Immunoglobulin A1 Protease, an Exoenzyme of Pathogenic *Neisseriae*, Is a Potent Inducer of Proinflammatory Cytokines

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Summary

A characteristic of human pathogenic *Neisseriae* is the production and secretion of an immunoglobulin (Ig)A1-specific serine protease (IgA1 protease) that cleaves preferentially human IgA1 and other target proteins. Here we show a novel function for native IgA1 protease, i.e., the induction of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 from peripheral blood mononuclear cells. The capacity of IgA1 protease to elicit such cytokine responses in monocytes was enhanced in the presence of T lymphocytes. IgA1 protease did not induce the regulatory cytokine IL-10, which was, however, found in response to lipopolysaccharide and phytohemagglutinin. The immunomodulatory effects caused by IgA1 protease require a native form of the enzyme, and denaturation abolished cytokine induction. However, the proteolytic activity is not required for the cytokine induction by IgA1 protease. Our results indicate that IgA1 protease exhibits important immunostimulatory properties and may contribute substantially to the pathogenesis of neisserial infections by inducing large amounts of TNF-α and other proinflammatory cytokines. In particular, IgA1 protease may represent a key virulence determinant of bacterial meningitis.

Key words: *Neisseria gonorrhoeae* • *Neisseria meningitidis* • inflammation • tumor necrosis factor α • virulence factor

The *Neisseriae* are gram-negative, diplococcal bacteria which primarily colonize human mucosal surfaces. Besides several commensal species, the genus includes two human pathogens, *Neisseria gonorrhoeae* (gonococci), the causative agent of gonorrhea, and *Neisseria meningitidis* (meningococci), one major cause of bacterial meningitis. Both pathogens exclusively colonize or infect humans as their natural hosts. Gonococci induce a local inflammatory response of the urogenital mucosa (1). Meningococci are mostly harmless colonizers of the respiratory tract (e.g., nasopharynx). Under some not yet fully understood circumstances, meningococci disseminate from locally infected tissues into the blood stream and penetrate the blood–brain barrier to cause severe inflammations in the central nervous system (2).

Various bacterial components have been implicated in the pathogenesis of neisserial infections (3–6). Pili and Opa proteins contribute to the attachment and the invasion of different host cells, and the outer membrane porin protein I or PorB was found to prevent the microbicidal activities of phagocytes (7, 5) and to induce apoptosis in target cells (8). Circumstantial evidence related to the unique association of IgA1 proteases with human pathogenic bacterial species (9, 6) suggests that this enzyme plays a significant role in pathogenesis. IgA1 proteases are produced by a variety of gram-positive and gram-negative human pathogens (10–12). Among those, the IgA1 proteases of the pathogenic *Neisseriae* and various *Haemophilus* species are structurally closely related (13, 14). They undergo frequent horizontal exchange across the species barrier, thus forming a polymorphic class of proteins (15, 16). The gram-negative bacterial IgA1 proteases are derived from a polyprotein precursor molecule which directs enzyme secretion by an autocatalytic process. While the neisserial IgA1 protease precursors include two additional secreted domains, an α-protein and a γ-peptide (13), the proteases of the two species probably exhibit similar functions.

IgA1 protease cleaves human IgA1 both in serum and at mucosal surfaces of the respiratory and urogenital tracts (10, 17). In particular, the neisserial IgA1 protease is a sequence-specific endopeptidase which cleaves single peptide bonds of distinct proline-rich consensus sequences that are found in the hinge region of human IgA1 but not IgA2 (10, 18). As a consequence, the enzymatic activity or characteristic cleavage products of IgA1 molecules were found in nasopharyngeal and vaginal secretions of individuals infected with pathogenic *Neisseriae* (11, 19, 20). As intact IgA1 protects against microorganisms infecting mucosal tissues by prevention of microbial adherence, toxin neutralization,

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/10/1049/10 $5.00
Volume 190, Number 8, October 18, 1999 1049–1058
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and activation of Fc-α receptor–dependent phagocytosis, the cleavage of IgA1 has been postulated to impair IgA1-mediated effector functions (11, 12). However, most individuals have neutralizing antibodies against IgA1 protease that may interfere with the function of the enzyme (21–23). Thus, the role of IgA1 protease in neisserial pathogenesis remains obscure.

Inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 are produced by activated monocytes/macrophages. They promote local inflammatory responses at the sites of microbial infections, and mediate adherence of leukocytes to the endothelial tissues and their transmigration by up-regulating the expression of adhesion molecules (24–27). Elevated levels of TNF-α, IL-1, IL-6, and IL-8 have been found in the cerebrospinal fluid (CSF)[1] and plasma of patients with meningitis (28–31) and were detected in the urine after the intraurethral challenge of patients with *N. gonorrhoeae* (32).

Besides LPS, several bacterial products that can stimulate cytokine synthesis in different cell types have been characterized (33). These cytokine-inducing agents include heat labile proteins, glycoproteins, lipoproteins, or peptides, which trigger cytokine synthesis by mechanisms distinct from LPS and therefore form an additional class of virulence factors termed modulins (34, 35).

The purpose of this study was to assess the immunogenic and immunomodulatory properties of the neisserial IgA1 protease. We demonstrated that purified IgA1 protease acts like a modulin by stimulating the release of TNF-α, IL-1β, IL-6, and IL-8 from PBMCs. These effects were independent of the proteolytic activity but required the native form of the enzyme.

