Selective Killing of Human Monocytes and Cytokine Release Provoked by Sphingomyelinase (Beta-Toxin) of *Staphylococcus aureus*

IWAN WALEV,^{1*} ULRICH WELLER,¹ SUSANNE STRAUCH,¹ TIMOTHY FOSTER,²
AND SUCHARIT BHAKDI¹

Institute of Medical Microbiology and Hygiene, University of Mainz, Mainz, Germany,¹ and Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin, Ireland²

Received 9 February 1996/Returned for modification 11 April 1996/Accepted 17 May 1996

The best-known activity of *Staphylococcus aureus* sphingomyelinase C, alias beta-toxin, is as a hemolysin that provokes hot-cold lysis of erythrocytes which contain substantial amounts of sphingomyelin in the plasma membrane. Sheep erythrocytes are most susceptible, and we found that one hemolytic unit, representing the toxin concentration that elicits 50% hemolysis of 2.5×10^8 erythrocytes per ml, corresponds to 0.05 enzyme units or to approximately 0.25 μg of sphingomyelinase per ml. The cytotoxic action of beta-toxin on nucleated cells has not been described in any detail before, and the present investigation was undertaken to fill this information gap. We now identify beta-toxin as a remarkably potent monocytocidal agent. At a concentration of 0.001 U/ml, corresponding to approximately 5 ng/ml, beta-toxin killed over 50% of human monocytes (10^6 cells per ml) within 60 min. By contrast, 1 to 5 μg of beta-toxin per ml had no cytotoxic effects on human granulocytes, fibroblasts, lymphocytes, or erythrocytes. A selective monocytocidal action was also observed with sphingomyelinase C from *Bacillus cereus* and a *Streptomyces* sp., whereas phospholipase A2 and phospholipase D at 100 U/ml were without effect. Monocytes succumbing to the action of beta-toxin processed and released interleukin-1 β , soluble interleukin-6 receptor, and soluble CD14 into the supernatant. Thus, monocyte killing by beta-toxin is associated with cytokine-related events that are important for the initiation and progression of infectious disease. These findings uncover a potentially important role for sphingomyelinase as a determinant of microbial pathogenicity.

Staphylococcal beta-toxin (beta-hemolysin, sphingomyelinase C) has been identified as an enzyme that specifically cleaves sphingomyelin (12, 13). Evidence that its enzymatic activity was responsible for cytotoxicity followed when it was shown that the susceptibility of erythrocytes from different species correlated with their content of sphingomyelin. Sheep erythrocytes, which contain the largest amounts of sphingomyelin (51% of total lipids), are most vulnerable, whereas dog and guinea pig erythrocytes that contain only 11% sphingomyelin are virtually resistant (35). Characteristically, hemolysis does not rapidly follow sphingomyelin degradation but is precipitated upon exposure of attacked cells to low temperature. One hypothesis proposes that “hot-cold” hemolysis is due to formation of fragile patches of sphingomyelin-depleted monolayers that become destabilized in the cold (35).

The predicted sequence of the *Staphylococcus aureus* beta-toxin is similar to that of the *Bacillus cereus* sphingomyelinase (17, 29). Overall, little is known about the action of sphingomyelinases on cells other than erythrocytes. The enzyme degrades sphingomyelin efficiently in the membrane in fibroblasts without affecting viability (9, 33). Regarding the significance of beta-toxin as a virulence factor, there is epidemiological evidence that toxin production is a frequent trait of *S. aureus* strains causing severe chronic skin infections despite its overall low occurrence in clinical isolates (16). Bovine mastitis isolates are also frequently beta-toxigenic (23). The significance of beta-toxin in intramammary infection was directly demon-

strated by comparing a non-beta-toxin-producing mutant with the parental strain in mouse mammary gland infection (8).

A search in the literature revealed the paucity of information that might explain the in vivo effect of the toxin. Only two leads were found. First, Wilkinson in 1975 reported that beta-toxin selectively inhibited locomotion of human monocytes but not of granulocytes (39), a finding that was essentially confirmed by Russell et al. (32). Second, Wadström and Mollby reported in 1972 that platelets were also susceptible to beta-toxin (36).

We are currently studying the effects of bacterial exotoxins on cells of the immune system and have reported on the monocytocidal and interleukin-1 (IL-1)-releasing action of *Escherichia coli* hemolysin and *S. aureus* alpha-toxin (5, 6, 38). In this article we describe a remarkable, selective cytotoxic activity of beta-toxin on monocytes and show that the dying cells release biologically important molecules including IL-1 β and the receptors for IL-6 and lipopolysaccharide (LPS).

