Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idotype and carrier antigens

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Keywords: antibody formation, cytokine, IFN-γ, IL-4, tumor antigen

Abstract
Defensins released by neutrophils are able to kill a broad spectrum of microbes. They also induce leukocyte migration in vitro and elicit inflammatory leukocyte responses at s.c. injection sites in mice. In vitro experiments showed that human defensins enhanced concanavalin A-stimulated murine spleen cell proliferation and IFN-γ production. This led us to examine the effects of human defensins on specific immune responses in vivo. BALB/c mice were immunized with 50 µg of keyhole limpet hemocyanin (KLH) adsorbed to aluminum hydroxide and administered with defensins in aqueous solution. Intraperitoneal administration of defensins significantly increased the production of KLH-specific IgG1, IgG2a and IgG2b antibodies 14 days after immunization. In vitro splenic KLH-specific proliferative responses were higher in mice treated with KLH and defensins than in those treated with KLH alone. Increased IFN-γ and, to a lesser extent, IL-4 production were also detected in the supernatants of ex vivo KLH-activated spleen cells from mice treated with defensins. Finally, defensins significantly enhanced the antibody response to a syngeneic tumor antigen, lymphoma Ig idiotype and also augmented resistance to tumor challenge. These results indicate that defensins act as potent immune adjuvants by inducing the production of lymphokines, which promote T cell-dependent cellular immunity and antigen-specific IgG production. Thus, defensins appear to function as neutrophil-derived signals that promote adaptive immune responses.

Introduction
Neutrophils have been recognized as the first line of host defense in infections. In the course of inflammation, neutrophils utilize oxygen-dependent and -independent mechanisms to kill ingested microbes, the latter pathway involving the discharge of antibiotic proteins and proteases present in neutrophil granules into phagosomes (1,2). Neutrophils also produce and release into the extracellular milieu several antimicrobial proteins including defensins and CAP37/azurocidin by the process of degranulation (3). Defensins constitute a family of cationic antimicrobial proteins that are stored in the cytoplasmic granules of neutrophils, some macrophages and intestinal Paneth cells (4,5). In human neutrophils, defensins comprise 30–50% of the granule proteins (6,7). Defensins have the ability to kill a broad spectrum of microbes including Gram-negative and -positive bacteria, fungi, and selected enveloped viruses. In addition to their antimicrobial activity, defensins have been reported to induce human monocyte migration in vitro (8). Recently we showed that both human defensins and CAP37/azurocidin purified from neutrophil granules are chemoattractants for resting...
human T cells in vitro and in vivo in mice (9). This suggests that defensins may play a role in the communication between cell types involved in innate or natural resistance such as neutrophils, and those responsible for adaptive immunity such as T and B cells. To analyze this further we examined whether and how well defensins could act as adjuvants with immunoenhancing effects.

Experiments were performed by immunizing BALB/c mice with keyhole limpet hemocyanin (KLH) adsorbed to aluminum hydroxide with or without injections of defensins. Immunization with protein adsorbed to aluminum hydroxide induces T cell-dependent immune responses and favors a T_{H2}-mediated humoral immunity. Such an approach has been used to examine antigen-specific cellular and humoral responses and adjuvant activity (10–13). IL-12 is known to induce differentiation of T_{H1} cells and the development of cell-mediated immune response (14,15). Since IL-12 was shown to be a potent adjuvant primarily by inducing synthesis of T_{H1}-related Ig production (11), we used IL-12 as a control in this study.

Methods

Reagents

KLH was obtained from Calbiochem (La Jolla, CA). Lipopolysaccharide (LPS; Escherichia coli; 055:B5) and concanavalin A (Con A) were purchased from Sigma (St Louis, MO). Murine IL-12 (sp. act. 4.6×10^{6} U/mg) was supplied by Genetics Institute (Cambridge, MA). Defensins were purified to homogeneity from granules of neutrophils obtained from normal donors as reported previously (9). Amino acid sequence analysis of the preparation revealed the presence of three related sequences; human neutrophil peptide (HNP)-1 which has Ala as the N-terminal residue (50%), HNP-3 which has Asp as the N-terminal residue (20%), and HNP-2 which is shorter than HNP-1 and HNP-3 by one N-terminal residue (30%). No other sequences were detected. The results of amino acid analysis corresponded well with amino acid composition of defensins suggesting that the preparation contained >95% defensins. Moreover, no protein contaminants were detected by matrix-assisted laser desorption/ ionization and time-of-flight (MALDI-TOF) mass spectroscopy. Endotoxin level in purified defensins was <0.1 endotoxin unit/20 µg of protein as measured by chromogenic Limulus amebocyte lysate assays. In some experiments the activity of defensin preparation was compared with a preparation of synthetic defensin (HNP-1) from American Peptide Company (Sunnyvale, CA).