Materials and Methods

Purification and Characterization of Recombinant IgA1 Protease. In this study, we used the recombinant form of IgA1 protease expressed in *Escherichia coli* strain H2053, which harbors plasmid pIP11 containing the *iga* gene of *N. gonorrhoeae* MS11 with the temperature-inducible bacteriophage λ promoter P_L, as described previously (18). In brief, the supernatant of a 4-liter stationary phase culture was recovered by centrifugation, supplemented with 0.1 M EDTA, and diluted 1:1 (vol/vol) with potassium phosphate buffer (20 mM, pH 7.0). The bacterial supernatant was applied to a cation exchange column. After washing, bound protein was eluted with 500 mM potassium phosphate, 8.6% glycerol. Fractions of 10 ml were collected, precipitated with ammonium sulphate, and then redissolved in gel filtration buffer. Gel filtration was performed with a preequilibrated Superdex column (Amersham Pharmacia Biotech). Protein-containing eluates were recognized by UV absorption, pooled, and precipitated with ammonium sulphate. Finally, the pellet was suspended in PBS and dialyzed against PBS, pH 7.4, and stored in aliquots at −70°C. Purity of the protein was assessed by SDS-PAGE with subsequent Coomassie staining showing the characteristic protein profile with the typical 109- and 106-kD protein bands as described previously (18). The protein concentration was determined using a commercial protein determination assay (Bio-Rad). Specificity for IgA1 protease was confirmed by immunoblotting using an IgA1 protease–specific rabbit antiserum (13). The cleavage activity of the recombinant IgA1 protease was determined with human IgA1 (20 ng/μl; Dakopatts) in Tris buffer (0.05 M Tris-HCl, 10 mM $CaCl_2$, and 10 mM $MgCl_2$, pH 7.5) at 37°C according to the method described previously (36). Heat denaturation (boiling for 10 min) completely destroyed the enzymatic activity. The enzymatic activity could also be blocked by addition of the specific peptide prolyl boronic acid inhibitor (37). 100 μM of the inhibitor (provided by A.G. Plaut, New England Medical Center, Boston, MA) abrogated cleavage of IgA at concentrations up to 10 ng per μl of IgA1 protease (data not shown).

Endotoxin Assays. The endotoxin contents of our IgA1 protease preparations were determined by *Limulus* amebocyte lysate assay (LAL; Haemochrom Diagnostica). All preparations contained <2 pg of endotoxin per μg protein. It is described that some proteins could interfere with the *Limulus* assay (38). To ensure that IgA1 protease is free of LPS, we have tested in addition the priming potency of IgA1 protease for neutrophils, which are as sensitive as the *Limulus* assay in detecting LPS. Different types of LPS can prime neutrophils to increase their oxidative response after stimulation with the agonist fMLP (Sigma Chemical Co.) (38). PMNs preincubated with 10 μg/ml IgA1 protease for 30 min stimulated with 1 μM fMLP showed no alteration of the oxidative response compared with the control when measured as luminol-dependent chemiluminescence in a six-channel Biolumat LB 9505 (Berthold; data not shown).

Cells. PBMCs were isolated from buffy coats or freshly citrated blood of adult, healthy volunteers by density gradient centrifugation over Ficoll/Isopaque (Amersham Pharmacia Biotech) and washed twice in Ca^2, Mg^{2+}–free PBS. For some experiments, lymphocytes and monocytes were separated (6×10^6 cells/ml in PBS plus 0.5% BSA) by counterflow centrifugation (elutriation) using the Beckman J6ML/JE-5.0 centrifuge system. Elutriation was performed at constant rotor speed with increasing flow rates, and lymphocytes were collected. To obtain purified T cells, the lymphocyte suspensions were positively sorted by anti-CD3 magnetic beads (MACS; Miltenyi Biotec). Purity of $CD3^+$ T cells was always >95% as determined by flow cytometry. Monocytes were prepared from PBMCs either by adherence to plastic dishes (1 h, 37°C) or by negative selection using the MACS monocyte isolation kit (Miltenyi Biotec). The purity of $CD14^+$ cells was always >85% as measured by flow cytometry using a PE-conjugated anti-CD14 antibody (Leu M3; Becton Dickinson). The remaining 15% consisted of $CD3^-$, $CD19^-$, and $CD56^-$ flow cytometric counting events.

The monocytic cell line MonoMac 6 (no. 124; American Type Culture Collection) was obtained from the German Collection of Microorganism and Cell Cultures (Braunschweig, Germany) and maintained in 24-well plates under low endotoxin conditions (<10 pg/ml) in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, and 9 μg/ml bovine insulin as described (39). The cell line normally expresses CD14 as controlled by flow cytometry.

Cell Viability. Cytotoxic effects of IgA1 protease preparations were excluded by staining of PBMCs with propidium iodide (10 μg/ml) and subsequent evaluation by flow cytometry. Cell viability of freshly isolated or cultured cells (24 h with 10 μg/ml IgA1 protease) was >95% in all experiments.

Cell Culture and Stimulation of Cytokine Release. All cell cultures were performed in RPMI 1640 supplemented with 20 nM

[1]*Abbreviation used in this paper:* CSF, cerebrospinal fluid.

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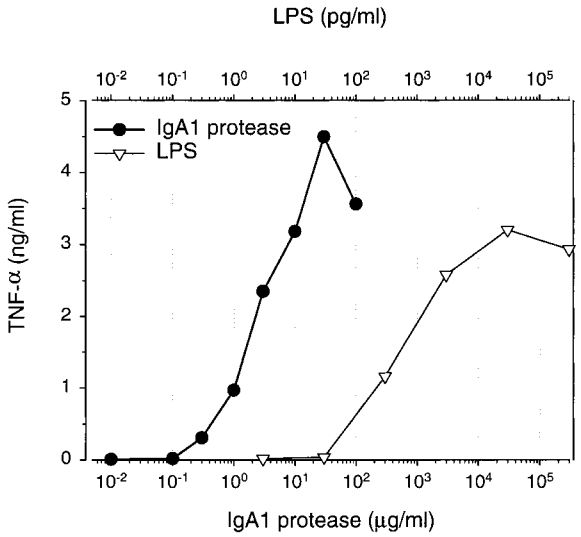

