CONCISE REPORT

In Vitro Tumor Cell Cytolysis Mediated by Peptide Defensins of Human and Rabbit Granulocytes

By Alan Lichtenstein, Tomas Ganz, Michael E. Selsted, and Robert I. Lehrer

We examined the activity of defensins, cysteine-rich cationic peptides that are abundant in the cytoplasmic granules of human and rabbit granulocytes, against various tumor targets. The three human defensins, HNP-1, HNP-2, and HNP-3, lysed human and murine targets in chromium release and dye exclusion assays. Defensin-mediated tumor cell lysis was concentration-dependent, inhibited by serum, and dependent on temperature-sensitive events. Lysis was first detected by three hours of incubation and it reached a plateau between eight and 14 hours. In vitro exposure of murine teratocarcinoma cells to HNP 1-3 abrogated their oncogenicity in vivo. Nonmalignant target cells were also susceptible to defensin-mediated lysis. Four rabbit granulocyte defensins exerted marked (NP-1, NP-2) or moderate (NP-3a, NP-3b) cytotoxic activity, whereas defensin NP-5 was not cytotoxic. When tumor cells were incubated with human defensins in combination with hydrogen peroxide, synergistic cytotoxicity was detected. As defensins are released from granulocytes by various stimuli, their release could contribute to extracellular cytotoxicity which is independent of reactive oxygen intermediates.

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MATERIALS AND METHODS

Purification of defensins. Rabbit defensins NP-1, NP-2, NP-3a, NP-3b and NP-5, were purified by a modification of our previously described protocol. Briefly, 1 to 2 x 10^10 peritoneal PMNs were disrupted by homogenization, extracted five times with 10% acetic acid at 8 °C and centrifuged for 20 minutes at 27,000 x g. Supernatants were pooled, lyophilized, resuspended in 30 mL of 10% acetic acid and subjected to gel filtration on a 4 x 150 cm Bio-Gel P-10 column equilibrated in 5% acetic acid. Defensin-containing fractions were pooled, lyophilized, and then separated by cation-exchange high-performance liquid chromatography (HPLC) on a 21.5 x 150-mm Bio-Sil TSK-CM-3-SW column. The sample was dissolved and applied to the column, in buffer A (0.05 mol/L sodium phosphate, pH 6.7, 10% acetonitrile) and eluted by application of a linear gradient of buffer B (0.05 mol/L sodium phosphate, pH 6.7, 3.5 mol/L sodium chloride, 10% acetonitrile). A 0% to 50% gradient was developed over 80 minutes at a flow rate of 6 mL/min. Near-baseline separation of the individual peptides was obtained in this step. Final purification was achieved by reverse-phase HPLC as described previously. The human defensins HNP-1, HNP-2, and HNP-3 were obtained from normal PMNs and purified to homogeneity as previously described, by applying sequential ion-exchange and reverse-phase HPLC followed by gel exclusion chromatography on a long Bio-Gel P-10 column. Lyophilized defensins were dissolved in 0.01% acetic acid at 1 mg/mL and stored at -20 °C until used.

Tumor lines. Suspension cultures of human (Raji, MOLT-4, K562, IM-9, U-937, and WIL-2) and murine (P815, YAC-1) lines were maintained in RPMI medium with 10% fetal calf serum (FCS, Reheis, Phoenix, AZ). The MOT teratocarcinoma was maintained in ascites form in C3H mice. The YAC-R15, U9TR, and U9NR16 lines were gifts from Dr S. Wright. YAC-R and U9NR, cloned from parental YAC-1 and U937 lines, had been rendered specifically resistant to lysis by natural killer cytolytic factor (NKCF). U9TR had been rendered specifically resistant to lysis by tumor necrosis factor (TNF).

Chromium release assay (CRA). Targets (10^6) were incubated with 50 μCi ₅¹Cr for one hour at 37 °C in RPMI-10% FCS. Labeled targets (10^6 in 0.1 mL of RPMI-1640) and the specified defensins (0.1 mL of RPMI) were incubated in microtitre plates at 37 °C in 5% CO₂. Subsequently, the plates were centrifuged (1,200 rpm x 10 min) and 0.1 mL of supernatant was counted in a gamma counter.

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Percent lysis was calculated as: 
\[
\frac{(cpm_{exp} - cpm_{control})}{cpm_{maximal} - cpm_{control}} \times 100. 
\]
Control release was determined from targets incubated in media alone (<35% incorporated counts), and maximal release was achieved by adding 1 mol/L HCl (>80% incorporated counts). Release from targets incubated in RPMI containing dilute acetic acid (the vehicle used in defensin stock solutions) did not differ from controls incubated in RPMI alone. The standard deviation (SD) of triplicate samples was always <5% of the mean.