Idiotypic IgM (38C13-Id) was rescued by somatic cell hybridization as described previously (16), and 38C13-Id and a control IgM(k) were purified from ascites and coupled to KLH using 0.1% glutaraldehyde.

Animals

Female BALB/c and C3H/HeN MTV− mice were obtained from the Animal Production Area (National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, MD), and were used at 8–12 weeks of age.

Immunization

Mice were immunized i.p. once on day 0 with 50 µg of KLH adsorbed to aluminum hydroxide dissolved in 0.2 ml of PBS containing 0.1% normal mouse serum. Some mice also received i.p. either 0.01 to 1 µg of human defensins or murine IL-12 dissolved in 0.2 ml of PBS containing 0.1% normal mouse serum. Control mice were injected i.p. with PBS containing 0.1% normal mouse serum. Fourteen days after KLH injection, mice were sacrificed, and sera and spleens were obtained. All experiments were performed with three mice per group and were performed 3–4 times with a representative experiment being shown. Mice were immunized i.p. with 50 µg 38C13-Id–KLH or control IgM–KLH in PBS in a volume of 0.2 ml as indicated, with or without defensins.

Measurement of KLH-specific antibodies

Serum levels of KLH-specific antibodies were measured by ELISA (17). Briefly, 96-well flat-bottom plates were coated with 10 µg/ml of KLH in carbonate buffer (0.1 M NaHCO_{3}, pH 9.6) for 2 h and washed 3 times with PBS containing 0.05% Triton X-100. This washing buffer was used for washing at all steps. The plates were then incubated with a blocking buffer (5% dry milk in PBS) for 1 h to saturate non-specific binding sites. Serial 5-fold dilutions of the sera in PBS + 2% BSA (Sigma) were added for 1 h followed by a 30 min incubation with isotype-specific biotinylated anti-mouse antisera (The Binding Site, San Diego, CA). After washing 5 times, streptavidin–peroxidase (The Binding Site) in PBS + 2% BSA was added for 30 min. The plates then were washed 7 times, the substrate ABTS (Kirkegaard & Perry, Gaithersburg, MD) was added, and the absorbance was determined at 405 nm with a reference wavelength of 490 nm.

Determination of phenotype of peritoneal cell population

Peritoneal cells of mice immunized with 50 µg of KLH (on day 0) and injected with 1 µg of defensins for five consecutive days were collected on day 14, and cell phenotyping was performed by using anti-mouse antibodies to B220, CD3, CD4 and CD8. Cells were analyzed with a FACScan flow cytometer to determine the percentage of positive cells.

Assays of lymphokine production and proliferation

Single cells prepared from spleens obtained from immunized mice were cultured in 24-well plates at a density of 2×10^{5} cells/ml in RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, 19 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO_{2}. KLH at 50 µg/ml was added to the cultures. The supernatants were harvested after 48 h, and tested for concentrations of IFN-γ and IL-4 by specific ELISA kits (IFN-γ, Genzyme, Cambridge, MA/IL-4; Endogen, Boston, MA). Sensitivity limits of IFN-γ and IL-4 were 27.0 and 5.8 pg/ml respectively. Triplicate cultures of 5×10^{5} spleen cells/well were cultured with KLH or medium alone in a final volume of 0.1 ml RPMI 1640 supplemented with 10% FBS, 4 mM glutamine, 19 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin in 96-well microculture plates at 37°C in 5% CO_{2}. After 5 days, cell proliferation was measured by colorimetric assay using MTT (Sigma) according to the standard method (18).