Figure 1. Purified IgA1 protease induces dose-dependent release of TNF-α from PBMCs. On the top axis, LPS concentrations were set according to the endotoxin content in the IgA1 protease protein on the bottom axis. TNF-α synthesis induced by LPS and IgA1 protease is shown by ▽ and ●, respectively. PBMCs were cultured for 18 h in the presence of IgA1 protease or LPS. TNF-α was measured in pooled cell-free culture supernatants by a specific ELISA. TNF-α levels in nonstimulated control cells (medium control) did not exceed 0.02 ng/ml. Data were confirmed by three experiments using PBMCs from different donors.

l-glutamine, 25 mM Hepes (GIBCO BRL), 500 U/ml penicillin, 500 μg/ml streptomycin, and 5% heat-inactivated human AB serum (pooled male serum, <<1 ng/ml endotoxin; Sigma Chemical Co.). This medium is further designated as culture medium.

A total of 10^5 cells was cultured in 200 μl culture medium in round-bottomed 96-well plates at 37°C, 5% CO_2. MonoMac 6 cells (10^5 cells/well) were incubated for 24 h in 96-well flat-bottomed plates with culture medium. Cell-free supernatants were harvested after 18 h (150 μl/well, three wells per independent experiment), pooled, and frozen in 50 μl aliquots at −20°C until assayed.

To investigate the effect of $CD3^+$ T lymphocytes on the IgA1 protease–induced cytokine production of $CD14^+$ monocytes, 2×10^4 $CD14^+$ cells were cultured either with or without $CD3^+$ cells keeping the monocyte/lymphocyte ratio constantly at 1:4. A total of 10^5 PBMCs were cultured as a control with a monocyte content ranging from 10 to 20%.

Cytokine ELISAs. The levels of the released cytokines TNF-α, IL-1β, IL-6, IL-8 (R&D Systems), and IFN-γ, GM-CSF, and IL-10 (PharMingen) in the culture supernatants were quantified with commercially available sandwich ELISA kits. The assays were performed as recommended by the manufacturer. In brief, polystyrene microtiter plates were coated with the catching mAb overnight, washed intensively (washing buffer: PBS, 0.02% Tween 20), and blocked with PBS, 5% AB serum. Different dilutions of all samples and the standard cytokine dilutions were assayed in duplicate or triplicate, respectively, with a total volume of 100 μl for 2 h at room temperature. After removing the unbound detecting mAb by washing (10 times), streptavidin-peroxidase solution was added to each well and incubated for 20 min. After 10 washes, the chromogenic substrate (TMB substrate with H_2O_2; Kirkegaard & Perry) was added to each well and color development was stopped after 20 min by adding 50 μl 1 M H_3PO_4. The absorbance of the samples was measured at 450 nm. The cytokine concentrations were determined by extrapolating from the standard curve. The results are expressed as means of triplicates in ng/ml. The detection limits of the ELISA kits were as follows: TNF-α, IL-1β, IL-10, GM-CSF, and IFN-γ ≤ 0.01 ng/ml; IL-6 and IL-8, ≤ 0.05 ng/ml.

Flow Cytometry. For immunofluorescence staining, we used the following: anti-CD14–PE as a monocytic marker (LeuM3, IgG2a; Becton Dickinson), and FITC-conjugated anti–HLA-DR (B.812.2, IgG2a; Coulter Immunotech) and anti-CD25 for detection of the IL-2 receptor (ACT-1, IgG1; Dako). Control mouse Ig (IgG1-FITC/IgG2a-PE) were obtained from Becton Dickinson. An FITC-conjugated goat anti–mouse F(ab) fragment (Coulter Immunotech) was used as a secondary antibody. The cells were washed, adjusted to 2×10^5/200 μl in PBS with 0.2% BSA, and incubated with the antibodies for 30 min on ice. After washing, the samples were analyzed on a FACScalibur™ (Becton Dickinson) using the CELLQuest™ program. The cells were gated due to their forward scatter/side scatter profile. Background levels of immunofluorescence were determined using isotype control Ig. For the detection of IL-2 receptor, cells were first labeled with unconjugated anti-CD25, washed, and then incubated with FITC-conjugated goat anti–mouse $F(ab)_2$ fragment (5 μg/ml).

Proliferation Assay. Proliferation assays with PBMCs (10^5 cells/well in culture medium) were performed at 37°C in 96-well round-bottomed plates at a total of 150 μl/well. The cells were cultured for various time periods (1–4 d) and pulsed with 18.5 kBq of [^{3}H]thymidine per well. Cells were then harvested on filters, and incorporation of [^{3}H]thymidine was measured using a Microbeta Scintillation Counter (Wallac Instruments). Unstimulated cells and PHA-stimulated (5 μg/ml) cells were used as controls.

Table I. *Dose-dependent Cytokine Induction by Purified IgA1 Protease and LPS from E. coli*

		IgA1 protease (μg/ml)				LPS (ng/ml)	
Cytokine*	Control‡	0.1	1.0	3.0	10	0.03	30
IL-1β	<0.01	0.02 ± 0.0	0.1 ± 0.0	0.31 ± 0.02	0.75 ± 0.05	<0.01	0.99 ± 0.09
IL-6	0.2 ± 0.0	0.3 ± 0.1	19.5 ± 0.5	14.3 ± 2.3	24.5 ± 1.7	ND	26.1 ± 2.5
IL-8	7 ± 0.5	15 ± 1.9	29.8 ± 3.6	33.1 ± 1.8	64.24 ± 6.1	21 ± 1.1	45.3 ± 2.6

*Cytokine levels in culture supernatants from PBMCs incubated for 18 h in the presence of different concentrations of IgA1 protease and *E. coli* LPS. Values are the means ± SD from triplicate wells. Amounts are given in ng/ml; IL-1β (detection limit 10 pg/ml), and IL-6 and IL-8 (detection limit 50 pg/ml).

