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Novel Path to Apoptosis: Small Transmembrane Pores Created by Staphylococcal Alpha-Toxin in T Lymphocytes Evoke Internucleosomal DNA Degradation

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Peripheral-blood human T lymphocytes were treated with *Staphylococcus aureus* alpha-toxin. Membrane permeabilization was assessed by measuring efflux of K^+ and Rb^+ and influx of Na^+, Ca^{2+}, and propidium iodide. Cellular ATP and [^{3}H]thymidine incorporation following lectin stimulation were measured as parameters for cell viability. Internucleosomal cleavage characteristic of programmed cell death was assessed by agarose gel electrophoresis and by quantifying low-molecular-weight, [^{3}H]thymidine-labeled DNA fragments. Nanomolar concentrations of alpha-toxin evoked protracted, irreversible ATP depletion in both activated and resting T lymphocytes. Toxin-damaged cells also lost their ability to incorporate [^{3}H]thymidine upon subsequent stimulation with phytohemagglutinin. These cells carried toxin hexamers, and their plasma membranes became permeable for monovalent ions but not for Ca^{2+} and propidium iodide. The permeabilization event was followed by internucleosomal DNA degradation characteristic of programmed cell death. Membranes of cells treated with high toxin doses (>300 nM) became permeable to both Ca^{2+} and propidium iodide. In this case, ATP depletion occurred within minutes and no DNA degradation was observed. When cells were suspended in Na^+-free buffer, alpha-toxin applied at low doses still bound and formed hexamers. However, these cells displayed neither DNA degradation nor loss of viability. The data indicate that formation of very small but not of large alpha-toxin pores may trigger programmed cell death in lymphocytes and that uncontrolled flux of Na^+ ions may be an important event precipitating the suicide cascade.

Alpha-toxin, the major cytolysin of *Staphylococcus aureus* (3, 7), damages cells by generating pores in the plasma membrane (7, 11, 27, 30). At low concentrations, the 34-kDa toxin monomer binds to susceptible cells by interaction with as-yet-unidentified acceptor molecules on the membrane. At high concentrations (>200 nM; 6 μg/ml), binding additionally occurs via nonspecific absorption to the lipid bilayer (9, 16). In rabbit erythrocyte membranes, both modes of binding result in the formation of hexameric pores with an effective diameter of 1 to 2 nm (7, 11). Aqueous pores of similar size have been produced through the action of alpha-toxin on liposomes (17) and planar lipid membranes (2, 20) in endothelial cells (25), polymorphonuclear granulocytes (26), platelets (6), and neuroendocrine cells (1). It is therefore generally assumed that, once formed, alpha-toxin pores will display effective diameters of 1 to 2 nm, allowing passage of mono- and divalent ions and of small macromolecules, including ATP, with molecular weights of ≤1,000 to 2,000 (7).

Recent studies in our laboratories have disclosed, however, that this original concept of homogeneous alpha-toxin pores requires revision. Thus, pores forming in keratinocytes at low toxin doses were found to permit passage of monovalent ions but not of Ca^{2+} or larger marker molecules such as propidium iodide (29). Formation of small pores was followed by breakdown of mitochondrial respiration and cell death. Formation of larger pores occurred at high toxin concentrations, and the working hypothesis was advanced that small pores may be created when the toxin binds to high-affinity, specific sites on the cells, whereas toxin molecules absorbed nonspecifically to the bilayer might be responsible for creating the large pores.

In the present study, peripheral-blood T lymphocytes treated with low toxin doses were also found to leak K^+ and take up Na^+ but not to accumulate Ca^{2+}. This finding prompted us to search for secondary reactions that might follow in the wake of formation of such small pores. One interesting aspect related to the possibility that programmed cell death might be triggered. This idea was originally expounded in a publication by Hameed et al. (15), who reported that internucleosomal DNA degradation occurred in cells treated with alpha-toxin and perforin. Their finding suggested that cells damaged by a pore-forming agent might ultimately die because membrane permeabilization would result in bypassing of regulatory mechanisms that normally hold the suicide cascade in check.

With regard to perforin, however, the data of Hameed et al. were soon contested. Several groups of investigators demonstrated that highly purified perforin killed target cells without inducing DNA degradation (10, 14). The conclusion was that the perforin preparation employed by Hameed et al. (15) had probably contained DNase contaminants. When we analyzed the commercial alpha-toxin employed by Hameed et al., we also found that it contained DNase contaminants (unpublished results). Even apparently pure alpha-toxin preparations, later obtained from the research laboratory of Behringwerke (Marburg, Germany), that exhibited a single, 34-kDa protein band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels contained DNase that created artifacts. Pilot experiments showed that application of such toxin preparations to human T cells at the high concentration used by Hameed et al. (200 μg/ml [15]) (see Fig. 1) induced extensive internucleosomal DNA degradation. However, when highly

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purified alpha-toxin preparations from our laboratory were used at the same concentration, no DNA degradation was observed. At this stage, therefore, the data of Hameed et al. with regard to alpha-toxin obviously also represented an artifact, and no link appeared to exist between the process of membrane permeabilization and triggering of programmed cell death.

The realization that pores of different sizes could be generated in nucleated cells at different doses of alpha-toxin now prompted us to reinvestigate the issue of DNA degradation. Experimental data obtained in this study led us to present a revised concept stating that formation of very small pores that essentially permit the flux of only monovalent ions may, indeed, trigger internucleosomal DNA degradation and programmed cell death in lymphocytes. These findings have wide implications for other situations in which cells are attached by agents that generate very small membrane lesions.

MATERIALS AND METHODS

Reagents. RPMI 1640, fetal calf serum (FCS) (heat inactivated for 45 min at 56°C), glutamine sodium pyruvate, penicillin-streptomycin, Ficoll (1.077 g/ml), phosphate-buffered saline (PBS), and Hanks' buffered saline without Ca^{2+} or Mg^{2+} (HBSS) were obtained from Biochrom (Berlin, Germany), and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer was from Boehringer (Mannheim, Germany). Phytohemagglutinin (PHA) was supplied by Wellcome (Burgwedel, Germany). Recombinant human interleukin-2 (IL-2) was from Boehringer, and [*methyl*-^{3}H]thymidine was from Amersham (Braunschweig, Germany). ^{125}I, $^{45}Ca^{2+}$, $^{86}Rb^+$, and $^{22}Na^+$ were obtained from Dupont de Nemours (Dreieich, Germany). Rotiszint eco plus liquid scintillator was supplied by Roth (Karlsruhe, Germany). Phenylmethanesulfonyl fluoride was from Merck (Darmstadt, Germany). Chemical reagents were purchased from Sigma (Munich, Germany). Fluo-3-acetoxymethylester (fluo-3/AM) and Pluronic F-127 were from Molecular Probes (Eugene, Oreg.).

