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Research Article

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Mechanism of Mammalian Cell Lysis Mediated by Peptide Defensins

Evidence for an Initial Alteration of the Plasma Membrane

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Abstract

Defensins induce ion channels in model lipid bilayers and permeabilize the membranes of *Escherichia coli*. We investigated whether similar membrane-active events occur during defensin-mediated cytolysis of tumor cells. Although defensin-treated K562 targets did not release chromium-labeled cytoplasmic components for 5–6 h, they experienced a rapid collapse (within minutes) of the membrane potential, efflux of rubidium, and influx of trypan blue. Defensin treatment also blunted the subsequent acidification response induced by nigericin, thereby further supporting the notion of enhanced transmembrane ion flow during exposure. These initial effects on the plasma membrane were not sufficient for subsequent lysis; a second phase of injury was required which involved the continued presence of defensin. The rapid membrane permeabilization phase was inhibited by azide/2-deoxyglucose, cytochalasin B, and increased concentrations of extracellular potassium and was unaffected by actinomycin-D, cycloheximide, and varying the calcium concentration. In contrast, the second phase was unaffected by cytochalasin B, inhibited by azide/2-deoxyglucose, enhanced by actinomycin D and cycloheximide, and varied with calcium concentration. These results indicate the initial adverse effect of defensins on mammalian cells occurs at the cell membrane. It is possible that the second phase of injury is mediated intracellularly by defensin that has been internalized through this leaky membrane. (*J. Clin. Invest.* 1991. 88:93–100.) Key words: cytotoxicity • membrane permeabilization • neutrophils • polymorphonuclear leukocyte granule proteins

Introduction

Neutrophils (PMNs) possess at least two mechanisms by which they can lyse mammalian target cells. One of these depends upon the production of toxic oxidative metabolites that are generated from the PMNs' respiratory burst (1, 2). However, a second mechanism, often detected during studies of PMN-induced antibody-dependent cellular cytotoxicity, is independent of the respiratory burst (3–5). This latter mechanism is less well understood, but some evidence (6, 7) suggests it is mediated by one or more toxic proteins released from PMN granules.

The most potent cytolytic proteins of PMN granules, at least for leukemia (8) and pulmonary (9) and endothelial (9) targets, are three small (mol wt 3,900) cationic peptides termed defensins or human neutrophil peptides 1, 2, and 3 (HNP 1–3).[1] The human defensins are secreted by activated PMNs (10) and could, therefore, function as cytotoxins during antibody-dependent cellular cytotoxicity. In addition, a synergistic cytotoxic effect occurs when target cells are exposed to a combination of defensins and toxic oxidants (8). It is, thus, conceivable that defensins may play a role in tissue injury seen during inflammatory or infectious processes.

To investigate the mechanism of defensin-induced injury, we have utilized continuously cultured tumor cells as model targets. In previous studies (11, 12), K562 cells exposed to defensins began to release radiolabeled chromium after a lag period of 3–4 h. Cytotoxicity depended upon energy metabolism and cytoskeletal activities of the target (12). These results suggested that HNP-mediated lysis was not due to a rapid creation of plasma membrane lesions and that endocytosis of HNP might have to occur before lysis could be realized. However, other evidence indicates defensins rapidly damage biological membranes. They are only active against enveloped viruses (13); they create ion channels in model lipid bilayers (14), and; their lethal effect on *Escherichia coli* is due to rapid sequential permeabilization of the outer and inner membrane (15). The results of the present study indicate the initial target structure for defensins on human tumor cells is also the plasma membrane. However, rapid membrane permeabilization, by itself, was insufficient for cell lysis. Ultimate target destruction also depended upon a second phase of injury which required the continued presence of HNP, thus explaining the lag in chromium release. The two phases of injury were independently regulated.

Methods

Tumor targets. The K562, Raji, L929, and YAC-1 lines were maintained in vitro by biweekly passage in RPMI media with 10% fetal calf serum (FCS, Reheis, Phoenix, AZ).

Reagents. Human defensins were purified from PMNs to homogeneity as previously described (9) by applying ion exchange and reverse-phase HPLC followed by gel exclusion chromatography on a long Bio-Gel P-10 column (Bio-Rad Laboratories, Richmond, CA). A mixture of the three defensins (HNP 1, 2, and 3 in a ratio of 2:2:1 by weight) was used in all experiments except in the binding assay where iodinated purified HNP-1 was used (see below). (2',7')-Bis-carboxy-ethyl)-(5,6)-carboxyfluorescein acetoxymethyl-ester (BCECF-AM) was purchased from Molecular Probes, Eugene, OR. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Rubidium release. Targets (10^5 in 0.1 ml) were incubated with 25 μCi of ^{86}RbCl in 0.1 ml of RPMI for 1 h and washed four times. Cells were then incubated with or without HNP in RPMI without FCS in

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1. *Abbreviations used in this paper:* HNP, human neutrophil peptide; TPP^+, tetraphenylphosphonium ion.

microtiter plates for varying durations. 4×10^4 targets were incubated per well. Plates were then centrifuged and 0.1 ml of cell-free supernatant was removed and assayed in a γ-counter. Percent specific release was calculated by: $(cpm_{exp} - cpm_{control}/cpm_{max} - cpm_{control}) \times 100$. Counts per minute-maximal was determined by lysis with detergent and control release was determined by incubating cells alone without HNP. Maximal release was always > 90% of incorporated counts and control release remained < 30% of incorporated counts during the first 35 min of incubation. Samples were run in quadruplicate and the standard deviation (SD) of replicates was always < 5% of the mean.

