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# Human T-Cell Response to Meningococcal Immunoglobulin $A_1$ Protease Associated α-Proteins

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A unique feature of the immunoglobulin $A_1$ ($IgA_1$) protease from pathogenic *Neisseriae*, i.e. *N. meningitidis* and *N. gonorrhoeae*, is its co-secretion with an amphipathic α-protein. Polymerase chain reaction (PCR) analysis of the respective $iga_\alpha$ gene region in 48 meningococcal strains revealed that this protein domain is conserved throughout all isolates in four different principal variants. Despite strain-dependent size and sequence variations, sequence analysis showed common structural characteristics. More than 80% of the amino acid sequence of all α-proteins is dependent on the five amino acids Q, E, A, K and R, resulting in a pI > 10. The sequences are highly conserved at the N-terminus and the C-terminus and contain long amphipathic α-helical stretches. These stretches have a strong probability of forming coiled coil conformations and comprise short repetitive sequence modules with pronounced similarities to T-cell epitopes. We therefore analyzed the T-cell response of 20 volunteer blood donors to four peptides, representing such predicted epitopes, and a recombinant meningococcal α-protein. Sixteen donors reacted against at least one peptide after culture of peripheral blood mononuclear cells in interleukin (IL)-2-rich medium, while two individuals showed a positive reaction only against an $IgA_1$ protease-derived control peptide. From one donor, we established and maintained T-cell clones specific for purified α-protein. Characterization of the T-cell clones revealed a CD3- and a CD4-positive phenotype and the secretion of IL-2 and interferon-γ (IFN-γ), but not of IL-4. This indicates an important role of α-proteins during meningococcal carriage and neisserial infection.

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## INTRODUCTION

A number of important mucosal pathogens secrete sequence-specific proteases capable of cleaving human immunoglobulin $A_1$ ($IgA_1$) antibodies within the 13 amino acid, proline-rich, hinge region [1]. A large body of indirect evidence indicates that these enzymes play a crucial role during infection, and they are therefore thought to be putative virulence factors. The group of micro-organisms producing $IgA_1$ proteases include the three principal causes of bacterial meningitis, i.e. *N. meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae*. While the streptococcal enzyme is a metallo-proteinase, the enzymes of the Gram-negative *Haemophilus* and *Neisseria* species represent serine proteinases. The serine-type $IgA_1$ proteases belong to a family of secreted proteins termed autotransporters (or 'type IV' transporters), which include various virulence factors of Gram-negative bacteria [2, 3]. One characteristic of autotransporters is the potential of their C-terminal domain to form a β-barrel within the outer membrane. This pore-like structure permits translocation of the N-terminally attached passenger domains, an $IgA_1$ protease and α- and γ-domains, which are then autoproteolytically released (Fig. 1) [4].

At least one substantial difference exists between the *Haemophilus* and *Neisseria* enzymes: despite remarkable sequence homologies, the *H. influenzae* enzyme lacks the co-secreted α-protein domain [5]. The term α-protein is descriptive of the pronounced α-helical structure of these domains [4]. The α-helix is predicted to be amphipathic and all α-proteins analysed so far exhibit an extremely positive charge with a pI > 10 [6, 7].

$IgA_1$ protease is unusual among neisserial antigens in being highly immunogenic in humans after clinical infection or nasopharyngeal carriage. While antibodies against other meningococcal surface markers, such as Opc and group A polysaccharide, decline over a 5-year period after vaccination, the antibody

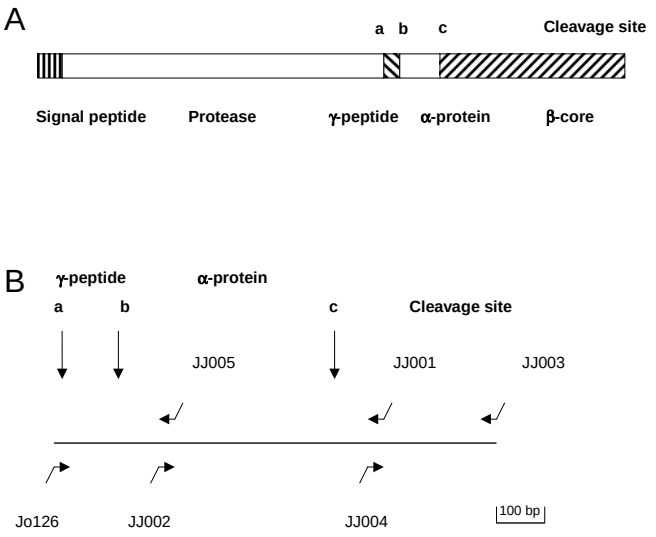


**Fig. 1.** Primer walking strategy for the polymerase chain reaction (PCR) and sequence analysis of gonococcal and meningococcal α-protein encoding *iga* gene segments. (A) Primary structure of the *iga*-encoded polyprotein including the immunoblobulin $A_1$ ($IgA_1$) protease and other domains. (B) Location of PCR primers (Table 2). Autoproteolytic cleavage sites (a, b, c) according to Pohlner *et al*. [4].

concentration against $IgA_1$ protease was found to remain constant or even to increase [8]. This observation might be caused by serial infections with *N. meningitidis* or *H. influenzae* [9] but could also highlight an immunodominance of secreted proteins compared with cell-bound antigens [10]. Antibody production by B cells is regulated by helper T cells, which recognize specific T-cell epitopes in combination with major histocompatibility complex (MHC) molecules. The persistent B-cell response towards $IgA_1$ protease may reflect the presence of immunodominant T-cell epitopes, effectively activating helper T cells [11]. No data are available on the T-cell response to meningococcal $IgA_1$ protease. By contrast, the T-cell recognition of the outer membrane proteins Opa, Opc and PorA has been the subject of several investigations [12–14]. Therefore, we decided to monitor human volunteers possessing high serum titres against $IgA_1$ protease, with regard to their $IgA_1$ protease-specific T-cell response. The co-secreted α-protein was chosen for two reasons. First, this approach allows discrimination between reactions to *Haemophilus* and *Neisseria* $IgA_1$ proteases. Second, because of its high content of amphipathic α-helices, the α-protein might be a promising target for T-cell recognition [15]. In the present investigation we report on the multiple sequence analysis of meningococcal α-proteins, the purification of one representative protein with typical features and the characterization of the T-cell response to α-proteins of individuals showing high serum titres against $IgA_1$ protease.

