NOVEL SURFACE EXPOSED IMMUNOGLOBULIN D-BINDING PROTEIN FROM MORAXELLA CATARRHALIS

Field of the invention

The present invention relates to a surface exposed protein, which can be detected in Moraxella catarrhalis, having an amino acid sequence as described in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, and to an immunogenic or IgD-binding fragment of said surface exposed protein, and to an immunogenic and adhesive fragment of said surface exposed protein.

Background of the invention

Moraxella catarrhalis is a Gram-negative diplococcus that for a long time was considered a relatively harmless commensal in the respiratory tract. At present, it is the third most frequent cause of otitis media and also a significant agent in sinusitis and lower respiratory tract infections in adults with pulmonary disease. M. catarrhalis is also one of the most common inhabitants of the pharynx of healthy children.

Two decades ago, Haemophilus influenzae and M. catarrhalis were shown to display a strong affinity for both soluble and surface-bound human IgD (1). The IgD-binding seems to be paralleled by a similar interaction with surface-bound IgD at the cellular level, a phenomenon that explains the strong mitogenic effects on human lymphocytes by H. influenzae and M. catarrhalis (2-4). An IgD-binding outer membrane protein from H. influenzae (protein D) was isolated and cloned, and shown to be an important pathogenicity factor (5). However, protein D does not bind to the majority of IgD myelomas tested, and it was suggested that encapsulated H. influenzae of serotype b expresses an additional IgD receptor (6).

Early studies demonstrated that the outer membrane proteins (OMPs) from a diverse collection of Moraxella

isolates exhibit a high degree of similarity (7). Investigators have primarily focused their research efforts on a selected group of proteins. Recent studies have demonstrated that the high-molecular-weight surface antigen, termed UspA or HMW-OMP, is actually comprised of two different proteins. These proteins are named UspA1 and UspA2 (8,9,10). The apparent molecular masses of these OMPs are greater than 250 kDa as determined by SDS-PAGE analysis. Reduction with formic acid yields bands of approximately 120 to 140 kDa, suggesting that the UspA proteins form an oligomeric complex composed of several monomeric subunits (11). The predicted mass of each protein, as deduced from the cloned genes, is 88 kDa and 62 kDa for UspA1 and UspA2, respectively. It is thought that the difference in the deduced mass and the mass determined using SDS-PAGE is due to a predicted coiled coil structure (9).

In a recent patent publication, an outer membrane protein of M. catarrhalis with a molecular mass of approximately 200 kDa was isolated (12). A sequence encoding a protein of approximately 200 kDa was also provided. The protein was shown to be immunogenic, but no further biological functions were presented. In addition, a 200 kDa protein is associated with hemagglutinating M. catarrhalis (13,14).

CopB is an 80 kDa surface exposed major OMP that shows a moderate antigenic conservation. In addition, OMP CD is a 46 kDa highly conserved protein with numerous surface exposed epitopes and OMP E a 47 kDa protein detected on a variety of heterologous strains. The lactoferrin-binding (LbpA and B) and transferrin-binding (TbpA and B) proteins have molecular sizes of 99-111 and 74-105 kDa, respectively.

Certain strains of Staphylococcus aureus produce immunostimulatory exotoxins such as toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin A (SEA), SEB and SEC, all of which are associated with food poisoning and toxic shock syndrome (TSS). These exotoxins have been

denominated as superantigens (SAg) due to their ability to activate a high frequency of T lymphocytes. SAg bind as unprocessed proteins to HLA class II molecules on APC and oligoclonally activate T cells expressing particular TCR Vβ chains. In vivo exposure to excessive amounts of SAg results in a strong cytokine production and includes IL-2, TNF-α and IFN-γ, which are associated with a toxic shock like syndrome.

Since the discovery of the first immunoglobulin-binding bacterial protein, S. aureus protein A (SpA) in 1966, this protein has been extensively characterized. The ability of SpA to bind the Fc part of IgG is well known, but SpA also binds a fraction of Ig-molecules of all classes due to the so called 'alternative' binding, which represents an interaction with the variable region of certain heavy chains. All IgG-binding capacity of *S. aureus* has been considered to be mediated by SpA. However, the existence of a second gene in S. aureus encoding an Ig-binding protein has also been reported.

Streptococcus pyogenes and Peptostreptococcus magnus are other examples of Ig-binding bacteria. S. pyogenes produces protein H belonging to the M family of proteins, and has strong affinity for the Fc region of IgG. Proteins expressed by some strains bind IgA instead of IgG or both IgG and IgA. Protein Bac or the B-antigen is an IgA-binding protein expressed by certain strains of group B streptococci. Finally, P. magnus expresses protein L that shows high and specific affinity for Ig light chains, especially k light chains, and thereby interacts with all classes of Ig.

IgD is a unique immunoglobulin that exists in both a soluble and a surface-bound form. Both forms are encoded by the same gene and are splicing products. All mature B lymphocytes have B cell receptors (BCR) consisting of membrane-bound IgD and IgM. Soluble IgD comprises approximtely 0.25 % of the total amount of serum-Ig. The main function of IgD seems to be as an antigen-receptor on the

B cell surface in order to optimize B cell recruitment and accelerated affinity maturation. Antigen is taken up through IgD by endocytosis followed by intracellular degradation and presentation on MHC class II for T cells, which in turn are activated and produce cytokines. Hereby, T cell help is obtained including numerous cytokines (e.g. interleukin-4) and co-stimulatory molecules such as CD28.

Despite macrophages, dendritic cells, and B cells all can present antigens to T lymphocytes, the B cells are 100-fold more efficient due to the importance of the antigen-presenting immunoglobulin on the surface. An attractive strategy in order to potentiate immunization is to directly target an antigen to the B cell receptor. It was early shown that the mouse antibody-response against bovine serum albumin (BSA) conjugated to anti-IgD monoclonal antibodies was 100-fold stronger compared to BSA administration without any antibody. In paralell, it has been demonstrated that a mouse myeloma antigen incorporated into the constant region of anti-IgD-antibodies targeted to the surface-bound IgD results in an up to 1000-fold more efficient antigen presentation on MHC class II (15).

Tolerance induction can be achieved experimentally by B cell activation through the IgD BCR without any additional T cell help. It would also be possible to treat autoimmune diseases by inducing B cell anergy and thus inhibit the production of auto-antibodies. In fact, SLE-prone mice administered dextran-conjugated anti-IgD antibodies exhibit a delayed development of autoimmunity. In yet another study it was shown that B cell activation via IgD decreases a T helper 2-induced IgE response suggesting a therapy for diminishing the IgE production in severely allergic individuals by displacing the antibody response from a Th2- to a Th1-response. By targeting antigens to the B cell receptor IgD, stimulation, tolerance, and a switch from IgE-production can be achieved.

In addition, polyclonal activation has been reported. The outcome is depending on the experimental model used. With different constructs including various repeating IgD-binding segments, it is possible to tailor the response.

The T cell is a significant player in the anti-tumour response since it recognizes tumour-specific antigens. However, the important T cells display commonly depressed activity in the cancer patient due to a general immunosuppression. A triggering of T helper cells would therefore be very beneficial. Vaccination against tumours using antigen presenting cells (APC) has recently been acknowledged (17). Immunization protocols with APC pulsed ex vivo with tumor antigens (peptides) have been shown to induce effective MHC class I presentation for cytotoxic T cells. It has also been demonstrated that EBV-transformed B cells are able to present melanoma antigens for tumour-infiltrating lymphocytes (TIL). In experimental models, it has also been shown that tumour cells transfected with MHC class II and B7 surface molecules, receptors that are abundant on B cells, would be a feasible approach for tumour vaccination. Interestingly, B16 melanoma bearing mice that were injected with B cells pulsed with a tumour lysate from the corresponding cell line showed a prolonged survival due to an increase in IFN-γ producing T cells. It was also demonstrated that the induced T helper cells evoked a stronger cytotoxic response against the solid tumours. Since myeloma antigen targeted to IgD induces a T cell response, the suggested approach using IgD-binding bacterial proteins conjugated to specific tumour antigens would be feasible.

To target an antigen (e.g. peptide derived from a microbe or a specific tumour) to IgD-bearing B cells in order to trigger both humoral and cellular immune responses a IgD-binding protein or a shorter IgD-binding peptide would be a very feasible vector. Several examples of successful strategies with a similar angle of approach exist. The humoral immune response in mice against bovine

serum albumin (BSA) conjugated to anti-IgD monoclonal antibodies is 100-fold stronger compared to when BSA is administered alone. A recent publication by Lunde et al. (15) describes that when a myeloma-derived peptide is integrated in the constant region of anti-IgD Fab´ fragments and injected into mice, a 1,000-fold more efficient antigen presentation is achieved against the antigen in question (15). In parallel, the Ig-binding fragment of S. aureus protein A fused with cholera toxin significantly increases both systemic and mucosal immune responses 10- to 100-fold against the cholera toxin (16). Finally, in a mouse tumour model consisting of the experimentally well defined B16 melanoma, activated B lymphocytes that are pulsed ex vivo with peptides derived from the tumour tissue can evoke a stronger anti-tumour response in vivo and consequently a prolonged survival (17).

Summary of the invention

In one aspect the present invention relates to a surface exposed protein, which can be detected in Moraxella catarrhalis, having an amino acid sequence as described in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants, or an immunogenic and adhesive fragment of said surface exposed protein.

In another aspect the present invention relates to an immunogenic or IgD-binding fragment of a surface exposed protein as defined above, which fragment can be detected in Moraxella catarrhalis, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof.

In a further aspect the present invention relates to an immunogenic or IgD-binding fragment as described

above, having an amino acid sequence as described in SEQ ID NO 10.

