



PROTEIN D - AN IgD-BINDING PROTEIN OF HAEMOPHILUS
INFLUENZAE

The present invention is related to a surface exposed protein named protein D which is conserved in many strains of Haemophilus influenzae or related Haemophilus species. Protein D is an Ig receptor for human IgD.

Several immunoglobulin (Ig) binding bacterial cell wall proteins have been isolated and/or cloned during the last two decades. The best characterized of these are protein A of Staphylococcus aureus and protein G of group G beta-hemolytic streptococci. The classical Fc-binding capacity of protein A involves IgG from humans and several mammalian species but the binding is restricted to human IgG subclasses 1, 2 and 4. Also other human classes of Ig (G, A, M, E) have been shown to bind to protein A, a reactivity that has been designed the alternative Ig binding which is mediated by Fab structures and characterized by a variable occurrence in the different Ig classes.

Protein G of group G streptococci binds all human IgG subclasses and has also a wider binding spectrum for animal IgG than protein A. On the IgG molecule the Fc part is mainly responsible for the interaction with protein G although a low degree of interaction was also recorded for Fab fragments. IgM, IgA and IgD, however, show no binding to protein G. Both protein A and protein G have acquired many applications for immunoglobulin separation and detection. (EP 0 200 909, EP 0 131 142, WO 87/05631, US 3,800,798, US 3,995,018.)

Certain strains of group A streptococci are also known to produce an IgG-binding protein which has been purified or cloned. The Ig-binding protein from group A streptococci is relatively specific for human IgG. Information about bacterial molecules that selectively bind IgA and IgM is more limited. However, IgA-binding proteins have been isolated from both group A and group B streptococci, two frequent human pathogens. The IgA receptor of

REPLACED SHEET	
☒	Description : paged ...1... to paged ...22...
☒	Claim(s) : paged ...23... to paged ...24...
☒	Drawing(s) : paged ...25... to paged ...26...
☒	Abstract : paged ...27... to paged ...28...
DATE OF RECEIPT	

INVENTOR

INVENTOR 15 FEB '96 12:03

group A streptococci has been named protein Arp. Certain strains of the anaerobic bacterium *Clostridium perfringens* preferentially bind IgM but also IgA and IgG. This binding is due to a cell surface protein (protein P). Recently a bacterial protein, protein L, with unique binding properties for L-chains was isolated from *Peptococcus magnus*. Protein L has been shown to bind IgG, IgA and IgM from human and several mammalian species. Among gram-negative bacteria, Ig receptors have been reported among veterinary pathogens. *Brucella abortus* binds bovine IgM and *Typhlocyba equigenitalis*, a venereal pathogen of horses, binds equine IgG. Recently *Haemophilus somnus* was reported to bind bovine IgG.

A decade ago *Haemophilus influenzae* and *Moraxella* (Branhamella) catarrhalis were shown to have a high binding capacity for human IgD (Forsgren A. and Grubb A, J. Immunol. 122:1468, 1979).

The present invention describes the solubilization and purification of a *H. influenzae* surface protein responsible for the interaction with IgD. It also describes the cloning, expression and nucleotide sequence of the IgD-binding protein gene of the *H. influenzae* in *Escherichia coli*. In addition it describes the Ig-binding properties of this molecule, named protein D, which were found to be different compared with previously isolated Ig-binding proteins. Protein D was found only to interact with IgD and not with other human immunoglobulin classes. Thus, protein D could be an important tool for studies, separation and detection of IgD in a way similar to the way in which protein A and protein G previously have been used for IgG. Protein D could also be a valuable tool alone and in combination with other molecules (for example proteins and polysaccharides) in the stimulation of the immune system through an interaction with B-lymphocytes. Protein D is not identical with any previously described protein from *H. influenzae*.

This Sheet Has Been Replaced



AWAPATENT

15 FEB '96 12:03 AWAPATENT



H. influenzae is a common human parasite and pathogen which colonizes the mucosa of the upper respiratory tract and causes disease by local spread or invasion. An important distinguishing feature between H. influenzae isolates is whether or not they are encapsulated. Encapsulated H. influenzae type b is a primary cause of bacterial meningitis and other invasive infections in children under 4 years of age in Europe and the United States. Non-encapsulated (non-typable) H. influenzae rarely cause invasive infection in healthy children and adults but are a frequent cause of otitis media in children and have been implicated as a cause of sinusitis in both adults and children. H. influenzae are also commonly isolated in purulent secretions of patients with cystic fibrosis and chronic bronchitis and have recently been recognized as an important cause of pneumonia.

A vaccine composed of purified type b capsular polysaccharide has proven effective against H. influenzae type b disease in children of 2 to 5 years of age. However, since children under two years of age respond poorly to this vaccine, conjugate vaccines with enhanced immunogenicity have been developed by covalently bonding the capsular polysaccharide to certain proteins. However, the polysaccharide vaccines, non-conjugated and conjugated, are of no value against nontypable H. influenzae disease. Hence, other cell surface components and in particular outer membrane proteins (OMPs) have been looked at as potential vaccine candidates both against type b and nontypable H. influenzae. (EP 0 281 673, EP 0 320 289.)

The outer membrane of H. influenzae is typical of gram-negative bacteria and consists of phospholipids, lipopolysaccharide (LPS), and about 24 proteins. Four different Haemophilus OMPs have been shown to be targets for antibodies protective against experimental Haemophilus disease. These include the P1 heat-modifiable major outer membrane protein, the P2 porin protein, the P6 lipoprotein and a surface protein with an apparent molecular weight of

98,000 (98 K protein). Of these at least antibodies to P2 have been shown not to protect against challenge with heterologous *Haemophilus* strains. (Loeb, M. R. Infect. Immun. 55:2612, 1987; Munson Jr, R. S. et al J. Clin. Invest. 72:677, 1983; Munson Jr, R. S. and Granoff, D. M. Infect. Immun. 49:544, 1985 and Kimura, A. et al, Infect. Immun. 194:495, 1985).