MATERIALS AND METHODS

Materials. Sphingomyelinase from *S. aureus*, from a *Streptomyces* sp., and from *B. cereus* were from Sigma (Deisenhofen, Germany). K^+ and Na^+ buffers were prepared as described elsewhere (38). The novel metalloprotease inhibitor TAPI (*N*-[D,L-[hydroxyaminocarbonyl)methyl]-4-methylpentanoyl]-L-3-[2'-naphtyl-L-alanine,2-aminoethyl amide] was prepared at Immunex Corp., Seattle, Wash. (22, 24). *N*-Acetyl-D-sphingosine (acetyl-ceramide), phospholipase A₂ from bee venom, phospholipase D from peanut, and LPS from *E. coli* serotype O26:BH6 were from Sigma.

Hemolytic activity of *S. aureus* sphingomyelinase. The commercial sphingomyelinase preparation from *S. aureus* was tested against erythrocytes of different species. As expected, human erythrocytes were not lysed by 1 U of beta-toxin per ml (5 $\mu\text{g}/\text{ml}$); however, the toxin provoked hot-cold hemolysis of sheep erythrocytes. The specific hemolytic activity determined with suspensions of 2.5×10^8 cells per ml was approximately 4,000 hemolytic units per mg; i.e., application of 0.25 $\mu\text{g}/\text{ml}$ evoked 50% lysis of the erythrocyte suspension. As shown below, the

* Corresponding author. Mailing address: Institute of Medical Microbiology and Hygiene, Hochhaus am Augustusplatz, D-55101 Mainz, Germany. Phone: (6131) 17-7341. Fax: (+6131) 39-2359.

commercial toxin preparation contained contaminants, so the true specific hemolytic activity of beta-toxin is higher. When the hemolytic activity was set in relation to enzyme activity, it was found that 1 U of enzyme activity corresponded to 20 hemolytic units. In the rest of the article, beta-toxin concentrations will generally be given in enzyme units (U) per milliliter. Unless otherwise stated, the commercial toxin preparation was used without further purification.

Cells. Human monocytes were isolated from citrated buffy coats which were kindly provided by the Blood Transfusion Center of the University of Mainz (Mainz, Germany). Monocytes were isolated as described elsewhere (11). Monocyte cell suspensions regularly contained 70 to 90% monocytes as determined by flow cytometry. They were adjusted with minimal Eagle's medium to densities of 1.5×10^6 cells per ml, and 1-ml aliquots of cell suspensions were applied per well to 24-well cell culture plates (Nunc AS, Roskilde, Denmark). After 1-h incubation of cells at 37°C at 5% CO₂, the cells were washed twice with Hanks balanced salt solution. The cells were then treated with sphingomyelinases or bacterial supernatants for 60 min at 37°C.

Human fibroblasts and T lymphocytes were isolated as described elsewhere (18, 37). Human granulocytes were isolated from heparinized blood essentially as described previously (3) except that erythrocytes were sedimented by addition of 1 volume of 5% dextran 200 (Serva, Heidelberg, Germany; prepared in saline) instead of gum arabic.

Bacterial strains. Strain 8325-4 (26) expressed both alpha-toxin and beta-toxin. It was derived from 8325 by curing three prophages (26). Strain DU1090 is a non-alpha-toxin-producing mutant of 8325-4 (*hla::Em'*) isolated by allelic replacement (28). DU5720 was derived from DU1090 by lysogenization with the converting bacteriophage 42E (8), which integrates with the *hly* gene (10) and thus fails to produce beta-toxin.

Bacterial strains were grown in Todd-Hewitt broth at 37°C for 12 h, and the supernatants were obtained by centrifugation at $10,000 \times g$ for 10 min.

Measurements of intracellular ATP. Chemiluminescent measurements of cellular ATP were performed as previously described by using the firefly assay (5).

Dye exclusion test. After treatment with sphingomyelinase or with bacterial culture supernatants, trypan blue (0.5%) was added to the medium and stain uptake was assessed by light microscopy.

Determination of lactate dehydrogenase. Lactate dehydrogenase was measured as previously described (37).

Assay of sphingomyelinase. The assay system for measurement of sphingomyelinase activity was similar to the one described by Gatt et al. (14) except for the incubation buffer. Samples were assayed for the ability to hydrolyze the sphingomyelin analog trinitrophenyl-aminolauroylsphingomyelin (TNAPAL-sphingomyelin; Sigma). Ten microliters of each sample was mixed with 190 μ l of incubation buffer containing 250 mM Tris-HCl, 10 mg of MgCl₂, 1% Triton X-100, and 0.02 mg of TNAPAL-sphingomyelin (pH 7.4). The reaction mixtures were slowly shaken at 37°C for 2 h. Reactions were stopped and extracted as described elsewhere (14), and absorbance of the trinitrophenyl-amino residue released into the organic phase was read spectrophotometrically at 330 nm.