Toxins. Alpha-toxin was fast protein liquid chromatography (FPLC) purified from a culture of *S. aureus* Wood 46 (22). A sample of highly purified alpha-toxin was also obtained as a gift from Behringwerke. To test for the presence of trace DNase contamination, 10 μg of high-molecular-weight genomic DNA was added to 0.1-ml solutions containing 300 μg of alpha-toxin per ml in RPMI 1640 for 40 min at 37°C. Thereafter, the samples were analyzed by agarose gel electrophoresis. No degradation whatsoever was seen when FPLC-purified alpha-toxin was used, whereas extensive degradation occurred with the preparation obtained from Behringwerke, although the latter also showed a single protein band of M_r 34,000 in SDS-PAGE gels. We interpreted this to indicate that our FPLC-purified toxin preparations contained essentially no DNase contamination. *Escherichia coli* hemolysin (HlyA) was prepared and used as described elsewhere (4, 18).

Isolation and culture of PBML and T lymphocytes. Peripheral-blood mononuclear leukocytes (PBML) from buffy coats were separated by centrifugation over a Ficoll step gradient for 20 min at 400 × *g*. Interphase cells were collected and washed twice with HBSS. T cells were separated from the remaining PBML by rosetting with sheep erythrocytes (Virion, Würzburg, Germany). All cells were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, 1% penicillin-streptomycin, and 2% glutamine (Fluka, Buchs, Switzerland).

Proliferation assay. Triplicate cultures with 10^5 cells in 200 μl of RPMI medium were treated with toxin. After 14 h, PHA was added at 1.25 μg/ml for 48 h. Subsequently, cells were labeled with 0.1 μCi of [^{3}H]thymidine for 14 h and harvested onto fiberglass filters. [^{3}H]thymidine incorporation was determined in a beta counter (Betaplate model no. 1205; Wallay OY, Turku, Finland). The stimulation index was calculated as [^{3}H]thymidine incorporation in toxin-treated samples relative to that in untreated samples.

Bioluminescence assay for determination of cellular ATP. Cells (10^5) in 200 μl of medium were lysed with 10 μl of 10% Triton X-100. Each lysate was transferred into a vial containing 155 μl of distilled water and 25 μl of luciferase (Boehringer), and chemiluminescence was measured in a luminometer (Bioluminat LB9500; Berthold, Wildbad, Germany). ATP contents of toxin-treated cells were expressed as percent luminescence relative to that of untreated cells (5).

Flow cytometric analysis of propidium iodide influx. Cells (10^5) in 200 μl of RPMI medium were incubated for 10 min with 2.5 μg of propidium iodide per ml at ambient temperature. Cells were washed once with PBS and analyzed by using a FACScan flow cytometer (Becton Dickinson and Co., Heidelberg, Germany). Excitation was with a 488-nm argon laser; fluorescence was measured at 585 nm (18).

Detection of membrane-bound alpha-toxin. Alpha-toxin was iodinated as described elsewhere (16) to a specific activity of 430 Ci/mmol. Cell membranes of rabbit erythrocytes treated with ^{125}I-alpha-toxin were prepared by being lysed and washed five times with 5 mM phosphate buffer, pH 7.4.

Membranes of toxin-treated T lymphocytes were solubilized by resuspending washed cells in lysis buffer (20 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 4 mM phenylmethanesulfonyl fluoride). After incubation for 30 min on ice, cellular debris was removed by centrifugation (13,000 × *g*, 30 min, 4°C) and the supernatant was applied to SDS gels.

K^+ efflux. For measuring the release of cellular K^+ into culture medium, 3×10^7 T lymphocytes were suspended in 1 ml of RPMI. At the times indicated, medium was withdrawn and centrifuged for 5 min at 250 × *g* followed by 5 min at 13,000 × *g*. Potassium concentrations were determined by flame photometry.

$^{86}Rb^+$ efflux. Loading with the isotope was achieved by incubating 5×10^7 T lymphocytes in 1 ml of RPMI, buffered with 25 mM HEPES, with 20 μCi of $^{86}Rb^+$ for 3 h at ambient temperature (12). Cells were then washed three times with HBSS. Cells (10^7) were incubated in 500 μl of RPMI with toxin for 20 min at 37°C. A 120-μl volume of cell suspension was pipetted onto 500 μl of an oil mix (10 volumes of dibutyl phthalate and 3 volumes of corn oil) and centrifuged at 1,500 × *g* for 10 min. The upper aqueous phase was removed, the wall of the vial was carefully rinsed with 300 μl of HBSS, and most of the oil was discharged. Cells were lysed with 200 μl of 0.2 N NaOH–1% SDS and transferred to scintillation vials containing 2 ml of scintillator, after which cellular $^{86}Rb^+$ levels were determined in a beta counter (Beckman, Fullerton, Calif.).

$^{22}Na^+$ Influx. T cells (0.5×10^8 to 1×10^8) were resuspended in 500 μl of choline buffer (140 mM choline chloride, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 100 μM amiloride, 20 mM Tris-HCl, pH 7.4) in order to minimize the physiologically rapid sodium exchange (13). Cells were incubated with toxin as indicated for 30 min at 37°C. Subsequently, 150 μl of choline buffer containing 1 μCi of $^{22}Na^+$ was added for 5 min at ambient temperature. Thereafter, cell-associated $^{22}Na^+$ was separated by centrifugation through the oil mix and counted as described above.

