Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 7882–7887, July 1996
Immunology

Pore-forming toxins trigger shedding of receptors for interleukin 6 and lipopolysaccharide

(cytolysin/cytokine/metalloproteinase/membrane receptor/inflammation)

IWAN WALEV*†, PETRA VOLLMER†‡, MICHAEL PALMER*, SUCHARIT BHAKDI*, AND STEFAN ROSE-JOHN‡§

*Institute of Medical Microbiology and Hygiene, and ‡I. Medical Clinic-Section Pathophysiology, Johannes Gutenberg University of Mainz, D-55101 Mainz, Germany

Communicated by Hans J. Müller-Eberhard, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany, April 17, 1996 (received for review September 30, 1995)

ABSTRACT **Cleavage of membrane-associated proteins with the release of biologically active macromolecules is an emerging theme in biology. However, little is known about the nature and regulation of the involved proteases or about the physiological inducers of the shedding process. We here report that rapid and massive shedding of the interleukin 6 receptor (IL-6R) and the lipopolysaccharide receptor (CD14) occurs from primary and transfected cells attacked by two prototypes of pore-forming bacterial toxins, streptolysin O and *Escherichia coli* hemolysin. Shedding is not induced by an streptolysin O toxin mutant which retains cell binding capacity but lacks pore-forming activity. The toxin-dependent cleavage site of the IL-6R was mapped to a position close to, but distinct from, that observed after stimulation with phorbol myristate acetate. Soluble IL-6R that was shed from toxin-treated cells bound its ligand and induced an IL-6-specific signal in cells that primarily lacked the IL-6R. Transsignaling by soluble IL-6R and soluble CD14 is known to dramatically broaden the spectrum of host cells for IL-6 and lipopolysaccharide, and is thus an important mechanism underlying their systemic inflammatory effects. Our findings uncover a novel mechanism that can help to explain the long-range detrimental action of pore-forming toxins in the host organism.**

Many membrane-associated, biologically important molecules can be released from cells by a proteolytic cleavage process called shedding. These include cytokines, receptors for cytokines and growth factors, and cellular adhesion proteins (1, 2). Soluble receptors for cytokines and growth factors retain their ligand-binding capacity and this can have different consequences. First, soluble receptors may act as competitive inhibitors of the cognate ligands. The soluble receptors for interleukin 1 (IL-1) and tumor necrosis factor (TNF) have been shown to inhibit IL-1 and TNF-specific responses (reviewed in ref. 2). Second, ligands bound to their soluble receptors may exhibit prolonged plasma half lives and thereby act as a depot for the free ligand. Such a biological activity has recently been ascribed to IL-4 (reviewed in ref. 2). Third, soluble cytokine receptors may also act as agonists (2). For example, soluble IL-6 receptor (sIL-6R) and soluble ciliary neurotrophic factor receptor bind by specific interactions to receptorless bystander cells, rendering the latter sensitive to the action of the respective cytokines (2, 3). An analogous phenomenon has been documented for the soluble lipopolysaccharide (LPS) receptor (sCD14), which binds to bystander cells and renders these susceptible to stimulation with LPS (4, 5).

Relatively little is known on the nature of the shedding proteases or their physiological inducers. One pathway for activation of these proteases involves activation of protein kinase C (PKC), since exposure of cells to phorbol ester causes shedding of many membrane proteins like TNF-α, the p60 TNF receptor, transforming growth factor α (TGF-α), and IL-6R from cells of monocytic, liver, or kidney origin (1, 2, 6–9). Shedding can, however, also be induced via two PKC-independent pathways, one of which is Ca^{2+} dependent (1). The membrane-bound shedding protease (or family of related proteases) has been characterized as a metalloproteinase that is inhibitable by the hydroxamic acid compound TAPI (TNF-α protease inhibitor), and to a lesser degree by conventional metalloproteinase inhibitors such as 1,10-phenanthroline (1, 8, 9). The specificity of the shedding protease has been studied by changing the cleavage site of several substrates including TNF-α, TGF-α, TNF-R, and IL-6R (1, 2, 10, 11). These studies indicated that there is no strict sequence specificity; deletion of a given cleavage site, however, led to complete abolishment of shedding of the respective protein (1, 2).

Investigations into the mechanisms by which endotoxin (LPS) and superantigens induce cytokine-overload reactions in the infected organism are legion. In contrast, the pathophysiological implications of cell damage provoked by proteinaceous cytotoxins have received relatively little attention. In the course of our studies on pore-forming bacterial proteins, we have found that these widespread toxins are endowed with a capacity to provoke a surprisingly wide spectrum of short- and long-range pathological reactions including the activation of PKC via a G-protein dependent pathway (12, 13).

In this study, we show that two prototypes of pore-forming toxins, streptolysin O (SLO) and *Escherichia coli* hemolysin (HlyA), induce rapid and massive shedding of CD14 and IL-6R, and that the cleaved sIL-6R is biologically active in transsignaling. The results uncover a novel mechanism by which pore-forming toxins can promote inflammatory processes.