‡Culture medium was used as a control.

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Statistical Analyses. The nonparametric Wilcoxon rank test was performed for comparison of cytokine data of PHA-, LPS-, and IgA1 protease–stimulated PBMCs. Statistical differences were considered significant at $P < 0.01$. Data from separate experiments were expressed as means ± SEM and were evaluated for statistical significance by Student's *t* test. Data from a single representative experiment are presented as means ± SD obtained by triplicate determination.

Results

Induction of Proinflammatory Cytokine Release from Human PBMCs by IgA1 Protease. To assess the production of the proinflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8, PBMCs from healthy human donors were incubated with purified IgA1 protease. LPS, PHA, and medium alone were used as control. Initial experiments showed that release of proinflammatory cytokines from PBMCs induced by IgA1 protease reached maximal levels between 12 and 24 h (data not shown). Furthermore, a rapid decrease in IgA1 protease–induced cytokine production could be observed after 24 h, and only small amounts of TNF-α were detectable after 72 h. Therefore, in subsequent experiments a single end-point of 18 h culture was chosen that allowed detection of TNF-α and other proinflammatory cytokines. IgA1 protease stimulated the release of proinflammatory cytokines by PBMCs in a dose-dependent manner (Fig. 1, and Table I). Significant amounts of TNF-α in the supernatants were already detectable in the presence of 1 μg/ml IgA1 protease, and highest levels were reached using 10 μg/ml. Similar dose–response patterns were obtained for other proinflammatory cytokines such as IL-1β, IL-6, and the chemokine IL-8. Nonstimulated PBMCs produced negligible amounts of TNF-α, IL-1β, and IL-6, but some IL-8, which was probably induced by adherence as previously postulated by Kasahara et al. (40).

To determine whether the cytokine induction was due to LPS present in the preparation of the recombinant IgA1 protease from *E. coli*, we tested the potential of *E. coli* LPS to induce TNF-α. The endotoxin content present in the protease preparation of 10 μg/ml did not exceed 20 pg/ml. As shown in Fig. 1, no significant cytokine production compared with the control was detected at 10–30 pg/ml LPS, indicating that endotoxin contamination was not the cause of cytokine induction by purified IgA1 protease. TNF-α synthesis in response to 10 μg/ml IgA1 protease reached the level synthesized by cells stimulated with a much higher dose of *E. coli* LPS (30 ng/ml). In subsequent experiments, low dose (30 pg/ml) and high dose (30 ng/ml) controls of LPS were always included.

The cytokine release of PBMCs showed similar variations between different donors upon stimulation with either IgA1 protease, LPS, or PHA. As shown in Fig. 2 (A–D), the median levels of the proinflammatory cytokines detected in the presence of 10 μg/ml IgA1 protease were approximately similar to those found after LPS stimulation and sometimes even higher than those incubated with the lectin PHA.

Heat denaturation abolished the cytokine-inducing capacity of IgA1 protease (data not shown). To test whether the

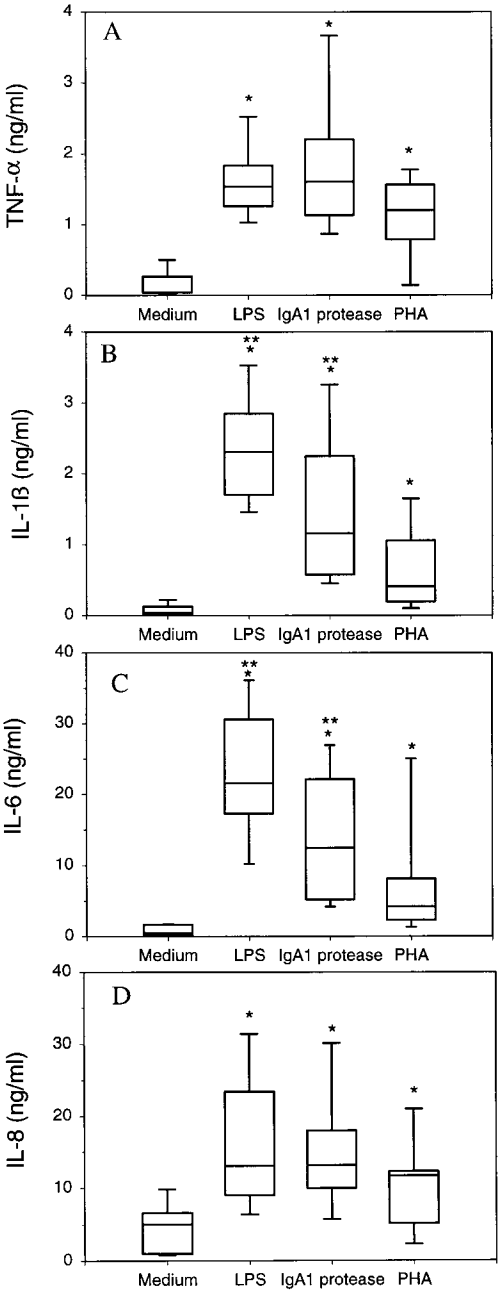

Figure 2. Comparison of cytokine production by PBMCs from different donors. PBMCs were cultured under low endotoxin conditions (<10 pg/ml as determined by the *Limulus* amebocyte lysate [LAL] assay). The indicated stimuli were added to the medium at the following concentrations: LPS, 30 ng/ml; PHA, 5 μg/ml; and IgA1 protease, 10 μg/ml. The cell-free supernatants were harvested after 18 h, pooled, and analyzed for the indicated cytokines by ELISA. Plots include data from $n = 15$ (for TNF-α [A] and IL-6 [C]) or $n = 12$ (for IL-1β [B] and IL-8 [D]) independent experiments. Boxes represent second and third quartiles; the lines within each box are the median values; the error bars represent 5 and 95% confidence. To compare cytokine levels in the different stimulation groups, the nonparametric Wilcoxon rank test was used. Results are considered as significantly different from values obtained with medium alone (*$P < 0.05$) and as significantly different from PHA (**$P < 0.01$).