Activation of monocytes. Monocytes were activated with LPS as described elsewhere (38).

Quantification of IL-1 β , IL-6R, and sCD14. After treatment, the cell supernatants were removed and centrifuged for 5 min at $13,000 \times g$. Quantification of IL-1 β , soluble IL-6 receptor (sIL-6R), and sCD14 in supernatants was undertaken by following the instructions supplied by the manufacturers of the enzyme-linked immunosorbent assay (ELISA) kits. The IL-1 β ELISA and sCD14 ELISA were obtained from Medgenix Diagnostics, Ratingen, Germany. The sIL-6R ELISA was supplied by Laboserv Diagnostica, Giessen, Germany.

Final purification of sphingomyelinase. Sphingomyelinase from *S. aureus* (solution in 50% glycerol containing 0.25 M phosphate buffer, pH 7.5) was further purified by ion-exchange chromatography on hydroxyapatite. The original buffer was changed to buffer A (100 mM NaCl, 20 mM Tris-HCl [pH 7.5]) by gel filtration on a Fast Desalting Column (Pharmacia, Freiburg, Germany) fitted to a fast protein liquid chromatography-system (FPLC; Pharmacia), 0.5-ml aliquots per run. Protein-containing peaks were pooled and applied to a 1-ml-column (HR 5/5; Pharmacia), filled with ceramic hydroxyapatite type I (Bio-Rad, Munich, Germany) equilibrated with the latter buffer. The column was washed with 3 ml of this buffer and eluted with a linear gradient (14 column volumes) to 50% buffer B (100 mM NaCl, 0.5 M NaPO₄ [pH 7.4]) at a flow rate of 2.5 ml/min; 0.5 ml samples were collected and assayed for purity by sodium dodecyl sulfate (SDS-PAGE) followed by silver staining.

RESULTS

Selective cytotoxicity of beta-toxin towards human monocytes. The toxin was first tested against human fibroblasts, T lymphocytes, and granulocytes. In these experiments, cells were either incubated for 2 h at 37°C with 1 U of sphingomyelinase per ml or, following a 60-min incubation at 37°C, they were placed on ice for another 60 min. In neither case was any ATP-depleting effect of sphingomyelinase observed, and the cells remained trypan blue negative. In marked contrast, when

FIG. 1. Rapid killing of monocytes by *S. aureus* beta-toxin. One million monocytes in 1 ml of Hanks balanced salt solution were treated with 0.1 U of sphingomyelinase from *S. aureus* at 37°C. At the indicated times, supernatants were removed and cells were analyzed either for trypan blue positivity or for relative cellular ATP content. The ATP chemiluminescence signals of untreated cells were expressed as percentages of the controls ($n = 3$). Bars, standard deviations.

monocytes were incubated with far-lower concentrations of beta-toxin, a rapid loss of cellular ATP with the concomitant appearance of trypan blue-staining cells was observed. Figure 1 depicts the kinetics of these processes when beta-toxin was applied at 0.1 U/ml. The very rapid kinetics of cellular killing were reminiscent of those observed with exotoxins that form transmembrane pores. Similar results were obtained with sphingomyelinase C from *B. cereus* and a *Streptomyces* sp. Although the cells became trypan blue positive, they did not release significant amounts of lactate dehydrogenase. Hence, cell death was not due to gross disruption of the bilayer.

Figure 2 depicts a dose-response curve, and it is apparent that remarkably low concentrations of beta-toxin were monocytoxic. The cells were found to be irreversibly damaged after exposure to 0.001 to 0.01 U of the enzyme per ml (5 to 50 ng/ml); they failed to replenish their ATP content and remained stainable with trypan blue following incubation in toxin-free medium for 3 to 16 h.

Two lines of evidence that the observed monocytoxic activity was truly attributable to beta-toxin were obtained. First, the commercial beta-toxin preparation was subjected to a final purification step by hydroxyapatite gel chromatography, and the monocytoxic activity was recovered in fractions displaying sphingomyelinase activity. The SDS-PAGE pattern of the commercial toxin sample is shown in Fig. 3, and three major protein bands of 30 to 40 kDa are visible. Hydroxyapatite chromatography led to clean separation of the middle band (M_r , ~35,000) from the other bands, and only the fractions containing this 35-kDa protein cleaved the synthetic sphingomyelinase substrate TNAPAL-sphingomyelin, displayed monocytoxic activity (Fig. 3), and provoked hot-cold hemolysis of sheep erythrocytes (not shown). The molecular mass of the protein (35 kDa) was in accord with published data for the purified enzyme (10, 20, 35). Thus, it was concluded that beta-