MATERIALS AND METHODS

Chemicals. Staurosporin, phorbol 12-myristate 13-acetate (PMA), pepstatin A, phenylmethylsulfonylfluoride, Nonidet P-40, and 1,10-phenanthroline were obtained from Sigma. Aprotinin, leupeptin, Pefabloc [4-(2-aminoethylbenzenesulfonylfluoride)], and complete protease inhibitor Cocktail were from Boehringer Mannheim. Protein A Sepharose CL-4B was obtained from Pharmacia. Pansorbin was purchased from Calbiochem. Tran[^{35}S]-label (44 TBq/mmol) was obtained

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HlyA, *Escherichia coli* hemolysin; IL, interleukin; IL-6R, IL-6 receptor; LPS, lipopolysaccharide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; s, soluble; SLO, streptolysin O; TNF, tumor necrosis factor; TAPI, TNF-α protease inhibitor; TGF, transforming growth factor.

*I.W. and P.V. contributed equally to this work

§To whom reprint requests should be addressed at: I. Medical Clinic-Section Pathophysiology, Johannes Gutenberg University of Mainz, Obere Zahlbacher Strasse 63, D-55101 Mainz, Germany. e-mail: rose@mzdmza.zdv.uni-mainz.de.

from ICN. DMEM with glutamax, MEM, and penicillin/streptomycin were from GIBCO. FCS was from Seromed (Berlin). The novel metalloproteinase inhibitor TAPI (*N*-{D,L-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}L-3-(2'naphthyl)-alanyl-L-alanine,2-amino-ethylamide) was prepared at Immunex (8). The preparation of the polyclonal monospecific antiserum against IL-6R has been described (14). The generation of the expression vector pCDM8-huIL-6R and pCDM8-huIL-6R ΔAB which lacks the PMA-induced cleavage site was described (11). Soluble human IL-6R was expressed as described (15). SLO and HlyA were purified as described (16, 17). Granulocyte/macrophage colony-stimulating factor and interferon-γ were from Essex Pharma (Munich) and Bioferon (Laupheim, Germany), respectively.

Monocyte Isolation and Treatment. Monocytes were isolated from citrated buffy coats, which were kindly provided by the Blood Transfusion Center of the University of Mainz. Monocytes were isolated as described (18). Monocyte cell suspensions regularly contained 70–90% monocytes as determined by flow cytometry. They were adjusted with MEM to densities of 1.5×10^6 cells/ml, and 1 ml aliquots of cell suspensions were applied per well to 24-well cell culture plates (Nunc). After 1 h incubation of cells at 37°C/5% CO_2, the plates were washed twice with Hanks' balanced salt solution. Experiments were performed both with freshly isolated monocytes and with macrophages, which were obtained by culturing the cells in MEM supplemented with 10% human serum from blood group AB donors and 2.5 ng of granulocyte/macrophage colony-stimulating factor per ml and 0.5 ng of interferon-γ per ml for 5 days. The cells were treated with HlyA (0.01–10 μg/ml) or SLO (1–5 μg/ml). At the times given in the figure legends, the medium was then removed and centrifuged for 5 min at 13,000 × *g*.

Quantification of sIL-6R and sCD-14. Quantification of sIL-6R and sCD14 in cell supernatants was undertaken following the instructions supplied by the manufacturers of the ELISA kits. The sIL-6R ELISA was supplied by Laboserv Diagnostica (Giessen, Germany). The sCD14 ELISA was obtained from Medgenix Diagnostics (Ratingen, Germany). Quantification of cellular IL-6R and CD14 was undertaken by solubilizing the cells in 20 mM Nonidet P-40 in the presence of a commercial protease inhibitor cocktail (Complete; Boehringer Mannheim) and analysis of the detergent extracts by ELISA.

Cell Culture and Transient Transfection of COS-7 Cells. COS-7 cells and HepG2-IL-6 cells (15) were grown in DMEM supplemented with 10% FCS, penicillin (60 mg/liter) and streptomycin (100 mg/liter) at 5% CO_2 and 37°C in a water-saturated atmosphere. Transient transfections of COS-7 cells were performed by using electroporation (Gene Pulser; Bio-Rad). Confluent growing cells (50–70%) were trypsinized, resuspended in 1 ml DMEM, pulsed (960 μF; 240 V; distance of electrodes, 4 mm) and further grown for 1–2 days.

Metabolic Labeling and Immunoprecipitation. Transiently transfected COS-7 cells were metabolically labeled for 2 h with 50 μ Ci (1 Ci = 37 GBq) of [^{35}S]cysteine/methionine per ml in cysteine/methionine-free MEM. Cells were chased in DMEM containing cysteine/methionine for 1 h. Culture supernatants were harvested and supplemented with 0.3% SDS/1% Nonidet P-40. Cells were lysed in buffer A (10 mM Tris·HCl, pH 7.4/0.4% Na-deoxycholate/60 mM EDTA/0.3% SDS/1% Nonidet P-40) containing protease inhibitors (2 g of aprotinin per ml/200 μg of EDTA per ml/0.5 μg of leupeptin per ml/0.7 μg of pepstatin per ml/0.2 mM phenylmethylsulfonylfluoride). Cell lysates and supernatants were pretreated with Pansorbin and subsequently incubated with the appropriate antisera for 1 h at 4°C. Immune complexes were precipitated with protein A-Sepharose and separated on 12.5% SDS/polyacrylamide gels. After intensifying fluorography with sodium salicylate, the gels were dried and exposed to x-ray film.