## MATERIALS AND METHODS

*Bacterial strains, plasmids and primers*. *N. gonorrhoeae* and *N. meningitidis* strains used in this study are listed in Table 1. The majority of these strains were isolated from nonsymptomatic carriers at different locations in Germany. Primers applied for cloning and sequencing of meningococcal α-proteins are shown in Table 2. For purification of the His-tagged α-protein of *N. menigitidis* B2b, the *Escherichia coli* DH5α::YZ88 strain (Z. X. Yan & T. F. Meyer, unpublished) was used as a recipient for the plasmid vector pRSETA (Invitrogen, Carlsbad, CA, USA).

*DNA isolation and recombinant DNA techniques*. Total DNA extraction from *Neisseriae* was carried out as described earlier [7]. Briefly, bacteria derived from a single colony were grown for 24 h at 37 °C on *N. gonorrhoeae* agar plates. The yield of one plate was resuspended in 1 ml of lysis buffer (100 mM NaCl, 20 mM Tris/HCl pH 7.5, 1 mM EDTA). After addition of 10 μg of lysozyme and 50 μl of Triton-X-100 (20%), the solution was incubated at 37 °C for 10 min. Proteinase K (10 μg) was added and a second incubation period followed for 10 min at 37 °C. The lysate was extracted twice with an equal volume of phenol–chloroform–isoamylalcohol (25 : 24 : 1) and once with an equal volume of chloroform. For precipitation of DNA at − 20 °C for 2 h, 0.8 vol of isopropanol and 0.1 vol of 3 M NaCl was added. The pellet was washed once with 70% ethanol and vacuum dried. The DNA was resuspended in 100 μl of $H_2O$, and, before photometric determination of purity and concentration, RNase A was added to a final concentration of 100 μg/ml.

All recombinant DNA techniques were performed according to standard protocols. Plasmid DNA was purified from *E. coli* by the alkaline lysis procedure. Competent *E. coli* cells for electroporation were prepared according to the protocol recommended for the Gene Pulser apparatus (Bio-Rad, München, Germany).

*Polymerase chain reaction (PCR) and sequence analysis*. $IgA_1$ protease-associated α-protein-encoding DNA segments were PCR amplified from meningococcal carrier and invasive strains by using the oligonucleotide primers indicated in Table 2. Purified chromosomal DNA (1–10 μg) was used as a template and denaturation was performed at 95 °C for 1 min, annealing for 1 min at 50 °C, and elongation at 1 min at 72 °C, for a total of 25 cycles. Primer JJ003 was used in cases where primer JJ001 led to multiple DNA amplification products. For sequence analysis, a primer-walking strategy was followed as schematically drawn in Fig. 1. To compare the α-protein-encoding DNA regions from all strains analysed, the size of the amplification products yielded with the primers JO126/JJ3 was included in Table 1. DNA analysis, as well as analysis of the derived protein sequences, e.g. multiple alignments and pattern searches, were performed using the GCG sequence analysis software (Genetics Computer Group, University of Wisconsin, Madison, WI, USA).

*Purification of His-tagged α-protein*. Recombinant B2b α-protein carrying a 39 amino acid N-terminal extension, including the affinity tag, αB2b (His), was purified from *E. coli* DH5α::pYZ88 by using the protocol supplied by the manufacturer (Quiagen, Hilden, Germany) with some modifications. Briefly, *E. coli* DH5α::pYZ88 were transformed with pRSETA::αB2b. A colony from a selective Luzis-Bertani (LB) plate was inoculated into 25 ml of LB broth containing ampicillin (100 μg/ml). The culture was grown, with shaking, at 28 °C to an optical density ($OD_{550}$) of ≈ 1.0–1.5. The culture was chilled on ice before centrifugation at 4 °C. Pelleted cells were lysed in 1 ml of buffer A (6 M guanidinium-HCl, 100 mM $NaH_2PO_4$, 10 mM Tris, pH 8.0). To complete lysis, this suspension was incubated for at least 2 h at room temperature with gentle agitation. The lysate was centrifuged at 16 000 ***g*** for 10 min and the clear supernatant was collected for purification of αB2b (His) protein. The fusion protein was purified by affinity chromatography as follows. A Ni-NTA-resin column (Quiagen) was equilibrated with buffer B (8 M urea, 100 mM $NaH_2PO_4$, 10 mM Tris, pH 8.0). Six-hundred

**Table 1.** *Neisseria* strains used for the amplification of immunoglobulin $A_1$ ($IgA_1$) protease-associated α-protein gene segments