FIG. 2. Dose dependency of monocytocidal action of beta-toxin. One million monocytes in 1 ml of HBSS were treated with sphingomyelinase at the given concentrations for 60 min at 37°C. Thereafter, supernatants were aspirated and cells were stained with trypan blue ($n = 3$). Bars, standard deviations.

toxin constituted the major or even sole component of the biologically active fraction. Second, culture supernatants of isogenic *S. aureus* strains expressing either both alpha-toxin and beta-toxin ($\alpha^+ \beta^+$), beta-toxin only ($\alpha^- \beta^+$), or neither toxin ($\alpha^- \beta^-$) were tested for cytotoxic activity against human monocytes and granulocytes and for hemolytic activity against rabbit erythrocytes. The supernatants were diluted in phosphate-buffered saline, and titers eliciting 50% ATP depletion or 50% hemolysis were determined. The $\alpha^+ \beta^+$ supernatant had a high monocytotoxic activity paired with marked hemolytic activity (titers, 4,096 and 128, respectively), whereas the $\alpha^- \beta^+$ strain was not hemolytic but retained monocytotoxic activity (titer, 4,096). The $\alpha^- \beta^-$ strain was neither hemolytic nor monocytotoxic. Assays for sphingomyelinase revealed an enzyme activity of approximately 1 U/ml in the culture supernatants of β^+ strains. Thus, lysis of rabbit erythrocytes was, as expected, a function of alpha-toxin, but monocytotoxic activity was mainly attributable to another factor. Upon depletion of beta-toxin gene function, all monocytotoxic activity was lost. Granulocytes were not killed in any instance, indicating lack of significant amounts of leukocidin in the culture fluids.

Monocytes attacked by beta-toxin rapidly secrete IL-1 β . Secretion of IL-1 β is a two-step process. First, stimulated monocytes accumulate the 34-kDa pro-IL-1 β intracellularly. Thereafter, pro-IL-1 β is cleaved by the interleukin-converting enzyme and the biologically active, mature 17-kDa IL-1 β is secreted to the extracellular medium (34). We previously observed that monocytes under attack by membrane-damaging cytotoxins rapidly processed and secreted IL-1 β , apparently because of activation of the interleukin-converting enzyme (5, 6, 38). To investigate the effects of beta-toxin on the processing of IL-1 β , monocytes were briefly challenged with LPS (60 min) in order to stimulate synthesis of pro-IL-1 β . The cells were then treated with 0.1 U of beta-toxin per ml, and release of IL-1 β into the cell supernatants was measured. The presence or absence of cycloheximide during toxin treatment had no

FIG. 3. Final purification of *S. aureus* sphingomyelinase by hydroxyapatite chromatography. The SDS-PAGE pattern of the commercial sphingomyelinase preparation (lane S) is shown together with molecular weight standards (lane M) in the inset. Three major polypeptide bands of approximately 30 to 40 kDa were present. This material was subjected to hydroxyapatite chromatography. The SDS-PAGE patterns before (lane a) and after (lane b) equilibration in chromatography buffer and of fractions 12 to 34 are shown together with results (A_{330}) of assays for sphingomyelinase activity using a colorimetric synthetic substrate. Determinations of monocytocidal activity were performed on the same samples, and titers are expressed as the dilutions eliciting 50% ATP depletion in the cells. Note the clear separation of the middle band of approximately 35 kDa from contaminants, the identification of this band as sphingomyelinase, and the correlation between sphingomyelinase and monocytocidal activities.

effect on the results, and data obtained in an experiment with cycloheximide are shown in Fig. 4. A very rapid release of mature IL-1 β that reached completion within 40 min was observed.

In previous studies, we have shown that monocytes harboring the IL-1 β precursor rapidly process and release mature IL-1 β upon treatment with pore-forming toxins (5, 6, 38). This is due to activation of the interleukin-converting enzyme, which in some way appears to be under a regulatory influence of cellular K^+ . Thus, presence of high K^+ in the medium was found to inhibit processing release of mature IL-1 β in cells permeabilized with *S. aureus* alpha-toxin (38). Since IL-1 β release provoked by exposure of monocytes to beta-toxin exhibited the same kinetics and independence of de novo protein synthesis as observed with pore-forming toxins, we tested whether IL-1 β maturation in beta-toxin-treated cells might also be influenced by K^+ . When cells were treated with beta-toxin in the presence of 140 mM KCl, it was indeed found that release of mature IL-1 β was totally inhibited (Fig. 4).

