (Kawai *et al.*, 1982). This haemagglutinin was extracted from whole-cells of *B.pertussis*, induced strong agglutination of human erythrocytes, and was thought to correspond to an ornithine-containing lipid. The effects of this proposed haemagglutinin on neutrophil aggregation is unknown.

6.1.2. Interspecies differences in neutrophil response to active factor(s)

A noteworthy observation was the apparent species-specificity of the aggregation response. Rabbit neutrophils were sensitive to the aggregating factor(s) in the *B.pertussis* X- and C-mode supernatant fractions whereas human and pig neutrophils were not. FHA also showed a similar pattern since it enhanced the aggregation of rabbit but not human neutrophils. FHA has an affinity for cholesterol (Irons and MacLennan, 1979) and the cholesterol content of the neutrophil surface may determine the species-specificity of FHA-induced aggregation. Whether or not the outer membranes of

rabbit and human neutrophils differ in their cholesterol content has not been reported. Alternatively, FHA may bind equally well to the different neutrophil species but with rabbit cells being the most sensitive to the subsequent effects of FHA. Purified PT showed no species-specificity; the toxin appeared to enhance aggregation of both rabbit and human neutrophils. The aggregation of rabbit peritoneal neutrophils was enhanced more than that of rabbit peripheral neutrophils by all active bacterial samples tested. Rabbit peritoneal neutrophils are more adhesive than rabbit peripheral cells *in vitro* (Bamberger *et al.*, 1985), which may explain the differences observed.

Part 2: The effect of B.pertussis components on the chemiluminescent response of neutrophils to fMLP

Chemiluminescence is an indirect measure of the bactericidal capacity of phagocytes. McCall et al. (1979) reported that the responses induced by fMLP in neutrophils are similar to those induced during an acute bacterial infection. Thus, alteration of neutrophil responses to fMLP may indicate an alteration of the normal neutrophil responses to infection.

6.2.1. Enhancing effect of filamentous haemagglutinin and LPS

Both the FHA and LPS of B.pertussis enhanced the chemiluminescence of rabbit peritoneal neutrophils in response to the pro-inflammatory mediator fMLP. There was a lag period of about 15 min before any FHA- or LPS-induced enhancement of chemiluminescence was observed. The minimum doses of FHA and LPS which caused a significant enhancement were 10 ng and 1 µg respectively. Both components caused a slight, but significant, stimulation of neutrophil chemiluminescence in the absence of fMLP. This is the first report of the effect of FHA on neutrophil reponse to a pro-inflammatory mediator.

The summed levels of neutrophil chemiluminescence generated in the presence of both fMLP and FHA or fMLP and LPS were significantly higher than the summed levels of neutrophil chemiluminescence generated in the presence of fMLP alone plus that of FHA or LPS alone. This suggests that there may be synergism between fMLP and FHA or LPS. Noticeably, there was a correlation between direct stimulus of neutrophil chemiluminescence by FHA or LPS in the absence of fMLP with

enhancement of chemiluminescence by FHA or LPS in the presence of fMLP. The rough LPS of E.coli J5, which contains only core glycolipid, and the smooth LPS of E.coli O111:B4 were both capable of inducing human neutrophil chemiluminescence directly in the presence of luminol (Henricks et al., 1983). This agrees with the present results showing that LPS caused a direct induction of chemiluminescence of rabbit peritoneal neutrophils. Henricks et al. (1983) reported that smooth E.coli LPS generated less chemiluminescence from human neutrophils than rough E.coli LPS. A similar pattern was found with Salmonella minnesota rough or smooth LPS and rabbit peritoneal neutrophils in the present work. Fish et al. (1987) suggested that FHA is the cell surface moiety responsible for hydrophobic adherence in B.pertussis. Possibly the interaction of neutrophils with both B.pertussis LPS or FHA is mediated by a similar non-specific, hydrophobic interaction and this may explain why both components had a similar effect on the chemiluminescent response of neutrophils to fMLP. Proctor (1979) reported that E.coli O111:B4 LPS did not induce chemiluminescence of human peripheral neutrophils directly. However, this study was done in the absence of luminol, which reduces the sensitivity of the method.

It would be of interest to know whether the neutrophil chemotactic response to fMLP is also influenced by FHA or LPS pre-treatment. Studies on the influence of FHA or LPS on neutrophil chemiluminescent response to other pro-inflammatory mediators (C5a or leukotriene B4) or phorbol myristate acetate may have given more information on how these B.pertussis components interact with neutrophils. If FHA or LPS also enhance the neutrophil chemiluminescent response to C5a and leukotriene B4, then this suggests that the B.pertussis

components are not exerting their effects solely by interfering with the fMLP receptor. Phorbol myristate acetate is a tumour-promoting agent which appears to exert its effects by activating the protein kinase C in target cells (Nishizuka, 1984). Information on the effect of FHA or LPS on phorbol myristate acetate-induced chemiluminescence of neutrophils may indicate whether protein kinase C activation was affected by FHA or LPS-treatment. Interestingly, PT inhibits neutrophil responses to fMLP, C5a, and leukotriene B4, but has no influence on neutrophil response to phorbol myristate acetate (Becker et al., 1986). This shows that bacterial components can affect some components of the neutrophil signal transduction pathways but not others.