| Stock no. | Species | Strain | PCR fragment (kbp) | Seq. | Source |
|---|---|---|---|---|---|
| N031 | *Nme* | REFK | 1.9 | | U. Berger |
| N032 | *Nme* | REFL | 1.9 | | U. Berger |
| N033 | *Nme* | REFX | 1.9 | | U. Berger |
| N038 | *Nme* | REFY | 1.9 | | U. Berger |
| N095 | *Nme* | C1938 | 1.9 | | U. Berger |
| N096 | *Nme* | B1939 | >1.9 | + | U. Berger |
| N098 | *Nme* | A | 1.9 | | U. Berger |
| N099 | *Nme* | D1941 | 1.1 | + | U. Berger |
| N100 | *Nme* | Y(D) | 1.1 | | U. Berger |
| N124 | *Nme* | MGCRopan | 1.9 | | M. Achtman |
| N271 | *Nme* | IHN5351 | 1.9 | | M. Achtman |
| N272 | *Nme* | HF 16 | 1.9 | + | M. Achtman |
| N419 | *Ngo* | MS11 | <0.8 | + | E.C. Gotschlich |
| N421 | *Nme* | Z4050 | 1.9 | | M. Achtman |
| N422 | *Nme* | Z4063 | 1.9 | | M. Achtman |
| N423 | *Nme* | B40 | 1.9 | | U. Berger |
| N424 | *Nme* | B380 | 1.9 | | U. Berger |
| N425 | *Nme* | Z4025 | 1.9 | | M. Achtman |
| N426 | *Nme* | B54 | 1.9 | | U. Berger |
| N427 | *Nme* | Z4096 | 1.9 | | M. Achtman |
| N428 | *Nme* | B48 | 1.8 | | U. Berger |
| N432 | *Nme* | B43 | 1.9 | | U. Berger |
| N485 | *Nme* | UM1 | 1.1 | | M. Achtman |
| N508 | *Nme* | AD9 | 1.1 | | M. Achtman |
| N565 | *Nme* | B15 | 1.1 | + | M. Achtman |
| N566 | *Nme* | B2B | 1.1 | + | M. Achtman |
| N577 | *Nme* | Poly3703 | – | | I. Ehrhard |
| N578 | *Nme* | Poly3664 | – | | I. Ehrhard |
| N579 | *Nme* | B3663 | 1.9 | | I. Ehrhard |
| N580 | *Nme* | B3633 | 1.9 | | I. Ehrhard |
| N581 | *Nme* | Y3622 | 1.9 | | I. Ehrhard |
| N582 | *Nme* | B3614 | 1.1 | + | I. Ehrhard |
| N583 | *Nme* | B3610 | 1.9 | | I. Ehrhard |
| N584 | *Nme* | B3594 | 1.9 | | I. Ehrhard |
| N585 | *Nme* | Y3576 | 1.1 | + | I. Ehrhard |
| N586 | *Nme* | X3573 | 1.9 | | I. Ehrhard |
| N587 | *Nme* | B3566 | 1.1 | + | I. Ehrhard |
| N588 | *Nme* | Poly3563 | – | | I. Ehrhard |
| N589 | *Nme* | B3555 | 1.9 | | I. Ehrhard |
| N590 | *Nme* | B3547 | 1.9 | | I. Ehrhard |
| N591 | *Nme* | B3542 | 1.9 | | I. Ehrhard |
| N592 | *Nme* | B3539 | 1.9 | | I. Ehrhard |
| N593 | *Nme* | B3533 | 1.9 | | I. Ehrhard |
| N594 | *Nme* | B3513 | 1.1 | | I. Ehrhard |
| N595 | *Nme* | Y3496 | 1.1 | | I. Ehrhard |
| N596 | *Nme* | C3477 | 1.1 | | I. Ehrhard |
| N597 | *Nme* | B3431 | 1.9 | | I. Ehrhard |
| N598 | *Nme* | C3391 | 1.9 | | I. Ehrhard |
| N599 | *Nme* | C3390 | 1.9 | | I. Ehrhard |

*Ngo*, *Neisseria gonorrhoeae*; *Nme*, *Neisseria meningitidis*; PCR, polymerase chain reaction; Seq., Sequence analyzed.

**Table 2.** Oligonucleotides for sequence analysis and cloning of IgA1 protease associated α-proteins.

| Oligonu-cleotide | Position | Additional Features | Sequence (5′-3′) |
|---|---|---|---|
| JO126 | *Ngo iga* + strand | | |
| | 5‘ cleavage site A | M13 RP | CAGGAAACAGCTATGACCATGCCGGAGAACCGCAGAACC GCCGCCGAGTCA |
| JJ001 | *Nme iga* -strand | | |
| | 3‘ cleavage site C | M13 FP | TGTAAAACGGCCAGTCATCTTCCAACGCATCCAAGG |
| JJ002 | *Nme iga* + strand | | |
| | 3‘ cleavage site B | M13 RP | CAGGAAACAGCTATGACCGCGCCGAACTTGCACGTC |
| JJ003 | *Ngo iga* - strand | | |
| | 3‘ cleavage site C | M13 FP | TGTAAAACGACGGCCAGTTTGACATCCAAACACTGT |
| JJ004 | *Nme iga* + strand | | |
| | 3‘ cleavage site C | M13 RP | CAGGAAACAGCTATGACCCCTTGGATGCGCTGGAAGATG |
| JJ005 | *Nme iga* - strand | | |
| | 3‘ cleavage site B | M13 FP | TGTAAAACGACGGCCAGTGACGTGCAAGTTCGGCGC |
| UW029 | *Nme iga* + strand | | |
| | 5‘ cleavage site B | *Eco*RI site | TGAGAATTCCGGTGGTATAGCCCTGCG |
| UW031 | *Nme iga* + strand | | |
| | 3‘ cleavage site C | *Bam*HI site | TCCGGATCCAGCCCGCAGGCAAATC |

microlitres of the cell lysate was applied to the column. Wash-buffer C (8 M urea, 100 mM $NaH_2PO_4$, 10 mM Tris, pH 6.3) was then applied and the protein was eluted with 2×150 μl of buffer E (8 M urea, 100 mM $NaH_2PO_4$, 10 mM Tris, pH 4.5). The eluate was dialyzed four to five times against ice-cold phosphate-buffered saline (PBS). The protein concentration was estimated using the Bradford protein assay (Bio-Rad), and the purified material was aliquoted and stored at − 70 °C. Purification from a 25-ml overnight culture resulted in 100–150 μg of purified His-tagged α-protein.