Northern Blot Analysis. RNA was prepared by using RNeasy (Quiagen, Hilden, Germany). Northern blotting was carried out as described (19). Nylon membranes (GeneScreen-Plus; NEN) were hybridized with a 0.9 kb *Hin*fI fragment of haptoglobin cDNA labeled by random priming (20).

RESULTS

PMA, SLO, and HlyA Induce Shedding of the IL-6R. As shown in Fig. 1*A*, treatment of human macrophages for 60 min with PMA, SLO, and HlyA led to the release of sIL-6R. To approximate the extent of shedding, detergent extracts of cells were applied to the ELISA. As shown in Fig. 1*B*, the relative cellular content of IL-6R was reduced by ≈35% and 50% after PMA and HlyA treatment, respectively. Similar results for HlyA were obtained with monocytes, but PMA-induced shedding in these cells was usually lower than in macrophages. The induction of shedding by HlyA (Fig. 2*A*) and SLO (data not shown) was dose-dependent; 0.3 unit of HlyA per ml (corresponding to 30 ng/ml) led to half maximal release of sIL-6R. Shedding of the IL-6R from macrophages was rapid and almost complete after 10 min. Shedding thus displayed more rapid kinetics than observed with PMA, where shedding

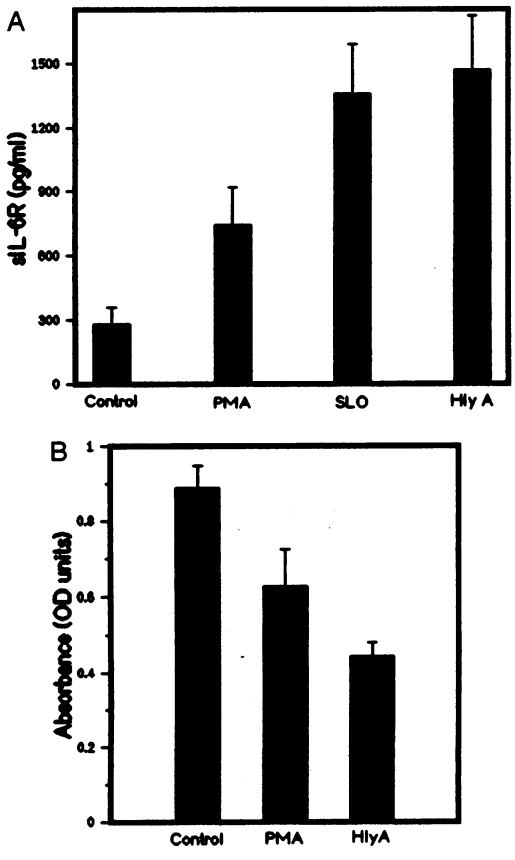

FIG. 1. (*A*) Shedding of human IL-6R induced by PMA, SLO, or HlyA. Macrophages (10^6) were treated with 10^{-7} M PMA, 1 μg of SLO per ml, or 0.1 μg of HlyA per ml for 60 min. sIL-6R concentrations in the supernatants of untreated (control) or stimulated cells were determined by ELISA ($n = 3 \pm$ SD). (*B*) ELISA determinations of cell-bound IL-6R present in detergent extracts. Human macrophages were incubated in buffer (control) or treated with 10^{-7} M PMA or 0.1 μg of HlyA per ml for 60 min. The supernatants were removed and the cells were then lysed with Nonidet P-40 in the presence of protease inhibitors. The detergent extracts were applied to the IL-6R ELISA, and reductions in cellular IL-6R of ≈30–50% were noted after PMA and HlyA treatment, respectively ($n = 3 \pm$ SD).