6.2.2. Inhibition of neutrophil chemiluminescence by pertussis toxin

PT suppresses certain neutrophil responses to fMLP and several other chemotactic factors (section 2.3.3.). The microbicidal responses of phagocytes can be separated into two processes: degranulation and the oxidative respiratory burst. The oxidative respiratory burst can be further separated into two processes: myeloperoxidase-associated reactions and reactions involving the generation of reactive oxygen radicals. PT inhibits both fMLP-induced neutrophil degranulation (Becker et al., 1985) and generation of the reactive oxygen radical superoxide (Lad et al., 1985). The effect of PT on neutrophil myeloperoxidase-associated reactions induced by fMLP has not been reported. Luminol-enhanced neutrophil chemiluminescence is dependent on a myeloperoxidase-associated reaction (DeChatelet et al.,

1982). The present results show that PT inhibits fMLP-induction of rabbit peritoneal neutrophil chemiluminescence in the presence of luminol. Thus, the myeloperoxidase-associated microbicidal responses of neutrophils induced by fMLP are also inhibited by PT.

PT ADP-ribosylates a membrane protein of molecular mass about 41 kDal in neutrophils (Bokoch and Gilman, 1984). This protein is a G-protein which is immunochemically distinct from G_i, G_o, and transducin (Gierschik et al., 1986). It has been suggested that this G-protein couples receptor-chemotactic factor interaction to phospholipase C activation (Becker et al., 1986; Bradford and Rubin, 1986). Thus, the inhibitory effects of PT on neutrophil response to fMLP may be due to ADP-ribosylation of a membrane protein which links the fMLP-receptor signal to phospholipase C activation. PT has no effect on either the number or affinity of the formyl-peptide receptors on neutrophils (Becker et al., 1985) supporting the theory that the fMLP-receptor signal transduction is impaired by PT-treatment.

FHA enhanced neutrophil chemiluminescent response to fMLP. Both FHA and PT are haemagglutinins and may induce similar responses in other cells. The haemagglutinating activity of PT resides in the B-oligomer of the toxin (Nogimori et al., 1984). The ADP-ribosylating activity of the toxin is found in the A-protomer of the toxin (Tamura et al., 1982). It would be of interest to assay the effect of purified B-oligomer of PT on neutrophil chemiluminescent response to fMLP in order to show whether this component alone, minus the inhibitory capacity of the A-protomer, enhances neutrophil chemiluminescence. FHA required only 15 min before an enhancing effect was observed. No enhancement of neutrophil

chemiluminescent response to fMLP by PT was observed before the inhibitory response of the toxin was noted after 30-40 min incubation with the neutrophils. This suggests that the binding of the B-oligomer of PT to the neutrophil outer membrane does not enhance neutrophil chemiluminescent response to fMLP. The binding of the B-oligomer of PT is required for the A-protomer to reach its site of action in target cells (Nogimori et al., 1984). Thus, pre-incubation of neutrophils with only the A-protomer of PT would be unlikely to have inhibitory effects on neutrophil chemiluminescent response to fMLP.

The chemiluminescence studies showed that inhibition of fMLP-induced chemiluminescence of rabbit peritoneal neutrophils by PT was detectable down to about 5 ng of toxin per vial. In its present form, an assay of PT-induced inhibition of neutrophil chemiluminescent response to fMLP is not as sensitive as the Chinese hamster ovary cell assay for PT. The latter assay involves a visual scoring of cell clustering induced by toxin-treatment and has been reported to detect toxin concentrations as low as 0.12 ng ml^{-1} (Hewlett et al., 1983). However, by the use of more cells, a higher concentration of luminol and by optimising the fMLP dose, the sensitivity of the chemiluminescence assay could probably be improved. Chemiluminescence has the advantages of being a faster and simpler technique than the Chinese hamster ovary cell assay; the former assay takes between 1-2 h whereas the latter assay takes between 16-24 h. In addition, chemiluminescence is less subjective and does not involve the possibility of human bias in scoring results.

6.2.3. Pertussis toxin suppression of FHA- and LPS-enhancement of neutrophil chemiluminescent response to fMLP

One of the most significant observations in this study was that PT inhibited whereas FHA and LPS enhanced rabbit peritoneal neutrophil chemiluminescent response to fMLP. Experiments were performed to see whether the effect on neutrophil chemiluminescence of the inhibitor, PT, or an enhancer, FHA or LPS, would predominate in a mixture of both. This is relevant to the question of which factor may predominate *in vivo*. The PT inhibitory effect overrode the enhancing effect of either FHA or LPS. This indicated that the capacity of both FHA and LPS to enhance the fMLP-induced chemiluminescence response was probably mediated mainly via the same pathway as the fMLP-receptor signal. Since PT could not totally inhibit the enhancing response of FHA or LPS, this may indicate that FHA and LPS are also causing chemiluminescence via some other route insensitive to inhibition by PT. Both FHA and LPS are capable of promoting neutrophil chemiluminescence directly. Thus, the use of PT as a probe suggests that the enhancement of neutrophil chemiluminescence by either FHA or LPS in the presence of fMLP is the sum of two different effects; one transduced by the same pathway as the fMLP-receptor signal and the second rather minor effect by some other pathway.

The inhibition of neutrophil chemiluminescent response to fMLP by a component, presumably PT, of an acellular pertussis vaccine was abolished after the vaccine was toxoided. The toxoided vaccine enhanced neutrophil chemiluminescent response to fMLP and FHA was implicated as the factor responsible. The results suggest that neutrophil chemiluminescent response to

fMLP can be used as a method of monitoring the toxoiding of an acellular pertussis vaccine.