*Peptide synthesis and purification*. The solid-phase technique was performed via standard Fmoc-chemistry [17]. All peptides were synthesized using a modified synthesis protocol on an ABI 433A peptide synthesizer (Applied Biosystems, Weiterstadt, Germany). Initially, TentaGel-SAC resin (Rapp Polymere, Tübingen, Germany) was loaded manually using 3 eq (equivalents) of the corresponding Fmoc-amino acid activated with 3 eq *N*,*N*-diisopropylcarbodiimide and 2.25 eq *N*-methylimidazole in methylene chloride (coupling time 2×3 h, resin loading: 0.24 mmol/g). Stepwise assembly of peptides was achieved via activation of the Fmoc amino acids with TBTU (2-[(1*H*-benzotriazole-1-yl)-(dimethylamino)methylene]-*N*-methylmethanaminium tetrafluoroborate *N*-oxide) for a coupling time of 30 min (single couplings, coupling concentration: 0.3 M in DMF [dimethyl formamide]). Deblocking of the Fmoc group was performed three times for 5 min using 25% piperidine/DMF. Peptide resin cleavage was achieved with a mixture of 5% water, 5% phenol and 2.5% tri-isopropylsilane in TFA (trifluoracetic acid) for 2.5 h at ambient temperature. After filtration, the resin was washed with methylene chloride (5×5 ml) and the combined washes concentrated by vacuum. Peptides were precipitated, washed with cold ether and dried.

Purification of the crude peptides was carried out by preparative HPLC on a PolyEncap A300, 10-μm column (250×20 mm I.D.) (Bischoff Analysentechnik, Leonberg, Germany) to yield final products with a 95% purity. The peptide masses were confirmed by matrix-assisted laser desorption ionization mass spectrometry (Voyager-DE, BioSpectrometry Workstation; Perseptive Biosystems, Inc., Framingham, MA, USA).

*Proliferation assay*. Peripheral blood mononuclear cells (PBMC) were isolated from fresh citrated blood of healthy adult volunteers by density-gradient centifugation over Ficoll/Isopaque (Pharmacia, Freiburg, Germany) and washed twice in $Ca^{2+}$-free PBS. The cells were resuspended in RPMI-1640 (Gibco Laboratories, Paisley, Strathclyde, UK) to a final concentration of $2\times10^6$ per ml. Cells ($1\times10^5$) were incubated in the presence or absence of antigen (20 μg/ml) in round-bottomed 96-well microculture plates. Cell proliferation was measured at three different time-points, on days 7, 10 and 13. After 7 days and after 10 days IL-2 was added at a concentration of 25 U/ml. Cultures were pulsed with 0.2 μCi of [$^3$H]-thymidine ([$^3$H]-TdR) 15–18 h before harvesting plates using a 96-well cell harvester. The filters were sealed in plastic bags after the addition of scintillation liquid. [$^3$H]-TdR incorporation was measured using a Wallac/LKB Betaplate counter (Wallac OY, Turku, Finland). The stimulation index (SI) was calculated as the ratio of counts/min (cpm) obtained in the presence of antigen versus the cpm obtained without antigen (medium). For each culture, the mean of three independent replicates was determined.

*Establishment of T-cell lines and T-cell clones*. PBMC were incubated in 96-well round-bottomed microculture plates with peptides (20 μg/ml) or purified α-protein (1 μg/ml) in a final volume of 200 μl. After 10 days of incubation, 100 μl of medium was replaced in each well by 100 μl of fresh RPMI medium containing 5 U of IL-2 (final concentration 25 U/ml). On day 19, medium was replaced with RPMI without IL-2. After overnight incubation, cultures were used for proliferation assays. For this purpose, autologous antigen pulsed (2 h at 37 °C) and then irradiated (5000 rads) PBMC were used as feeder cells. T cells ($2\times10^4$) were added to $5\times10^4$ feeder cells and incubated for 3 days. Proliferation was measured as described above.

Antigen-specific T-cell clones were obtained from stable T-cell lines by limiting dilution [18]. Selected lines exhibited a cpm value of $>10^4$ and a minimum SI of 2.0 after two rounds of restimulation (40-day culture). For limiting dilution (0.5 T cells/well) autologous feeder cells were irradiated (5000 rads) and placed in each well of 96-well round-bottomed microculture plates ($5\times10^4$ feeder cells/well). Phytohaemagglutinin (PHA; Sigma, Dreieich, Germany) was added to a final concentration of 1 μg/ml. After 10 days of incubation, the culture medium was replaced by fresh RPMI medium containing 25 U/ml of IL-2. Clones, which were usually detectable on day 12, were subsequently split every 3 days with addition of IL-2, and restimulated every 12 days with autologous feeder cells and PHA (1 μg/ml). Proliferation experiments were performed as described above for T-cell lines.

*MHC typing*. MHC DRB alleles of the donors were determined using the Biotest DRB SSO typing kit (Biotest, Langen, Germany).

*Fluorescence-activated cell sorter (FACS) analysis*. FACS analysis of T-cell clones was performed in a flow cytometer (FACSort; Becton-Dickinson, San Jose, CA, USA) by the use of CD3/CD4, anti-T-cell receptor (TCR) antibodies and – as a negative control – $IgG_1/IgG_2$ fluorescence dye-stained antibodies from the same supplier. Equal amounts of cells were washed with 200 μl of PBS + 0.2% bovine serum albumin (PBS/BSA) and incubated on ice for 15 min in the same volume of PBS/BSA containing 10 μl of each antibody preparation. After two washes with 200 μl of PBS/BSA, cells were resuspended in 300 μl of PBS and fluorescence was determined by flow cytometry. Data were collected and analysed using CELLQUEST software (Becton-Dickinson).

*Cytokine enzyme-linked immunosorbent assay (ELISA)*. The levels of the cytokines IL-2, IFN-γ and IL-4 in the culture supernatants were quantified using sandwich ELISA kits (IL-2: R & D Systems, Wiesbaden, Germany; IFN-γ and IL-4: Pharmingen, San Diego, CA, USA). The assays were performed as recommended by the manufacturers. In brief, polystyrene microtitre plates were coated with the capture monoclonal antibody (MoAb) overnight, washed 10 times (buffer: PBS + 0.02% Tween-20) and blocked with PBS + 5% AB serum. All test samples and the standards (IL-2 range: 50 ng/ml–3.2 pg/ml; IL-4 range: 3 ng/ml–46.9 pg/ml; IFN-γ range: 1.5 ng/ml–23.4 pg/ml) were incubated in duplicate or triplicate in a total volume of 100 μl for 2 h at room temperature. After removing the unbound detecting MoAb by washing (10×), streptavidin–peroxidase solution was added to each well and incubated for 20 min. After 10 wash cycles, the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB; Kierkegaard & Perry, Gaithersburg, MD, USA) was added to each well and colour development was stopped after 20 min by adding 50 μl of 1 M $H_3PO_4$. The absorbance of the samples was measured at 450 nm in an ELISA plate reader using 570 nm as reference. The cytokine concentration of the sample was determined by extrapolation from the standard curve. Results are expressed (in pg/ml) as the mean of duplicate or triplicate samples.