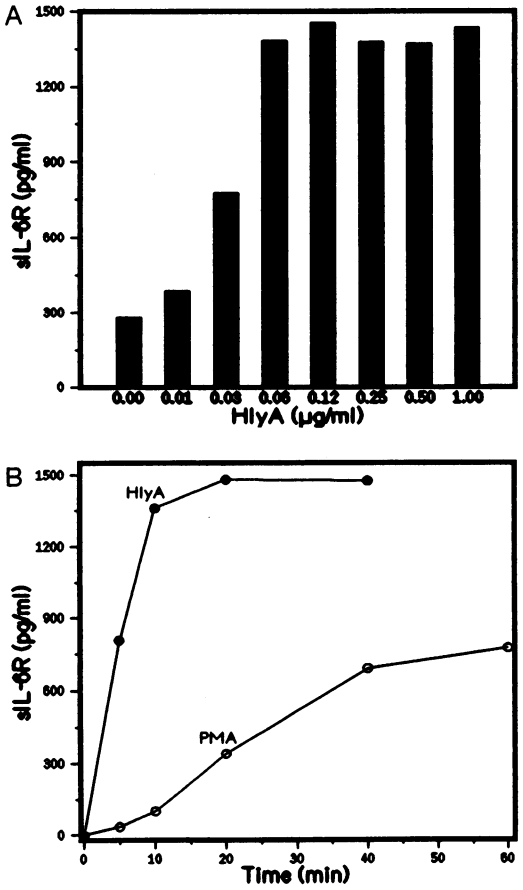

FIG. 2. (*A*) Dose-response curve of sIL-6R generation. Macrophages (10^6) were treated with increasing amounts of HlyA. sIL-6R concentrations in the supernatants were measured 60 min after toxin treatment by ELISA. (*B*) Time-dependency of sIL-6R generation. Macrophages (10^6) were treated with 0.1 μg of HlyA per ml. Supernatants were analyzed by sIL-6R ELISA at the given times. Note the more rapid release of sIL-6R induced by HlyA compared with 10^{-7} PMA. Like HlyA, SLO also induced very rapid shedding of sIL-6R (not shown).

required 40–60 min to reach completion (refs. 21 and 22; Fig. 2*B*). To examine the properties of the protease involved in toxin-induced shedding of IL-6R, inhibition experiments were conducted using the inhibitor of PKC, staurosporin, which has been shown to abolish PMA-induced shedding of the IL-6R (22), and with various protease inhibitors. It was found that the following inhibitors did not suppress shedding: *N*-ethylmaleimide (1 mM), Pefabloc (3 mM), aprotinin (10 μg/ml), pepstatin (10 μg/ml), and EDTA (2 mM; tested only in experiments with SLO since HlyA function is calcium-dependent) (23). The metalloproteinase inhibitor 1,10-phenanthroline was the only agent among conventional inhibitors that weakly suppressed shedding. Fifty percent inhibition was observed at 3 mM 1,10-phenanthroline, a concentration that far surpasses that normally used in inhibition experiments (1 mM) (24). In contrast, TAPI, the novel inhibitor of the TNF-α shedding proteinase, completely inhibited shedding of IL-6R at a concentration of 300 μM (Fig. 3). Staurosporin failed to inhibit toxin-induced shedding of the IL-6R, whereas induction of shedding by PMA was completely blocked.

Cleavage of IL-6R Induced by PMA or Bacterial Cytolysins Occurs at Different Sites. COS-7 cells transfected with the transmembrane form of the human IL-6R were metabolically labeled by using [^{35}S]methionine/cysteine. As shown in Fig. 4, treatment of the cells with PMA or bacterial toxins led to the loss of this 80-kDa IL-6R from the cells and to the appearance

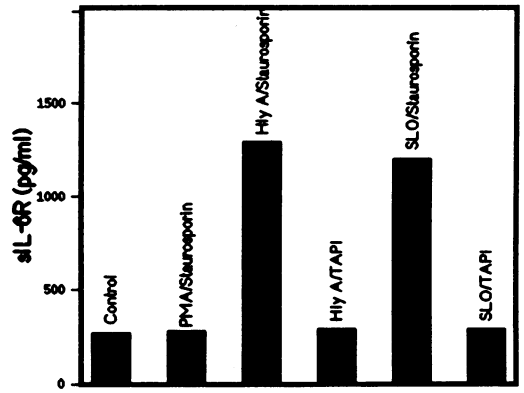

FIG. 3. Inhibition of shedding of IL-6R by TAPI and staurosporin. Macrophages (10^6) were treated with PMA, HlyA, or SLO in the presence of 1 μg of staurosporin per ml or 300 μM TAPI. Supernatants of untreated (control) and treated cells were analyzed by sIL-6R ELISA.

of the soluble 55-kDa IL-6R in the medium. No significant size difference in the sIL-6R proteins released from the cells after treatment with PMA, SLO, or HlyA was detected. We have previously shown that the IL-6R is cleaved between Gln-357 and Asp-358 close to the putative transmembrane domain (11). As expected, cells transfected with the mutant IL-6R ΔA,B lacking 10 amino acid residues around this cleavage site showed no shedding after stimulation with PMA (Fig. 5). However, when the same cells were treated with SLO or HlyA, shedding occurred to a comparable extent as with the unaltered IL-6R (Fig. 5).