Shedding of receptors for IL-6 and LPS by monocytes under attack by beta-toxin. We also tested whether biologically important molecules such as receptors for IL-6 and LPS might be released from monocytes under attack by beta-toxin. It is

FIG. 4. Monocytes under attack by sphingomyelinase release IL-1 β . Cells were stimulated with 1 μ g of LPS per ml for 60 min in order to stimulate synthesis of the IL-1 β precursor. Thereafter, cells were treated with 0.1 U of sphingomyelinase per ml in the presence of 10 μ g of cycloheximide per ml, and mature IL-1 β was quantified in the cell supernatants at the indicated times. Release of IL-1 β was observed after 20 min and was maximal at 40 min in the presence of 140 mM NaCl (Na⁺ buffer). No IL-1 β release was observed in buffer controls without sphingomyelinase. IL-1 β release was totally inhibited when cells were treated with sphingomyelinase in buffer containing 140 mM KCl (K⁺ buffer).

known that shedding of these molecules occurs through the action of intrinsic membrane-bound metalloproteinases, and shedding probably represents an important mechanism underlying the systemic effects of IL-6 and LPS (2, 31). Cells were treated with 0.1 U of beta-toxin per ml for 60 min, and the supernatants were assessed for sIL-1R and sCD14 by ELISA. It was found that both of these soluble receptor molecules were indeed shed in large amounts into the supernatants. Concentrations of sIL-6R were 280 ± 30 pg/ml in buffer controls without beta-toxin and $1,370 \pm 120$ pg/ml in supernatants of toxin-treated cells ($n = 3$). Concentrations of sCD14 were 5 ± 1.2 and 51 ± 6 ng/ml in the respective samples. The shedding effect was totally inhibitable by addition of TAPI, a specific metalloprotease inhibitor (22, 24). TAPI had no effect on the cytotoxic action of beta-toxin. Conventional inhibitors of metallo-, serine-, thiol-, or carboxyl-proteinases (1) did not inhibit the toxin-induced shedding process (not shown).

IL-1 β release and cell death do not appear to be mediated by ceramide. Degradation of sphingomyelin by sphingomyelinase leads to generation of ceramide, which in turn is an intracellular messenger (30). In endothelial cells and U-937 cells, ceramide reportedly triggers apoptosis (15, 27). To discern whether either IL-1 β processing or monocyte killing might be mediated by ceramide, cells were incubated with the membrane-permeable ceramide analog *N*-acetyl-sphingosine (27, 40), and IL-1 β and ATP determinations were performed. It was found that incubation with ceramide (1 to 100 μ M) provoked neither IL-1 β release nor cell death in our experimental system.

DISCUSSION

In this study, staphylococcal sphingomyelinase, alias beta-toxin, has been identified as a remarkably potent monocytoxic agent. The cytotoxic effect is observed at subnanomolar toxin concentrations: 50% killing of adherent monocytes occurred at toxin concentrations of only 0.001 U/ml (≤ 5 ng/ml). On a molar basis, beta-toxin was significantly more potent than alpha-toxin (5). The effect displayed a puzzling selectivity and was not observed when fibroblasts, lymphocytes, or granulocytes were used as targets.

It is known that *S. aureus* beta-toxin efficiently degrades sphingomyelin in fibroblasts (9, 33); hence, lack of cytotoxicity in fibroblasts cannot be explained by lack of degradation of the enzyme substrate. In contrast to sheep erythrocytes, sphingomyelin is not a dominant lipid component of monocyte membranes. The sphingomyelin contents of monocytes and granulocytes are reportedly 10.9 and 20.8% of total membrane lipids, respectively (19, 21). Therefore, the higher susceptibility of monocytes can neither be explained by an unusually high content of the enzyme substrate. Ceramide, which has been reported to induce apoptosis in endothelial cells and U-937 cells (15, 27), is apparently also not responsible: monocytes were not killed following incubation with the membrane-permeable ceramide analog *N*-acetyl-sphingosine. It is known that this analog penetrates into cells, and we have confirmed that this causes inhibition of superoxide formation in neutrophils and monocytes (25, 40). Incidentally, the generation of ceramide in fibroblasts was also without effect on cell viability within the period of observation. Overall, we must concede that the cytotoxic effect of sphingomyelinase on monocytes remains unexplained.

We are confident that the monocytocidal effects observed here were truly due to beta-toxin and not to another toxic bacterial product. First, the commercial toxin preparation displayed all functional properties characteristic of sphingomyelinase C. It provoked hot-cold hemolysis of sheep erythrocytes with the expected high titer, without causing hemolysis of rabbit erythrocytes. This indicated that the preparation did not contain significant amounts of alpha-toxin. It was devoid of cytotoxic activity against human granulocytes; thus, relevant contaminations with leukocidin were also absent. After hydroxyapatite chromatography, fractions displaying a single protein band with the correct molecular weight of sphingomyelinase exhibited monocytoxic activity, induced hot-cold hemolysis of sheep erythrocytes, and displayed sphingomyelinase activity as assayed with the use of a chromogenic substrate. Second, culture supernatants of isogenic staphylococcal strains expressing both alpha-toxin and beta-toxin, only beta-toxin, or neither toxin confirmed that monocytocidal activity was indeed primarily a consequence of beta-toxin expression. Finally, sphingomyelinases C from *B. cereus* and a *Streptomyces* sp. were also found to selectively kill human monocytes, whereas phospholipases A and D were not monocytocidal. The monocytocidal activity of bacterial supernatants was slightly higher than would have been expected from the measured sphingomyelinase activity. This may have been due to synergism between beta-toxin and other components in the *S. aureus* supernatants.