## RESULTS

### *PCR amplification and sequence analysis of meningococcal α-proteins*

Because it was not clear from the literature whether meningococci –although *a priori* $IgA_1$ protease positive – generally express α-proteins, we first decided to analyse a selection of *Neisseria meningitidis* strains using the PCR. The primers were derived from the sequence of the known $IgA_1$ protease gene from *Neisseria gonorrhoeae* MS11 [4]. They were designed to allow amplification of the α-protein DNA sequence within the $IgA_1$ protease gene of the gonococcus (Table 2). Of the 49 strains analysed (Table 1), 46 yielded a single PCR product. Whether the three negative strains, 3563, 3664 and 3703, contain no α-protein-like sequence, or PCR conditions or primers used were not appropriate, was not further addressed. The PCR fragments obtained could, however, be divided into four classes according to their molecular size. The majority of strains exhibited a single band with a size of ≈1100 bp or 1900 bp. Exceptions were *N. gonorrhoeae* MS11 and *N. meningitidis* B1939. *N. gonorrhoeae* MS11 was used as a control and showed a size significantly smaller than 1100 bp, whereas *N. meningitidis* B1939 exhibited an α-protein DNA fragment much larger than all other meningococci. This result confirmed earlier data of our group [6, 7].

Out of every group of α-proteins, the PCR product of at least one strain was sequenced and analysed. The derived amino acid sequences were compared with α-protein sequences available in protein databases. As shown in Fig. 2, all α-protein sequences are similar. They do not contain cysteines, phenylalanines or tryptophans and the five amino acids Q, E, A, K and R, organized in module-like motifs, account for nearly 80% of the sequence. The occurrence of these repeats reflect the hydrophobic heptad repeat structure of the α-proteins, which is probably involved in oligomerization [6]. It also mirrors the basic pI-value of >10, which might be crucial for its biological function. Similar stretches of local amino acid repeats could be found in various proteins, especially of human origin, i.e. myosin, tropomyosin, actin, radixin or others. The most striking similarity, however, was between a region of the *N. meningitidis* B1939 α-protein (AAELSAKQKVE) and a region of human parathymosin (AAELSAKDLKE). Computer structure prediction analysis using the COILS algorithm [19] revealed that the α-helical regions of the neisserial α-proteins have a high probability of forming a coiled coil structure (data not shown). Within these stretches of local amino acid repeats, the sequences contain MHC class II binding motifs [16, 20, 21]. Peptides corresponding to these motifs were synthesized.

The results of the PCR amplification experiments and the subsequent sequence analysis clearly indicate that α-proteins are typical of meningococci. However, the majority of meningoccocal α-proteins analysed contained no autoproteolytic cleavage site C of the gonococcal $IgA_1$ protease [4]. Meningococcal $IgA_1$ proteases may therefore recognize an additional, so far unknown, cleavage site with a still-unknown consensus. Alternatively,