According to the ELISA measurements, 30–50% of the cell-associated IL-6R was released as a soluble protein,

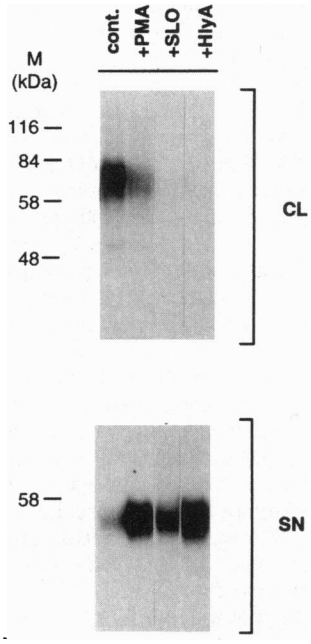

FIG. 4. Shedding of IL-6R by PMA, SLO, and HlyA. COS-7 cells (2×10^6) were transiently transfected with pCDM8-huIL-6R and seeded on to 35-mm plates. One to 2 days after transfection, cells were labeled for 2 h with 50 Ci tran[^{35}S]-label in methionine/cysteine-free medium. Cells were chased in complete medium for 1 h and further incubated for 1 h with 10^{-7} M PMA or for 10 min with 10 μg of SLO per ml or 1 μg of HlyA per ml. Cell lysates (CL) and supernatants (SN) of untreated (control) and stimulated cells were immunoprecipitated and analyzed by SDS/PAGE and fluorography. Treatment of cells with PMA or either bacterial cytolysin caused shedding of sIL-6R (molecular mass, 55 kDa) into the supernatant.

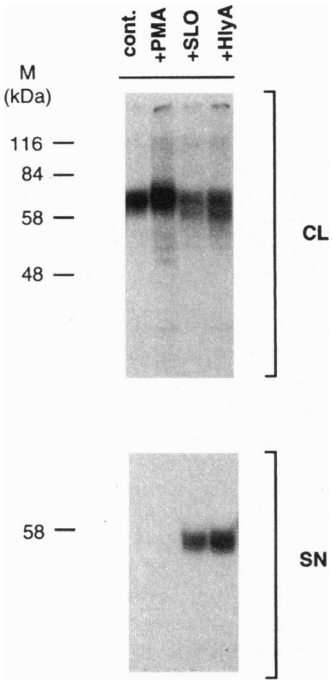

FIG. 5. Shedding of the human IL-6R lacking the PMA-dependent cleavage site. COS-7 cells (2×10^6) were transiently transfected with pCDM8-huIL-6R-A,B. Labeled cells were chased for 1 h and incubated with PMA, SLO, or HlyA as indicated in Fig. 4. Cell lysates (CL) and supernatants (SN) were immunoprecipitated and analyzed by SDS/PAGE and fluorography. PMA failed to induce shedding of the IL-6R mutant lacking the PMA-specific cleavage site, but shedding occurred on cells treated with both bacterial toxins.

whereas the pulse-chase experiments showed 50–100% shedding of the IL-6R. It should be considered that the ELISA procedure measures total IL-6R in the cell lysate and supernatant, whereas immunoprecipitation after pulse-chase labeling only follows the fate of the IL-6R synthesized during the pulse period. This difference probably accounts for the apparent discrepancy between the results of ELISA measurements and immunoprecipitation.

In any event, our data demonstrate that toxin-induced cleavage of the IL-6R is a posttranslational event, and that cleavage occurs at a shedding site distinct from that identified following phorbol ester stimulation.

Released sIL-6R Is Biologically Active. To test whether the sIL-6R released by the cells treated with PMA, SLO, or HlyA was biologically active, we employed the cell line HepG2-IL-6 which does not express the IL-6R at the cell surface (15). These human hepatoma cells secrete large amounts of human IL-6, and induction of acute phase proteins like haptoglobin or α_1 antichymotrypsin is solely dependent on the presence of soluble human IL-6R (15). As shown in Fig. 6, incubation of HepG2-IL-6 cells with supernatants from IL-6R transfected COS-7 cells that had been treated with PMA, SLO, or HlyA led to stimulation of synthesis of human haptoglobin mRNA. This demonstrated that the sIL-6R released from cells after stimulation with PMA, SLO, or HlyA was biologically active.

SLO and HlyA Trigger the Release of Soluble CD14. Shedding of CD14 is probably of central importance for the induction of systemic inflammatory responses to LPS (4). As shown in Fig. 7*A*, treatment of human monocytes with SLO or HlyA also led to release of sCD14. As in the case of IL-6R, the toxin-induced shedding of CD14 was not affected by the addition of staurosporin, but could be completely inhibited by the addition of TAPI (Fig. 7*B*). With regard to other protease inhibitors, the same results were obtained as described above for IL-6R. Analysis of cell extracts indicated a reduction in

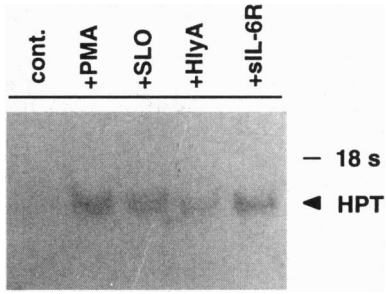

FIG. 6. Biologic activity of released sIL-6R. The biological activity of the released sIL-6R was determined by measuring the induction of synthesis of haptoglobin (HPT) in HepG2-IL6 cells. HepG2-IL-6 cells were stimulated for 18 h with supernatants from control COS-7-IL-6R cells or with supernatants of COS-7-IL-6R cells that had been treated with with 10^{-7} M PMA, 1 μg of SLO per ml, 0.1 μg of HlyA per ml. As a positive control, HepG2-IL-6 cells were incubated with 10 ng of sIL6-R. The expression of haptoglobin (HPT) was determined by Northern blot analysis.