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enzymatic activity is involved in that process, PBMCs were incubated in the presence of 100 μM of a specific IgA1 protease inhibitor (peptide prolyl boronic acid [37]). Although at this concentration the inhibitor completely blocked the cleavage of IgA1, it altered neither the LPS- nor the IgA1 protease–induced production of proinflammatory cytokines (data not shown). This demonstrates that the native form but not the enzymatic activity of IgA1 protease is required for the induction of proinflammatory cytokines.

Cytokine Release Induced by IgA1 Protease Is Augmented in the Presence of T Cells. IgA1 protease triggers a strong release of proinflammatory cytokines from PBMCs after only a short incubation period, indicating that monocytes might be the main producer of these mediators. To determine the source of cytokine release, we cultivated lymphocyte-depleted peripheral blood monocytes (purity varied between 85 and 95%). As demonstrated in Fig. 3, IgA1 protease induced significantly lower amounts of TNF-α, IL-1β, IL-6, and IL-8 compared with LPS, which readily stimulated TNF-α production in purified monocytes. Direct comparison with PBMCs revealed that IgA1 protease–stimulated PBMCs can elicit up to 20-fold higher amounts of these cytokines compared with the respective lymphocyte-depleted monocyte cultures, which was particularly true for IL-6 and IL-8 (data not shown; see also Fig. 2). We also tested the monocytic cell line, MonoMac 6. This cell line shows phenotypic and functional characteristics of mature blood monocytes and produces IL-1, IL-6, and TNF-α upon stimulation with LPS or PMA (39, 41). Compared with LPS, we found a significantly lower production of these cytokines and IL-8 after stimulation with IgA1 protease (Table II).

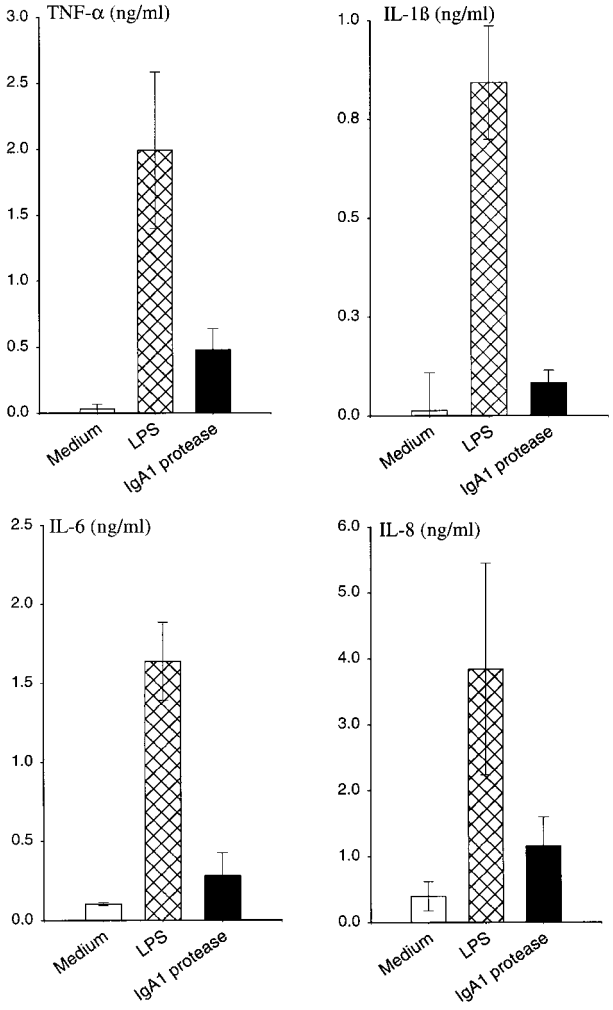

Figure 3. Production of proinflammatory cytokines by purified monocytes in medium alone (white bars) and in response to 30 ng/ml LPS (hatched bars) or 10 μg/ml IgA1 protease (black bars). The level of each cytokine in the supernatant of stimulated monocyte cultures (10^5) was determined after 18 h. The results shown here are mean values from three different experiments ± SEM.

Direct contact with T cells can augment monocyte cytokine production (42). To examine the necessity of cell contact for TNF-α release in the different types of stimulation, monocytes stimulated with IgA1 protease, LPS, or PHA were incubated with autologous active and fixed (15 min, paraformaldehyde at 0°C) lymphocytes at a monocyte/lymphocyte ratio of 1:2. In parallel, the lymphocytes and monocytes were incubated alone. Such lymphocytes and purified monocytes produced only low levels of TNF-α upon stimulation with 10 μg/ml IgA1 protease (Fig. 4). The same results were obtained after stimulation with PHA. The addition of viable lymphocytes increased the TNF-α secretion in nonstimulated, IgA1 protease–, and PHA-treated monocytes, whereas the addition of prefixed lymphocytes did not. Monocytes stimulated with IgA1 protease or PHA also showed a significantly stronger TNF-α induction in the presence of lymphocytes compared with the control. Only LPS was able to induce TNF-α in purified monocytes alone, and this was not further enhanced by lymphocytes.