To determine whether defensins activate normal murine
spleen cells, we examined the in vitro effect of defensins on the proliferation of mitogen-stimulated spleen cells. Triplicate cultures of $5 \times 10^6$ spleen cells obtained from untreated mice were incubated with Con A, LPS or medium along with various doses of defensins in 96-well microculture plates at 37°C in 5% CO₂. After 3 days, the cell proliferation was measured by MTT assay. Spleen cells prepared from untreated mice were incubated with Con A along with various doses of defensins in 24-well plates at a density of $2 \times 10^6$ /ml of RPMI 1640 supplemented with 10% FBS at 37°C in 5% CO₂. After 48 h, culture supernatants were harvested and assayed for IFN-γ by ELISA.

**Cell staining and flow cytometric analysis**

Single cells from spleens and peritoneal cells obtained from immunized mice were counted and adjusted to $1 \times 10^6$/ml. The cells then were incubated with the appropriate directly labeled antibodies. The antibodies used were: goat anti-mouse IgM from Fisher Scientific (Pittsburgh, PA), B220 (6B2; directed against a murine B cell marker) from Pharmingen (San Diego, CA), CD4 (L3T4; Pharmingen) and CD8 (Ly-2; Pharmingen). After incubation the cells were washed and then fixed with 1% paraformaldehyde, and the cells were analyzed using an Epics flow cytometer (Coulter, Hialeah, FL).

**Measurement of serum anti-idiotypic antibody**

As described previously (19), mouse serum was serially diluted over microtiter plates coated with affinity-purified 38C13-Id. Binding of antibodies in the serum to 38C13-Id was detected by horseradish peroxidase-conjugated goat anti-mouse IgG antibodies. Serum anti-idiotypic antibody levels were determined by comparing sera titration curves with a standard curve obtained with a known concentration of a mixture of purified monoclonal anti-idiotypic antibodies. Antibody levels are expressed in µg/ml serum for individual mice. In each ELISA, sera obtained from mice immunized with control IgM–KLH were included as negative controls. Such sera never showed any titratable binding activity on 38C13-Id.

**Tumor model**

The carcinogen-induced 38C13 B cell lymphoma has been previously described (20). The 38C13 tumor secretes and expresses IgM(k) on the cell surface. Inoculation with as few as $10^5$ tumor cells i.p. into normal syngeneic C3H/HeJ mice results in uniformly progressive tumor growth and death of the host. Tumor cells from a common frozen stock were passaged in vitro for 3 days before use.

**Statistical analysis**

The statistical significance of the experimental differences between test and control groups was determined by Student's t-test or Mann-Whitney test as indicated. Significance was defined at the $P < 0.05$ level. For tumor challenge experiments mice were checked daily to determine the date of death. Statistical comparisons of survival curves were performed using BMDP IL software (BMDP Statistical Software, Los Angeles, CA) to generate non-parametric Mantel–Cox log-rank $P$ values. Mice surviving >90 days after tumor challenge were euthanized and were reported as long-term survivors.

**Results**

**In vitro effect of defensins on mitogen-stimulated spleen cells**

To determine whether human defensins could activate murine immune cells, we examined the in vitro effect of defensins on the proliferation and lymphokine production by murine normal spleen cells. Defensins were not mitogenic by themselves, but they enhanced the proliferation of Con A-stimulated murine spleen cells in a dose-dependent manner. Defensins did not increase proliferation of LPS-stimulated cells (Fig. 1A). Defensins also enhanced IFN-γ production by Con A-stimulated murine spleen cells (Fig. 1B). These results suggest that human defensins can co-stimulate normal murine T cells.

**Defensins up-regulate anti-KLH IgG1, IgG2a and IgG2b antibody responses in vivo**

Previously reported data revealed that mouse serum anti-KLH antibody levels usually are maximal from 7 days to at least 5 weeks after injection of a single dose of KLH adsorbed to aluminum hydroxide (10). We therefore chose to assay the antibody response 14 days after KLH injection and determined KLH-specific Ig subclasses by ELISA (Fig. 2). Mice injected once with KLH alone exhibited relatively high serum levels of IgG1 antibody and low levels of IgG2a and IgG2b. Administration of 1 µg of defensins i.p. daily from day –1 to day 5 significantly ($P < 0.05$) augmented the level of KLH-specific IgG1, IgG2a and IgG2b antibodies in the serum at day 14.