CD14 content of ≈30% following toxin-induced shedding (Fig. 7*C*).

Although CD14 is a glycosylphosphadidylinositol-anchored protein, it has been established that the soluble form of CD14 is generated by proteolysis (4). Our observation that the inhibitor of metalloproteinase TAPI completely inhibited shedding of CD14 was entirely in line with this finding. From the presented data it appears that SLO and HlyA trigger cleavage of CD14 and IL-6R by similar or identical mechanisms.

Shedding of IL-6R and CD14 Is Dependent on Pore-Forming Activity of SLO. It was of interest to determine whether shedding of IL-6R and CD14 was dependent on the pore-forming activity of SLO. A mutant toxin containing a single amino acid substitution has been constructed that fully retains cell-binding activity but lacks pore-forming activity due to faulty polymerization (M.P. and S.B., unpublished data). It was found that the mutant SLO toxin applied at concentrations up to 50 μg/ml entirely failed to induce shedding of IL-6R or CD14. Hence, shedding was dependent on the pore-forming function (data not shown).

DISCUSSION

Mechanism of Shedding Induced by Bacterial Cytolysins. As is the case with other shedding stimuli, the mechanisms underlying the cleavage of IL-6R and CD14 induced by pore-forming toxins await elucidation. Toxin-induced shedding displayed a number of characteristic features. First, it was not inhibitable by staurosporin and hence did not involve PKC. Second, as shown with the bindable but noncytotoxic SLO mutant, shedding was dependent on transmembrane pore formation and was Ca^{2+}-independent. The kinetics of shedding were more rapid than those observed with other stimuli, and they closely followed the kinetics of pore formation. Third, the cleavage site used for shedding of the IL-6R after stimulation with PMA was obviously different from the one used after stimulation with PMA, because an IL-6R mutant lacking the PMA-dependent cleavage site was still shed from toxin-treated cells. The monospecific antibody employed for immunoprecipitation of the IL-6R recognizes an epitope 21 amino acids upstream of the PMA specific cleavage site (14). Interestingly, shedding of the IL-6R is not prevented by this antibody (J. Müllberg and S.R.-J., unpublished data). The sIL-6R released after treatment with SLO or HlyA was recognized by this antibody, so the alternative cleavage site must lie within the stretch of 21 amino acid residues. Since the IL-6R ΔA,B mutant, which lacks 5 amino acids upstream of the cleavage site (Fig. 5), and even a mutant that lacks further 4

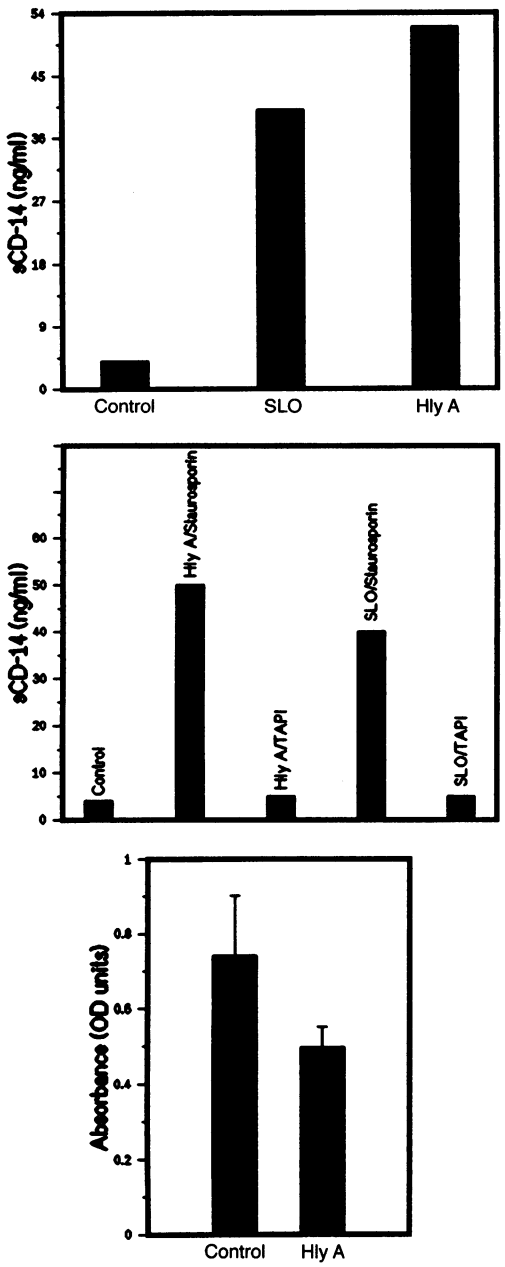

FIG. 7. (*A*) Generation of sCD14 from monocytes by SLO or HlyA. Monocytic cells (1.5×10^6) were treated with 1 μg of SLO per ml or 0.1 μg of HlyA per ml for 60 min. Supernatants of untreated (control) and treated cells were measured for sCD14 by ELISA. Results are mean values from three independent experiments. (*B*) Inhibition of generation of sCD14 by TAPI and staurosporin. Monocytic cells (1.5×10^6) were treated with HlyA or SLO in the presence of 1 μg staurosporin per ml or 300 μM TAPI. Supernatants were analyzed by sCD14 ELISA. Results are the mean values from two experiments. (*C*) Reduction in content of cellular CD14 in monocytic cells after treatment with HlyA. The same protocol was used as in *A*. Supernatants were discarded and the cells were solubilized in 20 mM Nonidet P-40. ELISA measurements of CD14 in the detergent extracts indicated ≈30% reduction in cellular CD14 content after toxin treatment ($n = 3 \pm$ SD).