Analysis of nontypable *H. influenzae* has shown that there are marked differences in OMP composition among strains (See e.g. Murphy et al. "A subtyping system for nontypable *Haemophilus influenzae* based on outer membrane proteins" J Infect Dis 147:838, 1983; Barenkamp et al. "Outer membrane protein and biotype analysis of pathogenic nontypable *Haemophilus influenzae*" Infect Immun 30:709, 1983).

If a surface exposed antigen (immunogen) which is conserved in all strains of *H. influenzae* could be found it would be an important tool in developing a method of identifying *H. influenzae* in clinical specimens as well as a vaccine against *H. influenzae*. The present invention shows that protein D with an identical apparent molecular weight (42,000), reacting with three different monoclonal antibodies and human IgD, was found in all 116 *H. influenzae* strains (encapsulated and nonencapsulated) studied, as well as in two other related *Haemophilus* species, namely *H. haemolyticus* and *H. aegypticus*.

Thus, according to the invention there is provided a surface exposed protein, which is conserved in many strains of *Haemophilus influenzae* or related *Haemophilus* species, having an apparent molecular weight of 42,000 and a capacity of binding human IgD. The invention also comprises naturally occurring or artificially modified variants of said protein, and also immunogenic or IgD-binding portions of said protein and variants. The protein is named protein D and has the amino acid sequence depicted in Fig. 9.

This Sheet Has Been Replaced



INVENTENT

AWAPATENT

15 FEB '96 12:04

There is also provided a plasmid or phage containing a genetic code for protein D or the above defined variants or portions.

Further there is provided a non-human host containing the above plasmid or phage and capable of producing said protein or variants, or said portions thereof. The host is chosen among bacteria, yeasts or plants. A presently preferred host is *E. coli*.

In a further aspect the invention provides for a DNA segment comprising a DNA sequence which codes for protein D, or said variants thereof, or for said portions. The DNA sequence is shown in Fig. 9.

In yet another aspect, the invention provides for a recombinant DNA molecule containing a nucleotide sequence coding for protein D, or said variants or portions, which nucleotide sequence could be fused to another gene.

A plasmid or a phage containing the fused nucleotide defined above could also be constructed.

Further such a plasmid or phage could be inserted in a non-human host, such as bacteria, yeasts or plants. At present, *E. coli* is the preferred host.

The invention also comprises a fusion protein or polypeptide in which protein D, or said variants or portions, could be combined with another protein by the use of a recombinant DNA molecule, defined above.

Furthermore, a fusion product in which protein D, or said variants or portions, is covalently or by any other means bound to a protein, carbohydrate or matrix (such as gold, "Sephadex" particles, polymeric surfaces) could be constructed.

The invention also comprises a vaccine containing protein D, or said variants or portions. Other forms of vaccines contain the same protein D or variants or portions, combined with another vaccine, or combined with an immunogenic portion of another molecule.

This Sheet Has Been Replaced



AWAPATENT

AWAPATENT

15 FEB '96 12:04



There is also provided a hybridoma cell capable of producing a monoclonal antibody to an immunogenic portion of protein D, or of naturally occurring or artificially modified variants thereof.

5 Further there is provided a purified antibody which is specific to an immunogenic portion of protein D or of naturally occurring or artificially modified variants thereof. This antibody is used in a method of detecting the presence of *Haemophilus influenzae* or related *Haemo-*
10 *philus* species in a sample by contacting said sample with the antibody in the presence of an indicator.

The invention also comprises a method of detecting the presence of *Haemophilus influenzae* or related *Haemo-*
15 *philus* species in a sample by contacting said sample with a DNA probe or primer constructed to correspond to the nucleic acids which code for protein D, or for naturally occurring or artificially modified variants thereof, or for an immunogenic or IgD-binding portion of said protein or variants.

20 Protein D, or said variants or portions, is also used in a method of detecting IgD. In such a detecting method the protein may be labelled or bound to a matrix.

Finally, the invention comprises a method of separating IgD using protein D, or said variants or portions,
25 optionally bound to a matrix.

MATERIALS AND METHODS

Bacteria

116 *H. influenzae* strains representing serotypes a-f and nontypable and in addition bacterial strains represent-
30 ing 12 species related to *H. influenzae* were obtained from different laboratories in Denmark, Sweden and the U.S.A.

Culture conditions

All strains of *Haemophilus*, *Eikenella* and *Acinobacillus*
35 *lus* were grown on chocolate agar. *H. ducreyi* were grown in microaerophilic atmosphere at 37°C and all other *Haemophilus* strains in an atmosphere containing 5% CO₂. 30

isolates of *H. influenzae* were also grown overnight at 37°C in brain-heart infusion broth (Difco Lab., Inc. Detroit, Mi.) supplemented with nicotinamide adenine dinucleotide and hemin (Sigma Chemical Co. St Louis, Mo.), each at 10 µg/ml.

Immunoglobulins and proteins

IgD myeloma proteins from four different patients were purified as described (Forsgren, A. and Grubb, A., J. Immunol. 122:1468, 1979). Eight different human IgG myeloma proteins representing all four subclasses and both L-chain types, three different IgM myeloma proteins and one IgA myeloma protein were isolated and purified according to standard methods. Human polyclonal IgG, serum albumin and plasminogen were purchased from Kabi Vitrum AB, Stockholm, Sweden, and human IgE was adapted from Pharmacia IgE RIACT kit (Pharmacia Diagnostic AB, Uppsala, Sweden). Bovine serum albumin, human and bovine fibrinogen and human transferrin were purchased or obtained as a gift.