In still a further aspect the present invention relates to an immunogenic and adhesive fragment of a surface exposed protein as defined above, which fragment can be detected in Moraxella catarrhalis, having a capacity of binding erythrocytes and epithelial cells.

In still another aspect the present invention relates to an immunogenic and adhesive fragment as defined above, having an amino acid sequence as described in SEQ ID NO 8.

In one aspect the present invention relates to a DNA segment comprising a DNA sequence, as shown in SEQ ID NO 2, which DNA sequence codes for a surface exposed protein of Moraxella catarrhalis as defined above, or naturally occurring or artificially modified variants of said DNA sequence.

In yet another aspect the present invention relates to a DNA segment comprising a DNA sequence which codes for an immunogenic or IgD-binding fragment as defined above.

In a further aspect the present invention relates to a DNA segment as defined above, comprising a DNA sequence, as shown in SEQ ID NO 11, which DNA sequence codes for an immunogenic or IgD-binding fragment as defined above.

In still a further aspect the present invention relates to a DNA segment comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined above.

In another aspect the present invention relates to a DNA segment as above, comprising a DNA sequence, as shown in SEQ ID NO 9, which DNA sequence codes for an immunogenic and adhesive fragment as defined above.

In a further aspect the present invention relates to a vaccine containing a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid

sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, or an immunogenic and adhesive fragment of said surface exposed protein.

In another aspect the present invention relates to a vaccine containing an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis, which has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, preferably a vaccine containing an immunogenic or IgD-binding fragment having an amino acid sequence as described in SEQ ID NO 10.

In still another aspect the present invention relates to a vaccine containing an immunogenic and adhesive fragment of a surface exposed protein of Moraxella catarrhalis as defined above, preferably a vaccine containing an immunogenic and adhesive fragment having an amino acid sequence as described in SEQ ID NO 8.

In one preferred embodiment said vaccines are combined with another vaccine and in another preferred embodiment said vaccines are combined with an immunogenic portion of another molecule.

In one aspect the present invention relates to a plasmid or phage comprising a DNA sequence, which codes for a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants.

In another aspect the present invention relates to a plasmid or phage comprising a DNA sequence, which codes

for a an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, preferably a plasmid or phage comprising a DNA sequence, which codes for a an immunogenic or IgD-binding fragment having an amino acid sequence as described in SEQ ID NO 10.

In still another aspect the present invention relates to a plasmid or phage comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined above, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells or naturally occurring or artificially modified variants of said fragment, preferably a plasmid or phage comprising a DNA sequence, which codes for a an immunogenic and adhesive fragment having an amino acid sequence as described in SEQ ID NO 8.

In yet another aspect the present invention relates to a non human host comprising at least one plasmid or phage as defined above, and capable of producing said protein or variants, or said immunogenic or IgD-binding fragment of said protein or variants, or said immunogenic and adhesive fragment of said protein, which host is chosen among bacteria, yeast and plants. In one embodiment the host is E. coli.

In one aspect the present invention relates to a recombinant DNA molecule comprising a DNA sequence coding for a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or for an immunogenic or IgD-

binding fragment of said protein, or variants, which DNA sequence is fused to another gene.

In another aspect the present invention relates to a recombinant DNA molecule comprising a DNA sequence coding for an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, which DNA sequence is fused to another gene, preferably a recombinant DNA molecule comprising a DNA sequence coding for an immunogenic or IgD-binding fragment having an amino acid sequence as described in SEQ ID NO 10.

In still another aspect the present invention relates to a recombinant DNA molecule comprising a DNA sequence coding for an immunogenic and adhesive fragment of a surface exposed protein as above, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, which DNA sequence is fused to another gene, preferably a recombinant DNA molecule comprising a DNA sequence coding for an immunogenic and adhesive fragment having an amino acid sequence as described in SEQ ID NO 8.

In yet another aspect the present invention relates to a plasmid or phage comprising said fused DNA sequence as defined above.

In a further aspect the present invention relates to a non-human host comprising at least one plasmid or phage as defined above, which host is chosen among bacteria, yeast and plants. In one embodiment the host is E. coli.

In one aspect the present invention relates to a fusion protein or polypeptide, in which a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively

binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants, is combined with another protein by the use of a recombinant DNA molecule as defined above.

In another aspect the present invention relates to a fusion protein or polypeptide, in which an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis, which has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, is combined with another protein by the use of a recombinant DNA molecule as defined above.

In still another aspect the present invention relates to a fusion protein or polypeptide in which an immunogenic and adhesive fragment of a surface exposed protein as defined above, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, is combined with another protein by the use of a recombinant DNA molecule as defined in above.

In yet another aspect the present invention relates to a fusion product, in which a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, is covalently or by any other means bound to a protein, carbohydrate or matrix.

In a further aspect the present invention relates to a fusion product in which an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis and has a capacity

of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, is covalently or by any other means bound to a protein, carbohydrate or matrix.

In still another aspect the present invention relates to a fusion product in which an immunogenic and adhesive fragment of a surface exposed protein as defined above, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, is covalently, or by any other means, bound to a protein, carbohydrate or matrix. Preferably, a fusion product in which an immunogenic or IgD-binding fragment, having an amino acid sequence described in SEQ ID NO 10, is covalently, or by any other means, bound to a protein, carbohydrate or matrix. Preferably, a fusion product in which an immunogenic and adhesive fragment, having an amino acid sequence described in SEQ ID NO 8, is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

In one aspect the present invention relates to a method of detecting IgD using a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, optionally labelled and/or bound to a matrix.

In a further aspect the present invention relates to a method of detecting IgD using an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modi-

fied variants of said fragment, optionally labelled and/or bound to a matrix.

In another aspect the present invention relates to a method of detecting IgD using an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis, having an amino acid sequence as described in SEQ ID NO 10, and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, optionally labelled and/or bound to a matrix.

In a further aspect the present invention relates to a method of separating IgD using a surface exposed protein of Moraxella catarrhalis, said protein an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, optionally bound to a matrix.

In yet another aspect the present invention relates to method of separating IgD using an immunogenic or IgD-binding fragment of a surface exposed protein, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, optionally bound to a matrix.

In another aspect the present invention relates to a method of separating IgD using an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis, having an amino acid sequence as described in SEQ ID NO 10, and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, optionally labelled and/or bound to a matrix.

In one aspect the present invention relates to a method of isolation of a surface exposed protein of

Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants. Said method comprises the steps:

a) subjecting a suspension of Moraxella catarrhalis to an extraction process by adding a zwitterionic or non-ionic detergent, optionally in the presence of EDTA;

b) applying the extract comprising the IgD-binding protein of Moraxella catarrhalis from step a) to an adsorption column;

c) eluating the IgD-binding protein; and

d) separating the IgD-binding protein.

In another embodiment the concentration of the detergent in step a) of the method is within the range 0.1-5%, preferrably 3%.

In yet another aspect the present invention relates to a method for treatment of an autoimmune disease comprising extra corporal circulation of the blood trough a material comprising a surface exposed protein as defined above, or a fragment thereof as defined above, for removal of IgD from the blood.

In one aspect the present invention relates to a purified antibody which is specific to an immunogenic portion of a surface exposed protein Moraxella catarrhalis, said protein having an amino acid sequence as described in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants.

In another aspect the present invention relates to a purified antibody as described above, which is specific to an immunogenic or IgD-binding fragment as defined above, having a capacity of selectively binding membrane

bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment.

In still another aspect the present invention relates to a purified antibody as described above, which is specific to an immunogenic or adhesive fragment as defined above, having a capacity of binding erythrocytes and epithelial cells.

Description of the figures

Fig. 1. Chromatography and rechromatography on a Sephacryl S-400 column of Empigen® soluble extract from M.catarrhalis after ion exchange chromatograpy. The solid line indicates protein content of the first chromatography and the broken line rechromatography of the first peak. Vo specifies the void volume.

Fig. 2. Analysis on SDS-PAGE of fractions representing different purification steps of MID. The fractions are shown for crude extract in 3 % Empigen®, after an ion-exchange chromatography on Q-sepharose column, and after the 1st and 2nd gelfiltrations on a Sephacryl S-400 column. Two gels were run simultaneously, one was stained with Coomassie blue (Stain) and one was blotted onto Immobilon-P membranes, probed with human IgD(κ) myeloma protein (IgD), anti-UspA (αUsp), or anti-CopB (αB) monoclonal antibodies followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. Molecular weights of marker proteins are indicated to the left.

Fig. 3. Binding of MID to human myeloma sera representing different immunoglobulin classes. All sera were diluted in two-fold steps (4 to 0.3 µg) and applied to a nitrocellulose membrane. After saturation, washing and blocking, an [125I]-MID-labeled probe was added. After overnight incubation and additional washings, specific MID-IgD binding was visualized by autoradiography.

Fig. 4. IgD-bearing B cells specifically bound FITC-conjugated MID. PBLs stained with RPE-conjugated mAbs against CD19+ (A) or CD3+ (D) followed by incubation with

MID-FITC were compared to PBLs incubated with anti-CD19 mAb in addition to an anti-IgD mAb (B). Double staining with CD3+ and anti-IgD mAb is demonstrated in (E). In (C), a panel with PBLs pre-incubated with a rabbit immunoglobulin fraction against human IgD followed by addition of anti-CD19 mAb and MID-FITC is shown. A control sample with no antibodies or MID-FITC is also included (F). PBLs were isolated from heparinized human blood using Lymphoprep one-step gradients. Lymphocytes (2.5 x 105) were incubated with the appropriate antibodies, washed and further incubated with MID-FITC (10 µg/ml). All incubations were performed on ice and after final washings, PBLs were analysed by flow cytometry. In this particular experiment, 68 % of the total lymphocyte population was gated and analysed. Less than 2 % of the cells were labeled when isomatched mAbs were included as negative controls. A pre-immune rabbit serum did not significantly block MID-FITC binding to the IgD BCR (not shown). An experiment with a typical donor out of three separate ones analysed is shown.