amino acids (11) (not shown) were still cleaved after exposure of the cells to the cytolysins, the alternative cleavage site can be narrowed down to the 12 amino acids 337-Asn-Lys-Asp-Asp-Asp-Asn-Ile-Leu-Phe-Arg-Asp-Ser-348.

The nature of the protease responsible for shedding in toxin-treated cells is unknown. Using a universal chromogenic substrate or a specific substrate harboring the cleavage region of IL-6R, we were unable to detect protease activity in supernatants of toxin-treated cells (data not shown). Therefore, shedding was apparently not due to release or secretion of an intracellular protease. By exclusion, it seems reasonable to assume that a membrane-associated metalloproteinase was responsible for shedding. This assumption is compatible with the finding that toxin-dependent shedding of both IL-6R and CD14 was inhibitable by TAPI, the recently described specific inhibitor of the intrinsic shedding metalloproteinase (11, 21, 22), but not by other conventional protease inhibitor except 1,10-phenanthroline when the latter was applied at very high concentrations.

Taking the above findings into account, we propose that shedding of IL-6R and CD14 is due to manifestation of activity of membrane-located metalloproteinases. Factors responsible for transition of the protease from a latent or inactive to an active state are unknown. A possibility we are considering is that activity becomes manifest when the protease gains contact with its substrate. This can be triggered by diverse stimuli, whereby the unifying event may be the perturbation of vertical or lateral membrane organization. Pore-forming toxins force lipids and integral membrane proteins aside and cause rapid loss of membrane lipid asymmetry (23). It is easy to envisage that both processes may cause the protease to come into contact with its substrates. The fact that the kinetics of shedding are rapid and closely follow membrane permeabilization would be compatible with this concept. It is not known whether activation of PKC is accompanied by similar perturbation of membrane structure; however, activated PKC is translocated from the cytosol and enters into tight association with the lipid bilayer (25). In this regard there is some resemblance to membrane-inserting cytolysins. However, it cannot be excluded that phosphorylation of membrane-associated proteins by PKC leads to the activation of the shedding protease.

That shedding induced by PMA and by bacterial toxins leads to different cleavage events could basically be due to two facts. First, different but related proteases may exist. Alternatively, depending on the mode of membrane perturbation, a single protease may gain access to slightly different regions within a cleavage domain of a membrane protein. It may be remarked that shedding at different sites within the same molecule has been documented for the Alzheimer protein where cleavage at two sites leads to amyloidogenic and nonamyloidogenic pathways (26).

Implications of Shedding of IL-6R and CD14 Induced by Pore-Forming Toxins. The generation of soluble forms of IL-6R and CD14 has far reaching physiological consequences. Both soluble proteins bind their ligands and elicit a specific signal on cells that would otherwise be completely unresponsive. This phenomenon, recently designated transsignaling (2), is characteristic of the IL-6 type cytokine family. Attack by pore-forming bacterial toxins on cells expressing IL-6R or CD14 will therefore dramatically widen the spectrum of responsive cells and thereby provoke a spectrum of long-range effects within the host organism. IL-6R and CD14 probably represent only two of many other biologically relevant moieties whose rapid shedding is induced by pore-forming toxins. We consider the extent of shedding to be massive, equalling or even surpassing that induced by PMA. Rough estimates obtained by ELISA determinations of cell extracts indicated loss of 30–50% of the respective molecules from toxin-treated cells. The consequences of shedding of biologically active proteins can obviously be multifaceted. Membrane-bound growth factors are activated and systemically available after cleavage (27). Cytokine receptors which do not belong to the IL-6 type family of cytokines show antagonistic activity since they compete with the membrane associated counterparts for binding of the ligand. Soluble adhesion proteins have been shown to act as antagonists of cell–cell contact which is

believed to be pivotal for the function of the immune system (28, 29). Altogether, the demonstration that pore-forming toxins induce rapid shedding of biologically important molecules in functionally active form thus has widespread implications regarding their roles in the pathogenesis of local and systemic infections. It can be anticipated that the sheddding phenomenon described herein occurs as a consequence of attack of pore-forming proteins of the immune system, complement (30) and perforin (31) on target cells.