¹²⁵I-IgD binding assay

The binding assay was carried out in plastic tubes. Briefly 4×10^8 bacterial cells in a volume of 100 µl phosphate buffered saline (PBS) with the addition of 5% human serum albumine (HSA) were mixed with 100 µl of ¹²⁵I-IgD in the same buffer (radioactivity was adjusted to 7-8x10⁴ cpm, i.e. approx. 40 ng). After 0.5 h incubation at 37°C, 2 ml of ice-cold PBS (containing 0.1% Tween 20) was added to the tubes.

The suspension was centrifugated at 4,599xg for 15 min and the supernatant was aspirated. Radioactivity retained in the bacterial pellet was measured in a gamma counter (LKB Wallac Clingamma 1271, Turku, Finland). Residual radioactivity from incubation mixtures containing no bacteria, i.e. background, was 2.5 percent. Samples were always tested in triplicates and each experiment was repeated at least twice, unless otherwise stated.

This Sheet Has Been Replaced





Monoclonal antibodies

Inbred female BALB/c mice (age 8 to 14 weeks) were immunized by an intraperitoneal injection of 25 µg purified protein D (25 µg/50 µl) in Freund's complete
5 adjuvant (300 µl) followed by two intraperitoneal injections of protein D (15 µg) in Freund's incomplete adjuvant (300 µl) 3 and 7 weeks later. In week 9 the mice were bled from the tails, serum was separated and tested for anti-protein D activity in an enzyme-linked immuno-
10 sorbent assay (ELISA). The best responding mouse was boosted by an intravenous injection of protein D (2 µg) in 150 µl PBS. One day after the last injection, the spleen was excised and spleen cells were prepared for the production of monoclonal antibodies (De St Groth SF,
15 Scheidegger SJ J Immunol Methods 35:1, 1980). After 10 to 14 days (mean 12 days) the hybridomas were tested for the production of antibodies against protein D in an enzyme-linked immunosorbent assay (ELISA), and the hybrids producing the highest titers of antibodies were cloned and
20 expanded by cultivation in RPMI medium containing 10% fetal bovine serum. Totally 68 clones producing antibodies to protein D were obtained. Three of the hybridomas were selected for further growth in the same medium. All cell lines were frozen in the presence of dimethyl sulfoxide
25 and 90% fetal bovine serum in liquid nitrogen.

SDS-PAGE and detection of protein D on membranes

SDS-PAGE was, using a modified Laemmli gel, prepared and run according to the procedure of Lugtenberg et al., (FEBS Lett 58:254, 1975) using a total acrylamide concentration of 11%. Samples of crude Sarcosyl extracts of H.
30 influenzae and related bacterial species were pretreated by 5-min boiling in sample buffer consisting of 0.06M of Tris hydrochloride (pH 6.8), 2% (w/v) SDS, 1% (v/v) β-ME, 10% glycerol, and 0.03% (w/v) bromphenol blue. Electro-
35 phoresis was performed at room temperature using PROTEIN II vertical slab electrophoresis cells (Bio-Rad Laboratories, Richmond, CA) at 40 mA per gel constant current.



Staining of proteins in gels was done with comassie brilliant blue in a mixture of methanol, acetic acid and water essentially as described by Weber and Osborn (J. Biol. Chem. 244:4406, 1969). Protein bands were also
5 transferred to nitrocellulose membranes (Sartorius, West Germany) by electrophoretic transfer from SDS-poly-acrylamide gels. Electrophoretic transfer was carried out in a Trans-Blot Cell (Bio-Rad) at 50 V for 90 min. The electrode buffer was 0.025M Tris, pH 8.3, 0.192M glycine,
10 and 20% methanol. The membranes were then washed for 1 h at room temperature in 1.5% ovalbumin-Tris balanced saline (OA-TBS), pH 7.4, to saturate additional binding sites.

After several washings with Tris balanced saline (TBS), the membranes were incubated overnight at room
15 temperature in 1% OA-TBS buffer containing IgD (20 µg/ml) to detect IgD-binding bands, then washed twice with TBS. The membranes were then incubated with peroxidase conjugated goat anti-human IgD (Fc) (Nordic Immunology, Tiiburg, The Netherlands) for 1-2 hrs at room temperature;
20 after several washings with Tween-TBS the membranes were developed with 4-chloro-1-naphthol and hydrogen peroxide. Protein D was also identified using anti-protein D mouse monoclonal antibodies 16ClO, 20G6 and 19B4 at 1:50 dilution in 1% OA-TBS. Protein 1 and 2 of H. influenzae
25 were identified using anti-P2 mouse monoclonal 9F5 (Dr. Eric J. Hansen, Dallas, Texas, USA) at a 1:1000 dilution and rabbit anti-P1 serum (Dr. Robert S. Munson, St. Louis, Mo, USA) at a 1:200 dilution.
Solubilization and purification of protein D from H.
30 influenzae

Briefly 3 g of bacteria were suspended in 10 ml of 10 mM HEPES Tris buffer (pH 7.4) containing 0.01M EDTA and sonicated three times in a sonifier (MSE) for 1 min while cooling in an ice bath. Following sonication Sarcosyl
35 (Sodium Lauryl Sarcosinate) was added to a final concentration of 1% (w/v). The suspensions were incubated at room temperature for 1 h using a shaker and then sonicated

10

again 2x1 min on ice and reincubated at room temperature for 30 min. After centrifugation at 12,000 g for 15 min at 4°C the supernatant was harvested and recentrifugated at 105,000 g for 1.5 h at 4°C.