Chromium release assay. Targets were chromated as previously described (7) and then washed four times. Labeled targets (10^4 in 0.1 ml of RPMI) were incubated with or without defensin (in 0.1 ml of RPMI) in microtiter plates at 37°C for varying durations. After incubation, plates were centrifuged, 0.1 ml of supernatant was removed, and counted in a γ-counter. Percent specific chromium release was calculated as described above for specific ^{86}Rb release. Maximal (> 90% of incorporated counts) and control values (always < 25% of incorporated counts) were also determined as for ^{86}Rb release. In some experiments (see Tables II and IV), the chromium release assay was performed in 10 × 75 borosilicate test tubes with 10^5 chromated targets incubated per tube. The test tubes were centrifuged at 30 or 60 min, HNP-containing media was removed, and targets were resuspended for continuation of the assay. Both cell-free supernatant and pellet were then counted in a γ-counter. Simple percent chromium release (in contrast to specific chromium release) was calculated as: $cpm_{supernatant}/cpm_{supernatant} + cpm_{pellet}) \times 100$.

Protein synthesis. Protein synthesis was determined as previously described (12) by assaying [^{3}H]leucine (New England Nuclear, Boston, MA) incorporation into TCA-precipitable material. After pulsing with 2 μCi/well (10^5 targets per well) of [^{3}H]leucine for 1 h, cells were precipitated with 5% TCA, redissolved in 0.1 N NaOH, added to Aquasol (New England Nuclear), and counted in a scintillation counter.

Membrane potential measurements. Membrane potential of K562 cells was assayed by the uptake of [^{3}H]tetraphenylphosphonium ion ([^{3}H]Ph_4P^+, TPP^+) as previously described (16, 17). Cells (4×10^7/ml in 0.1 ml) were diluted in 0.9 ml of low K^+ buffer (118 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 20 mM Hepes, 9 mM Na_2CO_3, pH 7.4) or high-K^+ buffer (low-K^+ buffer in which 118 mM KCl replaced the NaCl) containing 20 μM [^{3}H]TPP^+ (0.125 Ci/mmol as bromide salt, New England Nuclear). Cells were equilibrated at 37°C with buffer and [^{3}H]TPP^+ for 20 min before addition of HNP. At varying intervals after adding HNP (or media as a control), 0.1 ml of cell suspension was filtered onto glass microfiber filters and the filters were washed with 5–10 ml of either high- or low-K^+ buffer, respectively. Filters were then transferred to vials containing scintillation fluid and counted in a beta scintillation counter. The values of [^{3}H]-Ph_4P^+ taken up by cells in high-K^+ buffer were used as background and were subtracted from values obtained from cells in low-K^+ buffer. The corrected values were converted into concentration using the determined K562 intracellular volumes and the concentration values were converted into millivolts of membrane potential by the Nernst equation. The cell volume of K562 targets was determined to be 6±1.5 μl/10^6 cells (mean and range) as determined by a cell counter (Coulter Corp., Hialeah, FL). The membrane potential of resting K562 cells remained constant over an incubation period of 60 min (incubation in RPMI).

Binding assay. The iodination of HNP-1 was performed as previously described (12). Viable targets, typically 5×10^5 in a final volume of 1 ml, were exposed to HNP-1 that had been trace-labeled with ^{125}I. Incubations were performed in triplicate in 1.5-ml conical polypropylene centrifuge tubes (West Coast Scientific, Berkeley, CA) that contained 0.4 ml of silicone oil (Versilube F50, General Electric Co., Waterford, NY). After incubation, the tubes were centrifuged for 90 s at ~ 12,000 *g* in an Eppendorf model 3200 microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY). Separation of cells from supernatant was complete within 30 s. After the supernatant and oil layers were removed, the tip of the tube was amputated with a razor blade, and the radioactivity of the pellet was assayed in a γ-counter (model 1191, Tracor, Inc., Austin, TX). The mean coefficient of variation for counts per minute from the triplicate samples was < 5%. Control experiments, with [^{14}C]sucrose as an extracellular fluid marker, confirmed that negligible quantities of supernatant fluid contaminated the tumor cell pellet by this technique. Binding experiments were performed in RPMI media. The trace-labeled HNP-1 induced chromium release from K562 cells at 6 h with comparable efficiency compared to native preparations. The percent HNP bound was determined after incubating ^{125}I-HNP with K562 cells for varying intervals at 37°C.

Measurement of intracellular pH. Measurements of intracellular pH (pH_i) were made as previously described (18). Briefly, the pH-sensitive dye BCECF was incorporated into K562 cells by incubating 10^7 cells with 2 μM BCECF-AM for 30 min at 37°C in a solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM sodium pyruvate, 10 mM Hepes, 5 mM glucose, and 0.1% bovine serum albumin (BSA). The solution was adjusted to pH 7.35–7.45 with saturated Tris base. Cells were then washed twice and resuspended in the same solution except that BSA was excluded. The dye signal was calibrated by releasing the dye from K562 cells with 50 μM digitonin and measuring the fluorescence at pH 6.1–8.2. A linear response was obtained in the pH range 6.3–8.0. Fluorescence was continuously recorded in a fluorescence spectrophotometer (model 650-40, Perkin-Elmer Corp., Norwalk, CT) with excitation and emission wavelengths of 500 and 530 nm and slits of 3 and 12 nm. Approximately 10^6 cells were used in each experiment. In preliminary experiments, the spontaneous release of BCECF-AM from resting K562 cells incubated in the buffer over 60 min was < 5%.