We thank Dr. R. Black and Dr. K. Mohler (Immunex, Seattle, USA) for the gift of TAPI and Dr. J. Müllberg (Immunex, Seattle, USA) for helpful discussions and support. We thank S. Strauch and N. Voltz for able technical assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Bonn, Germany (SFB 311-D09; Ro632/6-1), the NMFZ, Mainz, Germany and the Verband der Chemischen Industrie, Frankfurt/Main, Germany.

1. Massagué, J. & Pandiella, A. (1993) *Annu. Rev. Biochem.* **62,** 515–541.
2. Rose-John, S. & Heinrich, P. C. (1994) *Biochem. J.* **300,** 281–290.
3. Sui, X., Tsuji, K., Tanaka, R., Tajima, S., Muraoka, K., Ebihara, Y., Ikebuchi, K., Yasukawa, K., Taga, T., Kishimoto, T. & Nakahata, T. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2859–2863.
4. Bazil, V. (1995) *Immunol. Today* **16,** 135–140.
5. Loppnow, H., Stelter, F., Schonbeck, U., Schluter, C., Ernst, M., Schutt, C. & Flad, H. D. (1995) *Infect. Immun.* **63,** 1020–1026.
6. Ehlers, M. R. & Riordan, J. F. (1991) *Biochemistry* **30,** 10065–10074.
7. Arribas, J. & Massagué, J. (1995) *J. Cell Biol.* **128,** 433–441.
8. Mohler, K. M., Sleath, P. R., Fitzner, J. N., Cerretti, D. P., Alderson, M., Kerwar, S. S., Torrance, D. S., Otten-Evans, C., Greenstreet, T., Weerawarna, K., Kronheim, S. R., Petersen, M., Gerhart, M., Kozlosky, C. J., March, C. J. & Black, R. A. (1994) *Nature (London)* **370,** 218–220.
9. Crowe, P. D., Walter, B. N., Mohler, K. M., Otten-Evans, C., Black, R. A. & Ware, C. F. (1995) *J. Exp. Med.* **181,** 1205–1210.
10. Brakebusch, C., Varfolomeev, E. E., Batkin, M. & Wallach, D. (1994) *J. Biol. Chem.* **269,** 32488–32496.
11. Müllberg, J., Oberthur, W., Lottspeich, F., Mehl, E., Dittrich, E., Graeve, L., Heinrich, P. C. & Rose-John, S. (1994) *J. Immunol.* **152,** 4958–4968.
12. Grimminger, F., Sibelius, U., Bhakdi, S., Suttorp, N. & Seeger, W. (1991) *J. Clin. Invest.* **88,** 1531–1539.
13. Bhakdi, S., Grimminger, F., Suttorp, N., Walmrath, D. & Seeger, W. (1994) *Med. Microbiol. Immunol.* **183,** 119–144.
14. Stoyan, T., Michaelis, U., Schooltink, H., Van Dam, M., Rudolph, R., Heinrich, P. C. & Rose-John, S. (1993) *Eur. J. Biochem.* **216,** 239–245.
15. Mackiewicz, A., Schooltink, H., Heinrich, P. C. & Rose-John, S. (1992) *J. Immunol.* **149,** 2021–2027.
16. Bhakdi, S. & Martin, E. (1991) *Infect. Immun.* **59,** 2955–2962.
17. Bhakdi, S., Roth, M., Sziegoleit, A. & Tranum-Jensen, J. (1984) *Infect. Immun.* **46,** 394–400.
18. Denholm, E. M. & Wolber, F. M., (1991) *J. Immunol. Methods* **144,** 247–251.
19. Rose-John, S., Rincke, G. & Marks, F. (1987) *Biochem. Biophys. Res. Commun.* **147,** 219–225.
20. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
21. Müllberg, J., Schooltink, H., Stoyan, T., Günther, M., Graeve, L., Buse, G., Mackiewicz, A., Heinrich, P. C. & Rose-John, S. (1993) *Eur. J. Immunol.* **23,** 473–480.
22. Müllberg, J., Schooltink, H., Stoyan, T., Heinrich, P. C. & Rose-John, S. (1992) *Biochem. Biophys. Res. Commun.* **189,** 794–800.
23. Bhakdi, S. & Tranum-Jensen, J. (1987) *Rev. Physiol. Biochem. Pharmacol.* **107,** 147–223.
24. Barrett, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues*, ed. Barret, A. J. (Elsevier, Amsterdam), pp. 1–55.
25. Nishizuka, Y. (1988) *Nature (London)* **334,** 661–665.
26. Haass, C. & Selkoe, D. J. (1993) *Cell* **75,** 1039–1042.
27. Dinarello, C. A. & Margolis, N. H. (1995) *Curr. Biol.* **5,** 587–590.
28. Springer, T. A. (1994) *Cell* **76,** 301–304.
29. Seth, R., Raymond, F. D. & Makgoba, M. W. (1991) *Lancet* **338,** 83–84.
30. Müller-Eberhard, H. J. (1988) *Annu. Rev. Biochem.* **57,** 321–347.
31. Liu, C. C., Persechini, P. M. & Young, J. D. (1995) *Immunol. Rev.* **146,** 145–175.