We also examined the effects of the number of daily defensin injections on the production of specific antibodies. As shown in Fig. 3, a significant ($P < 0.05$) up-regulation of production of KLH-specific IgG2a and IgG2b antibodies by defensins was observed in mice injected with defensins for at least 3 days. Thus, defensins can act as adjuvants that promote humoral immune responses in vivo. In addition, we compared the ability of purified defensins and a preparation of synthetic defensin (HNP-1) to enhance anti-KLH IgG response. The preparations of natural and synthetic defensins both significantly augmented the production of anti-KLH IgG (in comparison with PBS) (Fig. 4). There was no difference in stimulatory activity between ‘natural’ and synthetic defensin. These data show the synthetic defensin to have biological activity and argue against a role for contaminants in the purified defensin preparation.

**Comparison of the immunoenhancing effects of defensins and IL-12**

It is well established that IL-12 promotes the development and activation of Tₛ Th1 cells (14,15), resulting in the production of high levels of IgG2a and IgG2b antibody subclasses (10–12). Figure 5 shows the effects of various doses of defensins or IL-12 on the production of KLH-specific Ig subclasses. The administration of defensins enhanced the synthesis of KLH-specific IgG1, IgG2a and IgG2b antibodies in a dose-dependent manner. In contrast to defensins, IL-12 markedly inhibited KLH-specific IgG1 antibody production. On the other hand, IL-12 injection up-regulated the synthesis of KLH-specific IgG2a antibody to a significantly ($P < 0.05$) greater extent than in mice treated with defensins. IL-12 and defensin administration enhanced KLH-specific IgG2b antibody production to a similar extent. The pattern of antigen-specific Ig
Defensins promote Ig responses in vivo

Fig. 1. The in vitro effect of defensins on the proliferation and IFN-γ production by mitogen-stimulated spleen cells. (A) Spleen cells from untreated mice were incubated with 1 µg/ml of Con A or LPS along with various concentrations of neutrophil defensins. After 3 days, proliferation was measured by colorimetric MTT assays. (B) Spleen cells from untreated mice were incubated with 1 µg/ml of Con A along with various concentrations of defensins. After 48 h, culture supernatants were harvested and assayed for IFN-γ by ELISA. Data show means and SD of values in triplicate cultures. Statistically significant differences *P < 0.05 and **P < 0.01 relative to cells treated with Con A alone.

Production induced by IL-12 was identical to those previously reported for this selective activator of Th1 cells (11,12). Defensins, by contrast, enhanced Ig subclasses that are presumed to be augmented by both Th1 and Th2 cytokines. Administration of both IL-12 and defensin together had additive immunoenhancing effects (data not shown).

Cytokine production by spleen cells

Cytokines released by activated T cells promote the development of antibody responses. We therefore examined the influence of defensins on cytokine production by ex vivo KLH-activated spleen cells from immunized mice. As shown in Table 1, relatively low levels of IFN-γ were produced by spleen cells of mice treated with PBS or KLH alone. By contrast, there was ~3-fold up-regulation of IFN-γ production by spleen cells from mice treated with 0.1 and 1 µg of defensins when compared with the response of mice treated with KLH alone. Spleen cells from mice treated with KLH alone produced significantly (P < 0.05) higher levels of IL-4 when compared with the value of non-immunized mice. The IL-4 synthesis was further increased by spleen cells from mice treated with a low dose (10 ng) of defensins (P < 0.05). However, spleen cells from mice treated with the highest dose (1 µg) of defensins had a decreased capacity to produce IL-4 (P < 0.05). Based on reports that in mice IFN-γ and IL-4 favor the induction of IgG2a and IgG1 respectively (21–23), our results indicate that defensins enhanced the ability of spleen cells to produce antigen-specific IFN-γ, a Th1-type cytokine, while the effect of defensins on the production of antigen-specific IL-4, a Th2 cytokine, varied depending on concentrations administered.

Effect of defensins on proliferation of spleen cells in response to KLH

KLH-specific in vitro proliferation of spleen cells from immunized mice was determined by colorimetric MTT assays (Fig. 6). There was a dose-dependent increase in the proliferation of spleen cells from mice treated with 0.01–1 µg of defensins along with KLH when compared with those treated with KLH alone.