Part 3: Neutrophil chemiluminescent response to whole-cells of B.pertussis

The bactericidal response of neutrophils against intact B.pertussis was measured using chemiluminescence. Inhibition of neutrophil bactericidal response to B.pertussis would be of obvious survival advantage to the bacteria in vivo. The effect of individual B.pertussis components on neutrophil chemiluminescent response to whole-cells of B.pertussis was investigated using antigenically-modulated strains, transposon-induced mutants deficient in one or more virulence factors, and purified components of B.pertussis.

6.3.1. Effect of B.pertussis antigenic modulation

B.pertussis 18334 and Tohama X-mode strains induced higher levels of rabbit peritoneal neutrophil chemiluminescence than their respective C-mode strains. There was a positive correlation with strain hydrophobicity; both X-mode strains were significantly more hydrophobic than either C-mode strain. This agrees with the findings of Robinson et al. (1983) who also reported that B.pertussis becomes more hydrophobic during C- to X-mode variation.

The association of Escherichia coli with neutrophils, in the absence of antibodies and complement, is governed by hydrophobic interactions and mannose-specific structures such as type-1 fimbriae (Ohman et al., 1985). Topley et al. (1984) reported that the high surface hydrophobicity of unopsonized E.coli was associated with expression of type-1 fimbriae and the induction of a strong neutrophil chemiluminescent response. In addition, when fimbriation was suppressed the

bacteria became more hydrophilic and the neutrophil chemiluminescent response was greatly diminished. Piliation and hydrophobicity of non-mucoid Pseudomonas aeruginosa has been positively correlated with the susceptibility of the bacterium to phagocytosis (Speert et al., 1986). The neutrophil chemiluminescence experiments with B.pertussis were performed in the absence of antibodies and complement. The hydrophilicity results suggest that hydrophobic interactions may also be important in the association of B.pertussis with neutrophils. Fish et al. (1987) reported that FHA is the cell surface moiety responsible for hydrophobic adherence in B.pertussis. However, FHA is not a fimbrial component (Ashworth et al., 1982b). Thus, the loss of FHA from the cell surface of B.pertussis may account for the increased hydrophilicity of the C-mode strains and the poor capacity of these strains to stimulate neutrophil chemiluminescence.

A microorganism can sometimes avoid killing by phagocytes by avoiding phagocytosis. A correlation between increased bacterial hydrophilicity and increased resistance to phagocytosis by rabbit peritoneal neutrophils has been reported with Salmonella typhimurium (Stendahl et al., 1973). The smoothness of Salmonella typhimurium LPS appeared to be an important factor in determining the organism's hydrophilicity and resulting resistance to phagocytosis. The LPS of (virulent) phase I and (avirulent) phase IV B.pertussis are similar (Le Dur et al., 1980). This suggests that the LPS of B.pertussis would not change during antigenic modulation, and that changes in LPS are not responsible for the changes observed in cell hydrophilicity during antigenic modulation.

Neutrophil chemiluminescence has been associated with direct bacterial killing (Horan et al., 1982). Increased production of neutrophil chemiluminescence thus probably indicates increased levels of killing. The chemiluminescence results appear to indicate that rabbit peritoneal neutrophils might take up and kill X-mode organisms more easily than they kill C-mode organisms. This is contrary to the known virulence of the strains; X-mode organisms are more virulent than C-mode organisms (Lacey, 1960; Idigbe et al., 1981). X-mode strains have at least two factors (adenylate cyclase and PT) which can interfere with neutrophils. This suggests that since X-mode strains are virulent, adenylate cyclase and PT must be of prime importance in vivo.

The chemiluminescence studies were done with unopsonized B.pertussis organisms. However, B.pertussis may be opsonized prior to encounters with neutrophils in vivo. Opsonized B.pertussis induce higher levels of neutrophil chemiluminescence production than unopsonized B.pertussis (Pertilla et al., 1986). In the presence of serum opsonins, the neutrophil chemiluminescence patterns for the X- and C-mode organisms may be altered. The expected pattern of a negative correlation of B.pertussis virulence with neutrophil chemiluminescent response to whole-cells of B.pertussis may then be observed. Murine alveolar macrophages phagocytosed and killed opsonized B.pertussis phase IV organisms more easily than phase I organisms (Muse et al., 1979). This suggests that a similar correlation with virulence would be found with antigenically-modulated B.pertussis strains.

Opsonization may be important in determining the interaction of B.pertussis with neutrophils. The opsonization of smooth

Salmonella typhimurium by IgG and complement increased the organism's hydrophobicity and susceptibility to phagocytosis (Stendahl, 1983). However, the binding of secretory IgA to rough Salmonella typhimurium bacteria reduces the hydrophobic interaction of the organism with neutrophils, making the organism less likely to be phagocytosed (Magnusson et al., 1979). B.pertussis is a pathogen of the upper respiratory tract and IgA antibodies may be implicated in early immunity to pertussis and in the prevention of attachment (Bienenstock and Befus, 1980; Manclark and Cowell, 1984). The titres of IgA, IgG, and IgM against FHA increase during a natural pertussis infection (Granstrom et al., 1982). Burstyn et al. (1983) reported that IgA against FHA and LPS was present in convalescent sera and suggested that this may be presumptive evidence of recent pertussis infection. Thus, specific antibodies and complement are presumably also very important in determining the interaction of B.pertussis with neutrophils, and not simply hydrophobicity.