Ca^{2+} flux. Two assays were used to detect levels of cellular

Ca^{2+}. The first employed flow cytometry of cells loaded with Fluo-3/AM (19). The dye was dissolved at 1 mM in dimethyl sulfoxide containing 37.5 mg of Pluronic F-127 per ml. A 4-μl volume of the solution was added to 10^7 lymphocytes in 1 ml of RPMI containing 10% FCS and kept at 37°C for 20 min. A 4-ml volume of HBSS was then added, and cells were incubated another 40 min. After being washed twice with culture medium, cells were resuspended at 10^6/ml in medium. The concentration of cytosolic calcium was calculated as described elsewhere (28). The mean fluorescence value at 530 nm was analyzed by the FACScan flow cytometer. For calibration, the maximum fluorescence was measured immediately after permeabilization with 0.5 μg of *E. coli* hemolysin HlyA. Thereafter, fluorescence was determined in the presence of 2 mM $MnCl_2$. The second assay consisted of measuring levels of $^{45}Ca^{2+}$ that became cell associated. In the latter experiments, 2×10^6 cells in 1 ml of HBSS without Ca^{2+} or Mg^{2+} were incubated with toxin for 30 min at 37°C and then with 1 μCi of $^{45}Ca^{2+}$ for 15 min at room temperature. Cells were pelleted, washed with HBSS, and lysed with 200 μl of 1% Triton X-100, and then bound $^{45}Ca^{2+}$ was determined in a beta counter.

Tritium release assay. Isolated T cells were prepared at 2×10^6/ml and stimulated with 1 μg of PHA per ml. After 18 to 20 h, cells were washed three times and resuspended in fresh medium with 10 U of human recombinant IL-2 (Boehringer) per ml. On day 6 of cell culture, 0.6 μCi of [^{3}H]thymidine per ml was added. After overnight labeling, cells were washed three times with medium and resuspended at 10^6/ml in fresh medium containing IL-2. During the tritium release assay, 1 ml of the cell suspension was incubated in 24-well plates. Following incubation with alpha-toxin, samples of 200 μl were withdrawn and centrifuged for 10 min at $150 \times g$. The pelleted cells were lysed with 200 μl of lysis buffer (0.2% Nonidet P-40, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA), vortexed, and centrifuged for 10 min at $13,000 \times g$. The supernatant containing cytoplasmic DNA was withdrawn. The pellet containing nuclear DNA was solubilized in 200 μl of 1 N NaOH. The amounts of ^{3}H recovered in pellet and supernatant were determined after addition of 2 ml of scintillator in a beta counter. Tritium release was calculated as the ratio of disintegrations per minute in the cytoplasmic DNA to the sum of disintegrations per minute in cytoplasmic and nuclear DNAs.

Electrophoretic analysis of DNA fragmentation. After incubation of T lymphocytes at 10^6/ml under the conditions indicated, cells were washed with cold PBS and resuspended in TE (1 mM EDTA, 10 mM Tris-HCl, pH 7.4), and then Nonidet P-40 was added to 0.2%. After incubation for 20 min on ice, the lysate was centrifuged for 20 min at $27,000 \times g$, 4°C. The supernatant received 0.1% SDS and 10 μg of proteinase K (Boehringer) per ml and was digested for 12 h at 56°C. The DNA was precipitated with 2.5 volumes of ethanol at −20°C and pelleted (20 min at $27,000 \times g$, 4°C). The dried DNA was dissolved in TE as described above and digested for 30 min with 10 μg of heat-pretreated RNase per ml at 37°C. The DNA samples were loaded onto a 1% agarose gel in 89 mM Tris-HCl–89 mM borate–8.9 mM EDTA, pH 8.0, with 0.5 μg of ethidium bromide per ml and separated by electrophoresis at 50 V for 3 h. The length of fragmented DNA was estimated by comparison with a size marker (Bethesda Research Laboratories, Gaithersburg, Md.).

RESULTS

Reduction in levels of cellular ATP evoked by alpha-toxin. Lymphocytes were incubated with alpha-toxin at the concentrations given in Fig. 1, and relative ATP levels in cell lysates were determined after various times by the luciferase assay. A dose response was observed with respect to both kinetics and extent of ATP depletion. Approximately 50% ATP reduction was noted at toxin concentrations of 10 to 30 ng/ml after 2 h. Thus, the susceptibility of lymphocytes to attack by alpha-toxin is similar to that observed for human platelets (6) and monocytes (5). Reductions in ATP levels caused by 30 to 300 ng of alpha-toxin per ml were minimal at 5 to 10 min and approached 35 and 60% after 30 min at doses of 30 and 300 ng/ml, respectively.

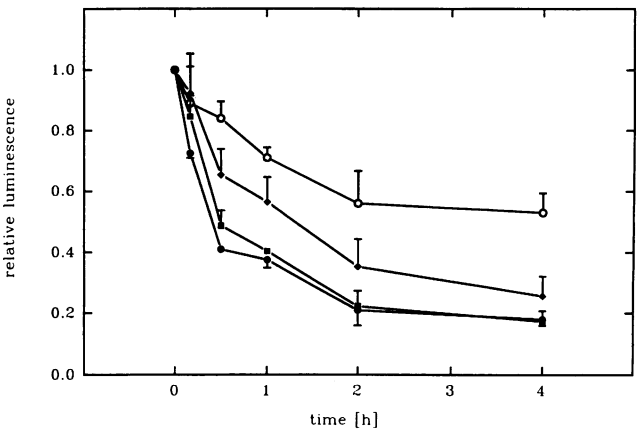


FIG. 1. Kinetics of ATP depletion in toxin-treated T lymphocytes. Cells were treated with 10 (○), 30 (◆), 300 (■), and 1,000 (●) ng of alpha-toxin per ml. Relative luminescence was determined at various times after addition of the toxin. Data represent means ± standard deviations (error bars) for three independent experiments.

Loss of cellular ATP correlates with loss of viability as determined by measurements of [^{3}H]thymidine incorporation. In order to determine whether the noted reduction in cellular ATP levels reflected irreversible damage to the cells, lymphocytes were treated overnight with alpha-toxin and then stimulated for 48 h with PHA. We found a toxin-induced reduction in [^{3}H]thymidine incorporation that displayed dose dependency identical with that of the drop in cellular ATP (Fig. 2). Thus, significant repair of toxin lesions with regaining of function was not noted.

Alpha-toxin forms hexamers in lymphocyte membranes. To confirm that cell damage inflicted by low doses of alpha-toxin was associated with formation of hexamers, cells were treated with 370 ng of radioiodinated alpha-toxin per ml. SDS-PAGE of membrane solubilizates followed by autoradiography documented the presence of hexamers on the membranes (Fig. 3). Binding of radiolabeled toxin was markedly reduced in the presence of a 100-fold excess of unlabeled toxin, indicating binding to a limited number of sites under the given conditions.