- 5 Sarcosylextracts prepared of *H. influenzae*, strain NT 772 as described above were applied to SDS-PAGE. After electrophoresis narrow gel strips were cut out, protein was transferred to membranes and the IgD-binding band was detected by Western blot assay using IgD and peroxidase
- 10 conjugated goat anti-human IgD as described above (see SDS-PAGE and detection of protein D on membranes). By comparison with the IgD-binding band on the membrane (Western blot) the appropriate band in the gel could be identified and cut out. Electrophoretic elution of the
- 15 IgD-binding molecules (protein D) was performed and SDS was removed from the protein containing solution by precipitation in potassium phosphate buffer using a method from Susuki and Terrada (Anal. Biochem. 172:259, 1988). Potassium phosphate in a final concentration of 20 mM was
- 20 added and after incubation at 4°C overnight the SDS-precipitate was removed by centrifugation at 12,000 g. Thereafter the potassium content was adjusted to 60 mM and after 4 hrs at 4°C centrifugation was performed as above. Finally the supernatant was concentrated and extensive
- 25 dialysis was performed.

Dot blot assay

- Proteins were applied to nitrocellulose membranes (Schleicher & Schuell, Dassel, West Germany) manually by using a dot blot apparatus (Schleicher & Schuell). After
- 30 saturation, the membranes were incubated overnight at room temperature in 1% OA-TBS containing ^{125}I -labeled protein probe (5 to 10×10^5 cpm/ml), washed four times with TBS containing 0.02% Tween-20, air dried, and autoradiographed at -70°C by using Kodak CEA.C X-ray films and Kodak X-Omat
- 35 regular intensifying screen (Eastman Kodak, Rochester, NY).

This Sheet Has Been Replaced



AWAPATENT

AWAPATENT

15 FEB '96 12:06

11

Amino acid sequence analysis

Automated amino acid sequence analysis was performed with an Applied Biosystems 470A gas-liquid solid phase sequenator (A) with online detection of the released
5 amino acid phenylthiohydantoin derivatives by Applied Biosystems Model 120A PTH Analyzer.

Bacterial strains, plasmids, bacteriophages and media used for cloning of protein D

H. influenzae, nontypable strain 772, biotype 2, was
10 isolated from a nasopharyngeal swab at the Department of Medical Microbiology, Malmö General Hospital, University of Lund, Sweden. *E. coli* JM83 were used as recipient for plasmids pUC18 and pUC19 and derivatives thereof. *E. coli* JM101 and JM103 were used as hosts for M13mpl8 and mp19
15 bacteriophages. *H. influenzae* was cultured in brain-heart infusion broth (Difco Lab., Inc. Detroit, Mi.) supplemented with NAD (nicotine adenine dinucleotide) and hemin (Sigma Chemical Co., St Louis, Mo.), each at 10 µg/ml. *E. coli* strains were grown in L broth or 2xYT media. L agar
20 and 2xYT agar contained in addition 1.5 g of agar per litre. L broth and L agar were, when so indicated, supplemented with ampicillin (Sigma) at 100 µg/ml.

DNA preparations

Chromosomal DNA was prepared from *H. influenzae*
25 strain 772 by using a modification of the method of Berns and Thomas (J Mol. Biol. 11:476, 1965). After the phenol:chloroform:isoamylalcohol (25:24:1) extraction step the DNA was ethanol precipitated. The DNA was dissolved in 0.1xSSC (1xSSC:0.15 M NaCl and 0.015 M sodium citrate) and
30 RNase treated for 2 h at 37°C. The RNase was removed with two chloroform:isoamylalcohol (24:1) extractions. The DNA was banded in a CsCl-ethidium bromide equilibrium gradient.

Plasmid DNA and the replicative form of phage M13
35 from *E. coli* JM101 were obtained by the alkaline lysis procedure followed by further purification in a CsCl-ethidium bromide gradient. In some cases plasmid DNA was

This Sheet Has Been Replaced



AMAPATENT

AMAPATENT

15 FEB '96 12:07



prepared using a Quiagen plasmid DNA kit (Quiagen GmbH Düsseldorf, FRG).

Single-stranded (ss) DNA from phage M13 clones was prepared from single plaques (Messing, J. Meth. Enzymol. 101C:20, 1983).

Molecular cloning of the protein D gene

A *H. influenzae* genomic library was constructed starting from 40 µg of *H. influenzae* strain 772 DNA which was partially digested with 1.2 units *Sau3A* for 1 h at 37°C. The cleaved DNA was fractionated on a sucrose gradient (Clark-Curtiss, J. E. et al., J. Bacteriol. 161:1093, 1985). Fractions containing DNA fragments of appropriate sizes (2-7 kilobasepairs (kbp)) were pooled and the DNA was ligated to dephosphorylated *Bam*HI digested pUC18 under standard conditions (Maniatis, T. et al., Molecular cloning: A laboratory manual, 1982). The ligation mixture was transformed into competent *E. coli* JM83 by high voltage electroporation with a Gene PulserTM/Pulse controller apparatus, both from Bio-Rad Lab. (Richmond, CA). The bacteria were plated onto L agar supplemented with ampicillin and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Colony immunoassay

For colony immunoblotting, *E. coli* transformants, cultivated overnight on L agar, were transferred to nitrocellulose filters (Sartorius GmbH, Göttingen, FRG) by covering the agar surfaces with dry filters. The plates were left for 15 min before the filters were removed and exposed to saturated chloroform vapour for 15 min. Residual protein binding sites on the filters were blocked by incubating the filters in Tris balanced saline containing ovalbumine for 30 min (TBS-ova; 50 mM Tris-HCl, 154 mM NaCl, 1.5% ova.; pH 7.4). After blocking, the filters were incubated in turn with (i) culture supernatants containing mouse monoclonal antibodies (MAbs) directed against protein D at a dilution of 1:10 in TBS-ova, (ii) horseradish peroxidase conjugated rabbit anti-mouse IgGs (DAKOPATTS



A/S, Glostrup, Denmark) in TBS-ova at a dilution of 1:2000 in TBS-ova, and (iii) 4-chloro-1-naphthol and H_2O_2 . The filters were washed 3x10 min in wash buffer (TBS-0.05% Tween 20) between each step. All incubations were done at room temperature.