The effects of defensins on splenic and peritoneal cell subpopulations

In order to determine the effect of defensins on various subsets of immune cells, spleen cells were stained with mAb specific for IgM, B220, CD4 and CD8. There was no increase over controls in the total number of spleen cells of mice treated with KLH alone or KLH plus defensins by day 14 (Table 2). Treatments with KLH plus 0.1 or 1 µg of defensins resulted in increased numbers of IgM+ and B220+ cells (P < 0.05). The number of CD4 and CD8 T cells was significantly (P < 0.05) lower in spleens from mice treated with KLH and 1 µg of defensins than in spleens treated with KLH alone (data not shown). Figure 7 shows a representative result in which a higher percentage of B220+/surface IgM+ cells was observed in the spleen from mice treated with KLH and 1 µg of defensins when compared with mice treated with KLH alone. Consequently administration of defensins along with KLH increased both the splenic B cell populations and anti-KLH antibody levels. FACScan analysis of peritoneal cells of mice immunized with KLH also demonstrated a significant increase in mature (B220+) positive B lymphocytes in mice injected with 1 µg of defensin at 14 days. Moreover, synthetic defensin induced a significant increase of B220+ cells, although at lesser extent than defensin purified from neutrophils (Table 3).

Defensins enhance antibody responses to a syngeneic tumor antigen and tumor idiotype-specific resistance

To evaluate the potency of defensins, we tested the ability of defensins to enhance the antibody response to a less...
Defensins promote Ig responses in vivo

Fig. 2. Serum KLH-specific IgG1, IgG2a and IgG2b antibodies of mice treated with neutrophil defensins or PBS (control). Mice were immunized i.p. with 50 µg of KLH on day 0. 1 µg of defensins was injected i.p. for 7 days from day –1 to day 5. On day 14, KLH-specific antibodies in sera were assayed by ELISA. Data show means and SD of values for three mice. Statistically significant differences *P < 0.01 and **P < 0.05 relative to control mice.

Immunogenic syngeneic tumor antigen. The unique amino acid sequences comprising the variable regions of Ig receptor heavy and light chains on clonal B cell malignancies can serve as antigenic determinants (idiotypes) and thus as tumour-specific antigens (24,25). In the 38C13 lymphoma model, syngeneic Ig isolated from this tumor is completely non-immunogenic. However, immunization with Id conjugated to KLH (Id–KLH) elicits idiotype-specific antibodies and this humoral response is sufficient to mediate resistance to lymphoma challenge (26). Mice were given a single immunization with Id–KLH i.p., with or without doses of defensins, ranging from 0.01 to 1 µg/day, administered concurrently for 7 days (day –1 to day 5). Control animals received a single immunization with a control IgM–KLH alone. In the experiment shown in Fig. 8, serum samples were collected from four to five randomly selected individual mice per group 2 weeks after immunization. Baseline antibody levels elicited by Id–KLH immunization alone (24 ± 5.4 µg/ml; mean ± SEM) were significantly (P < 0.05) enhanced by the addition of defensins at doses of 0.1 and 1 µg (mean 349 ± 156 and 270 ± 114 µg/ml respectively). The specificity of the induced antibodies for 38C13 idiotype was demonstrated by the lack of binding of these sera to a control IgM protein (data not shown). Ten mice per group were also challenged i.p. with viable 38C13 lymphoma cells from a single preparation obtained at time zero. A tumor dose as low as 100 cells is lethal in 100% of control immunized mice. A single immunization with Id–KLH produces resistance against subsequent challenge with this minimum lethal dose and previous studies have established the magnitude of the challenge dose (20). Therefore, in this experiment a dose of 10^4 tumor cells, 100 times the minimum lethal dose, was used to allow detection of improvements in survival afforded by the addition of defensins. Compared with Id–KLH alone, significantly prolonged survival was observed for mice which received defensins at the 1 µg dose (Table 4, group 1; range 18–>90 versus 18–19 days).
**Defensins promote Ig responses in vivo**

**Fig. 3.** Effect of number of daily injections of neutrophil defensins on KLH-specific IgG2 production. Mice were immunized i.p. with 50 µg of KLH on day 0. Defensins (1 µg) was injected i.p. for 1 day (day 0), 3 days (day –1 to 1), 5 days (day –1 to 3) or 7 days (day –1 to 5). Control mice were injected with PBS alone. On day 14, KLH-specific antibodies in sera were assayed by ELISA. Data show means and SD of values for three mice. Statistically significant differences \(^* P < 0.05\) and \(^{**} P < 0.01\) relative to mice treated with KLH alone.