To examine the particular role of T cells for IgA1 protease–induced TNF-α release, monocytes were incubated

Table II. *Release of Proinflammatory Cytokines in the Monocytic Cell Line, MonoMac 6*

	Proinflammatory cytokines* induced by:				
Cytokine‡	Control§	IgA1 protease (10 μg/ml)	LPS (30 ng/ml)	PHA (5 μg/ml)	PMA (100 ng/ml)
TNF-α	<dl	0.03	0.12	<dl	0.29
IL-1β	<dl	0.02	0.06	<dl	ND
IL-6	<dl	0.59	23.37	<dl	4.73
IL-8	2.02	12.21	84.96	11.28	67.96

*Cytokine concentrations in culture supernatants are given in ng/ml. <dl, below detection limit.

‡MonoMac 6 cells were incubated at 10^6 cells/ml for 24 h, and supernatants were analyzed for cytokine production. Values are means from duplicate assays.

§Corresponding culture medium was used as a control.

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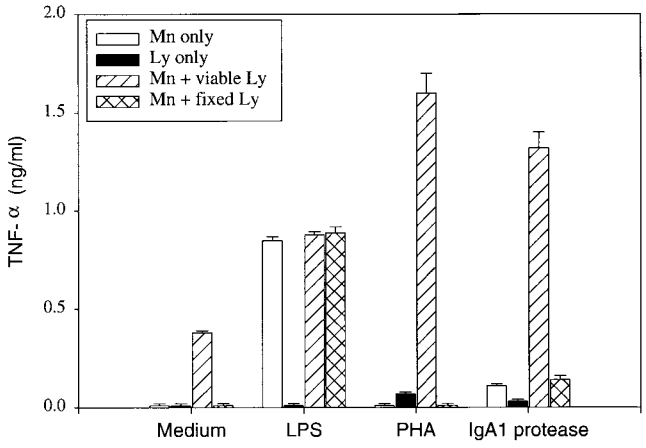

Figure 4. PBLs enhance TNF-α release of monocytes. PBMCs were separated by counterflow elutriation and adherence into monocytes (Mn) and lymphocytes (Ly) as described in Materials and Methods. After 1 h incubation, the autologous lymphocytes were added to the monocytes at a monocyte/lymphocyte ratio of 1:2. Fixed lymphocytes were treated with 2% paraformaldehyde (15 min at 0°C). TNF-α levels in the supernatants were measured 18 h after stimulation with 30 ng/ml LPS, 5 μg/ml PHA, or 10 μg/ml IgA1 protease.

with autologous $CD3^+$ T lymphocytes at a monocyte/lymphocyte ratio of 1:4. While T lymphocytes produced no or very low amounts of TNF-α, the monocytes released reduced levels of TNF-α in response to IgA1 protease compared with crude PBMCs. Such reduced TNF-α secretion of monocytes was enhanced in the presence of autologous purified T cells (Fig. 5). However, when the two cell populations were separated by a permeable membrane during the cell culture, the TNF-α release by monocytes was not increased in response to IgA1 protease (data not shown). These results suggest that direct contact between T cells and monocytes is necessary for a maximum of cytokine production by monocytes in response to IgA1 protease.

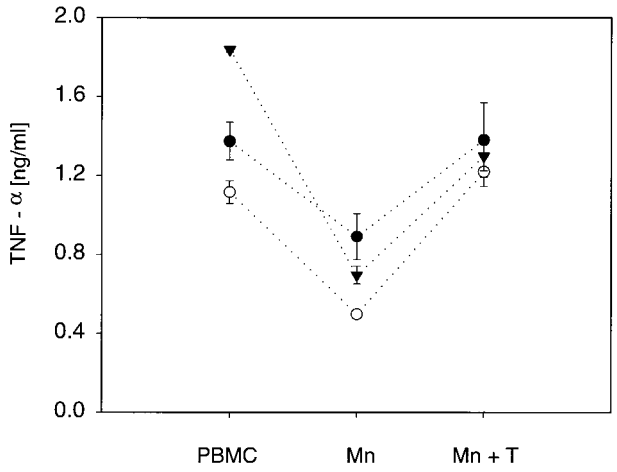

Figure 5. Purified $CD3^+$ T lymphocytes enhance TNF-α production of $CD14^+$ monocytes. The autologous $CD3^+$ T lymphocytes (T) were added to the monocytes (Mn) at a monocyte/lymphocyte ratio of 1:4. TNF-α levels in the supernatants were measured after 18 h of culture with 10 μg/ml IgA1 protease. The results shown here are mean values from three experiments from different donors ± SEM (▼, ○, and ●).

It is well known that lymphokines such as GM-CSF or IFN-γ can augment cytokine release in monocytes (43, 44). We found increased levels of GM-CSF in PBMC cultures after incubation with IgA1 protease for 18 h, whereas IgA1 protease failed to induce release of IFN-γ after that time (Fig. 6, A and B). Very low levels of IFN-γ were finally detectable after 4 d (data not shown). However, the IFN-γ levels in these cultures were always more than 100-fold lower than peak levels of IFN-γ in PHA-stimulated PBMC cultures. Furthermore, in the first 4 d of culture, neither cell proliferation nor increase of IL-2 receptor–positive lymphocytes could be measured after incubation with IgA1 protease (data not shown). These results suggest that viable T cells are required for the cytokine release by monocytes stimulated with IgA1 protease, but unlike PHA, IgA1 protease does not seem to provide a strong activation signal for T lymphocytes.