Assay for trypan blue internalization. Targets (10^5 in 0.1 ml) were incubated with or without defensins (in 0.1 ml) in microtiter plates. Assays were run in RPMI without FCS. After varying durations of incubation at 37°C, 20 μl of 0.4% trypan blue was added to each well and total viable (nonstaining) targets were counted by light microscopy.

Results

Early events of defensin-mediated injury. In previous studies (11, 12) with a chromium release assay, HNP-mediated lysis was first detected by 4 h and increased in linear fashion over the next 14 h. These kinetics suggested lysis was not due to the rapid creation of large plasma membrane pores as can be detected during exposure to perforin (19, 20). However, defensins can rapidly (within minutes) permeabilize the outer and inner membranes of *E. coli* (15) and form anion-selective channels in lipid bilayer model membranes (14). We, thus, more closely examined the early effects of HNP on the plasma membrane of K562 cells (Fig. 1). Within minutes, defensin significantly decreased the membrane potential of targets, induced the efflux of rubidium from prelabeled cells, and, after a short lag period of 10–15 min, permeabilized the cells for trypan blue (mol wt 960). Collapse of the membrane potential was maintained for at least 30 min (latest time point tested) and specific rubidium release increased linearly over the first 15 min. The internalization of trypan blue increased until ~ 25–30 min, reaching a plateau level where ~ 60% of cells were permeabilized. As we have previously reported (11), there was no detectable chromium release from HNP-treated targets until 4 h of incubation (Fig. 2). Thus, the early deleterious effects of defensin on the plasma membrane were significant enough to allow transit of small molecules but were insufficient for release of larger chromium-labeled cell components until much later time points.

When targets were incubated with defensin in the presence of 5% FCS, trypan blue internalization was abrogated (Table I) as was collapse of the membrane potential and specific rubid-

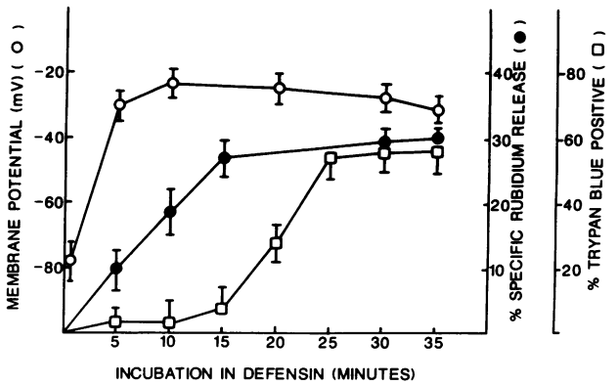

Figure 1. Early events induced by HNP. K562 cells were incubated with media or HNP (100 μg/ml) for varying durations. Membrane potential (○), percent specific rubidium release from prelabeled cells (●), and internalization of trypan blue (□) were assayed as described in Methods. The membrane potential of K562 cells incubated in media alone remained −75 mV±10 over the 35-min duration. Likewise, K562 cells incubated in media alone over 35 mins demonstrated < 5% trypan blue positivity. Results are mean±SD of five experiments.

ium release (not shown). This is probably due to the ability of serum albumin to bind HNP and protect targets (12). Loss of membrane integrity for trypan blue correlated with the concentration of HNP used and the temperature of the assay (Table I).

Further evidence of an initial membrane-localized effect of defensin was obtained from continuous recordings of the intracellular pH (pH_i) of K562 cells (Fig. 3). The pH_i of resting K562 cells was determined to be 6.95. When untreated targets were initially exposed to the potassium-hydrogen exchanger nigericin, a rapid acidification was detected (from pH_i 6.94–6.45 over 2 min) due to the efflux of potassium and influx of protons. However, if K562 cells were first incubated with HNP

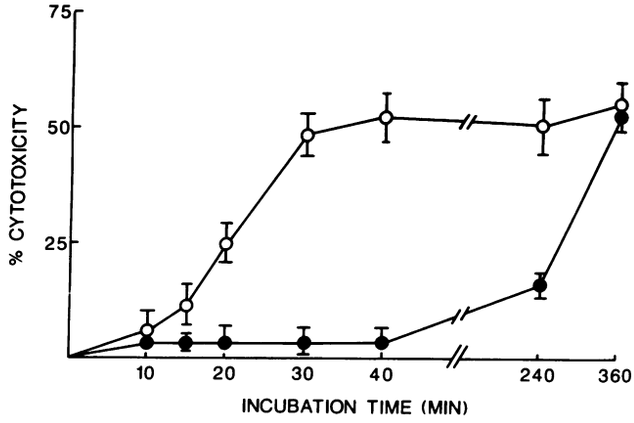

Figure 2. Comparison of kinetics of chromium release versus trypan blue internalization in HNP-treated targets. K562 cells were incubated with media or HNP (100 μg/ml) for varying durations and percent cytotoxicity in the chromium release assay (●) was compared to "percent cytotoxicity" in the trypan blue exclusion assay (○) and presented as mean±SD of five experiments. In the chromium release assay, percent cytotoxicity = percent specific chromium release (see Methods). In the trypan blue assay, percent cytotoxicity = (1− no. trypan-negative cells-experiment/no. trypan-negative cells-control) ×100.