Toxin pores restrict the passage not only of LDH but also of propidium iodide. Cell supernatants were examined for lactate dehydrogenase (LDH). As expected, low, cytocidal doses of alpha-toxin (300 ng/ml) induced virtually no leakage of LDH. At very high doses, considerable amounts of LDH were recovered in the supernatants (data not shown). Similar results were obtained when cells were incubated for 90 min with *E. coli* hemolysin (HlyA).

To discern whether pores formed by low doses of alpha-toxin would permit influx of the relatively small marker propidium iodide, cells were incubated in the presence of propidium iodide with 300 ng of alpha-toxin per ml and the relative numbers of cells exhibiting red fluorescence were

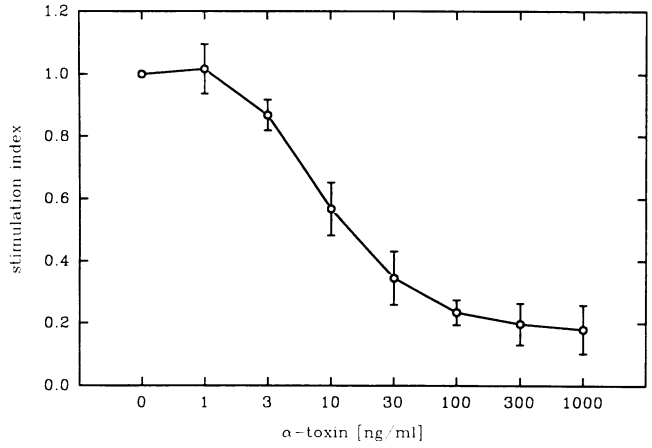


FIG. 2. Effects of *S. aureus* alpha-toxin on [^{3}H]thymidine incorporation in stimulated T lymphocytes. Cells were suspended in RPMI containing 10% FCS and treated with alpha-toxin. Thereafter, PHA was added and the stimulation index was determined by a proliferation assay. Data represent mean values (n = 3) obtained with three different donors. Error bars indicate standard deviations.

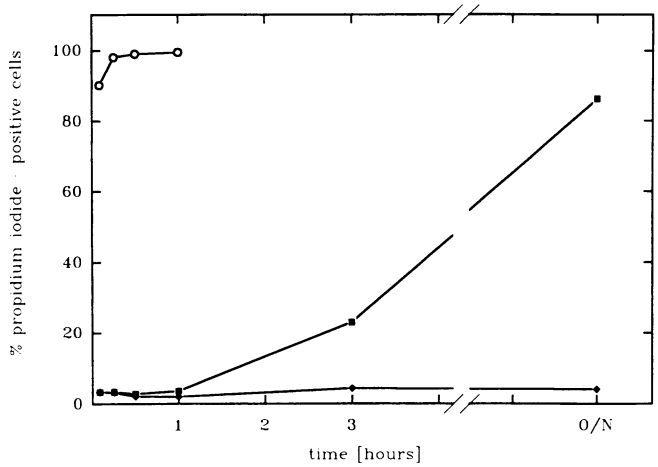


FIG. 4. Membrane permeability of T cells to propidium iodide at various times after treatment with alpha-toxin. T cells (10^5) were treated with 300 ng (■) or 300 μg (○) of alpha-toxin per ml or were left untreated (◆). After the indicated times, the percentage of propidium iodide-positive cells was determined by flow cytometry. O/N, overnight.

determined by flow cytometry. As shown in Fig. 4, the cell membranes remained impermeable to propidium iodide after 60 min of incubation with alpha-toxin. Only approximately 20% of the cells displayed red fluorescence at 3 h, whereas approximately 90% had taken up the marker after an overnight incubation. Thus, low but lethal doses of alpha-toxin rendered cells only slowly permeable to propidium iodide, and the kinetics of this event lagged far behind the kinetics of ATP depletion. In contrast, cells permeabilized with the very high dose of 300 μg of alpha-toxin per ml exhibited red fluorescence within minutes.

Low doses of alpha-toxin induce efflux of $^{86}Rb^+$ and K^+ and influx of $^{22}Na^+$. The following experiments confirmed that formation of hexamers at low toxin doses, although not creating propidium iodide-permissive pores, did lead to membrane permeabilization. Concentrations of K^+ in cell supernatants were determined by flame photometry. Additional experiments were performed in which loss of $^{86}Rb^+$ from the cells was quantified. As shown in Fig. 5, alpha-toxin applied at 30 and 300 ng/ml rapidly induced leakage of potassium from the cells. At 300 ng/ml, approximately 70% efflux was noted 5 min after toxin application. After 20 min, efflux was essentially

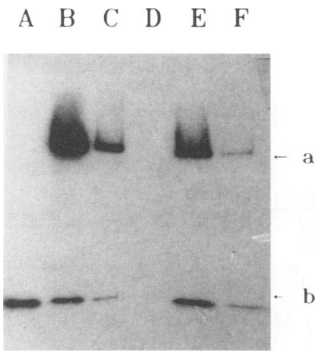


FIG. 3. Autoradiographic analysis of ^{125}I-labeled alpha-toxin bound to rabbit erythrocytes or T lymphocytes. After incubation, erythrocyte membranes were washed in 5 mM phosphate buffer. Lymphocytes were washed in PBS. Membrane pellets or cells were solubilized in sample buffer containing 4% SDS at 20°C and subjected to SDS-PAGE under reducing conditions. The gel was autoradiographed at −70°C for 6 h. Hexamers (a) and monomers (b) are indicated. Lanes: A, 3.7 ng of labeled toxin; B and C, 10^8 and 10^7 erythrocytes, respectively, lysed with 370 ng of labeled toxin per ml; D, 3.3×10^6 T lymphocytes without toxin; E, as lane D but after 90 min of incubation with 370 ng of labeled toxin per ml; F, as lane E, but cells were pretreated with 40 μg of unlabeled toxin per ml prior to addition of 370 ng of labeled toxin per ml.