Effect of IgA1 Protease on IL-10 Production by PBMCs. IL-10 is a cytokine with immunosuppressive and antiinflammatory effects on monocytes. Reportedly, IL-10 synthesis is delayed relative to the synthesis of inflammatory cytokines TNF-α, IL-1β, and IL-6 (45). In contrast to the induction of proinflammatory cytokines, IgA1 protease failed to induce IL-10, whereas LPS as well as PHA could stimulate IL-10 production in PBMCs (Fig. 7). Kinetic experiments revealed that IgA1 protease does not induce IL-10 production in PBMCs within a period up to 96 h. In contrast, culture supernatants of PHA- or LPS-stimulated PBMCs contained high IL-10 levels at different time points (data not shown).

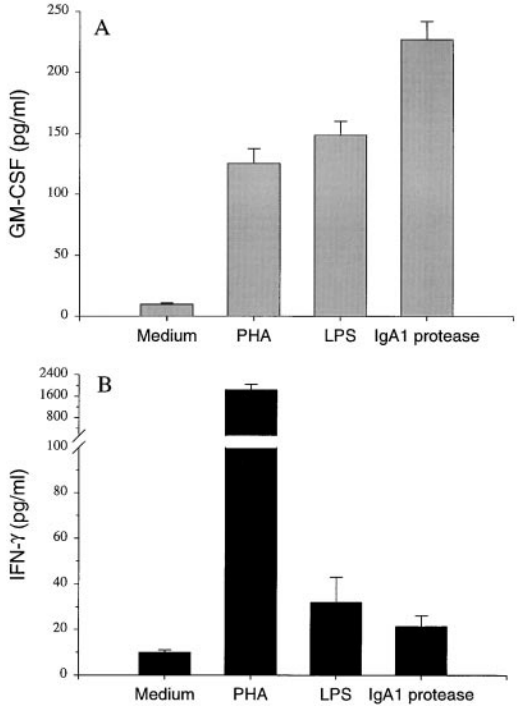

Figure 6. GM-CSF (A) and IFN-γ (B) release of PBMCs in response to IgA1 protease (10 μg/ml), PHA (5 μg/ml), and LPS (30 ng/ml). The levels of these cytokines were determined in supernatants after 18 h. The results (means ± SD of three determinations) were confirmed by three independent experiments.

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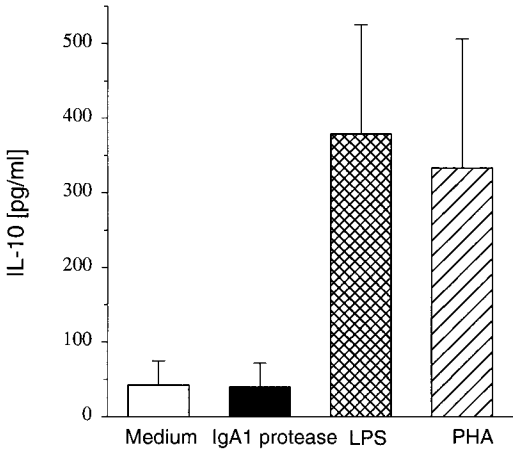

Figure 7. IL-10 release by PBMCs stimulated with IgA1 protease (10 μg/ml), LPS (30 ng/ml), or PHA (5 μg/ml) after 18 h incubation. Culture medium alone was used as a control. The means ± SEM of five independent experiments with PBMCs from five different donors are shown.

Discussion

IgA1 protease, a putative virulence factor of human pathogenic *Neisseriae*, was initially discovered as an enzyme that cleaves human IgA1 molecules, thus interfering with the biological functions of IgA1 (10). Here we report on a novel property of neisserial IgA1 protease, the induction of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, and the chemokine IL-8 in PBMCs.

The stimulation of cytokine synthesis by IgA1 protease occurred in the nanomolar range and increased in a dose-dependent manner. The level of cytokine release was substantial but varied to some extent depending on the donor. The rapid cytokine induction within 24 h indicates a role of monocytes/macrophages in this process. However, compared with PBMCs, significantly lower amounts of TNF-α and IL-8 and practically no IL-6 and IL-1β were elicited by purified monocytes in response to IgA1 protease, whereas LPS induced a strong cytokine secretion that included IL-6 and IL-1β. The monocytic cell line MonoMac 6 also showed a low production of TNF-α, IL-1β, IL-6, and IL-8 in response to IgA1 protease. These results suggest that the presence of lymphocytes is required for full cytokine induction by IgA1 protease.

It is known that the activation of monocytes/macrophages and the release of monocyte-derived cytokines can be provoked by either direct cell–cell contact with activated antigen-specific T cells or secreted lymphokines (42–44, 46). This is consistent with our finding that IgA1 protease–induced TNF-α secretion by monocytes was enhanced by the addition of lymphocytes or $CD3^+$ T cells to the cultures. To investigate the role of activated T cells in this process, we measured IFN-γ secretion, CD69, and IL-2 receptor expression, representing typical markers of activated T cells. Although CD69 was slightly upregulated, the low number of IL-2 receptor–positive cells suggests that no large T cell population was activated or that only partial activation occurred (46; and data not shown). The T cell cytokine IFN-γ can enhance the cytokine expression in monocytes synergistically in combination with GM-CSF (43, 44). Although GM-CSF was detected after IgA1 stimulation, the release of IFN-γ was low and the addition of anti–IFN-γ antibodies had no effect on the monokine production (data not shown). However, the fact that cocultivation with T cells separated from the monocytes by a permeable membrane could not enhance the production of proinflammatory cytokines indicates that T cells may also participate by a yet unknown contact-dependent mechanism or by nonclassical presentation of antigen (47–49). We also did not find binding of IgA1 protease to T cells, whereas $CD14^+$ monocytes were clearly stained with FITC-labeled IgA1 protease (data not shown).