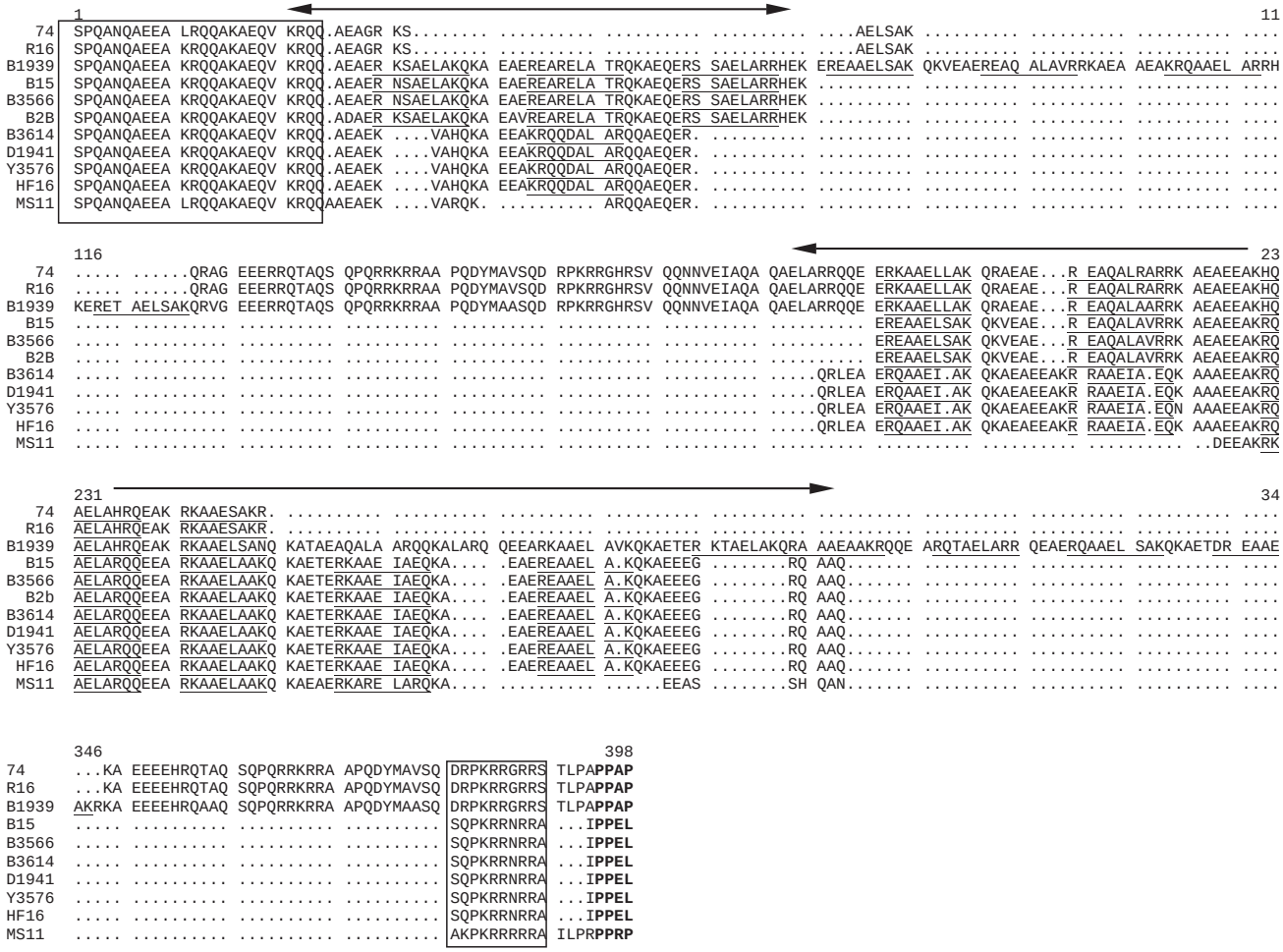

**Fig. 2.** Alignment of α-protein sequences from different gonococcal and meningococcal strains. Amino acid data were derived from DNA sequencing and compiled with data of earlier reports MS11, 74, and R16 AZE Gonococcal sequences. Boxes indicate highly conserved N- and C-terminal regions of the α-proteins. Bold letters indicate the putative autoproteolytic site C. Repetitive sequence modules are underlined. Arrows indicate regions of amphipathic α-helices.

autoproteolytic cleavage fails to occur between the α-protein and the β-core, leaving the α-protein attached to the transporter domain. Despite several attempts, we were not able to detect a cell-bound or a soluble protein corresponding to an α-protein/β-core fusion in any of the isolates. This may suggest the existence of a novel IgA$_1$ protease-recognition site.

*T-cell proliferation induced by synthetic peptides from N. meningitidis α-protein sequences*

PBMC were isolated from 20 healthy donors (10 male, 10 female) whose sera exhibited detectable antibody responses against purified gonococcal IgA$_1$ protease. To measure the

**Table 3.** Sequences of peptides used for T-cell proliferation assays

| Peptide no. | Origin | Sequence |
|---|---|---|
| P106 | *Ngo* IgA$_1$ protease (control) | NQSASFSSGRNVSDITANIT |
| P110 | *Nme* α-protein | HEKEREAAELSAKQKVEAER |
| P111 | Human parathymosin | EKSVEAAAELSAKDLKEKKE |
| P120 | *Nme* α-protein | ARKAAELAAKQKAET |
| P121 | *Nme* α-protein | AAELLAKIQRAEAE |

*Ngo, Neisseria gonorrhoeae*; *Nme, Neisseria meningitidis*.

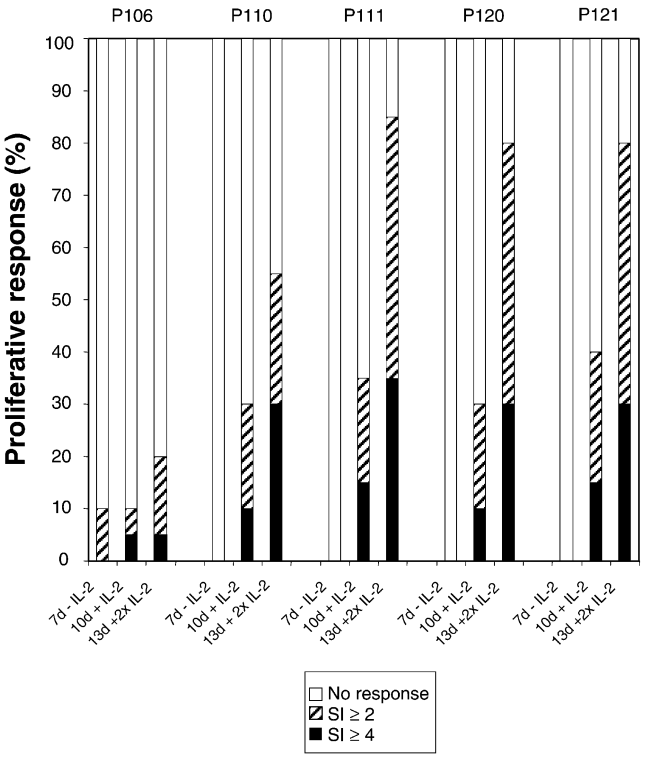


**Fig. 3.** T-cell proliferation against synthetic peptides of α-proteins. Peripheral blood mononuclear cells (PBMC) from 20 volunteer donors were stimulated with five different peptides (P106, P110, P111, P120 and P121) in the presence or absence of interleukin-2 (IL-2). Eighteen hours before harvesting, [$^3$H]-thymidine was added and proliferation was determined based on incorporated radioactive label. The stimulatory index (SI) was determined as the ratio between wells that contained peptide and control wells containing the solute. Results are expressed as the mean SI of triplicate values. d, days.