Fig. 5. Schematic map of the *mid* gene showing the cloning strategy. Oligonucleotide primers used for DNA amplification are indicated by arrows placed above (PCR) and below (inverse PCR [IPCR]) the relevant sequences. Degenerated primers based upon the amino acid sequences outlined in Table II and specific primers are shown by broken and solid lines, respectively.

Fig. 6. Nucleotide sequence of the *mid* gene from M. catarrhalis Bc5 together with the deduced amino acid sequence. Putative -35, -10 regions, a possible ribosome binding site (RBS), inverted repeat, the predicted signal peptide, and two alternative start-codons at amino acid positions 1 and 17 are indicated. The stop-codon and the inverted repeat is also shown.

Fig. 7. The degrees of identity and similarity between MID isolated from five *M. catarrahlis* strains and UspA1 and A2 from ATCC 25238 are demonstrated. The iden-

tity and similarity were calculated using the software Needle.

Fig 8. Comparison of the amino acid sequence of MID with the protein presented in US 5,808,024.

Fig. 9. Recombinantly expressed MID retained its IgD-binding capacity. The left panel shows a Coomassie brilliant blue stained gel and the right panel a Western blot probed with human IgD. Native MID protein (MID) was run and compared to cytoplasmic (C), periplasmic (P), and membrane (M) fractions. Numbers on the left indicate a molecular weight standard. E. coli BL21DE3 containing pET16-MID were induced for 4 h by IPTG. Cellular fractions were collected and proteins were separated by two SDS-PAGE that was run in parallel and either stained with Coomassie brilliant blue or blotted onto an Immobilon-P membrane. The membrane was probed with human IgD followed by incubation with a horseradish peroxidase-conjugated secondary antibody.

Fig. 10. MID764-913 (fragment E) and MID902-1200 (fragment F) is responsible for erythrocyte hemagglutination and IgD-binding, respectively. A series of truncated MID proteins (designated A to I) were manufactured. Recombinant proteins containing histidine tags in their C-terminals (A to H) or fused with maltose binding protein (I) were produced in *E. coli* and purified on nickel and amylose resin columns, respectively.

Fig. 11. MID962-1200 (fragment F2) has a conserved IgD-binding capacity compared with full length MID1-2139. Equimolar concentrations (range 240 to 0.06 nmol) of purified full length MID1-2139 and 8 truncated MID fragments (F1 to F8) were analysed for IgD-binding by dot blots. The proteins MID902-1130 (F8), MID985-1130 (F7), and MID1000-1200 (F4) did not attract IgD, whereas all other fragments bound IgD. DNA encoding for the various truncated MID proteins were cloned into the expression vector pET26b(+) and produced in *E. coli*. The recombinant proteins containing His-tags were purified and dot blot-

ted onto a nitrocellulose membrane. The membrane was probed with human IgD followed by secondary HRP-conjugated polyclonal antibodies that were used for detection.

Fig. 12. A tetrameric structure of MID962-1200 (F2) is a prerequisite for optimal IgD-binding. (A), SDS-PAGE of MID962-1200 after treatment at 60 °C separates monomers and tetramers. After heat treatment at 100 °C monomers only can be detected. (B), Corresponding Western blot with IgD as probe reveals weak IgD-binding to monomers. (C), Mean IgD-binding to tetramers and monomers, respectively in 6 different experiments. IgD-binding is shown as arbitrary units/µg protein. MID962-1200 was treated in SDS-sample buffer at 60 °C or 100 °C for 10 min, and subjected to SDS-PAGE and Western blot analysis. The resulting Coomassie-stained gel and Western blot were analysed by densitometry. The percentage of protein migrating as tetramer or monomer was calculated and compared with the IgD-binding capacity.

Fig. 13. [125I]-labelled recombinant MID764-913 (fragment E) is specifically attracted to erythrocytes and epithelial cells. [125I]-labelled MID and a series of truncated [125I]-MID fragments (C, E, F, G, and I) were added to human erythrocytes (A). The recombinant [125I]-labelled MID fragments were also added to epithelial cells (B). All truncated MID proteins (except fragment I) were produced in *E. coli* followed by purification on nickel resins. Fragment I was a fusion protein with MBP and consequently purified on an amylose resin. The recombinant proteins were labelled with [125I] and added to erythrocytes or the epithelial cell line A549. After several washings, bound radioactivity was measured in a γ-counter. Data are presented as mean values of 2 experiments with duplicates. Error bars indicate SD.

Fig. 14. Adhesion of MID-expressing *M. catarrhalis* to epithelial cells depends on the amino acid residues MID764-913 (fragment E). A decreased adhesion to epithelial cells was observed with MID-expressing bacteria coa-

ted with rabbit anti-MID1-2139 or anti-MID764-913 polyclonal antibodies compared to a pre-immune serum or anti-MID1011-1446 (fragment G) pAb. Bacteria were pre-incubated with the pre-immune serum or specific antisera for 1 h at 4 °C. Bacteria were added to the epithelial cells followed by centrifugation and incubation for 30 min at 37 °C. After washings, cells were treated with trypsin-EDTA and the suspensions were plated on blood agar plates. Colony forming units were counted after an overnight incubation. The adherence ratio (cfu added/ cfu adhered) was calculated. Results are shown as mean values of 4 separate experiments with duplicates. Error bars indicate SD. *** $P \leq 0.001$, ** $P \leq 0.01$ and * $P \leq 0.05$.

Description of the invention

MID is not identical to previously well characterized outer membrane proteins of M. catarrhalis. It is not recognized by monoclonal antibodies derived against the UspA or CopB outer membrane antigens. MID also has a different migration pattern in SDS-PAGE and a different composition as shown by amino acid and DNA sequence analysis. MID appeares as a 200 kDa band in accordance with the Mw from the deduced amino acid sequence, but also as an extra band with an estimated molecular mass of more than 1,000 kDa. The extra band indicates that native MID is an oligomeric complex in a similar fashion as UspA (11). This is further supported by the fact that MID was eluted immediately after the void volume from a Sephacryl S-400 column with a fractionation range of up to 8,000 kDa. The amino acid sequences for MID shows 11.1 and 6.7% identity, respectively, with the USPA1 and USPA2 outer membrane proteins from M. catarrhalis (Fig 7).

In a recent patent publication, an outer membrane protein of M. catarrhalis with a molecular mass of approximately 200 kDa was isolated (12). A sequence encoding a protein of approximately 200 kDa was also provided. However, that protein sequence is not identical to the sequence provided by us and shows only 45.9 to 54.4% iden-

tity with MID (Fig 7). The protein was shown to be immunogenic, but no further biological functions were presented. In addition, a 200 kDa protein is associated with hemagglutinating M. catarrhalis (13,14).

Experimental part

The present investigation describes the isolation, purification, characterization, cloning and expression of the novel Ig-binding protein named MID of M. catarrhalis, which has affinity for human IgD, of an immunogenic or IgD-binding fragment of said surface exposed protein, and of an immunogenic and adhesive fragment of said surface exposed protein.

Materials and methods

Bacteria and plasmids

M. catarrhalis, strain Bc5, was a clinical isolate from a nasopharyngeal swab culture at our Department. 118 strains isolated from blood, nasopharynx, and sputum were obtained from Sweden, Denmark, Finland, Hungary, Japan, and USA. Sequenced strains and plasmids used for expression are shown in Table I.

Tabell I: Bacterial strains and plasmids used in this study

Strains or plasmid	Description (site of isolation)	Reference or source
Strains		
DH5α	E. coli	Novagen
BL21DE3	E. coli	Novagen
BBH17	M. cararrhalis (sputum)	Christensen (Denmark)
Bc5	M. cararrhalis (nasopharynx)	Dept. Clinical Microbiology, Malmö, Sweden)
NCTC 4103	M. cararrhalis (nasopharynx)	CCUG (Gothenburg, Sweden)

Strains or plasmid	Description (site of isolation)	Reference or source
RH1	M. cararrhalis (blood)	Christensen (Denmark)
RH4	M. cararrhalis (blood)	Christensen (Denmark)
Plasmid		
pET16(b)	Expression vector	Novagen
pET16-MID	PET16(b) with the ORF of *mid*	This study

Bacteria were grown overnight in Nutrient Broth (Oxoid, Basingstoke Hampshire, England), harvested and washed in phosphate-balanced saline (PBS), pH 7.2 by centrifugation.

Immunoglobulins, sera and other proteins

The Ig preparations IgG1(κ), IgG1(λ), IgG2(κ), IgG2(λ), IgG3(κ), IgG3(λ), IgG4(κ), IgG4(λ), IgA1(κ), IgA1(λ), IgA2(κ), IgA2(λ), IgM(κ), IgM(λ), IgD(κ), IgD(λ) and IgE(κ) were all of human origin and purchased from The Binding Site (Birmingham, England). IgD myeloma sera IgD(κ) and IgD(λ) were from the same company and IgD-standard serum OTRD 02/03 was from Behringwerke AG (Marburg, Germany). Myeloma sera IgD(λ)A, IgD(λ) B, IgG A, IgG B, IgG C, IgM, IgA A and IgA B were obtained from the Department of Clinical Chemistry, Malmö, Sweden. The concentration of respective immunoglobulins was according to the suppliers.