The profile of the cytokine response of PBMCs to IgA1 protease shows some similarities to LPS stimulation. Although the IgA1 protease was produced as a recombinant protein in *E. coli*, our preparations contained usually <2 pg LPS/μg protein. This LPS content could not sufficiently induce the observed cytokine production. Furthermore, effects which are typically triggered by LPS, such as priming of phagocytes for an enhancement of the oxidative burst, could not be confirmed in cells incubated with IgA1 protease (38; and data not shown). On the other hand, LPS does not require T cell engagement, suggesting that IgA1 protease functions by a different mechanism. Both molecules can have synergistic effects in vitro (data not shown) and may also act concertedly in vivo. Activated monocytes/macrophages also produce the antiinflammatory cytokine IL-10. This cytokine mediates important autoregulatory effects by downregulating inflammation, and its maximal production is delayed compared with the initial burst of proinflammatory cytokines (45). IL-10 production was augmented by stimulation with LPS or PHA, but not with IgA1 protease (50). Thus, IgA1 protease seems unique in stimulating an asymmetric synthesis of proinflammatory cytokines from monocytes and fails to induce strong regulatory feedback loops.

Besides the neisserial IgA1 protease reported here, other bacterial products have been found to induce proinflammatory cytokines. These components have been classified as a separate class of bacterial virulence factors termed modulins (51, 34). Examples include the listeriolysin from *Listeria monocytogenes* (52), the *E. coli* hemolysin (53), and the streptococcal pneumolysin (54). Other proteins with enzymatic activity, such as the urease of *Helicobacter pylori* (55) and the sphingomyelinase from *Staphylococcus aureus* (56), also modulate cytokine production. Only a few modulins, like the anthrax lethal toxin from *Bacillus anthracis* (34) and the clostridium toxin from *Clostridium difficile* (57), act in the attomolar or femtomolar range, while most other modulins including IgA1 protease exhibit their activity in the nanomolar range. Some modulins, such the staphylococcal toxic shock syndrome toxin 1 (TSST-1) from *S. aureus* or the erythrogenic exotoxin (ET) from *Streptococcus pyogenes* possess superantigenic activity, thereby inducing T cells to proliferate and to produce cytokines (58). IgA1 protease is different from those superantigenic factors because it is not a T cell mitogen.

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The property of neisserial IgA1 protease to induce proinflammatory cytokines may be highly relevant for the disease-causing properties of gonococci and meningococci. A hallmark of the pathophysiology in these diseases is the intense inflammatory response associated with the substantial release of proinflammatory cytokines. TNF-α, IL-1, IL-6, and IL-8 have been detected in local secretions and sera during mucosal infections with gram-negative species, including *N. meningitidis* and *N. gonorrhoeae* (29, 35). Increased levels of IL-8 and TNF-α in particular in plasma and in urine, and smaller amounts of IL-1β and IL-6 were found in experimental gonorrhea after intraurethral application of gonococci (32).

High levels of these cytokines were also found in the CSF and in the serum during meningeal inflammation. These mediators promote the inflammatory response with subsequent tissue damage and in some cases septic shock (28–30). The level of TNF-α in the CSF directly correlates with the severity of the damage to the blood–brain barrier (29).

The most important bacterial component of gram-negative bacteria known to stimulate the release of inflammatory cytokines is LPS. Indeed, meningococcal LPS was found to be a major cytokine-inducing component in bacterial meningitis, since the cytokine profile and levels found in serum or CSF correlated with the LPS concentrations. However, Prins et al. (59) could not confirm the relationship between levels of cytokines and bacterial endotoxin produced by various strains of young meningitis patients, suggesting that factors other than endotoxin may be involved in cytokine induction during acute meningococcal septic shock. In vivo, LPS and IgA1 protease might be released together and act synergistically. Strikingly, though, the three major causative agents of bacterial meningitis, *N. meningitidis*, *H. influenzae*, and *Streptococcus pneumoniae*, all produce an IgA1 protease (11, 13, 14, 60). Therefore, IgA1 protease may constitute an underestimated virulence factor in sepsis and meningeal infection.

Besides the cleavage of IgA1, several additional functions have been attributed to neisserial IgA1 protease. IgA1 protease was recently shown to cleave LAMP-1, a lysosomal protein associated with the endosomal maturation (36, 61), but only one report suggested that, as a consequence, IgA1 protease facilitates the intracellular survival of pathogenic *N. gonorrhoeae* (61). Furthermore, other human proteins have been found to be proteolytic substrates in vitro. However, the biological relevance of their cleavage remains unclear. In a parallel study, we recently observed that IgA1 protease efficiently protects phagocytes from TNF-α–mediated apoptosis (our unpublished results). Considering the strong potential of IgA1 protease to induce TNF-α, this observation is intriguing since *Neisseria* is capable of entering phagocytic cells by an active mechanism (62). Hence, the pathogens may have an interest in preventing lethal effects of TNF-α on their intracellular niches.

The induction of proinflammatory cytokines may contribute to the characteristic inflammatory reaction caused by the pathogenic *Neisseriae*. Understanding the mechanism of IgA1 protease in the regulation of these cytokines has potential therapeutic significance and is currently in progress. Future research should reveal strategies for blocking this cytokine-inducing pathway in response to pathogenic *Neisseriae* and probably to other IgA1 protease–secreting human pathogenic bacteria.

We thank Dr. Susanne Beck for providing the recombinant IgA1 protease, Dr. Robert Hurwitz for IgA1 protease purification and characterization, and Ilona Harm, Uwe Koscy, and Petra Lindenberg for technical assistance. We are also grateful to Drs. T. Aebischer, S. Gödert, and A. Walduck for helpful discussions.

This work was supported by a grant from the Fonds der Chemischen Industrie to T.F. Meyer.

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Submitted: 22 April 1999 Revised: 8 July 1999 Accepted: 12 August 1999

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