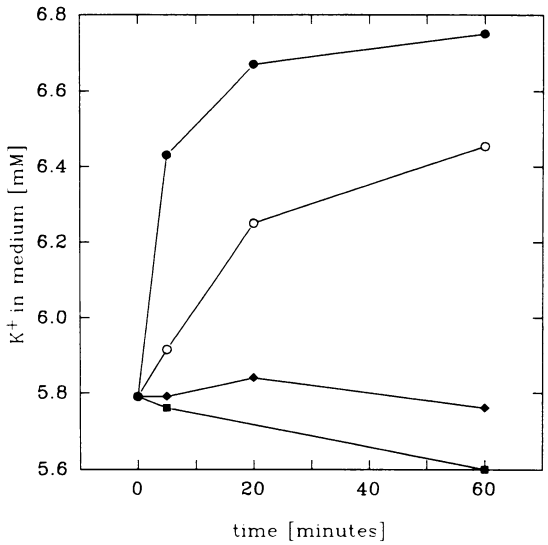
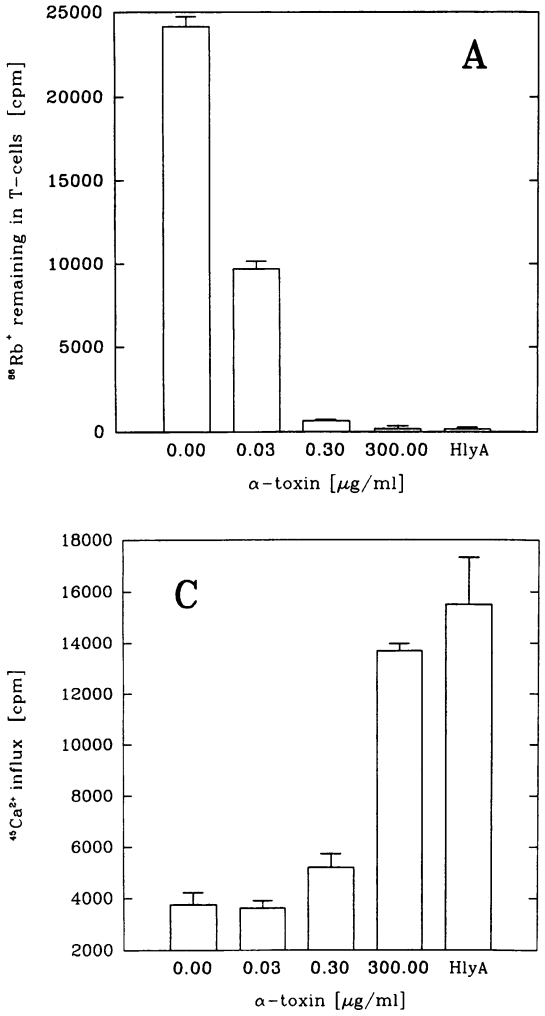


FIG. 5. K^+ release from alpha-toxin-treated cells. T cells (3×10^7) in 1 ml of culture medium were treated with alpha-toxin at concentrations of 30 (○) or 300 (●) ng/ml or with 150 mM NaN_3 (■) or left untreated (◆). K^+ concentrations in the medium were determined by means of flame photometry at various times after addition of the toxin. Data represent mean values for duplicate determinations from one of three similar experiments.



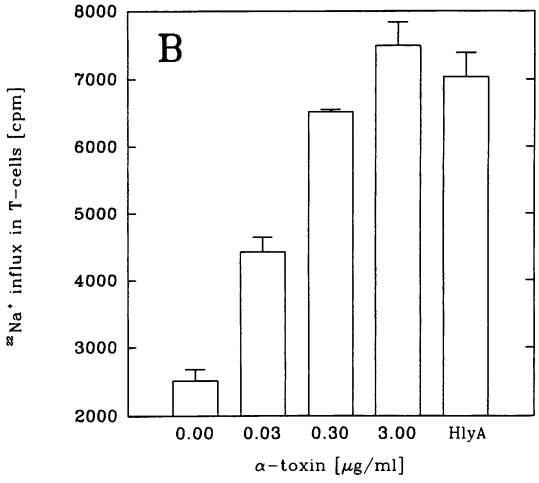


FIG. 6. (A) $^{86}Rb^+$ efflux from T lymphocytes after addition of alpha-toxin or HlyA. Cells (2.4×10^6) loaded with $^{86}Rb^+$ were incubated with various concentrations of alpha-toxin or 200 ng of HlyA per ml as a positive control at 37°C for 20 min. Remaining cellular $^{86}Rb^+$ was determined in triplicate samples after separation of cells from the medium. Bars indicate standard deviations. (B) $^{22}Na^+$ influx in T lymphocytes after addition of alpha-toxin or HlyA. Cells (2×10^7) were incubated with various concentrations of alpha-toxin or 200 ng of HlyA per ml in choline buffer for 30 min at 37°C. Subsequently, $^{22}Na^+$ was added at 1.5 μCi/ml for 5 min at room temperature. Data represent mean levels ± standard deviations (error bars) of cellular $^{22}Na^+$ measured in triplicate samples. (C) Determination of $^{45}Ca^{2+}$ bound to T lymphocytes after addition of alpha-toxin or HlyA. Cells (2×10^6) were incubated for 30 min at 37°C with toxins as indicated in HBSS without Ca^{2+} or Mg^{2+}. $^{45}Ca^{2+}$ was then added at 1 μCi/ml for 15 min. Bound $^{45}Ca^{2+}$ was determined after cell lysis by liquid scintillation. Data are given as means ± standard deviations (error bars) for triplicate determinations.

complete at this dose. No potassium efflux occurred in cells that had been poisoned with 150 mM NaN_3, although ATP levels in these cells did fall to less than 10% after 60 min of incubation (data not shown). From this, we concluded that efflux of K^+ from toxin-treated cells was not due to breakdown of K^+ transporter function secondary to ATP depletion.

Figures 6A and B depict the results of experiments measuring $^{86}Rb^+$ and $^{22}Na^+$ levels. The loss of $^{86}Rb^+$ from cells paralleled potassium leakage, and this was mirrored by an equivalent flux of $^{22}Na^+$ into the cells.

Transmembrane flux of monovalent ions is not accompanied by significant net influx of extracellular calcium. An unexpected discrepancy was noted when calcium flux was measured. Virtually no accumulation of cell-bound $^{45}Ca^{2+}$ was observed when toxin at 30 ng/ml was applied to the cells. Only a very small increase of cell-associated $^{45}Ca^{2+}$ was noted at 300 ng/ml. When toxin was applied at 300 μg/ml, there was a clear net influx of $^{45}Ca^{2+}$ that approached levels found when HlyA was used as the permeabilizing agent (Fig. 6C).