Antibodies

Horseradish peroxidase (HRP)-conjugated goat anti-human IgD was from Biosource (Camarillo, CA). Fluoresceinisothiocyanate (FITC)-conjugated mouse anti-human IgD, unlabelled rabbit anti-human IgD, and HRP-labelled rabbit anti-mouse Ig were purchased from Dakopatts (Gentofte, Denmark). Goat anti-human IgD and HRP-conjugated rabbit anti-human polyvalent immunoglobulins was from Sigma (St. Louis, MO). Phycoerythrin (RPE)-conjugated mouse anti-human CD3 and CD19 were from Becton Dickinson

(San José, CA). Mouse monoclonal antibodies 17C7 (UspA) and 10F3 (CopB) were kindly provided by Dr. Eric J. Hansen, Department of Microbiology, University of Texas (Dallas, TX).

Antisera

Rabbits were immunized intramuscularly with 200 µg of purified MID (Forsgren *et al.*, 2001), recombinant MID fragments, or recombinant UspA1 emulsified in complete Freunds adjuvans (Difco, Becton Dickinson, Heidelberg, Germany) and boosted on days 18 and 36 with the same dose of protein in incomplete Freunds adjuvans. Blood was drawn 2 to 3 weeks later. The anti-UspA1 polyclonal antibodies reacted with both recombinant UspA1 and UspA2 as examined by Western blots.

SDS-PAGE and detection of proteins on membranes (Western blot)

SDS-PAGE was run using a commercial electrophoresis system consisting of 10 % Bis-Tris gels with running (MES), sample (LDS), and transfer buffer as well as a blotting instrument (Novex, San Diego, CA). Briefly, samples were boiled for 10 min followed by electrophoresis at room temperature using Protein II vertical slab electrophoresis cells (Novex) at 150 constant voltage. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Sundbyberg, Sweden). In addition, electrophoretical transfer of protein bands from the gel to an immobilon-P membrane (Millipore, Bedford, MA) was carried out at 30 V for 2-3 h. After transfer, the immobilon-P membrane was blocked in PBS with 0.05% Tween 20 (PBS-Tween) containing 5% milk powder. After several washings in PBS-Tween, the membrane was incubated for 1 h in room temperature with purified IgD myeloma protein (0.5 µg/ml, hu IgD(κ) myeloma; The Bindingsite, Birmingham, UK) in PBS-Tween including 2 % milk powder. HRP-conjugated goat anti-human IgD diluted 1/1000 in the same buffer was added after several washings in PBS-Tween. In some experiments, IgD myeloma protein was replaced by myeloma

protein of other immunoglobulin classes and HRP-labeled anti-human polyvalent immunoglobulins (Sigma) was used as a secondary layer. Mouse mAbs 17C7 and 10F3 were used to detect Moraxella outer membrane proteins UspA1, 2 and Cop B, respectively (7,8). In these experiments, HRP-labeled rabbit anti-mouse immunoglobulins were used as a secondary layer. After incubation for 40 min at room temperature and several additional washings in PBS-Tween, developement was performed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden). Western blots were analysed by a Personal Molecular Imager FX (Bio-Rad).

Enzyme linked immunosorbent assay (ELISA)

ELISA was used to quantitate the immunoglobulin D-binding protein. Extracts of M. catarr*halis* diluted in five-fold steps in 0.1 M Tris-HCl, pH 9.0 were added in 100 µl volumes to microtiter plates (F96 Maxisorb, Nunc-Immuno module, Roskilde, Denmark), which were sealed and incubated at 4 °C overnight. After washing the plate four times in PBS-Tween, blocking buffer PBS-Tween containing 1.5 % ovalbumin, was added. The plate was incubated for 1 h at room temperature and further washed four times with PBS-Tween. IgD(κ) myeloma protein, 0.05 µg in 100 µl PBS-Tween containing 1.5% ovalbumin was added to each well and after incubation for 1 h at room temperature the plate was washed four times with PBS-Tween. After 1 h incubation with HRP-conjugated goat anti-human IgD diluted 1/1000 in the same buffer and subsequent washing with PBS-Tween, tetramethylbenzidine (20 mM) in 0.1 M potassium citrate solution, pH 4.25, mixed with hydrogen peroxide (final concentration 0.002%) was added. After 30 min, the enzymatic reaction was stopped by adding 2 M sulphuric acid. The optical density (OD) was then measured at 450 nm in an automated ELISA reader (Multiskan Plus, Labsystems, Finland).

Dot blot assay

Purified MID (0.0005-0.2 µg) in a volume of 100 µl in 0.1 M Tris-HCl, pH 9.0 were manually applied to nitrocellulose membranes (Schleicher & Schuell, Dessel, Germany) by using a dot blot apparatus (Schleicher & Schuell). After saturation, the membranes were incubated for 2 h at room temperature in PBS-Tween containing 1% ovalbumin and 5% milk powder and washed four times with PBS-Tween. Human myeloma protein 0.5 µg in 100 µl PBS-Tween was added and after 2 h of incubation, followed by several washings in PBS-Tween, HRP-labelled anti-human light chains (κ and λ) (Dakopatts) in dilution 1/200 was used as a secondary antibody. Development was performed as described above for the Western blots. In another set of experiments, dilutions of human myeloma sera in a volume of 100 µl in 0.1 M Tris-HCl, pH 9,0 was first applied to the membranes. After saturation, incubations, blocking, and washing steps were performed as described above. Thereafter, [^{125}I]-labeled protein MID probe (5 to 10 x 10^5 cpm/ ml) in PBS-Tween was added. After overnight incubation, the membrane was washed four times with PBS-Tween, air dried, and exposed to Kodak CEA.C x-ray films at -70 °C using Kodak X-Omat regular intensifying screen (Eastman Kodak, Rochester, NY).

Extraction of IgD-binding protein

M. catarrhalis bacteria (1-5 x 10^{11} colony forming units (cfu)/ ml) were suspended in 0.05 M Tris-HCl-buffer (pH 8.8) containing 0.1-5 % Empigen (Calbiochem Novabiochem, Bedford, MA). In some experiments Empigen was replaced by CHAPS (Sigma), n-Octyl-p-D-glucoside (Bachem, Budendorf, Switzerland) or Triton X-100 (Sigma). All these detergents at a concentration of 0.1-5 % were tested with or without 0.01 M EDTA. The bacterial suspensions were mixed by magnetic stirring for 2 h at 37°C. After centrifugation at 8000 x g for 20 min at 4°C, the supernatants were filtrated with sterile filters (0.45 µm; Sterivex-HV, Millipore).

Purification of IgD-binding protein

M. catarrhalis extract in 3 % Empigen® was applied to a Q-sepharose column (Amersham Pharmacia Biotech) equilibrated with 0.05 M Tris-HCl (pH 8.8) containing 0.1 % Empigen®. The column was eluted using a 0 to 1 M NaCl linear gradient in the same buffer. Fractions showing most IgD-binding activity as detected by ELISA and Western Blot were pooled, dialyzed in Spectraphor membrane tubes (molecular weight cut off 25,000; Spectrum, Laguna hills, CA) against 0.05 M Tris-HCl, pH 8.8, concentrated on YM100 disc membranes (molecular weight cut off 100,000; Amicon, Beverly, MA) and then applied to gel-chromatography. The gel-filtration of IgD-binding protein was done on a Sephacryl S-400 high resolution column (20 by 900 mm; Amersham Pharmacia Biotech), equilibrated with 0.05 M Tris-HCl, pH 8.8 containing 0.1% Empigen®. Fractions containing the strongest IgD-binding activity were concentrated and re-chromatographed as described above.

Peptide cleavage and amino acid sequence analysis

Purified MID in 0.05 M Tris-HCl (pH 8.8) containing 0.1% Empigen® was treated with trypsin or chymotrypsin in an enzyme-protein-ratio of 1:10 at 37°C overnight. The cleavage mixtures were subjected to SDS-PAGE and peptide bands transferred to Immobilon membranes were automatically sequenced or exposed to Western blot analysis as described above. In order to get an N-terminal sequence of the protein, deblocking of intact MID from a possible pyroglutamate group was attempted. Two different protocols were used to deblock both soluble and membrane-bound protein. Automated amino acid sequence analysis was performed with an Applied Biosystems (Foster City, CA) 470A gas-liquid solid phase sequenator with on-line detection of the released aminoacid phenylthiohydantoin derivatives by Applied Biosystems model 120A PTH analyser.

Labeling of protein MID

Purified MID was radioiodinated ([^{125}I]; Amersham, Buckinghamshire, England) to high specific activity with

lactoperoxidase. The preparations contained approximately 0.05 mol iodine per mol protein. FITC (Sigma) was conjugated to purified MID using a standard protocol. Briefly, MID (2 mg/ml) in 0.1 M carbonate buffer, pH 9.5, was incubated with 0.15 µg/ml FITC solubilized in DMSO. After 45 min at room temperature and constant stirring, the sample was diluted and subjected to a PD10 column (Pharmacia Biotech) pre-equilibrated with PBS, pH 7.4. The resulting MID-FITC was used for binding studies.

DNA isolation and sequencing

Genomic DNA was extracted from five M. catarrhalis strains (see Table I) using a genomic DNA preparation kit (Qiagen, Hilden, Germany) and was subsequently used as template for amplification of the MID gene by PCR. Degenerate primers were synthesized according to the amino terminal sequences of the four peptide fragments (Table II).

Table II. Amino acid sequences derived from highly purified MID after protease digestions.