Colonies were also checked for IgD binding by incubating other filters with purified human myeloma IgD:s, rabbit anti-human IgD (δ -chains) (DAKOPATTS), horseradish peroxidase conjugated goat anti-rabbit Ig:s (Bio-Rad Lab.) and 4-chloro-1-naphthol and H_2O_2 as above.

Restriction endonuclease analysis and DNA manipulations

Plasmid and phage DNA were digested with restriction endonucleases according to the manufacturers' instructions (Boehringer Mannheim mbH, Mannheim, FRG, and Beckman Instruments, Inc., England). Restriction enzyme fragments for subcloning were visualised with low energy UV-light and excised from 0.7-1.2% agarose gels (Bio-Rad) containing 0.5% ethidium bromide. The DNA bands were extracted with a GenecleanTM kit (BIO 101 Inc., La Jolla, Ca.) as recommended by the supplier.

Ligations were performed with 14 DNA ligase (Boehringer Mannheim) under standard conditions (Maniatis et al., 1982). The ligation mixtures were used to transform competent E. coli cells.

Progressive deletions of the recombinant plasmid pHIC348 for the sequencing procedure were produced by varying the time of exonuclease III digestion of KpnI-BamHI-opened plasmid DNA (Henikoff, S. Gene 28:351, 1984). For removal of the resulting single-stranded ends, mung bean nuclease was used. Both nucleases were obtained from Bethesda Research Laboratories Inc. (Gathersburg, Md.).

Protein D extraction from E. coli

Cells of E. coli expressing protein D were grown in L broth supplemented with ampicillin to early logarithmic phase and then subjected to osmotic shock. After removal of periplasmic fraction the cells were lysed with NaOH



(Russel, M. and Model, P., Cell 28:177, 1982) and the cytoplasmic fraction was separated from the membrane fraction by centrifugation. The periplasmic and cytoplasmic proteins were precipitated with 5% tri-chloro acetic acid.

DNA sequencing and sequence manipulations

The nucleotide sequence was determined by direct plasmid sequencing (Chen, E. Y. and Seeburg, P. H. DNA 4:165, 1985) of subclones and deletion derivatives of plasmid pHIC348 using the chain termination method with α [35 S]-dATP (Amersham) and SequenaseTM, version 2 (United States Biochemical Corp., Cleveland, Ohio) following the protocol provided by the supplier. Part of the sequencing was done on single-stranded M13 DNA carrying inserts derived from pHIC348. Autoradiography was performed with Fuji X-ray film.

RESULTS

Distribution of protein D in Haemophilus influenzae

A total of 116 H. influenzae strains obtained from culture collections and freshly isolated from nasopharyngeal swabs were selected for IgD-binding experiments. Eleven of the strains were encapsulated representing serotypes a-f, and 105 strains were non-encapsulated (nontypable). These 105 strains belonged to biotype I (21 strains), biotype II (39 strains), biotype III (14 strains), biotype IV (2 strains) and biotype I (5 strains). Of the non-encapsulated strains 31 were not biotyped (NET) but tested for IgD binding.

Approximately 4×10^8 cfu of H. influenzae bacteria grown on chocolate agar were mixed and incubated with 40 ng of radiolabeled human myeloma IgD. Thereafter a larger volume (2 ml) of PBS containing Tween 20 was added, bacteria were spun down and radioactivity of pellets was measured. All H. influenzae isolates bound IgD to a high degree (38-74%) (Fig. 1). There was no difference in IgD-binding capacity between different serotypes (a-f) of encapsulated H. influenzae. Nor was there any difference



between different biotypes of non-encapsulated strains. 30 strains representing different sero- and biotypes were also grown in brain-heart infusion broth. When those bacteria grown in liquid medium were compared with the same bacteria grown on chocolate agar, no difference in IgD-binding capacity could be detected.

Protein D was solubilized from all 116 *H. influenzae* strains by sonication and Sarcosyl extraction. Subsequently the extracts containing protein D were subjected to SDS-PAGE. Proteins were stained or electroblotted onto nitrocellulose membranes and probed with human IgD myeloma protein and three different mouse monoclonal antibodies recognizing protein D. Many protein bands could be detected in all SDS-gels but electrophoresis of extracts from all *H. influenzae* isolates gave a protein band with an apparent molecular weight of 42,000 (42 kilodaltons). IgD and also all three anti-protein D monoclonal antibodies (16C10, 20G6 and 19B4) bound to the same band after electrophoresis of all extracts and subsequent transfer to membranes and blotting.

Bacterial strains of 12 different species taxonomically related to *H. influenzae* (*H. ducreyi*, *H. paraphrophilus*, *H. parasuis*, *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, *H. aphrophilus*, *H. segnis*, *H. aegypticus*, *H. haemoglobinophilus*, *E. corrodens*, *A. actinomycetemcomitans*) were tested for their capacity to bind ^{125}I labeled human IgD. In addition crude Sarcosyl extracts from the same bacteria were tested by Western blot analysis with IgD and the three anti-protein D monoclonal antibodies (MAbs 16C10, 20G6, 19B4).