*Table I. Serum, Temperature, and Concentration Dependence of HNP-mediated Permeabilization for Trypan Blue**

Concentration of HNP	Percent trypan blue positive		
	Media	Media + 5% FCS	Media + 4°C
0	5±1	4±0.5	2±0.5
10	7±1	5±1	4±0.7
25	18±2‡	4±1	3±0.4
50	34±3‡	6±2	4±1
100	48±5‡	5±0.5	4±1

* K562 cells were incubated with increasing concentrations of HNP or without HNP (control) for 30 min in RPMI media (37°C), media + 5% FCS (37°C), or RPMI media at 4°C. Results are the percentage of targets staining with trypan blue, mean±SD for four experiments.
‡ Significantly greater than control (HNP = 0), $P < 0.05$.

for 7 min and then exposed to nigericin, the subsequent decrease in intracellular pH was blunted (Fig. 3). The absolute change in pH was less marked and the acidification occurred at a slower rate (pH_i 6.65–6.4 over 3 min). The depressed acidification response to nigericin was also seen when the order of treatments was reversed (defensin-treated cells tested before non-treated cells). In addition, the blunted response in defensin-treated cells was detected when incubation with HNP was for 3 (earliest time tested) or 10 min. Also note in Fig. 3 the modest decrease in pH_i of K562 cells during the exposure to HNP. These data collectively suggest defensin rapidly induces proton influx and potassium efflux from targets, resulting in a lower intracellular potassium concentration, which, in turn, is responsible for the blunted acidification response to nigericin.

Effect of altering extracellular potassium concentration on HNP-induced permeabilization for trypan blue. In prior studies with model planar lipid bilayer membranes, HNP formed voltage-dependent ion channels (14). To test the voltage dependence of membrane channels that allow trypan blue internalization, we decreased the membrane potential of defensin-treated K562 cells by incubating them in the presence of increasing extracellular potassium concentration. Such treatment over 30 min in the absence of HNP did not result in any toxicity. However, when the extracellular potassium concentration was increased to 80 and 130 mM, targets were protected from HNP-mediated permeabilization for trypan blue (Fig. 4). Altering the

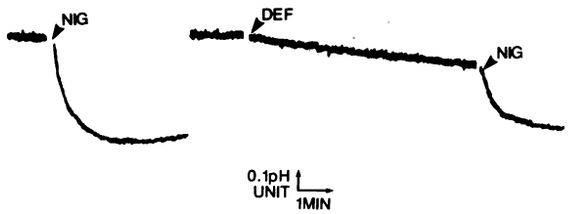

Figure 3. Blunted acidification response to nigericin in HNP-treated cells. Intracellular pH was continuously recorded in K562 cells loaded with BCECF as described in Methods. Nigericin (*NIG*, 1 μg/ml) and defensin (*DEF*, 100 μg/ml) were added as indicated. The figure compares the nigericin-induced acidification of control K562 cells (incubated in media) to K562 cells first incubated with defensin for 7 min. Similar results were obtained in two other experiments when defensin treatment was for 3 or 10 min.

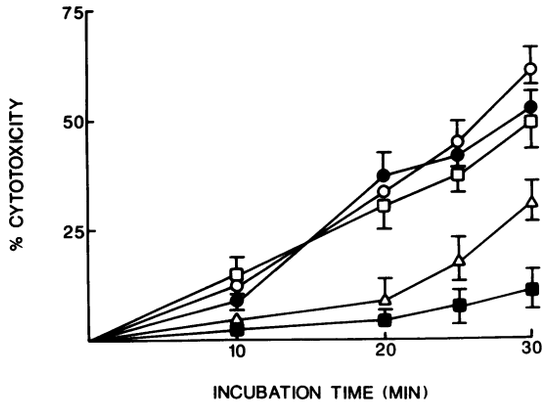

Figure 4. Modulation of HNP-induced permeabilization for trypan blue by altering the extracellular concentration of potassium. K562 cells were incubated with or without HNP (100 μg/ml) for 10, 20, or 30 min in buffer containing varying concentrations of KCl. At each time point, percent cytotoxicity was determined by the dye exclusion assay and calculated as described in the legend of Fig. 2. Results are mean±SD of three experiments. Extracellular concentration of KCl: ○, 5 mM; ●, 25 mM; □, 50 mM; △, 80 mM; ■, 130 mM.

extracellular potassium concentration did not change the binding of HNP to targets. The percentage of counts per minute bound after 30 min of incubation with HNP was 41±5% (mean±SD of three separate experiments) in 5 mM KCl, 45±3% in 25 mM KCl, 38±4% in 50 mM KCl, 43±4% in 80 mM KCl, and 48±6% in 130 mM KCl.

Initial membrane permeabilization in HNP-treated targets is not sufficient for subsequent cytolysis. To evaluate whether the early HNP-induced adverse effects on plasma membranes was sufficient for cell death detected as chromium release at 6 h, we incubated targets with or without 100 μg/ml of HNP for 30 or 60 min, assayed aliquots for trypan blue internalization, and then washed and resuspended the cell mixtures in HNP-free media for continued incubation to a total of 6 h at which time isotope release or trypan blue exclusion was determined (Table II). In agreement with Figs. 1 and 2, a significant percentage of defensin-treated targets were trypan blue positive relative to untreated controls at the 30- and 60-min time points (Table II, groups A and B). Defensin-treated cell mixtures that were washed and resuspended at 30 min did not demonstrate significant chromium release when assayed at 6 h (group C). Even extending the assays to 24 h (not shown in Table II) did not allow detection of isotope release which was greater than control groups (26% vs. 29%). In contrast, if targets were incubated with defensin for 60 min and then washed and resuspended, a significant increase in isotope release over control was evident at 6 h (group D, 29% vs. 14%). When incubated with defensin for the entire 6 h, targets demonstrated greater cytotoxicity (relative to controls) in both assays (group E). Thus, although incubation with HNP for 30 min results in loss of membrane integrity in about 60% of targets (trypan blue positivity), this effect is insufficient, by itself, to cause subsequent isotope release when assayed 5½ h later. A second critical phase of injury was required which depended upon the continual presence of HNP and which was initiated, at least in some targets, between 30 and 60 min of incubation.