Peptide sequence	Protease
TAQANTESSIAVG	Trypsin
GNTATNFSVNSGDDNALIN	Trypsin
QGINEDNAFVKGLEK	Trypsin
PSTVKADN	Chymotrypsin

In some of the PCR reactions (High Fidelity PCR System; Roche, Bromma, Sweden), specific primers were used in combination with the degenerate ones. DNA sequences flanking the central region of the gene, where the peptide fragments originated from, were isolated using inverse PCR (IPCR). Briefly, genomic DNA was cleaved with the following restriction enzymes used separately; *Eco*RV, *Sph*I and *Pst*I for the isolation of the start codon, and *Acc*I, *Asu*I and finally *Hinc*II for the isolation of the stop codon sequences. The resulting fragments were reli-

gated upon themselves (Rapid DNA Ligation Kit; Roche) and the DNA was used in IPCR. To amplify the start and stop codon areas of the gene, specific primers were designed and used in a long template PCR (LTPCR) (Expand Long Template PCR System; Roche). All PCR products were cloned into pPCR-Script-Amp (Stratagene, La Jolla, CA) and sequenced using the Big Dye Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, England). Primers for amplification of genomic DNA were designed using the Oligo Primer Analysis software (Molecular Biology Insights, Cascade, Co). The signal peptide was deduced using the SignalP V1.1 World Wide Web Prediction Server Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/ SignalP/).

PCR amplification of the mid gene

The complete 6.4 kb open reading frame of the *mid* gene was amplified by PCR using M. catarrhalis Bc5 strain genomic DNA as template. The oligonucleotide primers containing *Bam*HI restriction enzyme recognition sequences were 5'-cgggatccgatggccgtggcggaatatgcc-3' (primer A, SEQ ID NO 3) and 5'-cgcggatccgaaaagtgaaaacctgcaccaactgctgc-3' (primer B, SEQ ID NO 4) generating a PCR product of 6391 base pairs. *Bam*HI-digested insert was ligated into pET16(b) and the resulting plasmid pET16-MID was transformed into DH5α. Both strands of the cloned PCR product were sequenced.

To examine the full length *mid* gene in other M. catarrhalis strains, the primers A and B were used. In addition, primers used for narrowing down the sequence encoding the signal peptide were either primer A or 5'-tgtcagcatgtatcatttttttaaggtaaaccaccatg-3' (primer C; detecting the upper start codon, SEQ ID NO 5) in combination with 5'-catcaattgcgatatgtctgggatcttg-3' (primer D; located at a conserved region just outside the signal peptide, SEQ ID NO 6) generating 192- and 266-base pair long PCR products (using Bc5 genomic DNA as template), respectively. Furthermore, primer A or C in combination

with 5'-cttcaccccatcagtgccatagacc-3' (primer E, SEQ ID NO 7) were used for confirming the existence of the *mid* gene resulting in 1355- and 1429-base pair long fragments, respectively. The expand long template PCR system was used in all reactions and conditions were as recommended by the manufacturer (Roche, Bromma, Sweden).

Expression of the mid gene product in E. coli and cell fraction

To express the *mid* gene product, pET16-MID was transformed into the expression host BL21DE3, containing a chromosomal copy of the T7 RNA polymerase gene under *lacUV5* control. The recombinant bacteria were grown in LB medium supplemented with 2% of glucose and ampicillin. Overexpression was achived by growing cells to logarithmic-growth phase at OD_{600} of 0.6 followed by addition of 1 mM IPTG. After 4 h of induction, bacteria were sonicated according to a standard protocol and the resulting proteins were analysed by SDS-PAGE.

Localisation of recombinant protein from pET16-MID was carried out by osmotic shock as described. Briefly, broth cultures of induced and uninduced cells were harvested and resuspended in 30 mM Tris-HCl, pH 8, containing 20% sucrose. EDTA was added to a final concentration of 1 mM and the solution was slowly stired at room temperature for 10 min. After centrifugation at 10,000g for 10 min at 4°C, cells were resuspended in ice-cold 5 mM $MgSO_4$ and stired for 10 min on ice. During this step, the periplasmic proteins were released into the buffer. The supernatant containing the periplasmic fraction was collected by centrifugation. Bacteria were completely lysed by lysozyme at a final concentration of 100 mg/ml followed by sonication. Finally, the soluble cytoplasmic and insoluble membrane fractions were collected.

Truncated MID-derived recombinant proteins

The different truncated MID fragments designated A to I with their specific sizes and primers for generating the proteins are shown in Figure 10. The open reading

frame of the *mid* gene from *M. catarrhalis* Bc5 (in pET26-MID) (Forsgren *et al.*, 2001) was used as template. All MID constructs, except for MID367-590 (C), were amplified by PCR using specific primers introducing *Bam*HI and *Hind*III restriction enzyme sites. Due to an internal *Hind*III restriction enzyme site in fragment C, an *Xho*I site was used instead of *Hind*III at the 3´ end. All PCR products, except for MID1616-2139 (I), were cloned into pET26 (Novagen, Madison, WI). The PCR product encoding for the I fragment was cloned into pMAL-c2 (New England Biolabs, Beverly, MA). To avoid presumptive toxicity, the resulting plasmids were first transformed into the non-expressing host *E. coli* DH5α. Thereafter, plasmids encoding for fragments A to D, G and H were transformed into *E. coli* BL21(DE3), whereas the host BL21(DE3)-pLysS was used for vectors containing fragments E and F. Both *E. coli* strains were incubated in the presence of kanamycin, whereas chloramphenicol also was supplemented when BL21(DE3)-pLysS transformants were used. Fragment I was expressed in DH5α. Bacteria were grown to mid-log phase followed by induction with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG). After 3.5 h, transformants were sonicated and the overexpressed proteins were purified according to the manufacturers´ instructions. Resulting recombinant proteins having a histidine tag or fused to maltose binding protein were purified on resins containing nickel amylose, respectively. The concentrations of the eluted proteins were determined using the BCA Protein Assay Kit (Pierce). Thereafter, recombinant proteins were analysed by SDS-PAGE and Western blots.

Hemagglutination

Human erythrocytes were obtained from freshly drawn heparinized human blood. The erythrocytes were washed twice in PBS (pH 7.2) and suspended in PBS at a final concentration of 1 %. Bacteria cultured in Nutrient Broth were harvested by centrifugation, washed and suspended to 1-2 x 109/ml in PBS. Bacteria and erythrocyte suspension

(50 µl of each) were mixed in round bottom microtiter plates (Sarstedt, Newton, NC). In some experiments, erythrocytes were mixed with MID-Sepharose or BSA-Sepharose in 150 µl PBS. Agglutination was read by the naked eye.

Cell line and adherence assay

The lung carcinoma cell line A549 (type II alveolar epithelial cells; CCL-185) was obtained from ATCC. The cells were cultured in RPMI 1640 medium (Gibco BRL, Life Technologies, Paisley, Scotland) supplemented with 10 % fetal calf serum, 2 mM L-glutamine, and 12 µg/ml gentamicin (referred to as culture medium). On the day before adherence experiments, cells were harvested, washed twice in gentamicin-free RPMI 1640 and added to 12-well tissue culture plates (Nunc, Roskilde, Denmark) at a concentration of 1 x 104 cells/ well in 2.0 ml gentamicin free culture medium. Cells were thereafter incubated overnight at 37 °C in 5 % CO2. On the day of experiments, *M. catarrhalis* (≈ 2 x 108) in PBS, 0.15 % gelatin (Sigma) was inoculated onto the monolayers. In neutralization experiments with specific antisera, bacteria were pre-incubated with polyclonal antibodies (dilution 1/250). After 1 h at 4 °C, bacteria were added to the epithelial cells. In all experiments, tissue culture plates were centrifuged at 3,000g for 5 min and incubated at 37 °C, 5 % CO2. After 30 min, the infected monolayers were rinsed twice with PBS, 0.15 % gelatin with gentle rocking to remove nonadherant bacteria and then treated with trypsin-EDTA (0.05% trypsin, 0.5 mM EDTA) to release them from the plastic support. Thereafter, the resulting cell/ bacteria suspension was seeded to agar plates containing 1.1 % isovitalex, 7.8 % human blood, and finally 0.9 % proteose peptone. Data was calculated from duplicate cultures.

Flow cytometry analysis

Human peripheral blood lymphocytes (PBLs) were isolated from heparinized blood from healthy donors by cen-

trifugation on a step gradient of Ficoll-Isopaque (Lymphoprep; Pharmacia, Uppsala, Sweden). For flow cytometry analyses, a standard staining protocol was used with 0.5% BSA (w/v) in PBS as buffer. PBLs (2.5 x 10^5 in 100 µl) were labeled with anti-CD3 or anti-CD19 mAbs with or without FITC-conjugated anti-IgD mAb on ice for 30 min according to the manufacturer´s instructions. In blocking experiments, lymphocytes were also pre-incubated with anti-IgD immunoglobulins for 30 min. After two washes, 10 µg/ml of purified FITC-conjugated MID was supplemented to the cells followed by incubation for 45 min on ice. After 4 final washes with excess PBS 0.5% BSA, 105 cells for each sample were analysed in an EPICS®XL-MCL flow cytometer (Coulter, Hialeah, Florida). Where appropriate, rabbit and goat pre-immune sera and mouse IgG1 and IgG2a were included as negative controls (Dakopatts).

Results

Extraction and purification of MID

Solubilization of MID was a major obstacle in the process of purification. Amongst several detergents tested, only Empigen® and n-Octyl-b-D glucoside alone at a final concentration of 3% solubilized MID from a suspension of M. catarrhalis efficiantly as estimated by ELISA and Western blot. The two detergents were equally efficient. Triton X-100 alone did not solubilize MID, but Triton X-100 plus 0.01 M EDTA solubilized MID efficiently. C HAPS alone or CHAPS with EDTA or EDTA alone did not solubilize MID. In the following experiments, Empigen® extraction was used for solubilization and subsequent purification of MID. When the Empigen® extract of M. catarrhalis was applied to a Q-sepharose column, all IgD-binding material was eluted from the column with 0.1 % Empigen® in 0.05 M Tris HCl, pH 8.8. No additional IgD-binding material could be eluted when a NaCl-gradient up to 1 M was applied to the same column. After concentration of the IgD-binding material obtained after separation on Q-sepharose, fractionation of the extract was

achieved by gel filtration in the presence of 0.1% Empigen® on a Sephacryl S-400 column (Fig 1). Most IgD-binding material was eluted in this first peak immediately after the void volume. MID was further purified by rechromatography of the first peak under the same conditions.