Of all twelve species tested, only *H. haemolyticus* (5/5 strains) and *H. aegypticus* (2/2 strains) bound radiolabeled IgD, 21-28% and 41-48%, respectively, in the direct binding assay (Fig. 2). In Western blot analysis IgD and all three monoclonal antibodies detected a single band with an apparent molecular weight of 42,000 (42 kilodaltons).



None of the 6 strains of *H. paraphrophilus*, 11 *H. parainfluenzae*, 8 *H. aphrophilus*, and 3 *A. actinomycetem-comitans* bound radiolabeled IgD in the direct binding assay or reacted with IgD in Western blot analysis. However, extracts of all these strains reacted with two or three of the monoclonal antibodies in Western blot analysis showing a single 42 kilodaltons protein band. Western blot analysis of three strains of *E. corrodens* revealed a single high molecular weight band (90 kilodaltons) with MAb 16C10 in all three strains. In an extract of one of the strains, a single 42 kilodaltons band was detected with the two other monoclonal antibodies. Two strains of *H. ducreyi*, *H. parasuis* (2 strains), *H. parahaemolyticus* (2 strains), *H. sengius* (2 strains), *H. haemoglobinophilus* (1 strain) did not bind radiolabeled IgD in the direct binding assay and Sarcosyl extracts from the same bacteria did not reveal any protein band detectable by IgD or the three monoclonal antibodies.

Solubilization of protein D

Three different strains of *H. influenzae* (two non-typable strains, 772 and 3198 and one type B, Minn A.) were grown overnight in broth. Initially attempts were made to solubilize protein D according to a well established method for isolation of *H. influenzae* outer membrane proteins by sonication, removal of the cell debris by centrifugation and extraction of the supernatant with Sarcosyl followed by ultracentrifugation (Barenkamp SJ and Munson RS J Infect Dis 143:668, 1981). The pellets (cell debris) (d) and supernatants (s) after sonication as well as the pellets (p) and supernatants (ss) after Sarcosyl-treatment and ultracentrifugation were subjected to SDS-PAGE. Proteins were stained or electroblotted onto Immobilon membranes and probed with human IgD myeloma protein followed by incubation with peroxidase conjugated anti-human IgD-antibodies and substrate. As shown in Fig. 3 the sonication procedure solubilized proteins including protein D effectively. However, IgD-binding



molecules (protein D) could also be detected in the cell debris, i.e. were not solubilized by sonication. The yield of IgD-binding molecules in the supernatant varied between different experiments. Fig. 3 also shows that protein D mostly could be detected in the Sarcosyl soluble supernatant after ultracentrifugation. In contrast previously described outer membrane proteins of *H. influenzae* (protein 1 to 6) are readily solubilized by sonication and are considered Sarcosyl insoluble.

10 To improve the yield of protein D several extraction methods were tried. In subsequent experiments the bacterial cells were sonicated and the whole cell suspension sonicated and extracted in different detergents (Sarcosyl, NP-40, Triton X-100 and Tween 80). The cell debris was
15 removed by centrifugation (12,000 g) and the supernatant ultracentrifuged. The thus obtained cell debris (d), supernatants (s) and pellets (p) were analysed by SDS-PAGE, electroblotting onto membranes and subsequent probing with IgD. As shown in Fig. 4 Sarcosyl treatment
20 effectively solubilized protein D leaving little left in the cell debris and pellet. NP-40, Triton X-100 and Tween-80 solubilized protein D less effectively.

Attempts were also made to solubilize protein D from the bacteria with lysozyme and different proteolytic enzymes (papain, pepsin and trypsin) at different concentrations. Of the enzymes only lysozyme solubilized protein D (Fig. 4).

Purification of protein D

Protein D was solubilized by Sarcosyl extraction of
30 whole bacteria as described above and purification was performed by SDS-PAGE of the supernatant after ultracentrifugation. After electrophoresis narrow gel strips were cut out, proteins were transferred to membranes and the IgD-binding band (protein D) was detected by Western blot
35 assay. Gel slices containing a protein band corresponding to the IgD-binding molecules were cut out from the gel and solubilized by electronic elution. At reelectrophoresis



the purified protein, protein D (D), migrated as a single band (42 kilodaltons) (Fig. 5) without discernible breakdown products.

To confirm that protein D was not identical with the previously described outer membrane proteins 1 or 2 with molecular weights of 49 and 39 kilodaltons, respectively, debris (d) and supernatants (s) after Sarcosyl extraction of whole *H. influenzae* bacteria were subjected to SDS-PAGE, transferred to Immobilon filters and blotted with antibodies to protein 1 and protein 2 and also with human IgD. As can be seen in Fig. 5 protein D migrates differently from protein 1 and protein 2.

Binding properties of protein D

The interaction of protein D with human IgD was further verified in gel filtration experiments where ^{125}I -protein D was eluted together with IgD when a mixture of the two proteins was run on a Sephadex G-200 column (Fig. 6c). Protein D run alone on the same column was eluted slightly after the 43 kilodaltons standard protein (Ovalbumin) confirming the apparent molecular weight of 42 kilodaltons for protein D.

Radiolabeled protein D was also studied in different dot blot experiments to further examine the binding specificity of the molecule. Fig. 7 shows that protein D effectively bound two highly purified human IgD myeloma proteins. A distinct reaction could be detected at 0.15 and 0.3 μg of the two IgD proteins, respectively. Two additional IgD myeloma proteins which were tested with the same technique could also distinctly be detected at 0.3 μg (data not shown). In dot blots IgD-Fab fragments and IgD-Fc fragments bound protein D at 2.5 and 1.2 μg , respectively. In contrast 8 different IgG myeloma proteins representing all subclasses and L-chain types showed no visible reaction with protein D at 5 μg . Neither could any reaction between protein D and three monoclonal IgM, one monoclonal IgA preparation, polyclonal IgE or some additional proteins be detected. However, with polyclonal IgG a weak reaction was detected at 5 μg (Fig. 7).