In these experiments, we also noted an absence of cytotoxicity by dye exclusion when cells were treated with defensin for

*Table II. Loss of Membrane Integrity, by Itself, Is Insufficient for Lysis**

Group	Defensin	Duration of exposure to HNP	Percent recovery of cells not stained with trypan‡	Percent chromium release§
A	−	30 min	97±4	—
	+	30 min	42±5‖	—
B	−	60 min	95±2	—
	+	60 min	39±5‖	—
C	−	Wash at 30 min, assay 6 h	94±2	14±3
	+	Wash at 30 min, assay 6 h	90±3	16±2
D	−	Wash at 60 min, assay 6 h	93±3	14±2
	+	Wash at 60 min, assay 6 h	61±5‖	29±5‖
E	−	6 h	91±3	21±3
	+	6 h	37±5‖	50±6‖

* Targets were incubated with or without HNP (100 μg/ml) for 30 or 60 min, assayed for dye exclusion (groups A and B), washed, and resuspended in HNP-free media for a total incubation of 6 h at which time percent chromium release and dye exclusion (groups C, D, and E) were assayed. Data presented as mean±SD of three separate experiments.

‡ Percent recovery of viable cells (No. trypan-negative targets/total input of trypan-negative targets) × 100.

§ Percent chromium release ($cpm_{supernatant}/cpm_{supernatant} + cpm_{pellet}$) × 100.

‖ Significantly different ($P < 0.05$) from corresponding control group (no HNP).

30 min and then washed and resuspended and assayed at 6 h (percent recovery in group C). Since these same groups demonstrated a significant difference in trypan blue exclusion at the time of washing (97% vs. 42% viability at 30 min, group A), the data suggest that cells removed from defensin after 30 min and resuspended in fresh media are capable of repairing membrane damage.

Differential regulation of two phases of injury. To test the differential regulation of the two phases of injury, we used drugs that altered defensin-mediated lysis as detected in the chromium release assay at 6 h. In addition to cytochalasin B and the combination of azide and 2-deoxyglucose, which we previously showed (12) to protect targets against HNP-mediated lysis, we also studied the effects of actinomycin D, cycloheximide, and varying the concentration of calcium and magnesium. At the concentrations used, actinomycin D and cycloheximide each inhibited protein synthesis > 90% within 1 h of treatment but were not, by themselves, toxic to K562 cells. Table III demonstrates that both reagents significantly enhanced HNP-mediated cytotoxicity. Not only was lysis increased at two different concentrations of defensin, the kinetics of lysis were accelerated as significant cytotoxicity was evident by 2½ h.

The level of extracellular calcium also influenced defensin-mediated chromium release at 6 h. The addition of EGTA (5 mM) to mixtures of targets and defensin in complete media (calcium concentration = 0.4 mM), modestly but significantly

*Table III. Effects of Actinomycin D and Cycloheximide on HNP-induced Lysis**

Defensin	Reagent	Assay duration	Percent specific lysis
μg/ml		h	
40	—	2.5	0
40	Actinomycin D	2.5	24±3‡
40	Cycloheximide	2.5	29±4‡
40	—	6	24±2
40	Actinomycin D	6	47±5‡
40	Cycloheximide	6	42±5‡
100	—	6	46±4
100	Actinomycin D	6	63±5‡
100	Cycloheximide	6	69±4‡

* Chromated targets were incubated with defensin for 2.5 or 6 h in the presence or absence of actinomycin D (1 μg/ml) or cycloheximide (50 μg/ml). Data presented as percent specific lysis, mean±SD of three separate experiments.

‡ Significantly different ($P < 0.05$) from defensin-treated control in the absence of actinomycin D or cycloheximide.

increased cytotoxicity (68±3% specific chromium release at HNP = 100 μg/ml, $\bar{x}$±SD of three experiments) compared to assays run in media alone (50±5% specific release). EGTA did not induce chromium release when used alone (without HNP) and did not presensitize targets to enhanced HNP-mediated lysis when used in preincubation protocols (1 h of pretreatment followed by washing three times) indicating it did not cause an irreversible nonspecific effect on targets which leads to chromium release. These data suggested calcium protected defensin-treated targets. This was borne out by adding increasing concentrations of exogenous $CaCl_2$ to defensin-target mixtures. Cytotoxicity gradually diminished in a concentration-dependent fashion (Fig. 5). In contrast, identical concentrations of $MgCl_2$ had no effect on lysis.

Chelation of extracellular calcium also greatly accelerated the kinetics of HNP-mediated lysis. EGTA (5 mM) allowed

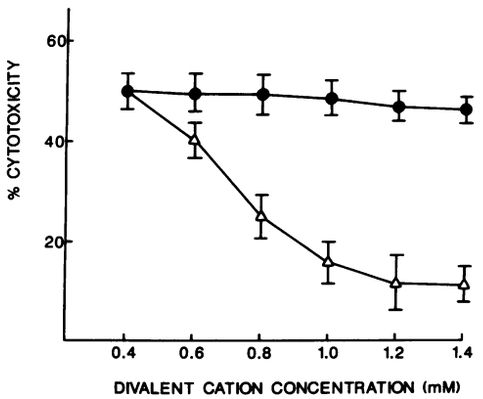

Figure 5. Effect of increasing concentration of $CaCl_2$ and $MgCl_2$ on HNP-induced target lysis. Chromated K562 cells were incubated in media (control) or HNP (100 μg/ml) in the presence of increasing concentrations of $CaCl_2$ (△) or $MgCl_2$ (●). After 6 h, percent cytotoxicity assayed. Percent cytotoxicity = percent specific chromium release presented as mean±SD of four experiments.