Dye exclusion assay. Targets (10^6) in 0.1 mL RPMI were incubated with 0.1 mL of defensins or in medium alone (control wells). Samples were run in quadruplicate in microtitre plates. The plates were incubated in 5% CO2 at 37 °C and surviving targets were counted by trypan blue exclusion. Percent cytotoxicity was calculated as: (mean no. of viable targets in control wells) – (mean no. of viable targets in test wells)/(mean no. targets in control wells) × 100.

Cytoxicity was also strictly dependent upon temperature-sensitive events (optimal lysis at 37 °C; no lysis at 25 °C).

Although their lysis was always significant, murine lines were consistently less sensitive than human lines as targets (Table 1). The order of sensitivity of human lines shown in Table 1 (WIL-2 > IM-9 > RAJ1 > K562 > MOLT-4) was consistently present in each of five experiments. Variant tumor lines, specifically resistant to NKCF (YAC-R, U9NR) or TNF (U9TR) were equally as sensitive as their respective parent lines to HNP-mediated lysis. Exposure of MOT cells to HNPs also destroyed their in vivo oncogenicity. Mice inoculated with tumor cells (10^4 or 10^5/group; eight mice/group) incubated in control media all died within 35 days after a period of progressive ascites. In contrast, MOT cells exposed to 100 μg/mL of HNPs 1-3 caused tumor outgrowth in three of eight mice injected with 10^4 MOT cells, and in none of eight injected with 10^5 cells.

Significant chromium release was not detected until three hours of incubation and rose to very high levels by eight hours (Fig 1). Whereas a plateau was reached in eight hours for some targets, lysis of others continued to increase linearly until 14 hours of incubation. Spontaneous release exceeding 35% precluded assaying groups past 14 hours of incubation.

Both dye exclusion and CRA confirmed HNP-mediated lysis of nonmalignant targets. Murine thymocytes and spleen cells as well as human lymphocytes and PMNs were sensitive to lysis and this was comparable to corresponding tumor cells (data not shown).

To examine the correlation between lysis and net charge of the defensins, each of the human peptides were tested separately and compared to similarly purified rabbit PMN peptides (Table 2). Within each species, the degree of lysis roughly correlated with the overall antimicrobial effects of the individual peptides, but there was little correlation with cationic charge. Note that although rabbit NP-5 was more cationic than any of the human defensins, it was

<table>
<thead>
<tr>
<th>Table 1. Lysis of Various Tumor Lines by Defensins*</th>
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<tr>
<td><strong>Concentration of Defensins (mcg/mL)</strong></td>
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<tr>
<td>Human</td>
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<tr>
<td>WIL-2 94 ± 4 (98 ± 4)</td>
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<tr>
<td>IM-9 88 ± 4.1</td>
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<tr>
<td>RAJ1 80 ± 4</td>
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<tr>
<td>K562 54 ± 3 (91 ± 5)</td>
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<tr>
<td>MOLT-4 48 ± 2.4</td>
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<tr>
<td>U937 46 ± 2.7</td>
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<tr>
<td>U9NR 44 ± 3.1</td>
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<tr>
<td>U9TR 49 ± 4</td>
</tr>
<tr>
<td>Mouse</td>
</tr>
<tr>
<td>YAC-1 35 ± 4.2</td>
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<tr>
<td>YAC-R 38 ± 2.5</td>
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<tr>
<td>MOT 30 ± 2.6</td>
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<td>P815 27 ± 1.7</td>
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*Tumor cells incubated with varying concentrations for six hours; specific lysis determined by CRA; figures in parentheses denote cytotoxicity, determined in dye exclusion assay, for WIL-2 and K562; mean ± SD of five separate experiments.

†Tumor line resistant to NKCF.

‡Tumor line resistant to TNF.
DEFENSIN-MEDIATED TUMOR LYSIS

Fig 1. Kinetics of lysis by defensins. Tumor lines incubated with defensins for one, three, eight, or 14 hours and chromium-release assayed. O — O, WIL-2; A — A, IM-9; O — O, RAJI; ■ — ■, MOLT-4; □ — □, K562.

Fig 2. Synergistic lysis of tumor cells. Chromium-labeled K562 targets incubated for four hours with H2O2 (10^{-7} to 10^{-2} mol/L), defensins (20 or 40 \mu g/mL) or both agents. Percent lysis determined after incubation and presented as mean ± SD of quadruplicate samples.

Ineffective as a lytic agent. Also, NP-2 is more lytic than NP-3a and NP-3b and carries the same charge.

Although a cationic charge is not sufficient to induce lysis, CRAs performed in the presence of the polyanion substance heparin suggest that net charge plays a role in the detected cytolyis. Defensin-mediated lysis decreased by 63% and 92% in the presence of 1 U/mL and 10 U/mL heparin, respectively.