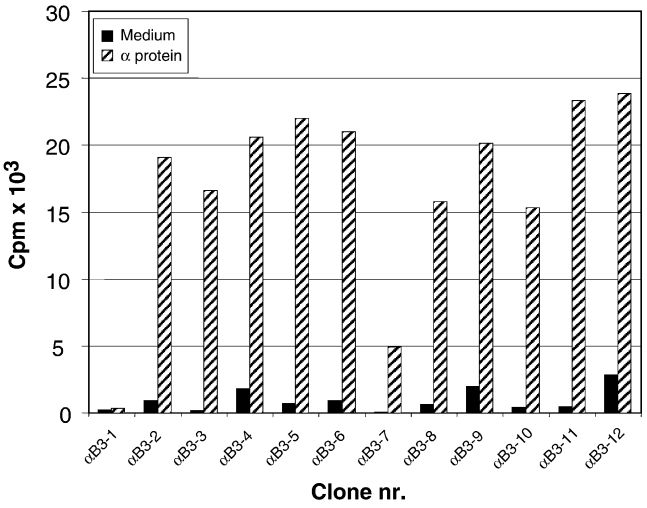


**Fig. 4.** Proliferative response of 12 individual T-cell clones against purified α-protein. Clones were obtained by limiting dilution of the α-protein reactive T-cell line from donor FL. Each bar represents the proliferation of $10^4$ cells that were incubated for 3 days with or without α-protein. Eighteen hours before harvesting [$^3$H]-thymidine was added. cpm, counts/min.

level of $IgA_1$ protease antibodies, ELISAs were performed according to Brooks *et al*. [9]. The titre was defined as the highest serum dilution to give an absorbance (*A*) at 405 nm of > 1.5 in duplicates. A donor was considered positive if the serum titre against $IgA_1$ protease preparation exceeded 1 : 800 [9]. The $IgA_1$ protease preparation used for testing was produced according the method of Pohlner *et al*. [4]. As a result of the purification procedure, it contained the protease itself and also the co-secreted α-protein.

For all donors, the titres against this preparation ranged from 1 : 800 to 1 : 12 800, consistent with previous reports suggesting a particularly pronounced immune response against $IgA_1$ protease [9, 11]. Of the 20 volunteer donors who were additionally MHC typed, only one exhibited nasopharyngeal carriage of *N. meningitidis*. This individual also exhibited the highest serum titre against $IgA_1$ protease (1 : 12 800). The highest antibody titre of noncarrier individuals was 1 : 6400.

The PBMC of all donors were incubated with synthetic peptides containing α-protein-derived sequences (Table 3). As a control, a peptide containing a sequence of the gonococcal $IgA_1$ protease domain was included. Peptides with sequences derived from α-proteins did not stimulate a response within the first 7 days (Fig. 3). However, two volunteers showed a positive reaction to the protease-derived control peptide. The proliferative response to this peptide was restricted to these two volunteers after day 10 and to two additional donors after day 13, whereas the response to the α-protein-derived peptides dramatically increased after a prolonged incubation period with two applications of IL-2 (Fig. 3). The majority of PBMC obtained from the volunteers with high serum titres against $IgA_1$ protease preparations exhibited a proliferative response to synthetic peptides with sequences derived from α-proteins. The SI increased to values higher than 8.0, whereas the response to the protease-derived control peptide typically did not exceed an SI of 2.0.

### *α-Protein-specific and peptide-specific T-cell lines and T-cell clones*

PBMC from those seven volunteer donors who showed at least a single high response (SI ≥ 4) to an α-protein-derived peptide were used for establishing T-cell lines and T-cell clones. In addition to the peptides P110, P111, P120 and P121, purified His-tagged meningococcal α-protein was used as a control antigen. While it was possible to establish stable T-cell lines against α-protein and the peptides for five of seven of the donors, T-cell clones could only be established from two donors.

Interestingly, one of the negative donors of whom we were unable to derive stable T-cell lines against either complete α-protein or synthetic peptides, had a significant serum antibody titre against $IgA_1$ protease (1 : 6400). This might indicate an immune reaction against the protease itself or the related γ-peptide.

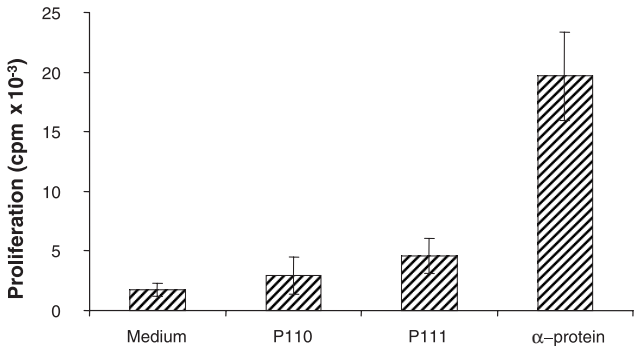


**Fig. 5.** Mean proliferative response of T-cell clone αB3-2 against different antigens. Proliferation of $10^4$ cells (24 repetitions) incubated for 3 days with or without antigen. Bars represent standard deviations of means.

One of the two positive donors (donor code MAH; MHC: DRB1, DRB4) was a nasopharyngeal carrier of meningococci and had a serum titre against IgA$_1$ protease of 1 : 12 800. The T-cell clone from this donor was obtained by limiting dilution of a T-cell line raised against P120 (data not shown). The second donor was female (donor code FL; MHC: DRB1, DRB3, DRB5), and also exhibited a high serum response against IgA$_1$ protease (1 : 1600), although no existing meningococcal carriage was detected. Clones were obtained from a T-cell line against purified His-tagged α-protein by limiting dilution. The T-cell line (FL-αB3) chosen for subsequent cloning exhibited a stable response against α-protein with an SI of 3.9 after 20 days and an SI of 4.0 after 40 days. This moderate stimulation might be a result of the short resting period (24 h) of T cells prior to the determination of proliferation. After limiting dilution by PHA, stimulation of 12 single cell clones was obtained, 10 of which exhibited high reactivity to meningococcal α-protein (Fig. 4).

One of the T-cell clones, clone αB3-2, was chosen by optical evaluation and expanded for further characterization. Clone αB3-2 was CD3 and CD4 positive, as determined by FACS analysis. Control experiments demonstrated the expression of the T-cell receptor as well as the absence of IgG$_1$/IgG$_2$ specificity. After antigen-specific stimulation, T-cell clone αB3-2 secreted IL-2 (5 pg/24 h × $10^4$ cells) as well as IFN-γ (305 pg/24 h × $10^4$ cells). After 24 h, we were unable to detect IL-4 in supernatants beyond the assay limit of 46.9 pg/ml. The antigen specificity was determined by proliferation to antigen-pulsed autologous feeder cells (Fig. 5). For this purpose, $2 \times 10^5$ cells were incubated for 3 days with antigen without IL-2 and pulsed for 16 h with [$^3$H]-TdR. The expanded T cells showed high reactivity with purified α-protein (SI = 11.3), but weaker reaction with the synthetic peptides P111 (SI = 2.6) and P110 (SI = 1.7).