Fig 2 shows that after purification MID appeared as two bands, one 200 kDa-band and a second band with an apparent molecular mass of more than 1,000 kDa. Western blot experiments were performed to confirm that MID was not identical to the previously described outer membrane proteins UspA1 and 2 with an apparent molecular mass varying from 350 to 720 kD (8-10) or CopB with a molecular weight of 80 kDa. The crude Empigen® extract of M. catarrhalis or partly purified preparations of MID were subjected to SDS-PAGE, transferred to Immobilon filters and blotted with antibodies to those Moraxella proteins and also with human IgD. As can be seen in Fig 2, MID (as revealed by IgD-binding) is not identical with the outer membrane proteins UspA and Cop B.

Three attempts were made to determine the amino-terminal amino acid sequence of purified MID. Approximately 1000 pmol of MID was applied each time in an automated amino acid sequencer. Inasmuch as no amino acid phenylthiohydantoin derivatives were obtained, the amino-terminal end of the singel MID polypeptide chain was probably blocked. It was recently determined that the moraxella UspA1 and UspA2 proteins, which are also resistant to Edman degradation, contained a pyroglutamyl residue that was removed by the treatment with pyroglutamate aminopeptidase. However, when MID purified from M. catarrhalis or recombinant MID was treated with this enzyme according to two different protocols (twice for each method) and then subjected to Edman degradation, no N-terminal amino acid sequence was obtained.

IgD-binding properties of MID

Crude Empigen® extracts of M. catarrhalis and highly purified MID subjected to SDS-PAGE and transferred to filters were exposed to highly purified commercially available Ig-preparations representing all human Ig-classes and subclasses (Table III).

Table III. Summary of Western Blot and dot blot analyses showing the binding specificity of highly purified commercially available myeloma immunoglobulin D preparations against a crude Empigen® extract of M. catarrhalis and highly purified MID.

Immunoglobulin	200 kDa-protein in crude extract	Purified MID
IgD(κ), IgD(λ)	+	+
IgG1(κ), IgG1(λ)	-	-
IgG2(κ), IgG2(λ)	-	-
IgG3(κ), IgG3(λ)	-	-
IgG4(κ), IgG4(λ)	-	-
IgA1(κ), IgA1(λ)	-	-
IgA2(κ), IgA2(λ)	-	-
IgM;(κ), IgM(λ)	-	-
IgE(κ)	-	-

Only the two IgD preparations interacted with the MID-band in the 200 kDa-position in a similar fashion as shown for IgD in Fig 2. When dot blot experiments were performed and purified MID in dilutions was first added to membranes and purified human myeloma proteins and secondary antibodies were subsequently applied, only the two IgD myelomas interacted with MID. One of the two myelomas detected as little as 0.001 µg of MID on the membrane. The specificity of the interaction between MID and IgD was further verified by using radiolabeled MID in other dot blot experiments. In Fig 3, it is demonstrated that MID effectively bound four IgD myeloma sera. A

distinct reaction could be detected in the range 0.03-4 μg of IgD. For the IgD standard serum (B.W.) reactivity was seen at even lower concentrations (not shown). In contrast, 6 different Ig myeloma sera representing IgG, IgA and IgM showed no visible reaction with MID at 4 μg.

Purified MID specifically attracted human soluble IgD as revealed in dot and Western blots (Fig 2 and 3, Table III). To test whether MID bound to the surface-expressed B cell receptor (BCR) IgD, human peripheral blood lymphocytes (PBLs) were isolated. FITC was conjugated to MID followed by incubation with PBLs for 45 min on ice. In parallel, PBLs were labeled with RPE-conjugated mAbs directed against the T cell marker CD3 or the B cell specific surface antigen CD19 and subsequently analysed by flow cytometry (Fig 4). Interestingly, a large fraction of $CD19^+$ lymphocytes bound significant amounts of MID-FITC (Fig 4A), whereas T cells ($CD3^+$ lymphocytes) only displayed a non-specific background binding (Fig 4D). The MID-FITC signal corresponded well with $CD19^+$ cells incubated with anti-IgD mAbs revealing IgD-postive B cells (Fig 4B). To further elucidate the specificity of MID-FITC binding to IgD bearing $CD19^+$ lymphocytes, PBLs were preincubated with a rabbit anti-human IgD immunoglobulin fraction. After incubation and washings, MID-FITC binding was analysed by flow cytometry according to the standard procedure. The antiserum almost completely inhibited specific MID-FITC binding to the IgD BCR when compared to cells incubated with the pre-immune serum. Mean fluorescence intensity decreased from 79.2 to 14.6 arbitrary units. Similar results were obtained with goat immunoglobulins raised against IgD (not shown). Thus, IgD-expressing B cells promoted specific MID-FITC binding to the surface-expressed BCR IgD.

Cloning of the gene encoding MID and DNA sequence analysis

Degenerate primers were designed according to the obtained amino terminal sequences of four peptide frag-

ments originating from MID (Table II) and were used in PCRs in all possible combinations. The specific primers 2982+ and 3692- (Fig 5) were synthesized using the deduced sequence of a distinctive PCR product generated with the degenerate primer pair 2629+/ 3693-. A PCR reaction using the specific primers in combination with the degenerate ones (718+ and 5772-) resulted in totally 5054 bp of the gene coding for MID. Flanking sequences surrounding the core of the *mid* gene were obtained by inverse PCR (IPCR). IPCR on *Eco*RV- and *Asu*I/ *Acc*I-digested M. catarrhalis genomic DNA with the primer-pairs 2982+/ 945- and 3668+/ 120-, respectively, provided the sequence for the start-codon area. In addition, IPCR on *Hinc*II-digested moraxella genomic DNA with the primer-pair 5898+/ 5511- generated the 3´ sequence including the stop-codon. The complete nucleotide sequence of the gene encoding MID in M. catarrhalis Bc5 is shown in SEQ ID NO 2 and the resulting amino acid sequence is shown in SEQ ID NO 1. Two alternative open reading frames were revealed and are indicated at amino acid positions 1 and 17, see Fig 6). Consequently, the length of the *mid* gene product was either 2123 or 2139 amino acids. In addition to a putative ribosome-binding site (AAGG), -10 (TAATTA) and -35 (TTGAAT) consensus sequence boxes were identified. Furthermore, 62 bases downstream of the TAA stop-codon an inverted repeat was found with the potential of stem-loop formation that is necessary for transcriptional termination. To get an overview of the similarity and identity between diferent *mid* genes, the sequences of the five ORF MID proteins were analysed. For 4 strains, the degree of identity and similarity was ≥ 75.8 % and ≥ 78.3 %, respectively (Fig. 7). In contrast, slightly lower values, ≥ 65.3 % and ≥ 71.2 %, respectively, were obtained for the fifth isolate (RH4). Identity and similarity with UspA1 was 5.5-11.1 % and 8.3-17.9 %, respectively, and with UspA2 6.5-7.5 % respectively 11.1-12.4 %.

The mid gene can be detected in all M. catarrhalis strains

By PCR analyses, the *mid-1* gene was detected in all 118 M. catarrhalis strains, whereas the Moraxella (neisseria)-related controls were negative. In addition, the size of the *mid-1* gene was confirmed using primers spanning the whole gene including the start and stop codons.

Analysis of the deduced amino acid sequence of MID differs from UspA1, UspA2 and the protein described in US 5,808,024

The open reading frame defined a protein with a calculated molecular mass of just below 220 kDa that readily corresponded to the empirical value of approximately 200 kDa found by SDS-PAGE. The N-terminal amino acid sequence showed the typical characteristics of a signal peptide with a potential cleavage site between amino acids 66 and 67. Despite that the first amino acid after the signal peptidase cleavage site most likely was a glutamine residue, any sequence could not be determined by Edman degradation. Furthermore, no amino acid sequence was obtained after pyroglutamate aminopeptidase treatment. The predicted amino acid sequence was also subjected to a hydrophobicity profile analysis by the method of Kyte and Doolittle and showed mainly hydrophilic properties except for the putative signal peptide that was strongly hydrophobic. The deduced amino acid sequence for MID differs significantly from those for the protein described in US 5,808,024 and also from the UspA-proteins (Fig 7 and 8).

The *mid* gene is distributed in all M. catarrhalis strains To investigate whether or not the *mid* gene existed in all *M. catarrhalis* strains, primers were chosen based upon a conserved area upstream of the open reading frame (ORF) and a conserved area downstream including the stop codon sequence (Forsgren *et al.*, 2001). The *mid* gene was detected in all 86 clinical isolates and 7 type strains analysed, and the length of the genomic

mid DNA was approximately 6,000 base pairs. The existance was further verified by Southern blots using a probe containing a sequence selected from the 3´-end of the gene. Southern blot experiments revealed that the moraxella strains contained only one *mid* gene.