**Fig. 4.** Different preparations of defensins augment KLH-specific IgG1 production. Mice were immunized with 50 µg of KLH (on day 0), injected i.p. with PBS, neutrophil defensins (1 µg) or synthetic defensin (HNP-1, 1 µg) daily for 5 consecutive days. On day 14, KLH-specific IgG in sera were assayed by ELISA. Scatter graph shows data for 10 mice in each group. Data were analyzed by Mann–Whitney test, the two-tailed \(P\) value was determined (<0.015 for synthetic defensin and <0.003 for purified defensins compared to mice injected with PBS).

**Fig. 5.** Comparison of serum KLH-specific IgG1, IgG2a and IgG2b antibody levels of mice treated with different doses of defensins or IL-12. Mice were immunized i.p. with 50 µg of KLH on day 0 and treated i.p. with various doses of neutrophil defensins or IL-12 or PBS alone for 7 days from day –1 to day 5. On day 14, KLH-specific antibodies in sera were assayed by ELISA. The results are shown as the ratio of OD to that of mice treated with KLH alone. Data show means and SD of values for three mice. Statistically significant difference \(^* P < 0.05\) and \(^{**} P < 0.01\) relative to mice treated with KLH alone. Statistically significant difference \(^{†} P < 0.05\) relative to mice treated with KLH and defensins.
Defensins promote Ig responses in vivo

Table 1. Antigen-specific cytokine release by mouse spleen cells

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>IFN-γ</th>
<th>IL-4</th>
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<tr>
<td>PBS</td>
<td>154 ± 34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>KLH</td>
<td>294 ± 161</td>
<td>97 ± 34&lt;sup&gt;id&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH + defensins (0.01 µg)</td>
<td>531 ± 365</td>
<td>196 ± 46&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH + defensins (0.1 µg)</td>
<td>1133 ± 405&lt;sup&gt;c&lt;/sup&gt;</td>
<td>144 ± 44</td>
</tr>
<tr>
<td>KLH + defensins (1 µg)</td>
<td>834 ± 95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21 ± 9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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<sup>a</sup>Spleen cells prepared from mice were incubated in vitro in 24-well plates at a density of 2 x 10⁶/ml in RPMI 1640 supplemented with 10% FBS at 37°C in 5% CO₂. The cultures were incubated with 50 µg/ml of KLH. After 48 h, cultures supernatants were harvested, and assayed for IFN-γ and IL-4 content by ELISA.

<sup>b</sup>Data show means ± SD for three mice. IFN-γ and IL-4 levels are expressed as pg/ml.

<sup>c</sup>Significantly different from the value of mice treated with KLH alone (P < 0.05).

<sup>d</sup>Significantly different from the value of mice treated with PBS alone (P < 0.05).

Table 2. IgM<sup>+</sup> and B220<sup>+</sup> cells in mouse spleens

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>Total cells (x 10⁷)</th>
<th>IgM&lt;sup&gt;+&lt;/sup&gt; cells (x 10⁶)</th>
<th>B220&lt;sup&gt;+&lt;/sup&gt; cells (x 10⁷)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9.3 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>KLH</td>
<td>9.9 ± 0.9</td>
<td>3.6 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>KLH + defensins (0.01 µg)</td>
<td>8.7 ± 2.0</td>
<td>3.3 ± 0.6</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>KLH + defensins (0.1 µg)</td>
<td>10.6 ± 1.3</td>
<td>4.3 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.2 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>KLH + defensins (1 µg)</td>
<td>10.6 ± 0.8</td>
<td>4.5 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Spleen cells were prepared from mice with PBS or KLH along with various doses of defensins or PBS on day 14 after KLH treatment. Splenic IgM<sup>+</sup> cells and B220<sup>+</sup> cells were evaluated by flow cytometric analysis as described in Materials and Methods.

<sup>b</sup>Data show means ± SD for three mice.

<sup>c</sup>Significantly different from the value of mice treated with KLH alone (P < 0.05).

**Fig. 6.** Effect of defensins on antigen-specific proliferation of spleen cells. Spleen cells from mice immunized with 50 µg of KLH along with PBS alone or various doses of neutrophil defensins were incubated with various concentrations of KLH for 5 days in vitro. Proliferation was measured by colorimetric MTT assays: ○, PBS; ▲, 0.01 µg defensins; ■, 0.1 µg defensins; ●, 1 µg defensins. The results are calculated as: OD (after in vitro culture in the presence of KLH) – OD (after in vitro culture in the absence of KLH). Data show means ± SD of values for three mice.