## DISCUSSION

*N. meningitidis* is one of the prominent causative agents of bacterial meningitis in humans. There is currently a world-wide upsurge of meningococcal infections [22]. No vaccine exists against serogroup B meningococci, and vaccines against other serogroup strains need further improvement. Therefore, the search for potential vaccine candidates is of critical importance.

Several meningococcal antigens have been tested for vaccine suitability. Vaccines containing capsular polysaccharides (CPS) elicited protection against meningococci of certain serotypes, such as A or C. Protection was, however, short-lived as plain carbohydrate antigens mainly induce low-affinity immunoglobulin M (IgM) antibodies and usually no memory [23, 24]. Vaccination with CPS conjugated to peptides containing T-cell epitopes may overcome this problem. The group B CPS was shown, however, to be nonimmunogenic in humans. This is probably caused by a molecular mimicry with host cell glycopeptides or glycolipids [25]. The conjugative CPS–peptide vaccine approach is therefore unlikely to provide protection against B-serotype meningococci and, at the same time, exclude immunological complications.

Outer membrane proteins (OMPs) also represent potential vaccine candidates. The immunogenicity of OMPs has been investigated and epitopes that effectively activated helper T-cell-mediated response were identified [12–14]. However, a major disadvantage of OMP antigens is their substantial intraspecies variability. Indeed, this variability is used for serological typing. OMP-based vaccination may result in only serotype-specific protection. To circumvent this problem, it has been proposed that T-cell epitopes should be identified within those regions of OMPs that are conserved between strains of most serotypes, including type B [13].

Recently, it has been shown that in human vaccinees responses against meningococcal IgA$_1$ protease remained constant, or even increased, over 5 years of investigation, whereas antibodies against group A polysaccharide and Opc protein decreased [8]. IgA$_1$ protease-specific antibodies are stimulated by clinical infection as well as by asymptomatic carriage [9]. As this clearly indicates that the IgA$_1$ protease is immunogenic, we decided to study a T-helper-mediated response to evaluate its potential as a vaccine antigen. Neisserial IgA$_1$ proteases consist of four structural domains [4]: the secreted protease domain; the β-domain (autotransporter), which remains anchored within the outer membrane; and the smaller co-secreted α- and γ-proteins. The protease domain exhibits antigenic heterogeneity amongst different meningococcal isolates [26], and the gonococcal protease domain was found to possess a mosaic-like structure implicating horizontal genetic exchange [7]. Moreover, there is significant sequence homology within the protease and the β-domains of IgA$_1$ proteases from *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae* [5, 27]. To exclude any cross-reactivity between *Haemophilus* and *Neisseria*, the co-secreted α-protein represented an ideal model antigen.

By PCR amplification we were able to show that a wide variety of meningococcal strains belonging to different serotypes possess α-protein-encoding DNA fragments. Thus, meningococci contain IgA$_1$ protease-associated α-proteins, consistent with the earlier notion that the expression of α-proteins is a unique characteristic of pathogenic *Neisseriae* [6]. Four different subgroups were identified and exemplary sequence analysis of

selected fragments revealed that, despite the observed length polymorphism, all α-proteins shared similar structural features. They comprised amphipathic, α-helical stretches that tended to form a coiled coil. α-helicity, with the periodic appearance of hydrophobic amino acids, is also a structural feature used for the prediction of T-cell epitopes presented by MHC class II molecules [15, 20]. Some of these modular sequence units were synthesized as peptides and tested for T-cell stimulation in PBMC from volunteer donors. The donors were chosen based on the criterion of a high antibody titre against entire $IgA_1$ protease preparations, so that former contact of individuals with the pathogen was probable. Initial experiments with four out of 20 of these volunteers indicated the property of purified α-protein to strongly stimulate blood cells (data not shown). PBMCs from 17 of 20 donors, exhibited significant proliferation in the presence of IL-2 with α-protein-derived peptides. Under the same conditions, there was only a weak reaction against a protease domain-derived control peptide. This indicates that α-induced proliferation is likely to represent a specific, sequence or epitope-dependent reaction, rather than a nonspecific stimulation caused by IL-2 addition. We were able to maintain antigen-specific T-cell lines and, by limiting dilution, an α-protein-specific single cell clone could be raised. This clone was highly reactive on purified α-protein ($SI > 11$), as well as on α-protein-derived peptides, although to a lesser extent ($SI = 2$). Whether this is the result of a dose effect, as the entire α-protein contains multiple potential T-cell epitopes, or whether our peptides did not perfectly match the epitope of the raised T-cell clone, has to be elucidated. The T-cell clone αB3-2 clearly belongs to the T helper 1 (Th1) subset because it exhibited a $CD3^+/CD4^+$ phenotype and secreted significant amounts of IL-2 and IFN-γ [28]. No IL-4 secretion was detected and controls with $IgG_1/IgG_2$-specific antibodies were negative.

Our results demonstrate that meningococcal $IgA_1$ protease-associated α-protein can effectively stimulate a helper T-cell response. This $CD4^+$ T-cell response is a prerequisite for an efficient humoral immune response generating lytic IgG and memory B cells. Moreover, effective T-cell help is obligatory for the induction of T-cell-dependent immunity and for the regulation of immunoglobulin class switching, affinity maturation and induction of memory. We therefore speculate that meningococcal α-proteins or α-protein-derived epitopes are promising candidates for further investigations concerning immunity against meningococcal infections and vaccination. In addition, we demonstrated an efficient tool for differentiating between a *Neisseria*- and a *Haemophilus*-induced immune response to $IgA_1$ proteases, which could facilitate future epidemiological investigations and lead to novel insights into the role of $IgA_1$ proteases and associated proteins in the infection process.

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