Cloning of the protein D gene

DNA isolated from *H. influenzae* 772 was partially digested with *Sau*3A and enriched for fragments in the size of 2 to 7 kilobasepairs (kbp) by fractionation on a sucrose gradient. These fragments were ligated to the BamHI-cut and phosphatase-treated vector pUC18. *E. coli* JM83 cells transformed with the ligation mixture by high voltage electroporation were plated selecting for resistance to ampicillin. Individual colonies were transferred to nitrocellulose filters and screened with a cocktail of monoclonal antibodies (MAbs) as described in Materials and Methods.

Among the 15,000 colonies tested, 60 were found positive. Eight positive colonies were picked, purified and subjected to another two rounds of screening. All clones remained positive during the purification. The purified clones were tested for IgD binding with human IgD, rabbit anti-human IgD and peroxidase conjugated goat anti-rabbit Ig:s in a colony immunoassay as described in Materials and Methods. All were positive regarding IgD binding. Additionally, the clones were found positive when screening with the three MAbs individually.

Restriction enzyme analysis of plasmid DNA from the positive clones showed that all but one clone carried a 3.3 kbp insert with two internal *Sau*3A sites. One clone contained an additional 2.0 kbp *Sau*3A fragment. One of the smaller recombinant plasmids, pHIJ32, was chosen for further characterization. A partial restriction enzyme map was established for the insert of *H. influenzae* DNA in pHIJ32 (Fig. 8). To identify the region coding for protein D, restriction enzyme fragments were subcloned into pUC18. The resulting transformants were tested for expression of protein D using colony immunoblot analysis as described above. These experiments showed that plasmids carrying a 1.9 kbp *Hind*III-*Cla*I fragment from one end of the insert allowed expression of IgD-binding protein. This recombinant plasmid, called pHIC348, was kept for further experi-



ments. The protein D gene cloned in pHIC348 is expressed from a promoter in pUC18. This was shown by cloning the HindIII-ClaI fragment of pHJ32 in the opposite orientation in pUC19. All transformants expressed IgD binding, as would be expected if the gene is under the control of an endogenous promoter. Transformants carrying the HindIII-ClaI fragment in the opposite direction to pHIC348 grew poorly and autolysed during cultivation. This was probably due to the lacZ promoter of pUC19 being oriented in the same direction as the promoter of protein D which led to an overexpression of protein D which was lethal to the cells. In pHIC348 the lacZ promoter was in the opposite direction of the protein D promoter.

DNA sequence analysis of the protein D gene

The nucleotide sequence of both strands of the insert from pHIC348 was determined either by direct plasmid sequencing of subclones and deletion constructs or by subcloning restriction fragments into phages M13mp18 and M13mp19. Commercially available universal and reverse M13 primers were used. Sequencing was done across all restriction enzyme sites used in subcloning and the sequencing strategy is outlined in Fig. 8.

The DNA sequence (Fig. 9) reveals an open reading frame of 1092 bp starting with an ATG codon at position 204 and finishing at position 1296 with a TAA stop codon. The open reading frame corresponds to a protein of 364 amino acid residues. Ten nucleotides upstream of the methionine codon is a sequence, AAGGAG, that is complementary to the 3' end of the 16S rRNA of *E. coli* (Shine, J. and Dalgarno, L. Proc. Natl. Acad. Sci. USA, 71:1342, 1974). The spacing between the centre of this putative ribosome-binding site (rbs) and the start codon is 13 bp in comparison to the average spacing of 10 bp in *E. coli*. The 5' flanking region, upstream of the proposed rbs, shows the presence of possible promoters. The sequences of the -10 region, TAAAAT (151-156), and the -35 region, TTGCTT (127-132), show homology to the consensus of *E.*



coli promoters (Rosenberg, M. and Court, D., Annu. Rev. Genet, 13:319, 1979) and are identical with promoters recognized by the E. coli RNA polymerase. The spacing between the putative -10 and -35 sequences is 18 bp, which
5 is comparable with the favoured value of 17 bp.

Between position 1341 and 1359 there is an inverted repeat with the potential to form a stem and loop structure. This repeat does not, however, resemble a typical rho-independent transcription terminator.

10 Protein D structure

The gene for protein D encodes for a protein of 364 amino acid residues deduced from the nucleotide sequence (Fig. 9). The N-terminal amino acid sequence has typical characteristics of a bacterial lipoprotein signal peptide
15 (Vlasuk et al., J. Biol. Chem. 258:7141, 1983) with its stretch of hydrophilic and basic amino acids at the N-terminus followed by a hydrophobic region of 13 residues, and with a glycine in the hydrophobic core. The putative signal peptide ends with a consensus sequence Leu-Ala-Gly-Cys,
20 recognized by the enzyme signal peptidase II (SpaseII). The primary translation product has a deduced molecular weight of 41,821 daltons. Cleavage by SpaseII would result in a protein of 346 amino acids with a calculated
molecular size of 40,068 daltons, in contrast to the
25 estimated size of the mature protein D of approximately 42 kilodaltons. Posttranslational modifications of the preprotein may account for this discrepancy. Several attempts to determine the amino-terminal amino acid sequence of protein D were performed by applying about
30 1000 pmoles thereof in an automated amino acid sequencer. Since no amino acid phenylthiohydantoin derivatives were obtained, the amino-terminal end of the single IgD-receptor polypeptide chain is probably blocked.