The monocytocidal action of beta-toxin displayed rapid kinetics and was complete within 30 min when the toxin was applied at only 0.1 U/ml. Cytotoxicity was reflected by an irreversible depletion of cellular ATP and by positive staining with trypan blue. We currently believe that the primary lesion induced by beta-toxin in monocyte membranes is the generation of small permeability defects because influx of trypan blue

occurs in the absence of lactate dehydrogenase efflux. From a functional aspect, beta-toxin attack on monocyte membranes appears to generate lesions resembling those produced by pore-forming toxins (7).

It is known that permeabilization of the plasma membrane can provoke an array of biologically significant reactions in dying cells (4). In the case of monocytes, two such reactions are the release of cytokines and the liberation of membrane-anchored receptor molecules. Examples for the latter are the receptors for IL-6 and LPS (2, 31). Shedding of these molecules occurs through cleavage by a membrane-bound metalloproteinase that is specifically inhibitable with TAPI (24). The events underlying activation of the protease are largely unknown. Phorbol esters cause activation, but physiological stimuli of microbial origin have not been identified. The significance of receptor shedding lies in the fact that the soluble receptor molecules will bind to many bystander cells, rendering these sensitive to the action of the respective ligands (2, 31).

Monocytes succumbing to the action of beta-toxin indeed were found to secrete IL-1 β and shed receptors for IL-6 and LPS. The rapid secretion of IL-1 β was obviously not related to de novo protein synthesis, since it occurred in the presence of cycloheximide. As was recently found for cells attacked by the pore-forming staphylococcal alpha-toxin, IL-1 β secretion must therefore have been due to increased processing of the precursor molecule through the action of the interleukin-converting enzyme. Raising the extracellular K⁺ concentration inhibited release of mature IL-1 β in beta-toxin-treated cells, a finding that was again analogous to that made of mature IL-1 β in beta-toxin-treated cells, a finding that was again analogous to that made with pore-forming toxins (38). Thus, it seems probable that sphingomyelinase somehow produces small permeability defects in monocyte membranes, with loss of K⁺-Na⁺ homeostasis then leading to activation of the interleukin-converting enzyme.

A second potentially important phenomenon induced by beta-toxin was the shedding of receptors for IL-6 and LPS. That shedding could be totally inhibited by TAPI indicated that sphingomyelinase-treated cells recruited the same mechanism for receptor shedding as previously found for phorbol esters (2, 31). The mechanism of protease activation caused by sphingomyelinase remains unknown.

The present findings explain the earlier data of Russell et al. (32) and Wilkinson (39), who observed a negative effect of beta-toxin on monocyte locomotion, and they should revive interest in the significance and mechanism of action of bacterial sphingomyelinases. Several pressing questions remain unanswered. What is the cause of the apparent selective cytotoxic action of sphingomyelinase C on monocytes? Is this action dependent solely on degradation of sphingomyelin? If so, how does cleavage of sphingomyelin so rapidly lead to breakdown of the membrane permeability barrier? These unresolved questions notwithstanding, the present work uncovers a potentially important role of sphingomyelinase in the initiation and progression of bacterial infections.

ACKNOWLEDGMENTS

This work was supported by funds from the Deutsche Forschungsgemeinschaft (SFB 311), the Verband der Chemischen Industrie, the EC Network-Project ERBCHRX-CT93-0255, and the Wellcome Trust (to T.F.).

We thank Stefan Rose-John for kindly supplying a sample of the protease inhibitor TAPI.