The effects of B.pertussis virulence factors on neutrophils are of obvious importance and should be taken into consideration when analysing the neutrophil chemiluminescence patterns induced by whole-cells of B.pertussis. Thus, B.pertussis Tohama mutants, deficient in one or more virulence factors and grown in X-mode, were assayed to determine the contribution of each virulence factor to the neutrophil chemiluminescence pattern obtained for the virulent parental organism. The adenylate cyclase- and haemolysin-deficient strain B.pertussis 348, the FHA-deficient strain B.pertussis 353, and the PT-deficient strain B.pertussis 357 induced similar chemiluminescence peak values which were not significantly different. All three

strains induced lower chemiluminescence peak values compared to the virulent B.pertussis parental strain. The loss of one or two virulence factors by the mutants may have lessened the surface hydrophobicity of the organism making the organism more hydrophilic and therefore resistant to neutrophil interaction. However, the hydrophilicity values obtained for the parental strains and the three mutant strains B.pertussis 348, 353, and 357 were not significantly different. This is contrary to the findings of Fish et al. (1987) who reported that B.pertussis strains deficient in FHA had increased hydrophilicity when compared to the parental strain which produced FHA. The hydrophilicity assay used for the present study may not have been sensitive enough to differentiate small differences in hydrophilicity, which may have been more readily detected by the differences observed in the neutrophil chemiluminescence studies. The avirulent strain B.pertussis 347 is very similar to a C-mode organism since both organisms are deficient in a range of virulence factors. The hydrophilicity value of B.pertussis 347 and the neutrophil chemiluminescence induced by the organism were similar to those of both B.pertussis Tohama or 18334 C-mode organisms.

The adenylate cyclase- and haemolysin-deficient strain, B.pertussis 348, induced lower levels of neutrophil chemiluminescence than the parental strain which produced both adenylate cyclase and haemolysin. This was contrary to expectations; presence of adenylate cyclase would be expected to cause a decrease in neutrophil chemiluminescence induced by intact bacteria. Studies by Confer and Eaton (1982) suggested that B.pertussis adenylate cyclase toxin inhibited both neutrophil killing of bacteria and induction of luminol-enhanced chemiluminescence by opsonized zymosan. The

protein concentration of the most active crude adenylate cyclase preparation they used to obtain an inhibitory effect on chemiluminescence was between about 2-32 μg ml^{-1}. The protein concentration per vial of the intact B.pertussis suspension used in the present chemiluminescence studies was about 11 μg. Any inhibitory effect of the adenylate cyclase toxin would probably be undetectable at such low protein concentrations. In addition, the bacteria used in the present study had been washed, a process which could have removed any surface-bound adenylate cyclase.

The effect of PT or FHA on neutrophil chemiluminescent response to whole-cells of bacteria has not been reported previously. Therefore, before any conclusions on the chemiluminescence patterns of the PT-deficient or FHA-deficient strains can be made, the effect of each component on chemiluminescence induced by whole-cells of B.pertussis must be determined. The results of such experiments are discussed in section 6.3.2.

6.3.2. Effect of purified virulence factors

PT in this study had a slight inhibitory effect on the neutrophil chemiluminescent response to whole-cells of B.pertussis. Possibly, this inhibition by PT reflects a partial suppression of phagocytosis which would be of advantage to the pathogen. Studies on the effect of PT on phagocytosis by other cell types have been performed, but the results appear contradictory. Meade et al. (1984) reported that PT inhibited phagocytosis by murine macrophages. However, Lad et al. (1986) reported that PT did not influence phagocytosis by human neutrophils. Differences in these

reports may reflect variations between the cell types used and in the experimental procedures.

The PT-deficient strain B.pertussis 357 did not induce greater chemiluminescence production or higher chemiluminescence peak values than the virulent parental strain. This is perhaps surprising since the toxin individually caused an overall inhibition of neutrophil chemiluminescence. FHA-treated neutrophils produced chemiluminescence more rapidly than untreated cells when intact B.pertussis was used as a stimulus. The rate of chemiluminescence production induced by the FHA-deficient strain B.pertussis 353 was similar to the parental strain which produced FHA. Perhaps the chemiluminescence assay was not sensitive enough to detect the influence of the low concentrations of PT or FHA found on whole-cells of B.pertussis. The results suggest that the way in which individual B.pertussis components, such as PT and FHA, are expressed on the bacterial cell surface may also influence the chemiluminescence patterns induced by whole-cells of B.pertussis.

B.pertussis LPS was assayed for its effects on neutrophil chemiluminescent response induced by intact B.pertussis. No studies of the effects of B.pertussis LPS on phagocytes have been reported previously. LPSs from other organisms have a variety of effects on phagocyte function. For example, E.coli O111:B4 LPS inhibited phagocytosis of opsonized microorganisms by both human neutrophils (Henricks et al., 1983) and human macrophages in vitro (Davis et al., 1980). Cohn and Morse (1960) reported that Salmonella abortus equi LPS enhanced killing of Staphylococcus albus by rabbit peritoneal neutrophils in the presence of 10% serum, but

reduced killing in the presence of 1% serum. In the present investigation, B.pertussis LPS inhibited the neutrophil chemiluminescent response to unopsonized, whole-cells of B.pertussis.

It would be of interest to study the effects of various toxins of B.pertussis on direct killing of B.pertussis by neutrophils. In addition, killing studies using antigenically-modulated strains and transposon-induced mutants of B.pertussis could be performed. Any results from such experiments could be compared with the present chemiluminescence results. Preliminary studies had shown that following incubation of B.pertussis with neutrophils, any ingested bacteria could be liberated by lysis of the neutrophils with saponin, an agent with an affinity for cholesterol. This lysis procedure did not influence the viability of the bacteria (data not shown). After neutrophil lysis, the viable count of the bacteria (section 4.15.) and the rate of bacterial killing could be obtained. For this assay it would probably be advantageous to use the nalidixic acid-resistant B.pertussis Tohama strain to minimise contamination.