Protein D expressed in E. coli JM83 carrying pHIC348
35 was analysed in immunoblotting experiments (Fig. 10). Cytoplasmic, periplasmic and membrane fractions from cells in late logarithmic phase were separated on a SDS-PAGE gel

AMAPATENT

AMAPATENT

15 FEB '96 12:11

and electroblotted to an Immobilon filter. A protein that binds all three anti-protein D monoclonal antibodies (16C10, 20G6 and 19B4) and radiolabeled IgD could be detected in all three fractions (lane 2-4) from *E. coli* JM83/pHIC348 as a single band with an estimated molecular weight of 42 kilodaltons, i.e. equal or similar to protein D prepared from *H. influenzae* (lane 1, Fig. 10).

The nucleotide sequence and the deduced amino acid sequence of *H. influenzae* 772 protein D were compared with other proteins of known sequence to determine homology by using a computer search in the EMBL and Genbank Data Libraries. Apart from similarities in the signal sequence no homology was found.

SUMMARY

A novel surface exposed protein of *H. influenzae* or related *Haemophilus* species is described. The protein named protein D is an Ig receptor for human IgD and has an apparent molecular weight of 42,000. Protein D can be detected in all of 116 encapsulated and non-encapsulated isolates of *H. influenzae* studied. The protein from all strains shows in addition to the same apparent molecular weight immunogenic similarities since protein D from all strains interacts with three different mouse monoclonal antibodies and monoclonal human IgD. A method for purification of protein D is described. Cloning of the protein D gene from *H. influenzae* in *E. coli* is described as well as the nucleotide sequence and the deduced amino acid sequence corresponding to a molecular weight of 41,821 daltons including a putative signal sequence of 18 amino acids containing a consensus sequence, Leu-Ala-Gly-Lys for bacterial lipoproteins.

This Sheet Has Been Replaced



23

CLAIMS



1. A surface exposed protein, which is conserved in
5 many strains of Haemophilus influenzae or related Haemo-
philus species, having an apparent molecular weight of
42,000 and a capacity of binding human IgD, or naturally
occurring or artificially modified variants thereof, or an
immunogenic or IgD-binding portion of said protein or
10 variants.

2. A protein according to claim 1, having the amino
acid sequence as described in Fig. 9, or naturally occur-
ring or artificially modified variants thereof, or an
immunogenic or IgD-binding portion of said protein or
15 variants.

3. A plasmid or phage containing a genetic code for a
protein of Haemophilus influenzae or related Haemophilus
species, said protein having an apparent molecular weight
of 42,000 and a capacity of binding human IgD, or for
20 naturally occurring or artificially modified variants
thereof, or for an immunogenic or IgD-binding portion of
said protein or variants.

4. A non-human host containing a plasmid or a phage
as defined in claim 3 and capable of producing said
25 protein or variants or a portion of said protein or
variants, which host is chosen among bacteria, yeasts and
plants.

5. A host according to claim 4, c h a r a c t e -
r i s e d in that it is E. coli.

30 6. A DNA segment comprising a DNA sequence which
codes for a protein of Haemophilus influenzae or related
Haemophilus species, said protein having an apparent
molecular weight of 42,000 and a capacity of binding human
IgD, or for naturally occurring or artificially modified
35 variants thereof, or for an immunogenic or IgD-binding
portion of said protein or variants.

This Sheet Has Been Replaced



AWAPATENT

AWAPATENT

15 FEB '96 12:12



7. A DNA-segment according to claim 6, wherein the DNA sequence is the one specified in Fig. 9.

8. A recombinant DNA molecule containing a nucleotide sequence coding for a surface exposed protein of
5 Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or for naturally occurring or artificially modified variants thereof, or for an immunogenic or IgD-binding portion of said protein or
10 variants, which nucleotide sequence is fused to another gene.

9. A plasmid or phage containing a fused nucleotide sequence according to claim 8.

10. A non-human host containing at least one plasmid
15 or phage according to claim 9, which host is chosen among bacteria, yeasts or plants.

11. A host according to claim 10, c h a r a c -
t e r i s e d in that it is E. coli.

12. A fusion protein or polypeptide in which a sur-
20 face exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion
25 of said protein or variants, is combined with another protein by the use of a recombinant DNA molecule according to claim 8.

13. A fusion product in which a surface exposed protein of Haemophilus influenzae or related Haemophilus
30 species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion of said protein or variants, is covalently or by any other means
35 bound to a protein, carbohydrate or matrix.

This Sheet Has Been Replaced



AWAPATENT

AWAPATENT

15 FEB '96 12:12



25

14. A vaccine containing a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion thereof.

15. A vaccine containing a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion thereof, combined with another vaccine.

16. A vaccine containing a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion thereof, combined with an immunogenic portion of another molecule.

17. A hybridoma cell capable of producing a monoclonal antibody to an immunogenic portion of a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD.

18. A purified antibody which is specific to an immunogenic portion of a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD.

19. A method of detecting the presence of Haemophilus influenzae or related Haemophilus species in a sample by contacting said sample with the antibody of claim 18 in the presence of an indicator.

This Sheet Has Been Replaced





20. A method of detecting the presence of Haemophilus influenzae or related Haemophilus species in a sample by contacting said sample with a DNA probe or primer constructed to correspond to the nucleic acids which code
5 for a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or for naturally occurring or artificially modified variants thereof, or for an immunogenic or IgD-
10 -binding portion of said protein or variants.

21. A method of detecting IgD using a surface exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or
15 naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion of said protein or variants, optionally labelled and/or bound to a matrix.

22. A method of separating IgD using a surface
20 exposed protein of Haemophilus influenzae or related Haemophilus species, said protein having an apparent molecular weight of 42,000 and a capacity of binding human IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding portion
25 of said protein or variants, optionally bound to a matrix.

30

This Sheet Has Been Replaced



35

AWAPATENT

15 FEB '96 12:13 AWAPATENT