REFERENCES

- Barrett, A. J. 1977. Introduction to the history and classification of tissue proteinases, p. 1–55. In A. J. Barrett (ed.), *Proteinases in mammalian cells and tissues*. Elsevier, Amsterdam.
- Bazil, V. 1995. Physiological enzymatic cleavage of leukocyte membrane molecules. *Immunol. Today* **16**:135–140.
- Bhakdi, S., S. Greulich, M. Muhly, B. Eberspächer, H. Becker, A. Thiele, and F. Hugo. 1989. Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. *J. Exp. Med.* **169**:737–754.
- Bhakdi, S., F. Grimminger, N. Suttrop, D. Walrmath, and W. Seeger. 1994. Proteinaceous bacterial toxins and pathogenesis of sepsis syndrome and septic shock: the unknown connection. *Med. Microbiol. Immunol.* **183**:119–144.
- Bhakdi, S., M. Muhly, S. Korom, and F. Hugo. 1989. Release of interleukin- β associated with potent cytotoxic action of staphylococcal alpha-toxin on human monocytes. *Infect. Immun.* **57**:3512–3519.
- Bhakdi, S., M. Muhly, S. Korom, and G. Schmidt. 1990. Effects of *Escherichia coli* hemolysin on human monocytes. Cytotoxic action and stimulation of interleukin-1 release. *J. Clin. Invest.* **85**:1746–1753.
- Bhakdi, S., U. Weller, I. Walev, E. Martin, D. Jonas, and M. Palmer. 1993. A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microbiol. Immunol.* **182**:167–175.
- Bramley, A. J., A. H. Patel, M. O'Reilly, R. Foster, and T. J. Foster. 1989. Roles of alpha-toxin and beta-toxin in virulence of *Staphylococcus aureus* for the mouse mammary gland. *Infect. Immun.* **57**:2489–2494.
- Byers, D. M., M. W. Morgan, H. W. Cook, F. B. Palmer, and M. W. Spence. 1992. Niemann-Pick type II fibroblasts exhibit impaired cholesterol esterification in response to sphingomyelin hydrolysis. *Biochim. Biophys. Acta* **1138**:20–26.
- Coleman, D. C., J. P. Arbutnot, H. M. Pomeroy, and T. H. Birkbeck. 1986. Cloning and expression in *Escherichia coli* and *Staphylococcus aureus* of the beta-lysin determinant from *Staphylococcus aureus*: evidence that bacteriophage conversion of beta-lysin activity is caused by insertional inactivation of the beta-lysin determinant. *Microb. Pathog.* **1**:549–564.
- Denholm, E., and F. M. Wolber. 1991. A simple method for the purification of human peripheral blood monocytes. *J. Immunol. Methods* **144**:247–251.
- Doery, H. M., B. J. Magnusson, I. M. Cheney, and J. Gulasekharan. 1963. A phospholipase in staphylococcal toxin which hydrolyzes sphingomyelin. *Nature* **198**:1091–1092.
- Doery, H. M., B. J. Magnusson, J. Gulasekharan, and J. E. Pearson. 1965. The properties of phospholipase enzymes in staphylococcal toxins. *J. Gen. Microbiol.* **40**:283–296.
- Gatt, S., T. Dinur, and Y. Barenholz. 1978. A spectrophotometric method for determination of sphingomyelinase. *Biochim. Biophys. Acta* **530**:503–507.
- Haimovitz-Friedman, A., C.-C. Kan, D. Ehleiter, R. S. Persaud, M. McLoughlin, Z. Fuks, and R. N. Kolesnick. 1994. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J. Exp. Med.* **180**:525–535.
- Hedstrom, S. A., and T. Malmqvist. 1982. Sphingomyelinase activity of *Staphylococcus aureus* strains from recurrent furunculosis and other infections. *Acta Pathol. Microbiol. Immunol. Scand B* **90**:217–220.
- Johansen, T., T. Holm, P. H. Guddal, K. Sletten, F. B. Haugli, and C. Little. 1988. Cloning and sequencing of the gene encoding the phosphatidylcholine-prefering phospholipase C of *Bacillus cereus*. *Gene* **65**:293–304.
- Jonas, D., I. Walev, T. Berger, M. Liebetrau, M. Palmer, and S. Bhakdi. 1994. Novel path to apoptosis: small transmembrane pores created by staphylococcal alpha-toxin in T lymphocytes evoke internucleosomal DNA degradation. *Infect. Immun.* **62**:1304–1312.
- Kennett, F. F., H. A. Schenkein, T. M. Ellis, and R. B. Rutherford. 1984. Phospholipid composition of human monocytes and alterations occurring due to culture and stimulation by C3b. *Biochim. Biophys. Acta* **804**:301–307.
- Low, D. K. R., and J. H. Freer. 1977. The purification of β -lysin (sphingomyelinase C) from *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **2**:139–143.
- Marinetti, G. V., and K. Cattieu. 1982. Composition and metabolism of phospholipids of human leukocytes. *Chem. Phys. Lipids* **31**:169–177.
- Mohler, K. M., P. R. Sleath, J. N. Fitzner, D. P. Cerretti, M. Alderson, S. S. Kerwar, D. S. Torrance, C. Otten-Evans, T. Greenstreet, K. Weerawarna, S. R. Kronheim, M. Petersen, M. Gerhart, C. J. Kozlosky, C. J. March, and R. A. Black. 1994. Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. *Nature* **370**:218–220.
- Möhlby, R. 1983. Isolation and properties of membrane damaging toxins, p. 619–662. In C. S. F. Easton and C. Adlam (ed.), *Staphylococci and staphylococcal infections*, vol. 2. Academic Press, London.
- Mullberg, J., F. H. Durie, C. Ottenevans, M. R. Alderson, S. Rose-John, D. Cosman, R. A. Black, and K. M. Mohler. 1995. A metalloprotease inhibitor blocks shedding of the IL-6 receptor and the P60 TNF receptor. *J. Immunol.* **155**:5198–5205.
- Nakamura, T., A. Abe, K. J. Balazovich, D. Wu, S. J. Suchard, L. A. Boxer, and J. A. Shayman. 1994. Ceramide regulates oxidant release in adherent human neutrophils. *J. Biol. Chem.* **269**:18384–18389.
- Novick, R. P. 1963. Properties of a cryptic high frequency transducing phage in *Staphylococcus aureus*. *Virology* **33**:155–166.
- Obeid, L. M., C. M. Linardic, L. A. Karolak, and Y. A. Hannun. 1993.