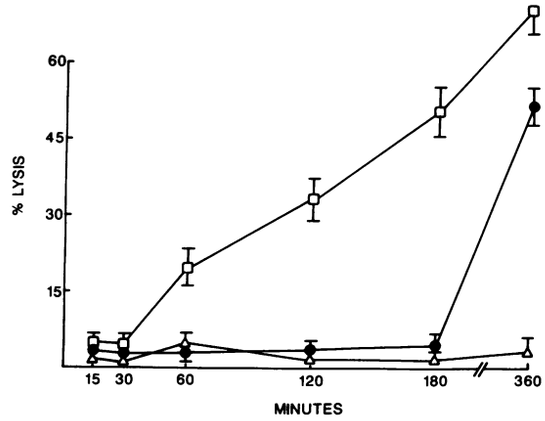

Figure 6. Effect of EGTA on kinetics of HNP-induced chromium release. Chromated K562 cells were incubated for 6 h with HNP (100 μg/ml, ●), EGTA alone (5 mM, △), or the combination of HNP and EGTA (□). Results are percent specific chromium release, mean±SD of three experiments.

significant cytolysis of defensin-treated targets as early as 60 min of incubation in contrast to the 4–6 h required for exposure to defensin alone (Fig. 6). These results further support the finding that a potentially lethal event is induced by HNP as early as 60 min after incubation.

The binding of radiolabeled defensin to K562 targets over a 1-h incubation was 58±5% of counts per minute bound (mean±SD of three experiments). The addition of cycloheximide (53±4% bound), actinomycin D (51±7%), 5 mM EGTA (57±4%), or 1.4 mM exogenous $CaCl_2$ (60±4%) did not significantly affect binding.

Modulation of the initial phase of HNP injury. As shown in Fig. 1 and Table II, the first phase of HNP-mediated injury plateaued by 30 min of incubation and the second phase was initiated between 30 and 60 min. The absence of overlap between the two phases allowed us to investigate their independent regulation.

Fig. 7 demonstrates that the combination of azide and 2-deoxyglucose as well as cytochalasin B were capable of protecting against defensin-induced loss of membrane integrity. In contrast, actinomycin D, cycloheximide, EGTA, and excess $CaCl_2$ had no effect. The drugs were used in concentrations that were not toxic, by themselves, to targets. It should also be noted that, in these experiments, targets were first exposed to drugs alone for 20 min and defensin was then added for 10, 20, 30, or 60 min more. It is certainly possible that the development of permeabilization would be sensitive to some reagents if longer incubations with the drugs were used. We utilized this protocol since the effects on defensin-mediated chromium release at 6 h also was determined by exposing cells to drugs for 20 min before addition of defensin.

Modulation of the second phase of defensin injury. To investigate the second phase of HNP-mediated injury, chromated targets were incubated with defensin or media (control) for 60 min, at which time HNP-containing media was washed away and targets were resuspended in media or various agents for continuation of the chromium release assay. Released isotope was assayed 5 h later (6-h chromium release assay). When targets were treated with defensin for 60 min followed by washing and resuspending in defensin-free media, significantly in-

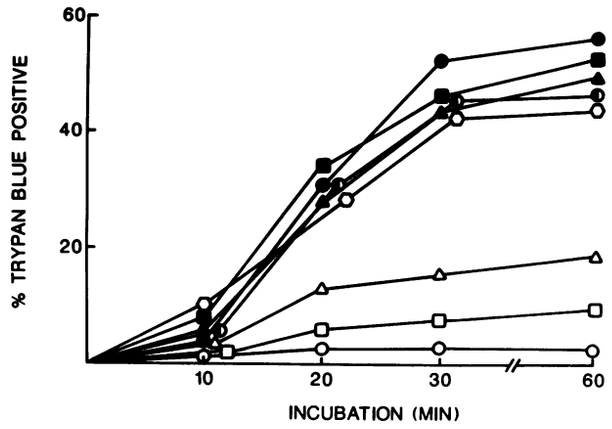

Figure 7. Modulation of the initial phase of HNP-induced injury. K562 cells incubated with various agents for varying intervals after which percent trypan blue-staining targets was enumerated. The experiment was repeated at least three times for each modulating agent and the results represent the means. The standard deviations, which are not shown, were always < 10% of the means. ○, media control; ●, HNP, 100 μg/ml; ■, HNP + EGTA (5 mM); ▲, HNP + $CaCl_2$ (1 mM); ◐, HNP + actinomycin D (1 μg/ml); ◇, HNP + cycloheximide (50 μg/ml); △, HNP + cytochalasin B (50 μM); □, HNP + sodium azide (5 mM)/2-deoxyglucose (10 mM).

creased chromium release over nontreated controls was evident at 6 h (group A, Table IV), which is consistent with the data presented in Table II. However, when HNP-treated cells were resuspended at 1 h in azide/2-deoxyglucose or excess $CaCl_2$ (groups B and E), cytotoxicity was prevented. Azide/2-deoxyglucose and excess calcium, thus, protect against the second phase of defensin-injury. If HNP-treated targets were resuspended at 1 h in cytochalasin B, resulting cytotoxicity at 6 h was unaltered (group C). However, resuspension at 1 h in EGTA, actinomycin D, or cycloheximide (groups D, F, and G) significantly enhanced cytolysis of defensin-treated targets while having little effect on control K562 cells incubated in media alone during the first hour. Thus, these latter reagents enhance the second phase of defensin-mediated injury.