Thus, the results indicate that the neutrophil chemiluminescence pattern induced by an individual bacterial strain, in the absence of opsonins, is highly dependent on physicochemical factors such as hydrophobicity. Other physicochemical aspects which may also influence the chemiluminescence results obtained for the intact bacterial studies include the surface charge of the organism (Stendahl, 1983). At the bacteria to neutrophil ratio used, no influence of bacterial-associated virulence factors on neutrophil chemiluminescence pattern generated was detected. However,

using purified virulence factors, different components of B.pertussis had effects on neutrophil chemiluminescent response to intact B.pertussis. The neutrophil chemiluminescence patterns induced by the different B.pertussis mutants may have been influenced by how the individual virulence factors are expressed by the bacteria. In conclusion, the neutrophil chemiluminescent response to bacteria appears to depend on both the physicochemical nature of the surface of the bacteria and on expression of the different virulence factors by the bacteria.

Part 4: The potential significance of the results on neutrophil responses in vivo

6.4.1. Importance of neutrophils in the control of respiratory tract infections

In humans, the inhibition of normal neutrophil responses by B.pertussis factors may be responsible for the host's susceptibility to pertussis and other secondary infections. The lodgement and replication of B.pertussis in the upper respiratory tract would probably initiate an inflammatory response, with the subsequent recruitment of neutrophils to the infected area. The production of anti-neutrophil factors by B.pertussis may therefore be important in pathogenesis of the disease.

The role of phagocytes in controlling upper respiratory tract infections has been poorly studied. Studies have tended to concentrate on the lower respiratory tract and on the role of pulmonary alveolar macrophages in the control of infection in this area (Green et al., 1977; Huber et al., 1977). However, certain observations suggest that neutrophils are also important in fighting infections of the respiratory tract. For example, patients with neutrophils which have chemotactic disorders are prone to recurrent respiratory infections including rhinitis and pneumonia (Gallin et al., 1980; Smith et al., 1972). Various studies have indicated that neutrophils are crucial in the early clearance of nontypable Haemophilus influenzae from murine lungs (Toews et al., 1985). Granulocytes are also recruited to the murine lung in the early clearance of Streptococcus pneumoniae and bacterial clearance is reduced in neutropenic animals

(Heidbrink et al., 1980). Both H.influenzae and S.pneumoniae can superinfect pertussis patients (Jamieson, 1973). Possibly inhibition of neutrophil responses by B.pertussis virulence factors contributes to the host's susceptibility to secondary infections by these and other organisms.

The majority of the studies performed were done using rabbit neutrophils. The human is the natural host for B.pertussis and the mouse is the most commonly used animal model for pertussis. Thus, the use of human or murine neutrophils would have made the results easier to discuss in relation to the human situation. However, Preston et al. (1980) suggested that pernasal pertussis infection in the rabbit would be more appropriate than murine intracerebral infection for a study of immunity to pertussis in the human child. In addition, rabbits have been infected with B.pertussis with the subsequent development of an upper respiratory tract infection (Ashworth et al., 1982a). Human neutrophils were difficult to obtain and murine neutrophils could not easily have been obtained in sufficient numbers to complete the quantity of assays performed using rabbit neutrophils. Thus, rabbit neutrophils were perhaps the next most appropriate experimental animal species to use for the studies performed.

6.4.2. Alteration of normal neutrophil responses by B.pertussis components

FHA had profound effects on rabbit neutrophil function. The aggregation of rabbit peritoneal and peripheral neutrophils was enhanced by FHA treatment which could indicate that margination and locomotion of rabbit neutrophils may be altered in vivo. FHA did not influence human peripheral

neutrophil aggregation although this does not rule out that it may influence human exudate neutrophil aggregation. During a pertussis infection, any neutrophils which were recruited to fight infection would of necessity have undergone emigration. PT appeared to enhance the aggregation of both rabbit and human neutrophils although relatively higher doses of toxin than of FHA were required to enhance rabbit peritoneal neutrophil aggregation. If PT does influence the aggregation of human peripheral neutrophils, it may alter the adhesive properties of the neutrophils *in vivo*. Thus, the alteration of neutrophil adhesiveness by circulating FHA or PT could inhibit neutrophil margination and locomotion *in vivo* thereby prolonging the survival of *B.pertussis* in the host.

FHA has been implicated as a potential adhesin of *B.pertussis* *in vitro* (Fish *et al.*, 1987) and may be an important adhesin *in vivo* (Tuomanen and Weiss, 1985). Scheffer *et al.* (1985) suggested that bacterial adhesins have a potent role in inducing the release of inflammatory mediators from neutrophils. My results show that FHA enhanced the chemiluminescent response of rabbit peritoneal neutrophils to a pro-inflammatory mediator (fMLP). In addition, FHA induced the chemiluminescence of these cells directly. FHA may also induce the release of inflammatory mediators from neutrophils. The effect of FHA on human neutrophil chemiluminescent response to fMLP was not investigated.