Expression of recombinant MID in E. coli

To confirm that the cloned *mid* gene corresponded to the purified IgD-binding protein, the gene including the predicted signal sequence and start codon was subcloned into the expression vector pET16(b) and thereby under the control of a T7 promoter. The resulting pET16-MID was subsequently transformed into E. *coli* BL21DE3 followed by induction with IPTG. Bacterial cells were lysed and subfractionated, and recombinant MID was localized by Western blots using human IgD as a probe. Important verifying characteristics of MID were provided from the expression experiments (Figure 9). Firstly, following induction, cells containing pET16-MID were able to produce recombinant MID confirming the correct reading frame of the gene. Secondly, recombinant MID (as shown by SDS-PAGE) displayed a molecular mass of approximately 200 kDa, corresponding to the 217 kDa calculated value from the amino acid sequence. Thirdly, the recombinant protein was indeed the *mid* gene product in E. coli as its IgD-binding phenotype was confirmed by Western blot analysis. Total protein from E. coli containing induced pET16(b)vector without insert did not display any IgD-binding capacity (data not shown). Fourthly, the subcellular localization of the recombinant protein showed that MID was equally located in the cytoplasmic and the membrane fractions, but not in the periplasmic space. The localization of MID´s in the membrane fraction correlated very well with the known outer membrane localization in M. catarrhalis.

IgD-binding is preserved in 238 amino acids of MID

To in detail determine the MID IgD-binding region, 9 sequences derived from the full length MID were cloned into pET26b(+) and expressed in *E. coli*. The recombinant

proteins covered the entire MID sequence and their individual lengths and positions were as demonstrated in Fig 10. The recombinant proteins comprising amino acid residues 69-1111 or 1011-2139 of MID did not bind IgD as revealed in Western and dot blots. In contrast, the protein MID902-1200 (protein fragment F1) attracted IgD, strongly suggesting that the single IgD-binding region of MID was within that particular sequence.

To pinpoint the sequence responsible for the IgD-binding, the truncated MID902-1200 was systematically shortened at the N- and C-terminal ends (Fig 11). Equimolar concentrations of the various recombinant proteins were compared to native full length MID1-2139 isolated from *M. catarrhalis*. The different recombinant proteins were diluted in four-fold steps, added to membranes and incubated with human IgD. On a molar basis, an essentially preserved IgD-binding capacity was detected for the truncated MID protein stretching from amino acid residue 962 to 1200. The shortest truncated protein still interacting with IgD was localized between MID985 and MID1142 (fragment F6). The IgD-binding property was lost when the N-terminus was reduced to the MID1000 residue (fragment F4) or when the C-terminal was shortened to MID1130 (fragment F7). Finally, a fragment (MID902-1130; F8) with a longer N-terminal and a shorter C-terminal (compared to MID985-1200; F3) was also manufactured and analysed. However, this truncated MID did not interact with IgD, suggesting that the binding capacity was depending on a longer C-terminal.

To further characterize the specific MID-dependent IgD-binding, an IgD ELISA was constructed using human IgD as bate. All the recombinant truncated MID fragments were subjected to ELISA followed by incubation with a specific rabbit anti-serum directed against MID902-1200. The ELISA was developed using HRP-conjugated goat anti-rabbit polyclonal antibodies. The same pattern as with the dot blot (Fig 11) was observed, i.e. fragments F4, F7, and F8 was

not attracted to the solid phase IgD, whereas the other fragments bound to a variable degree compared to full length MID (not shown).

Optimal MID962-1200 - IgD interaction is depending on a tetramer structure

To shed light upon the need for a tetramer structure in order to obtain an optimal IgD-binding, MID962-1200 (F2, SEQ ID NO 10) was incubated at 60 or 100 °C followed by analysis on SDS-PAGE and Western blots. MID962-1200 formed both a monomer and a tetramer after pre-treatment at 60 °C (Fig 12A). The tetrameric structure was, however, disrupted at 100 °C and resulted in a monomeric form, which displayed a considerably weaker binding to IgD when examined in Western blots (Fig 12A and B). To investigate the capability of the tetramer to bind IgD in comparison with the monomeric form, the MID962-1200 fragment, SEQ ID NO 10, was subjected to analysis at 60 °C in 6 different experiments. The heattreated protein was subjected to SDS-PAGE and the IgD-binding activity was analysed by Western blots. Resulting gels and filters were analysed by densitometry and the protein concentration (density) of the monomer was divided with the corresponding tetramer concentration. The obtained value (%) was related to the concentration (µg) of total protein loaded on the gels. Interestingly, when IgD-binding to the tetrameric respectively monomeric forms were compared, a 23-fold more efficient binding to IgD was found with the tetrameric MID962-1200 (Fig 12C).

M. catarrhalis IgD-binding protein (MID) hemagglutinates human erythrocytes

To investigate a putative involvement of MID in hemagglutination, a series of clinical isolates that either expressed MID or by phase variation had shut off the *mid* gene was selected. Interestingly, all out of 21 isolates expressing MID hemagglutinated human erythrocytes, whereas only four out of the MID-negative strains (n=21) hemagglutinated the red blood cells. An almost full

correlation between hemagglutinating capacity and MID expression was observed. UspA1/2 expression was similar and irrespective of the MID expression.

These initial experiments prompted us to examine whether or not purified MID protein from the model strain *M. catarrhalis* Bc5 (Forsgren *et al.*, 2001) hemagglutinates erythrocytes. To mimic the bacterial surface, MID was conjugated to Sepharose beads and incubated with the human erythrocytes. Bovine serum albumin (BSA) linked to Sepharose was included as a negative control. Interestingly, the human erythrocytes were hemagglutinated in the presence of MID-Sepharose, whereas BSA-Sepharose did not interfere with the erythrocytes (data not shown).

The hemagglutinating domain of MID is located between amino acid residues Alanine764 and Serine913

To dissect the molecule and pin-point the specific site of the molecule that was responsible for the hemagglutination, a series of truncated DNA fragments of the *mid* gene was cloned and recombinantly expressed in *E. coli* (Fig. 10). Polyclonal antibodies against the truncated MID proteins were raised in rabbits and used in an ELISA. In preparatory experiments, antibodies to MID and the MID-derived proteins were titrated to give similar values when tested in ELISA against respective antigens. The capacity of the truncated MID proteins to bind to lysed erythrocytes was then measured in ELISA using the specific antibodies at appropriate concentrations. MID or MID764-913 (fragment E) gave higher ELISA values (4 to 16 times) as compared to the other truncated MID proteins. Thus, the hemagglutinating structure of MID seemed to be located within amino acid residues 764-913 of MID (SEQ ID NO 8).

MID764-913 (fragment E, SEQ ID NO 8) binds directly to both eryhtrocytes and type II alveolar epithelial cells

To further confirm the importance of MID764-913 as an adhesin, MID and a selection of the truncated MID-derived proteins were radiolabelled and tested in direct

binding experiments with human erythrocytes and alveolar epithelial cells (Fig. 13). Both [125I]-MID and [125I]-MID764-913 strongly bound to erythrocytes, whereas the truncated MID fragments MID367-590 (fragment C), MID902-1200 (F), MID1011-1446 (G), and MID1616-2139 (I) did not bind above background levels (Fig. 13A). In parallel, the alveolar epithelial cell line A549 also attracted both the full length [125I]-labelled MID and the truncated MID764-913 (Fig. 13B). All the other fragments did not bind to the epithelial cells. Taken together, the fragment MID764-913 (SEQ ID NO 8) was the crucial part of the adhesin MID that mediated the attachment to mammalian cells.

Antibodies to full length MID1-2139 and MID764-913 inhibit adherence of M. catarrhalis *to type II alveolar epithelial cells*

To further analyse the influence of full length MID and MID764-963 on *M. catarrhalis* adherence to type II alveolar epithelial cells, a MID-expressing and a MID-deficient *M. catarrhalis* strain were preincubated with antibodies to MID and subsequently added to alveolar epithelial cells for adherence. As demonstrated in Fig. 14, polyclonal antibodies directed against full length MID1-2139 and MID763-913 (fragment E, SEQ ID NO 8) effectively inhibited adherence for the MID-expressing isolate. In contrast, pre-immune serum and a pAb directed against MID1011-1466 (fragment G) did not significantly interfere with adhesion.

References

1. Forsgren,A. and Grubb,A. (1979) Many bacterial species bind human IgD. J. Immunol. 122, 1468-1472.

2. Banck,G. and Forsgren,A. (1978) Many bacterial species are mitogenic for human blood lymphocytes. Scand. J. Immunol. 8, 347-354.

3. Calvert,J.E. and Calogeres,A. (1986) Characteristics of human B cells responsive to the T-independent mitogen Branhamella catarrhalis. Immunology 58, 37-41.

4. Forsgren,A., Penta,A., Schlossman,S.F. and Tedder,T.F. (1988) Branhamella catarrhalis activates human B lymphocytes following interactions with surface IgD and class I major histocompatibility complex antigens. Cell. Immunol. 112, 78-88.

5. Janson,H., Carlén,B., Cervin,A., Forsgren,A., Björk-Magnusdottir,A., Lindberg,S. and Runer, T. (1999) Effects on the ciliated epithelium of protein D-producing and -nonproducing nontypeable Haemophilus influenzae in nasopharyngeal tissue cultures. J. Infect. Dis. 180, 737-746.

6. Sasaki,K. and Munson Jr.,R.S. (1993) Protein D of Haemophilus influenzae is not a universal immunoglobulin D-binding protein. Infect. Immun. 61, 3026-3031.

7. Helminen,M.E., Beach,R., Maciver,I., Jarosik,G., Hansen,E.J. and Leinonen,M. (1995) Human immune response against outer membrane proteins of Moraxella (Branhamella) catarrhalis determined by immunoblotting and enzyme immunoassay. Clin. Diagn. Lab. Immunol. 2, 35-39.

8. Aebi,C., Maciver,I., Latimer,J.L., Cope,L.D., Stevens,M.K., Thomas,S.E., McCracken,G.H. and Hansen,E.J. (1997) A protective epitope of Moraxella catarrhalis is encoded by two different genes. Infect. Immun. 65, 4367-4377.