The question of calcium flux was further analyzed by flow cytometry. Cells were loaded with Fluo-3/AM and treated with 300 or 3,000 ng of alpha-toxin per ml. As shown in Fig. 7, there was only a minor, almost imperceptible, increase in green fluorescence, which indicated a rise in mean cytosolic concentrations on the order of 100 nM (for calculation of actual

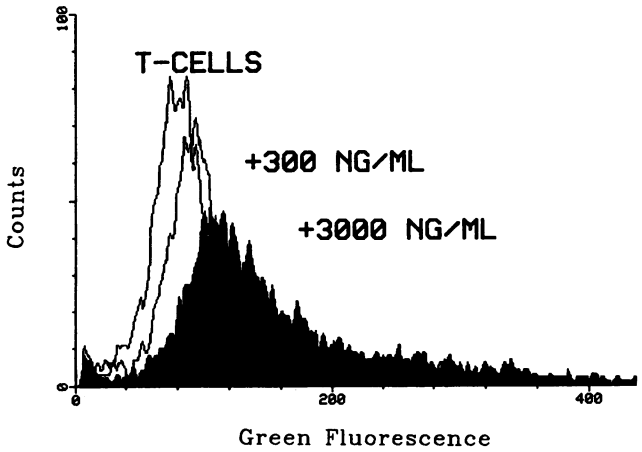


FIG. 7. Histograms depicting fluorescence of fluo-3/AM-loaded T cells treated with alpha-toxin at 300 and 3,000 ng/ml compared with that of untreated T cells.

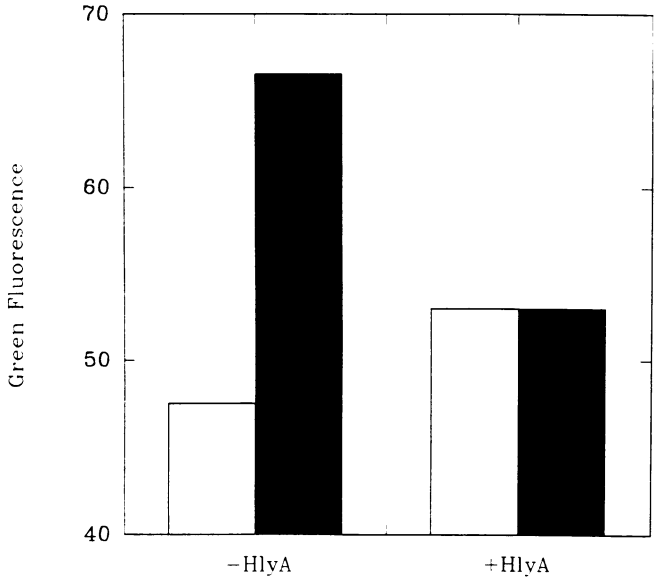


FIG. 8. Alpha-toxin-induced rise of cytosolic Ca^{2+} in T lymphocytes suspended in EGTA. Resting T cells were loaded with fluo-3/AM and incubated in RPMI–10% FCS–1 mM EGTA for 1 h with 3 μg of alpha-toxin per ml (shaded bars) or without toxin (unshaded bars). Note the slight increase in fluorescence corresponding to results shown in Fig. 7 for the toxin-treated cells. Cells from the same samples were posttreated with 1 μg of HlyA per ml, and green fluorescence was measured again after 1 min. Because of formation of Ca^{2+}-permissive pores, mean fluorescence values became identical in the two cell populations. Data represent means for duplicate determinations.

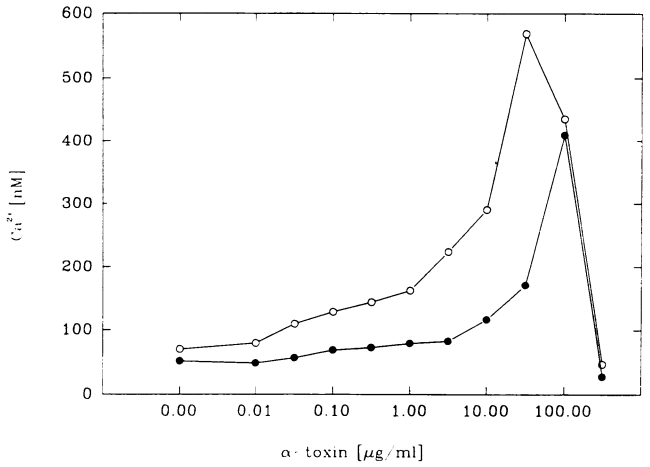


FIG. 9. Cytosolic concentration of Ca^{2+} in T lymphocytes treated with alpha-toxin. Resting T cells (○) or cells 6 days after stimulation with PHA (●) were loaded with fluo-3/AM and treated with alpha-toxin at the indicated doses. Mean concentrations of cytosolic Ca^{2+} were calculated on the basis of mean values for green fluorescence.

values, see Fig. 9). To obtain an indication of whether this was due to true Ca^{2+} influx or to mobilization of Ca^{2+} from intracellular stores, cells were suspended in 1 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid) and treated with alpha-toxin. The results are shown in Fig. 8 (leftmost pair of columns). Again, mean fluorescence of the cell population exhibited a slight increase following alpha-toxin treatment (shaded column). This indicated first that the rise in cytosolic Ca^{2+} levels derived from mobilization from intracellular storage pools and second that the liberated Ca^{2+} was not able to diffuse through the alpha-toxin pores. This was confirmed by posttreating the cells with HlyA, which formed Ca^{2+}-permissive pores (Fig. 8, rightmost pair of columns). HlyA caused equilibration of Ca^{2+} ions across the membrane, reflected by the fact that fluorescence in control cells was identical to that in alpha-toxin-treated cells. The overall increase in cellular Ca^{2+} probably stemmed from residual free Ca^{2+} in the culture medium, a major contribution stemming from Ca^{2+} contained in the HlyA preparation (4).

In the next experiments, the concentration of cytosolic Ca^{2+} was quantified flow cytometrically over a range of toxin concentrations by using both resting (Fig. 9, open circles) and stimulated (closed circles) T cells. In resting T cells, slight rises in cytosolic Ca^{2+} levels were observed at toxin doses of 30 to 3,000 ng/ml. As argued above, this was presumably due to mobilization of Ca^{2+} from intracellular stores. Higher doses had more pronounced effects, interpreted to stem partially from true Ca^{2+} influx. At 300 μg/ml, cells lost fluorescence, probably through leakage of the fluorescent dye from the cells. In stimulated T cells, the rise in intracellular Ca^{2+} evoked by 10 to 3,000 ng of toxin per ml was even more discrete, but essentially the same pattern was observed. The apparent difference in free Ca^{2+} levels in resting and stimulated T cells was considered to be insignificant in view of the limited accuracy of the fluorescence method at these very low calcium concentrations.

