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 10^9 peritoneal PMNs were disrupted by homogenization, extracted five times with 10% acetic acid and centrifuged for 20 minutes at 27,000 x g. Supernatants were pooled, lyophilized, and applied to the Bio-Gel P-2 column, in buffer A (0.05 mol/L hydrogen phosphate, pH 7.0, 10% acetonitrile) and subjected to gel filtration 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 HL-60) and murine (P815, YAC-1) lines were maintained in RPMI medium with 10% fetal calf serum (FCS, sera, Phoenix, AZ). The MOT teratocarcinoma was maintained in ascites form in C3H mice. The YAC-R1, U9TR, and U9NR lines had been rendered specifically resistant to lysis by tumor necrosis factor (TNF).

Chromium release assay (CRA). Targets (10^5) were incubated with 50 μCi 51Cr for one hour at 37°C in RPMI-10% FCS. Labeled targets (10^4 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: \[
\frac{\text{cpm}_{\text{sample}} - \text{cpm}_{\text{blank}}}{\text{cpm}_{\text{blank}}} \times 100.
\]
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 (1x10^6) 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 presence of 1% FCS decreased HNP-mediated lysis by CRAs in Table 1 were performed in the absence of serum. Assays, wherein lysis was achieved with optimal concentration (SD) of triplicate samples was always <5% of the mean.

**Oncogenicity assay.** MOT cells (2 x 10^5) were incubated in 5 mL of RPMI with 0.01% acetic acid (control) or in RPMI with 100 mcg/mL of HNPs 1-3. After eight hours in 5% CO_2 at 37 °C, significant isotope release was achieved by adding 1 mol/L HC1 (>80% incorporated counts), and maximal release was precluded assaying groups past 1 4 hours of incubation. Spontaneous release exceeding 35% 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 assays past 14 hours of incubation.

**Use of nonmalignant cells as targets.** Murine thymocytes and spleen cells were obtained from adult C3H mice as previously described.17 Peripheral blood PMNs and lymphocytes were harvested from gradients as previously described.18 Viability of targets was >90%.

**RESULTS**

Tumor lines incubated with a mixture of the three human defensins (HNP 1, 2, and 3 in a ratio of 2:2:1 by weight) for six hours at 37 °C demonstrated significant isotope release (Table 1). Tumor lysis was concentration-dependent and optimal lysis was achieved with 25 to 100 mcg/mL. Table 1 also demonstrates the lytic potency of HNPs in dye-exclusion assays, wherein cytotoxicity (in parentheses) was also concentration-dependent for WIL-2 and K562 targets. The CRAs in Table 1 were performed in the absence of serum. The presence of 1% FCS decreased HNP-mediated lysis by 30% and the presence of 5% FCS decreased it by 75%. Lysis 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. Mouse inoculated with tumor cells (10^6 or 10^7/mice/group) incubated in control media all died within 35 days after a period of progressive ascites. In contrast, MOT cells exposed to 100 mcg/mL of HNPs 1-3 caused tumor outgrowth in three of eight mice injected with 10^6 MOT cells, and in none of eight injected with 10^7 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 assays past 14 hours of incubation.

Table 1. Lysis of Various Tumor Lines by Defensins*

<table>
<thead>
<tr>
<th>Target</th>
<th>Concentration of Defensins (mcg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>WIL-2</td>
<td>94 ± 4 (98 ± 4)</td>
</tr>
<tr>
<td>IM-9</td>
<td>88 ± 4.1</td>
</tr>
<tr>
<td>RAJ1</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>K562</td>
<td>54 ± 3 (91 ± 5)</td>
</tr>
<tr>
<td>MOLT-4</td>
<td>48 ± 2.4</td>
</tr>
<tr>
<td>U937</td>
<td>46 ± 2.7</td>
</tr>
<tr>
<td>U9NR†</td>
<td>44 ± 3.1</td>
</tr>
<tr>
<td>U9TR‡</td>
<td>49 ± 4</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>YAC-1</td>
<td>35 ± 4.2</td>
</tr>
<tr>
<td>YAC-R†</td>
<td>38 ± 2.5</td>
</tr>
<tr>
<td>MOT</td>
<td>30 ± 2.6</td>
</tr>
<tr>
<td>P815</td>
<td>27 ± 1.7</td>
</tr>
</tbody>
</table>

* 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; ▲—▲, IM-9; ●—●, RAJI; ■—■, K562; □—□, MOLT-4.

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 μg/mL) or both agents. Percent lysis determined after incubation and presented as mean ± SD of quadruplicate samples.

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 unproven, 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.21 The undiminished sensitivity of tumor cells resistant to NKCF or TNF suggests that the mechanism differs qualitatively from that induced by those two cytotoxins.

Other cationic proteins are often cytotoxic.22 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,14 the human defensins did not show selective cytotoxicity for malignant targets. The use of less potent cytotoxic material in a longer assay14 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

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**Table 2. Comparison of Lysis by Individual Rabbit and Human Defensins**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Net Charge</th>
<th>% Specific lysis</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100 mg/mL</td>
<td>25 mg/mL</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HNP-1</td>
<td>+3</td>
<td>55.0 ± 2.3</td>
</tr>
<tr>
<td>HNP-2</td>
<td>+3</td>
<td>43.5 ± 2.3</td>
</tr>
<tr>
<td>HNP-3</td>
<td>+2</td>
<td>22.5 ± 2.7</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP-1</td>
<td>+9</td>
<td>84.3 ± 3.2</td>
</tr>
<tr>
<td>NP-2</td>
<td>+8</td>
<td>82.7 ± 2.5</td>
</tr>
<tr>
<td>NP-3a</td>
<td>+8</td>
<td>26.7 ± 0.7</td>
</tr>
<tr>
<td>NP-3b</td>
<td>+8</td>
<td>36.5 ± 3.5</td>
</tr>
<tr>
<td>NP-5</td>
<td>+4</td>
<td>5.1 ± 1.1</td>
</tr>
</tbody>
</table>

Individually purified human or rabbit defensins, 25 or 100 mcg/mL, were incubated with ¹⁵⁶Cr-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)].
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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