Since defensins might be secreted by PMNs in tandem with H2O2, we next examined possible interactions between these two potential cytolyisins. When K562 targets are incubated with reagent grade H2O2 in combination with 20 or 40 \mu g/mL of HNPs 1-3, tumor lysis is significantly greater than the sum of that achieved by either agent alone (Fig 2). This was most evident between H2O2 concentrations of 10^{-3} and 10^{-4} mol/L. Sodium azide (1 mmol/L) did not prevent synergistic killing, ruling out the possibility that the HNP preparation was contaminated with trace amounts of myeloperoxidase that could have reacted with H2O2 and augmented cytolyis.

DISCUSSION

Several studies have demonstrated that PMNs can lyse target cells by mechanisms not involving ROI production.

| Table 2. Comparison of Lysis by Individual Rabbit and Human Defensins |
|-----------------------------|-----------------------------|-----------------------------|
| Peptide | Net Charge | % Specific lysis*  |
|       | | 100 mcg/mL | 25 mcg/mL |
| Human | | | |
| HNP-1 | +3 | 55.0 ± 2.3 | 27.0 ± 2.8 |
| HNP-2 | +3 | 43.5 ± 2.3 | 26.2 ± 2.4 |
| HNP-3 | +2 | 22.5 ± 2.7 | 22.3 ± 2.6 |
| Rabbit | | | |
| NP-1 | +9 | 84.3 ± 3.2 | 70.1 ± 2.6 |
| NP-2 | +8 | 82.7 ± 2.6 | 68.3 ± 3.9 |
| NP-3a | +8 | 26.7 ± 0.7 | 9.5 ± 1.0 |
| NP-3b | +8 | 36.5 ± 3.5 | 16.1 ± 1.3 |
| NP-5 | +4 | 5.1 ± 1.1 | 0.9 ± 1.1 |

Individually purified human or rabbit defensins, 25 or 100 mcg/mL, were incubated with ^{31}Cr-labelled K562 targets for eight hours.

*Specific lysis is shown as the mean ± S.D. of three separate experiments. Net cationic charge at pH 7.4 was calculated from the published amino acid sequences of the defensins (8, 9), as follows: Net charge = \{(arginine + lysine residues) - (glutamic acid + aspartic acid residues)\}.

The present study demonstrates that defensins, recently characterized antimicrobial peptides of human and rabbit PMNs, can damage various normal and tumor cell targets in vitro. Defensin-mediated lysis was confirmed in three independent assays: isotope release, dye exclusion and in vivo oncogenicity. The presence of cytotoxic components such as defensins in the cytoplasmic granules of the PMN echoes the presence of other cytotoxic constituents in natural killer (NK) lymphocytes and certain macrophages. It is possible, but yet proven, that secretion of cytotoxic principles such as HNPs from PMNs underlies their ability to lyse adjacent targets. In addition, the data of Fig 2 suggest that small amounts of defensins may act synergistically with H2O2 to achieve heightened tumor lysis.

The mechanism of lysis is not yet known. The temporal delay in its onset (Fig 1) and its prominent temperature dependence suggest that more than a simple detergent effect is operative. The small size of the defensins excludes their functioning as enzymes such as the cytolytic serine protease reported from cytotoxic macrophages. The undiminished sensitivity of tumor cells resistant to NKCF or TNF suggests that the mechanism differs qualitatively from that induced by those two cytokotoxins.

Other cationic proteins are often cytotoxic. In addition, the presence of the polyanion heparin prevented HNP-mediated lysis, suggesting that charge of these molecules plays a role in target cytotoxicity. However, Table 2 indicates that the lytic effect of HNPs is not solely due to their cationicity as there was a poor correlation between charge and cytotoxicity of the individual defensins.

In contrast to the effects of rabbit macrophage defensins, the human defensins did not show selective cytotoxicity for malignant targets. The use of less potent cytotoxic material in a longer assay may have allowed the detection of selective cytotoxicity. In addition, the prerequisite for antibody-directed effector: target binding in PMN-mediated antibody-dependent cellular cytotoxicity (ADCC), as well as the tendency of inflammatory PMNs to bind to neoplastic
targets more effectively than to nonmalignant targets, even in the absence of antibody, could impart target specificity to an otherwise nonspecific molecular effector. Serum markedly inhibited the ability of HNPs to effect cytotoxicity. We attribute this to avid binding of HNPs by albumin and other macromolecular serum components (M. Selsted, unpublished data, April 1986). This suggests that HNP-mediated lysis might only occur if tight binding of targets to PMNs creates a contact zone that excludes macromolecular serum components. This possibility is currently under investigation.

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