Mechanism of resistance to defensin in L929 targets. We previously documented that murine L929 targets were quite resistant to the cytotoxic action of HNP when tested by chromium release at 6 h (12). Table V demonstrates that they are also resistant to early membrane permeabilization for trypan blue. In contrast, binding of radiolabeled defensin to L929 targets is comparable to sensitive YAC-1 and K562 cells.

Discussion

The results of this study indicate that, after binding of HNP to the target cell plasmalemma, small membrane channels that permit ion flux are rapidly created. Although we have not ruled out the possibility that the depressed acidification response to nigericin is due to an HNP-induced stimulation of cellular metabolism and a resulting increased acid load, the rapid collapse of the membrane potential and early leak of rubidium make it more likely that defensin induced proton influx and potassium efflux to account for those results. Moreover, these rapid changes were soon followed by permeabilization for trypan blue, further supporting the initial breach of membrane integrity. However, the breach is insufficient, by itself, for subsequent lysis (defined as chromium release at 6 h), as targets can be completely rescued by removing HNP-containing media at 30 min, a time when at least 50% are trypan blue-positive. A second phase of injury, which depends upon the continuing presence of HNP, is required for subsequent lysis and the two phases are independently regulated. The first is temperature dependent, dependent upon the extracellular concentration of potassium, inhibited by serum as well as the presence of azide/2-deoxyglucose and cytochalasin B, and unaffected by actino-

*Table IV. Modulation of the Second Phase of Injury**

Group	HNP	Resuspended at 1 h in	Percent chromium release‡	Percent specific lysis§
A	−	Media	12±2	—
	+	Media	26±4‖	15±2
B	−	Azide/2-deoxyglucose	15±3	—
	+	Azide/2-deoxyglucose	16±2	2±1
C	−	Cytochalasin B	14±2	—
	+	Cytochalasin B	28±3‖	14±2
D	−	EGTA	18±3	—
	+	EGTA	44±4‖	42±5
E	−	$CaCl_2$	9±2	—
	+	$CaCl_2$	12±2	3±1
F	−	Actinomycin D	14±3	—
	+	Actinomycin D	39±4‖	31±2
G	−	Cycloheximide	16±2	—
	+	Cycloheximide	43±3‖	39±3

* Chromated targets were treated with HNP (100 μg/ml) or media for 1 h and then washed twice and resuspended in media, azide/2-deoxyglucose (5 mM/10 mM), cytochalasin B (50 μM), EGTA (5 mM), $CaCl_2$ (1.2 mM), actinomycin D (1 μg/ml), or cycloheximide (50 μg/ml). 5 h later, percent chromium release and percent specific lysis were assayed. Data is mean±SD of three experiments.

‡ Percent chromium release ($cpm_{supernatant}/cpm_{supernatant}$ + cpm pellet) × 100.

§ Percent specific lysis = [percent chromium release-exp. group − percent release-control (1-h incubation without HNP)]/[percent maximal release (detergent) − percent release-control] × 100.

‖ Significantly different ($P < 0.05$) from corresponding control (1st h of incubation without HNP).

Table V. HNP-resistant L929 Targets Are Not Permeabilized by HNP‡§*

	K562		YAC-1		L929	
Time	Percent cytotoxicity	Percent binding	Percent cytotoxicity	Percent binding	Percent cytotoxicity	Percent binding
min						
20	24±2	32±4	25±3	27±2	2±1	30±3
40	51±4	50±3	55±5	42±3	1±1	48±5

* Different targets were incubated in HNP (100 μg/ml) or media for 20 or 40 min and percent cytotoxicity (by trypan blue exclusion) and percent binding of iodinated HNP were determined.

‡ Data presented as percent cytotoxicity (determined as described in Fig. 2), mean±SD of four experiments.

§ Data presented as percent of count per minutes bound (determined as described in Methods), mean±SD of four experiments.

mycin D, cycloheximide, or altering the calcium concentration. The second phase is inhibited by azide/2-deoxyglucose, enhanced by actinomycin D and cycloheximide, varies with calcium concentration, and is unaffected by cytochalasin B. The effects of cytochalasin B and azide/2-deoxyglucose are consistent with an earlier study (12) where we found that cytochalasin-sensitive events were complete by 1 h of incubation with HNP but azide/2-deoxyglucose continued to be inhibitory to lysis when added as late as 2 h after defensin.

Although membrane permeabilization is identical to membrane binding of HNP in its temperature, concentration, and serum dependence (12), they differ in that binding is independent of energy metabolism and cytoskeletal function (12). In addition, although resistant L929 cells were not permeabilized, their binding of I-HNP is comparable to that of sensitive targets (Table V). These data indicate binding can be dissociated from membrane permeabilization. On the other hand, the characteristics of permeabilization correlate closely with those of cytolysis at 6 h suggesting the initial membrane-active events are critical for lysis. The lack of HNP-induced permeabilization of L929 cells also correlates with the significant resistance of these targets to lysis, providing further support for the importance of the initial membrane injury. The absence of permeabilization in L929 cells may be due to a different biochemical structure of L929 cell membranes. Alternatively, since permeabilization is also determined by target-dependent processes, L929 metabolism may be singularly insufficient for generating membrane pores.