With a mixture of PT and FHA, the inhibitory effect of PT overrode the enhancing effect of FHA on the fMLP-induced chemiluminescence of neutrophils. Thus, neutrophil bactericidal response would probably be switched off during a pertussis infection. FHA had a lag period of about 15 min whereas PT had a lag period of about 30-40 min before any

effect on fMLP-induced chemiluminescence of neutrophils was observed. Both PT and FHA are found extracellularly *in vitro* and possibly *in vivo*, therefore both may act at a distance from the site of infection. Thus, although PT takes longer than FHA to alter fMLP-induced chemiluminescence, the time difference may not be relevant in the *in vivo* situation. FHA has been implicated as a major adhesin of *B.pertussis* and the results presented here suggest that FHA induced the generation of bactericidal products from neutrophils. One of the functions of PT may be to suppress any neutrophil bactericidal responses induced by FHA.

Previously PT has been shown to inhibit neutrophil response to fMLP in both human (Lad *et al.*, 1985) and rabbit neutrophils (Becker *et al.*, 1985). Thus, the chemiluminescence results showing that the toxin had an inhibitory effect on fMLP-induced chemiluminescence of rabbit peritoneal neutrophils are more easily discussed in terms of the disease in humans. Inhibition of neutrophil response to fMLP perhaps indicates an inhibition of the normal neutrophil responses to infection. Becker *et al.* (1986) suggested that the inhibitory effect of PT on the response of different neutrophil species to fMLP is closely related to the ADP-ribosylation of a G-protein common to the different neutrophil species. The chemiluminescence results indicate that PT inhibited the fMLP-induced chemiluminescence of rabbit peritoneal neutrophils. Although human neutrophils were not assayed, a similar inhibitory response would probably be found with these cells. Therefore, human neutrophil responses may also be inhibited in pertussis infection. In addition, PT induced a slight inhibition of the rabbit peritoneal neutrophil chemiluminescent response to intact *B.pertussis*. This would be

an obvious advantage to the organism *in vivo*. Since the mechanism of PT-induced inhibition of neutrophil chemiluminescence to intact bacteria is unknown, no predictions can be made using the results obtained. Chemiluminescence studies involving human neutrophils would have indicated whether PT also inhibited human neutrophil chemiluminescent response to intact *B.pertussis*.

The studies described in this thesis support the view that PT and FHA are important virulence factors of *B.pertussis*. PT is important primarily as the cause of the harmful effects of whooping cough (Pittman, 1979) and it may also, as this work has shown, inhibit neutrophil bactericidal responses to a pro-inflammatory mediator (fMLP) and intact *B.pertussis*. PT may also increase the adhesiveness of neutrophils and thus inhibit cell movement. Therefore, PT possibly acts as an aggressin by depressing host-defences. FHA, on the other hand, appears to enhance the bactericidal response to fMLP which PT inhibited. However, the inhibitory effect of PT overrode the enhancing effect of FHA. In addition, FHA may induce a faster bactericidal response to intact *B.pertussis*. If FHA is essential to the pathogen for lodgement, its apparent stimulatory effect on neutrophils (which is harmful to the pathogen) may be counteracted by PT, and probably by *B.pertussis* adenylate cyclase. It is clear from the above that the interaction of *B.pertussis* with neutrophils is complex and that a logical extension of the present work would be to investigate ingestion and killing (or survival) of *B.pertussis* in the presence and absence of opsonins of various specificities. Such studies would produce a better understanding of the pathogenesis of pertussis and might find applications in development of an improved vaccine.

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APPENDICES

8.1. Media and Diluents

Stainer-Scholte X-mode medium

A modification of the original Stainer and Scholte (1971) composition was used.

Chemical	Concentration (g l^{-1})
1. Monosodium l-glutamate	10.72
2. L-proline	0.24
3. NaCl*	2.50
4. KH_2PO_4	0.50
5. KCl	0.20
6. $MgCl_2.6H_2O$	0.10
7. $CaCl_2$	0.02
8. Tris	1.50
9. L-cysteine	0.04
10. $FeSO_4.7H_2O$	0.01
11. Ascorbic acid	0.02
12. Nicotinic acid	0.004
13. Glutathione	0.10

Ingredients 1-8 were dissolved in approximately 800 ml of distilled water. The pH was then adjusted to 7.6 with 2.5 N HCl and the total volume made up to 990 ml with distilled water. This preparation was then autoclaved at 121°C for 15 min at 15 p.s.i. and stored at 4°C until use. Ingredients 9-13 were dissolved in 10 ml of distilled water, sterilized by membrane filtration and then added before use.

*This was replaced by 5 g of $MgSO_4.7H_2O$ for C-mode medium.

Hepes buffered saline (HBS)

Chemical	Concentration (l^{-1})
NaCl	8.00 g
KCl	0.40 g
$MgCl_2.6H_2O$	0.20 g
$CaCl_2$	0.14 g
Glucose	1.00 g
HEPES	2.388 g
Phenol red	10 ml

Ingredients were dissolved in 900 ml of distilled, deionised water and the pH adjusted to 7.4 with 1 N NaOH. The volume was then made up to one litre with distilled, deionised water and the preparation autoclaved at 121°C for 15 min at 15 p.s.i. The divalent cation-free HBS-EDTA solution was prepared by the omission of the Ca- and Mg-containing salts and the addition of 0.292 g of monosodium EDTA.

Phosphate-buffered saline (Dulbecco A)

Chemical	Concentration (g l^{-1})
NaCl	8.00
KCl	0.20
Na_2HPO_4	1.15
KH_2PO_4	0.20

Phosphate-buffered saline tablets (Oxoid) were used. Each tablet was added to 100 ml of distilled water and the solution autoclaved at 121°C for 15 min at 15 p.s.i. The pH of the subsequent solution was 7.3.