Formation of small, non-Ca^{2+}-permissive pores in T lymphocytes triggers internucleosomal DNA degradation. Internucleosomal DNA degradation was first detected by agarose gel electrophoresis. PHA-stimulated T cells treated with 300 ng of alpha-toxin per ml displayed extensive DNA fragmentation, and the degradation patterns were as seen with cells killed by irradiation (Fig. 10A).

To obtain more quantitative data, we determined the generation of soluble DNA fragments by the tritium release assay. The experiments were conducted with cells that were prestimulated in the presence of [^{3}H]thymidine to incorporate the label. As shown in Fig. 10B, extensive ^{3}H release occurred in cells receiving 10 ng to 10 μg of alpha-toxin per ml. This process was abrogated at high toxin doses (>30 μg/ml).

Removal of Na^+ from the medium prevents DNA degradation and ATP depletion. A remarkable finding was made when cells were suspended in Na^+-free medium. In this case, treatment with 300 ng of alpha-toxin per ml still led to formation of hexamers on the membranes (Fig. 11A). However, DNA degradation did not occur (Fig. 11B). When these cells were transferred to normal medium 4 h later, they exhibited a normal capacity to incorporate [^{3}H]thymidine (Fig. 11C). Hence, a first correlation was established between the process of DNA degradation and cell death evoked by alpha-toxin.

DISCUSSION

This study disclosed that peripheral-blood human T lymphocytes are highly susceptible to the action of alpha-toxin, probably because of the presence of a limited number of specific high-affinity binding sites for the toxin. Toxin molecules binding at low concentrations to these sites ultimately form hexamers and render the membrane permeable for monovalent ions. Proteins do not egress from the cells, as inferred from absence of LDH in the supernatants. The

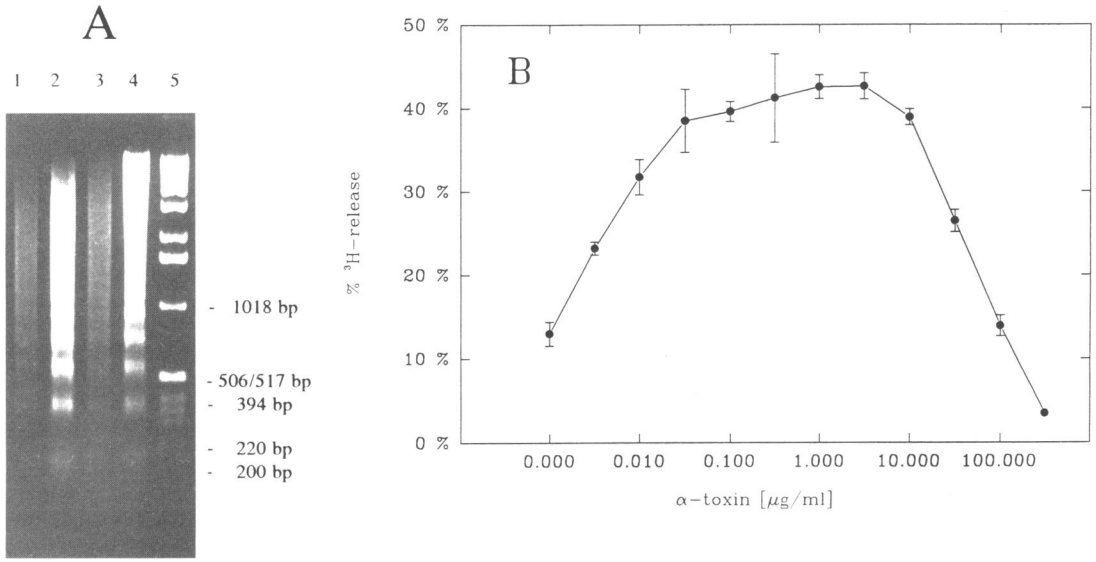


FIG. 10. (A) Electrophoretic analysis of DNA fragmentation in stimulated T cells. T cells (1.3×10^7) were stimulated with PHA and cultivated for 6 days in the presence of IL-2. They were then subjected to various treatments, and DNA was prepared 12 h thereafter. Lanes: 1, untreated control; 2, irradiation with 30 Gy; 3, 10 ng of alpha-toxin per ml; 4, 300 ng of alpha-toxin per ml; 5, DNA standard. The lengths of some fragments are indicated. (B) Dose-dependent induction of DNA fragmentation in T lymphocytes by alpha-toxin. T cells were activated with PHA and labeled with [^{3}H]thymidine. They were treated with alpha-toxin at the indicated concentrations, and release of [^{3}H]thymidine was measured after 4 h.

impairment of the intracellular ionic milieu apparently cannot be repaired; ATP levels drop, and the ability to incorporate [^{3}H]thymidine upon PHA stimulation is lost. These findings are all consistent with the hexamer pore concept of toxin action and allow lymphocytes to be added to the list of susceptible human cells.

The first unexpected finding related to the functional size of the pore. Collectively, all published data have been consistent with the notion that, once formed, the pores will permit free passage of calcium and even of small macromolecules such as ATP. In fact, this assumption underlies the widespread use of alpha-toxin as a membrane-permeabilizing agent (7, 8). Here, however, we observed a discrepancy between the easily documentable flux of monovalent ions across damaged membranes and the flux of $^{45}Ca^{2+}$. We were unable to detect significant association of $^{45}Ca^{2+}$ with cells permeabilized by low toxin doses. As the doses were increased to the range in which nonspecific binding may have occurred, calcium flux became detectable. Results obtained with another pore-forming toxin (*E. coli* hemolysin) indicated that the experimental method