The initial permeabilization phase of injury was antagonized by interferring with target cell energy metabolism. Similar results were obtained when *E. coli* were exposed to HNP (15) as mitochondrial poisons prevented outer and inner membrane permeabilization. Defensin-induced ion channels in model lipid bilayer membranes are strictly voltage dependent (14). Defensin injury to the plasmalemma may, thus, be specific for an "energized membrane," providing one possible explanation for the energy dependence. The data presented in Fig. 4 also supports this hypothesis since increasing the extracellular potassium concentration, which would decrease the membrane potential, protected targets from defensin. However, an alternative explanation for the results of Fig. 4 is that potassium efflux is involved in the HNP-mediated permeabilization for trypan blue.

The voltage-dependent channels induced in model membranes by human and rabbit defensins rapidly close when the voltage across the membrane is reversed (14). If HNP-induced channels created in tumor cell membranes are similarly voltage-dependent, one might expect channel closure when the membrane potential initially collapses in the first 5–10 min. It is unclear how channels would then remain open to allow subsequent internalization of trypan blue. One possibility is that the target's cytoskeleton interacts with HNP to stabilize it in the membrane and ultimately render its channel voltage insensitive. This would explain the inhibition of defensin-mediated trypan blue permeabilization mediated by cytochalasin B. Alternatively, the defensin-induced membrane channels may become voltage insensitive over time without a requirement for an active participation by the target cell. In fact, although current can be completely turned off by reversing the voltage at early times after addition of HNP to model membranes, a voltage-independent current gradually develops (14).

An alternate explanation for the inhibition of permeabilization by cytochalasin B is that cytoskeletal processes may serve to enlarge the initial pore such that substances of at least 960 mol wt (that of trypan blue) can gain access to the target. Since cytochalasin B also protects targets against lysis, this hypothesis implies that the size of the membrane pore is critical for determining the ultimate fate of the target. A threshold pore size would determine if and when critical cellular metabolites are lost or whether defensin molecules (mol wt 3,900) could easily gain entry to the interior of the target where a subsequent "lethal hit" may take place. After permeabilization, subsequent lysis depends upon the continued presence of defensin molecules, supporting this latter notion. The inhibition of the second phase of injury with azide/2-deoxyglucose also suggests that an active lethal hit occurs after permeabilization rather than a simple loss of metabolites.

When K562 targets were resuspended in fresh media after a 30-min incubation in HNP, a time at which at least 50% were trypan-blue-stained, efficient repair of membrane damage occurred. Since actinomycin D and cycloheximide enhance HNP-mediated lysis through their effects on the second phase of injury, it is possible that de novo protein synthesis is required for membrane repair. Alternatively, protein synthesis may protect targets against defensin by other mechanisms.

The effects of altered calcium concentration on HNP-induced injury may also be due to modulation of membrane repair. Chelation of calcium enhanced and the addition of exogenous calcium (but not magnesium) depressed HNP-mediated chromium release. These alterations in calcium concentration did not affect the initial permeabilization but effectively modulated the second phase of injury. In similar fashion, the absence of calcium markedly enhances the lysis of mammalian cells exposed to the membrane pore-forming proteins staphylococcal-alpha toxin (21) and the terminal attack complex of complement (22). The increased sensitivity of cells to lysis in the absence of calcium may be due to an impaired repair mechanism (21, 22). For example, when the number of C5b-9 complexes generated on nucleated targets is limited, membrane channels can form that permit ion flux but do not result in subsequent cytolysis (23). Targets are capable of repairing limited numbers of pores by endocytosis of the transmembrane channels (23) and endocytosis is triggered by the influx of calcium (22). In the absence of calcium, the channels will persist and targets will be more likely to die. Protection of targets by subphysiological concentrations of calcium (as well as by serum proteins [11, 12]) suggest that HNP-mediated cytolysis of tumor cells might not occur in vivo.

The adverse effects of defensins on microbes is somewhat similar to the effects on tumor cells. First, HNP sequentially permeabilizes the outer and inner membranes of *E. coli* which is critical for bacterial death (15). Secondly, *E. coli* (15) and *Candida albicans* (24) are susceptible to defensins only when they are actively undergoing energy metabolism and mitochondrial poisons prevent outer and inner membrane permeabilization of *E. coli* (15). These results are similar to the protection seen when tumor cells are exposed to defensins in the presence of azide combined with 2-deoxyglucose. Third, divalent cations appear to protect against the microbicidal as well as cytocidal effects of defensins.

However, there are also differences between the activity of defensins on microbial versus tumor cell targets. Outer and inner membrane permeabilization was sufficient for the bactericidal effects (15) whereas an event subsequent to permeabiliza-

tion was required for tumor cell lysis. This may be due to a greater capacity of mammalian cells to repair membrane damage. Also, while both calcium and magnesium cations protect against candidacidal effects of HNP-1 (24), only excess calcium protects against lysis of tumor cells. Finally, excess calcium protects against outer and inner membrane permeabilization of *E. coli* (15) while it had no effect on the permeabilization of K562 membranes. Thus, the data suggest similarities in the initial interaction between HNP and outer membranes of microbes and tumor cells. However, the regulation of these events and the subsequent phases of injury differ.

The membrane-permeabilization characteristics of defensins are similar to other channel-forming proteins. Common to these peptides is the ability to form an amphiphilic complex although they may achieve this by different means. The bee toxin mellitin (25) and the antibacterial magainin peptides (26) are capable of forming alpha helices of an amphiphilic nature. In contrast, complement (27) and staphylococcal alpha toxin (28) are hydrophilic polypeptides that self-associate on target cells to form membrane-penetrating amphiphilic complexes. The amphiphilic topology of the nonhelical defensins appears to be conferred by alternating sequences of hydrophilic and hydrophobic amino acid residues (29). Defensin molecules may also self-associate on target membranes although we have no direct evidence for this. If so, such polymerization would be calcium and magnesium independent.

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