8.2. ELISA buffers

0.05 M Carbonate Buffer

Chemical	Concentration (g l^{-1})
Na_2CO_3	1.59
$NaHCO_3$	2.93

Both ingredients were dissolved in one litre of distilled water. The pH of the resulting solution was approximately 9.6.

PBS-Tween

Chemical	Concentration (g l^{-1})
NaCl	8.0
KH_2PO_4	0.2
Na_2HPO_4	1.15
KCl	0.2

Ingredients were dissolved in one litre of distilled water. Tween 20 (polyoxyethylene sorbitan mono-laurate) was then added to a final concentration of 0.05% (v/v).

Citrate-Phosphate Buffer

Solution A: 0.1 M citric acid (21.01 g l^{-1} of distilled water).

Solution B: 0.2 M $Na_2HPO_4.2H_2O$ (35.6 g l^{-1} of distilled water).

A 0.15 M solution, pH 5.0, was prepared by the addition of 49 ml of Solution A to 59 ml of Solution B. The resulting solution was passed through a Whatman no. 1 filter paper before use (Whatman Ltd., Maidstone, Kent, England).

8.3. Buffers and gels for SDS-PAGE

Buffer		Per 100 ml
Solubilising Buffer:	glycerol	10 ml
	mercaptoethanol	5 ml
	SDS	3.0 g
	Bromophenol blue	0.01 g
	Tris buffer (pH 6.8)*	to 100 ml

*a 1 in 4 dilution of the upper buffer.

Lower buffer (pH 8.19)†:	Tris	18.1 g
	SDS	0.4 g
Upper buffer (pH 6.80)†:	Tris	6.06 g
	SDS	0.4 g

†pH was adjusted using HCl.

Acrylamide / Bis:	Acrylamide	30.0 g
	Bis	0.8 g
Running buffer (pH 8.3):	Tris	0.303 g
	glycine	1.44 g
	SDS	0.1 g

Gel

Lower gel (pH 8.19):	lower buffer	15 ml
	distilled H_2O	15 ml
	Acrylamide / Bis	30 ml
	10% (w/v) ammonium persulphate	0.30 ml
	TEMED	0.03 ml
Upper gel (pH 6.8):	upper buffer	5.0 ml
	distilled H_2O	12 ml
	Acrylamide / Bis	3.0 ml
	10% (w/v) ammonium persulphate	0.06 ml
	TEMED	0.04 ml

8.4. Standard curves

Figure 19. Cyclic AMP assay standard curve. Co/Cx represents the ratio of radioactive label bound in the absence of unlabelled cAMP (Co) over the ratio of radioactive label bound in the presence of standard or unknown unlabelled cAMP (Cx). Each point shown is a mean of 2 observations.