9. Cope,L.D., Lafontaine,E.R., Slaughter,C.A., Hasemann,C.A.Jr., Aebi,C., Henderson,F.W., McCracken,G.H.Jr and Hansen,E.J. (1999) Characterization of Moraxella

catarrhalis uspA1 and uspA2 genes and their encoded products. J Bacteriol 181, 4026-4034.

10. Klingman,K.L. and Murphy,T.F. (1994) Purification and characterization of a high-molecular-weight outer membrane protein of Moraxella(Branhamella) catarrhalis. Infect. Immun. 62,1150-1155.

11. WO 98/28333

12. Sasaki,K., Harkness,R.E., Loosmoore,S.M. and Klein, M.H. (1998) United States Patent 5,808,024.

13. Fitzgerald,M., Mulcahy,R., Murphy,S., Keane,C., Coakley,D. and Scott,T. (1997) A 200 kDa protein is associated with haemagglutinating isolates of Moraxella (Branhamella) catarrhalis. FEMS Immun. Med. Microbiol. 18, 209-216.

14. Tucker,K., Plosila,L., and Samuel,J. (1994) Correlation between hemagglutination and globotetraosylceramide binding by Branhamella catarrhalis. Abstract 117 of the 94th General meeting of the American Society for Microbiology.

15. Lunde E, Munthe LA, Vabo A, Sandlie I, Bogen B. (1999) Antibodies engineered with IgD specificity efficiently deliver integrated T-cell epitopes for antigen presentation by B cells. Nat Biotechnol. 17, 670-675.

16. Lycke N. (2001) The B-cell targeted CTA1-DD vaccine adjuvant is highly effective at enhancing antibody as well as CTL responses. Curr. Opin. Mol. Ther. 3, 37-44.

17. Ito O, Harada M, Takenoyama M, Tamada K, Li T, Abe K, Fujie H, Nomoto K. 1998 Vaccination with activated B cells pulsed with tumor-lysates can induce tumor-specific CD4+ T cells in vivo. Immunobiol. 199, 133-147.

CLAIMS

1. A surface exposed protein, which can be detected in Moraxella catarrhalis, having an amino acid sequence as described in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants, or an immunogenic and adhesive fragment of said surface exposed protein.

2. An immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof.

3. An immunogenic or IgD-binding fragment according to claim 2, having an amino acid sequence as described in SEQ ID NO 10.

4. An immunogenic and adhesive fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis, having a capacity of binding erythrocytes and epithelial cells.

5. An immunogenic and adhesive fragment according to claim 4, having an amino acid sequence as described in SEQ ID NO 8.

6. A DNA segment comprising a DNA sequence, as shown in SEQ ID NO 2, which DNA sequence codes for a surface exposed protein of Moraxella catarrhalis as defined in claim 1, or naturally occurring or artificially modified variants of said DNA sequence.

7. A DNA segment comprising a DNA sequence which codes for an immunogenic or IgD-binding fragment as defined in claim 2.

8. A DNA segment according to claim 7, comprising a DNA sequence, as shown in SEQ ID NO 11, which DNA sequen-

ce codes for an immunogenic or IgD-binding fragment as defined in claim 3.

9. A DNA segment comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 4.

10. A DNA segment according to claim 9, comprising a DNA sequence, as shown in SEQ ID NO 9, which DNA sequence codes for an immunogenic and adhesive fragment as defined in claim 5.

11. A vaccine containing a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, or an immunogenic and adhesive fragment of said surface exposed protein.

12. A vaccine containing an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment.

13. A vaccine according to claim 12 containing an immunogenic or IgD-binding fragment as defined in claim 3.

14. A vaccine containing an immunogenic and adhesive fragment of a surface exposed protein of Moraxella catarrhalis as defined in claim 4.

15. A vaccine according to claim 14 containing an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 5.

16. A vaccine according to any one of claims 11-15, combined with another vaccine.

17. A vaccine according to any one of claims 11-16, combined with an immunogenic portion of another molecule.

18. A plasmid or phage comprising a DNA sequence, which codes for a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants.

19. A plasmid or phage comprising a DNA sequence, which codes for an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment.

20. A plasmid or phage according to claim 19 comprising a DNA sequence, which codes for an immunogenic or IgD-binding fragment as defined in claim 3.

21. A plasmid or phage comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells or naturally occurring or artificially modified variants of said fragment.

22. A plasmid or phage according to claim 21 comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 5.

23. A non human host comprising at least one plasmid or phage as defined in any one of claims 18-22, and capable of producing said protein or variants, or said immunogenic or IgD-binding fragment of said protein or variants, or said immunogenic and adhesive fragment of said protein, which host is chosen among bacteria, yeast and plants.

24. A host according to claim 23, which is E. coli.

25. A recombinant DNA molecule comprising a DNA sequence coding for a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants, which DNA sequence is fused to another gene.

26. A recombinant DNA molecule comprising a DNA sequence coding for an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, which DNA sequence is fused to another gene.

27. A recombinant DNA molecule according to claim 26 comprising a DNA sequence, which codes for an immunogenic or IgD-binding fragment as defined in claim 3, which DNA sequence is fused to another gene.

28. A recombinant DNA molecule comprising a DNA sequence coding for an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, which DNA sequence is fused to another gene.

29. A recombinant DNA molecule according to claim 28 comprising a DNA sequence, which codes for an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 5, which DNA sequence is fused to another gene.

30. A plasmid or phage comprising a fused DNA sequence as defined in any one of claims 25-29.

31. A non-human host comprising at least one plasmid or phage as defined in claim 30, which host is chosen among bacteria, yeast and plants.

32. A host according to claim 31, which is E. coli.

33. A fusion protein or polypeptide, in which a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants, is combined with another protein by the use of a recombinant DNA molecule as defined in claim 25.

34. A fusion protein or polypeptide in which an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, is combined with another protein by the use of a recombinant DNA molecule as defined in claim 26.

35. A fusion protein or polypeptide according to claim 34 in which an immunogenic or IgD-binding fragment as defined in claim 3, is combined with another protein by the use of a recombinant DNA molecule as defined in claim 27.

36. A fusion protein or polypeptide in which an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 1, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, is combined with another protein by the use of a recombinant DNA molecule as defined in claim 28.

37. A fusion protein or polypeptide according to claim 36 in which an immunogenic and adhesive fragment as

defined in claim 5, is combined with another protein by the use of a recombinant DNA molecule as defined in claim 29.

38. A fusion product, in which a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

39. A fusion product in which an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 2, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

40. A fusion product according to claim 39 in which an immunogenic or IgD-binding fragment as defined in claim 3 is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

41. A fusion product in which an immunogenic and adhesive fragment of a surface exposed protein as defined in claim 2, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding erythrocytes and epithelial cells, or naturally occurring or artificially modified variants of said fragment, is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

42. A fusion product according to claim 41 in which an immunogenic and adhesive fragment as defined in claim 5 is covalently, or by any other means, bound to a protein, carbohydrate or matrix.

43. A method of detecting IgD using a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, optionally labelled and/or bound to a matrix.

44. A method of detecting IgD using an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 2, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment, optionally labelled and/or bound to a matrix.

45. A method of detecting IgD according to claim 44 using an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis as defined in claim 3, and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, optionally labelled and/or bound to a matrix.

46. A method of separating IgD using a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, optionally bound to a matrix.

47. A method of separating IgD using an immunogenic or IgD-binding fragment of a surface exposed protein as defined in claim 2, which fragment can be detected in Moraxella catarrhalis and has a capacity of selectively binding membrane bound or soluble IgD, or naturally oc-

curring or artificially modified variants of said fragment, optionally bound to a matrix.

48. A method of separating IgD according to claim 47 using an immunogenic or IgD-binding fragment of a surface exposed protein of Moraxella catarrhalis as defined in claim 3, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, optionally labelled and/or bound to a matrix.

49. A method of isolation of a surface exposed protein of Moraxella catarrhalis, said protein having an amino acid sequence as shown in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said protein, or an immunogenic or IgD-binding fragment of said protein or variants, said method comprising the steps:

a) subjecting a suspension of Moraxella catarrhalis to an extraction process by adding a zwitterionic or non-ionic detergent, optionally in the presence of EDTA;

b) applying the extract comprising the IgD-binding protein of Moraxella catarrhalis from step a) to an adsorption column;

c) eluating the IgD-binding protein; and

d) separating the IgD-binding protein.

50. A method according to claim 49, wherein the detergent is selected from the group comprising Empigen®, n-Octyl-β-D glucoside and Triton X-100+0,01M EDTA..

51. A method according to claim 49 or 50, wherein the concentration of the detergent in step a) is within the range of 0,1-5%, preferably about 3%.

52. A method for treatment of an autoimmune disease comprising extra corporal circulation of the blood through a material comprising a surface exposed protein as defined in claim 1, or a fragment thereof as defined in claims 2 or 3, for removal of IgD from the blood.

53. A purified antibody which is specific to an immunogenic portion of a surface exposed protein Moraxella catarrhalis, said protein having an amino acid sequence as described in SEQ ID NO 1, an apparent molecular weight of 200 kDa and a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants thereof, or an immunogenic or IgD-binding fragment of said protein or variants.

54. A purified antibody according to claim 48, which is specific to an immunogenic or IgD-binding fragment as defined in claim 3, having a capacity of selectively binding membrane bound or soluble IgD, or naturally occurring or artificially modified variants of said fragment.

55. A purified antibody according to claim 48, which is specific to an immunogenic or adhesive fragment as defined in claim 5, having a capacity of binding erythrocytes and epithelial cells.