- Programmed cell death induced by ceramide. *Science* **259**:1769–1771.
28. O'Reilly, M., J. C. S. de Azavedo, S. Kennedy, and T. J. Foster. 1986. Inactivation of the alpha-haemolysin gene of *Staphylococcus aureus* 9325-4 by site-directed mutagenesis and studies on the expression of its haemolysin. *Microb. Pathog.* **1**:125–138.
 29. Projan, S. J., J. Kornblum, B. Kreiswirth, S. L. Moghazeh, W. Eisner, and R. P. Novick. 1989. Nucleotide sequence. The β -hemolysin gene of *Staphylococcus aureus*. *Nucleic Acids Res.* **17**:3305.
 30. Pushkareva, M., L. M. Obeid, and Y. A. Hannun. 1995. Ceramide: an endogenous regulator of apoptosis and growth suppression. *Immunol. Today* **16**:294–302.
 31. Rose-John, S., and P. C. Heinrich. 1994. Soluble receptors for cytokines and growth factors: generation and biological function. *Biochem. J.* **300**:281–290.
 32. Russell, R. J., P. C. Wilkinson, R. J. McInroy, S. McKay, A. C. McCartney, and J. P. Arbutnot. 1975. Effects of staphylococcal products on locomotion and chemotaxis of human blood neutrophils and monocytes. *J. Med. Microbiol.* **8**:433–449.
 33. Slotte, J. P., and E. L. Bierman. 1988. Depletion of plasma-membrane sphingomyelin rapidly alters the distribution of cholesterol between plasma membranes and intracellular cholesterol pools in cultured fibroblasts. *Biochem. J.* **250**:653–658.
 34. Thornberry, N. A., H. G. Bull, J. R. Calaycay, K. T. Chapman, A. D. Howard, M. J. Kostura, D. K. Miller, S. M. Molineaux, J. R. Weidner, J. Aunins, K. O. Elliston, J. M. Ayala, F. J. Casano, J. Chin, G. J.-F. Ding, L. A. Egger, E. P. Gaffney, G. Limjuco, O. C. Palyha, S. M. Raju, A. M. Rolando, J. P. Salley, T. T. Yamin, T. D. Lee, J. E. Shively, M. MacCross, R. A. Mumford, J. A. Schmidt, and M. J. Tocci. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* **356**:768–774.
 35. Titball, R. W. 1993. Bacterial phospholipase C. *Microbiol. Rev.* **57**:347–366.
 36. Wadström, T., and R. Mollby. 1972. Some biological properties of purified staphylococcal haemolysins. *Toxicon* **10**:511–519.
 37. Walev, I., M. Palmer, E. Martin, D. Jonas, U. Weller, H. Höhn-Bentz, M. Husmann, and S. Bhakdi. 1994. Recovery of human fibroblasts from attack by the pore-forming α -toxin of *Staphylococcus aureus*. *Microb. Pathog.* **17**:187–201.
 38. Walev, I., K. Reske, M. Palmer, A. Valeva, and S. Bhakdi. 1995. Potassium-inhibited processing of IL-1 β in human monocytes. *EMBO J.* **14**:1607–1614.
 39. Wilkinson, P. C. 1975. Inhibition of leukocyte locomotion and chemotaxis by lipid-specific bacterial toxins. *Nature* **255**:485–491.
 40. Wong, K., X. B. Li, and N. Hunchuk. 1995. *N*-Acetylsphingosine (C_2 -ceramide) inhibited neutrophil superoxide formation and calcium influx. *J. Biol. Chem.* **270**:3056–3062.

Editor: V. A. Fischetti