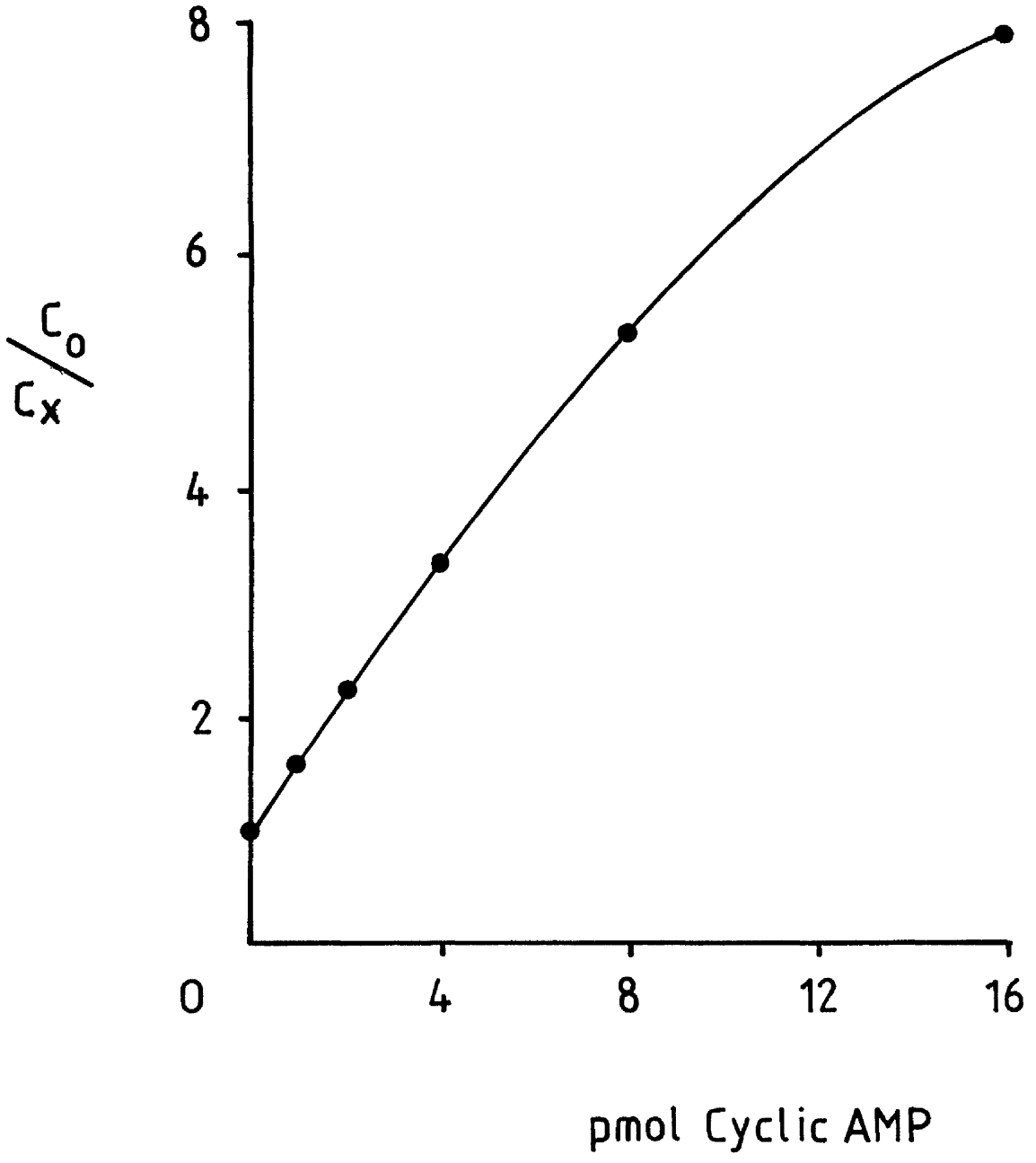
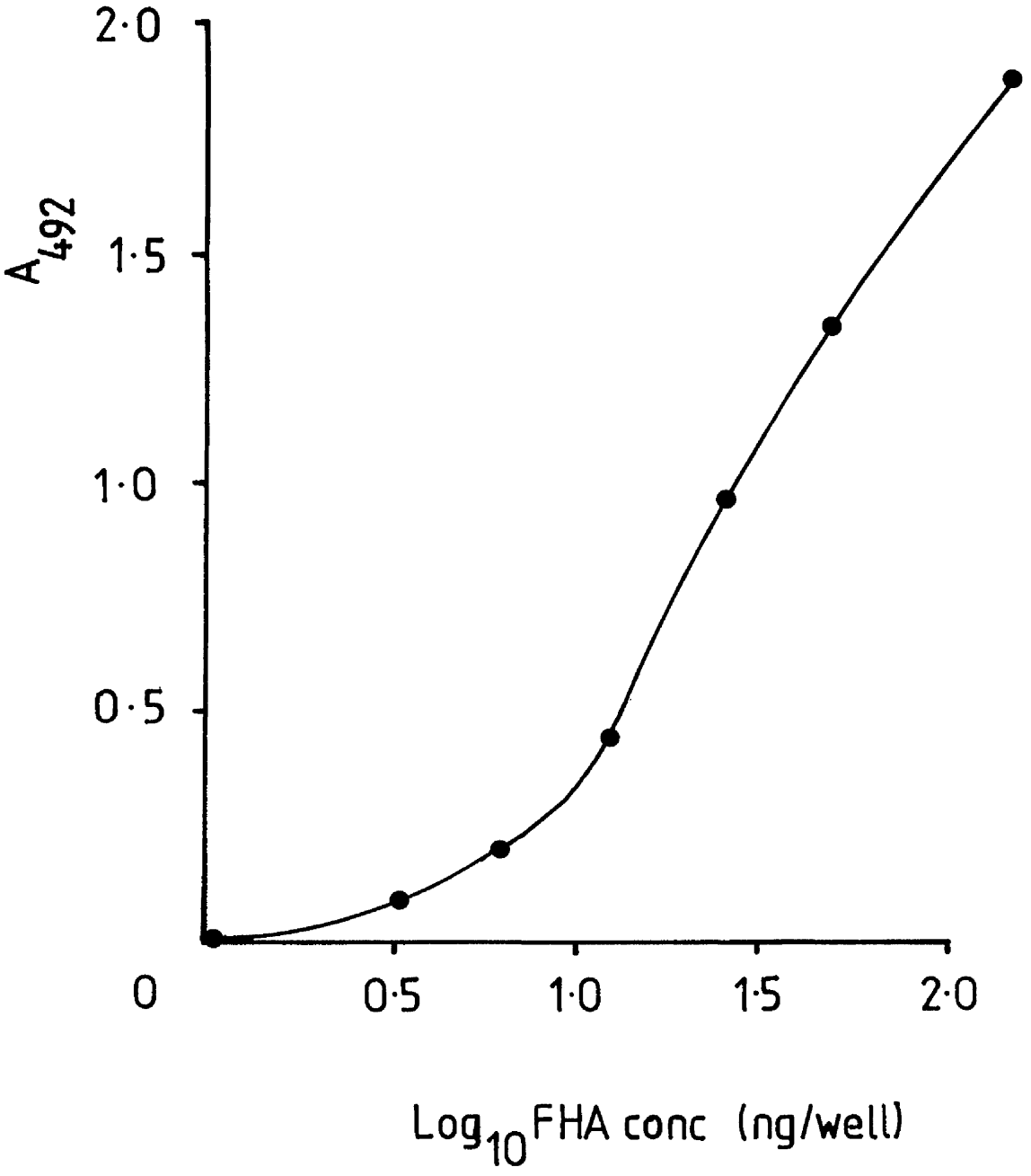

8
6
4
2
0
4
8
12
16
C_o / C_x
pmol Cyclic AMP

Figure 20. Filamentous haemagglutinin ELISA standard curve.

Each point shown is a mean of 2 observations.


2·0
1·5
1·0
0·5
0
A_{492}
0·5
1·0
1·5
2·0
Log_{10} FHA conc (ng/well)