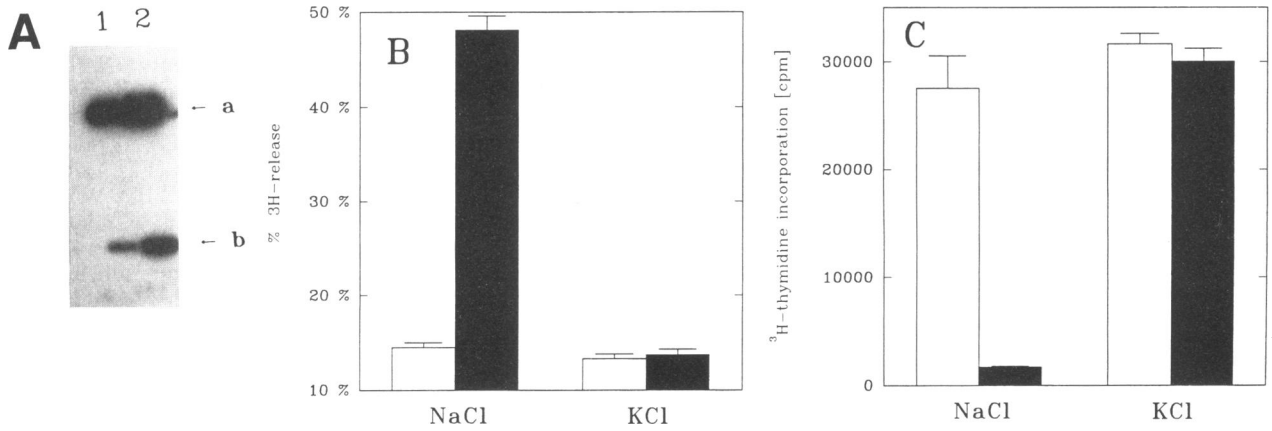


FIG. 11. Removal of Na^+ in culture medium prevents DNA degradation and cell death by alpha-toxin hexamers. Activated T lymphocytes were incubated in Na^+ and K^+ buffers, composed of 140 mM NaCl and 140 mM KCl, respectively, with 1.2 mM $CaCl_2$, 0.89 mM $MgCl_2$, 1 mg of glucose per ml, 1 μg of phenol red per ml, and 25 mM HEPES, pH 7.4. (A) Cells (4×10^6) were incubated in Na^+ (lane 1) or K^+ (lane 2) buffer with ^{125}I-alpha-toxin (300 ng/ml), and membranes were subjected to SDS-PAGE followed by autoradiography. Toxin hexamers (a) and monomers (b) are indicated. (B) Cells in Na^+ or K^+ buffer were incubated with 300 ng of alpha-toxin per ml (shaded bars) or without toxin (unshaded bars), and DNA fragmentation was measured after 4 h. (C) Cells were incubated as described in the legend to panel B. After 4 h they were spun down, resuspended in RPMI containing 10 U of IL-2 per ml plus 0.1 μCi of [^{3}H]thymidine per ml, and thymidine incorporation was determined after 16 h; data depict mean results and standard deviations (error bars) for triplicate determinations from one of two similar experiments.

itself was not at fault. Determination of propidium iodide influx extended these findings by showing that this small molecule did not penetrate the plasma membrane of most cells that had been exposed to low doses of alpha-toxin within the first 3 h. At this time, complete equilibration of K^+ and Na^+ and extensive reduction in ATP levels had occurred. Influx of propidium iodide was noted at much later time points, indicating either a gradual increase in the functional size of the pores over time or, more probably, simply a loss of membrane integrity in the dead cells.

We presently have no explanation for the discrepancy between the size of the alpha-toxin pores observed in lymphocytes and that of those observed in other experimental models. It should, however, be noted that a similarly detailed study on dose dependency of pore size in nucleated cells has only recently been undertaken with keratinocytes, for which similar findings were made (29). It seems possible that toxin molecules binding to the acceptor sites may generate small pores, whereas molecules binding via nonspecific absorption to the bilayer create larger membrane defects.

The finding of major interest relates to the question of whether pore formers can trigger programmed cell death. This idea was first advanced by Hameed et al. (15) on the basis of experiments utilizing perforin and commercial alpha-toxin. However, the cited results obtained for both these agents were probably incorrect because of contaminating DNases in the cytolysin preparations. Results from other investigators using pure perforin (10, 14), as well as our results reported herein for alpha-toxin, indicate that cells carrying transmembrane pores that are Ca^{2+} permissive die directly, probably because of massive Ca^{2+} influx and ATP leakage. No DNA degradation occurs, and programmed cell death plays no role in such cases.

The situation changes when alpha-toxin is applied at concentrations evoking formation of very small pores that are not Ca^{2+} permissive. These low doses were not utilized in the experiments of Hameed et al. (15). Somewhat ironically, genuine internucleosomal DNA degradation then occurs. The close correlation between induction of DNA degradation and creation of non-Ca^{2+}-permissive pores was established in the present study by detailed experiments using a variety of assays. Failure of cells to accumulate Ca^{2+} from the medium was faithfully reflected by their simultaneous inability to take up propidium iodide. Assessment of membrane permeability to propidium iodide could become a useful test to screen for small versus large membrane defects created by other pore formers.

An indication that DNA degradation may not be a trivial phenomenon stemmed from the finding that cells suspended in Na^+-free medium displayed neither DNA degradation nor loss of viability, despite hexamers being formed on the membranes. One possibility we are considering is that uncontrolled Na^+ influx could provoke leakage of Ca^{2+} ions from intracellular stores, this in turn leading to release and activation of relevant DNases (23). Flow cytometric analyses utilizing Fluo-3/AM yielded indications for release of Ca^{2+} from intracellular storage pools, and the concept of programmed cell death evoked by leakage of Ca^{2+} from mitochondria is supported by experimental data (24). The fact that alpha-toxin hexamers formed on T cells in the absence of extracellular Na^+ lack lethal effects if cells are transferred to Na^+-containing medium hours later indicates that nucleated cells have mechanisms for repairing such small membrane lesions. This issue will be addressed in a separate communication.

Since genuine programmed cell death is ATP dependent, it is understandable that transmembrane pores large enough to permit rapid leakage of the nucleotide should not cause apoptosis. This situation occurs when high doses of alpha-toxin are employed, or when pore-forming agents such as *E. coli* hemolysin or perforin are used, since both generate fairly uniform lesions exceeding 1 nm in effective diameter in target membranes. On the other hand, very small lesions that lead neither to flooding of cells with toxic levels of Ca^{2+} nor to loss of ATP may generally trigger programmed cell death. At this stage, attention is directed to a recent report by Ojcius et al. (21), who demonstrated that valinomycin and beauvericin, agents that selectively facilitate transmembrane movement of monovalent ions, induced apoptosis. Their and our results should now stimulate a search for other toxic agents that may similarly be killing cells by indirect activation of the suicide cascade. Such agents include not only toxins of microbial origin, but possibly any effector molecule of the immune system that creates small membrane permeability defects.

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