Furthermore, one long-term, tumor-free survivor was observed in this defensin-treated group. The small increase in survival among mice receiving lower doses of defensins with Id–KLH, compared with Id–KLH alone, was not statistically significant.

**Discussion**

Taub et al. showed that human neutrophils stimulated in vitro with IL-8 released a granule-derived factor(s) that induces in vitro human T cell and monocyte chemotaxis (27). This suggested that neutrophils could release chemoattractants that mediate T cell accumulation at sites of inflammation, and led to the biochemical identification of defensins and CAP37/azurocidin released by degranulation from IL-8-stimulated neutrophils as the T cell chemoattractants (9). Moreover, it has been reported that depleting rat neutrophils by treatment with anti-neutrophil antiserum (28,29) or suppression of neutrophil activities by infusion of anti-IL-8 mAb reduced subsequent delayed-type hypersensitivity responses (30). These results suggest that neutrophil-derived peptides may
Defensins promote Ig responses in vivo

Defensins play an important pathophysiological role in promoting T cell-dependent immune responses by activation of T cells. Our results demonstrate that neutrophil-derived defensins have potent immunoadjuvant activity promoting antigen-specific Ig responses and tumor immunity. This is reinforced by data showing that intranasal administration of human defensins to mice enhances the systemic antibody responses to co-administered ovalbumin (31).

IL-12, induced in the course of innate host response, is reported to act as an adjuvant by promoting specific cell-mediated immunity reactions (13,32–35). IL-12 is a known inducer of Th1 development but an inhibitor of Th2 differentiation as evidenced by increased Th1-type antibodies (IgG2a/IgG2b) and decreased Th2-type antibody (IgG1) (10–12). In this study we confirmed that IL-12 up-regulated KLH-specific IgG2a and IgG2b antibody subclasses, but down-regulated KLH-specific IgG1 subclass. In contrast, human defensins increased the production of KLH-specific murine IgG1, as well as IgG2a and IgG2b subclasses in vivo. The pattern of synthesis of Ig subclasses stimulated by defensins suggests that defensins up-regulate antigen-specific Ig synthesis mediated by both Th1 and Th2 dependent pathways.

Our study showed that there was a significant increase in B cell number, the ability of B cells to produce particular Ig subclasses is also dependent on cytokines produced by CD4 T cells (10,32,36–38). IFN-γ and IL-4 play pivotal roles in regulating Ig isotype switching.

**Fig. 8.** Neutrophil defensins augment the antibody response to lymphoma-derived Ig idiotype. Shown are individual serum anti-idiotypic IgG levels elicited by a single Id–KLH immunization i.p. on day 0 with or without concurrent administration of defensins. Defensins were administered i.p. at the doses indicated on day −1 through day 5. Random mice in each group were bled 2 weeks later and individual anti-idiotypic IgG levels were determined as described. Bars indicate the mean IgG levels of four to five mice.

**Table 3.** Defensins induce an increase in B cell infiltration in the mouse peritoneal cavity

<table>
<thead>
<tr>
<th>In vivo treatment</th>
<th>% CD3</th>
<th>%B220</th>
<th>%CD4</th>
<th>%CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLH + PBS</td>
<td>88.2 ± 9.0b</td>
<td>11.8 ± 8.8</td>
<td>70.0 ± 6.9</td>
<td>30.0 ± 6.0</td>
</tr>
<tr>
<td>KLH + neutrophil defensins</td>
<td>69.2 ± 8.9</td>
<td>30.8 ± 8.9c</td>
<td>69.7 ± 6.5</td>
<td>30.3 ± 3.5</td>
</tr>
<tr>
<td>KLH + synthetic defensin</td>
<td>73.5 ± 2.7</td>
<td>26.5 ± 4.3c</td>
<td>63.5 ± 7.8</td>
<td>36.5 ± 2.8</td>
</tr>
</tbody>
</table>

aMice were immunized with 50 µg of KLH with or without defensins (1 µg) i.p. (day 0). Defensins (1 µg) were consecutively injected i.p. (days 1–4). On day 14 peritoneal exudates were collected and phenotyping of cell populations was performed. Cells were analyzed using a FACScan flow cytometer to determine the percentage of positive cells.

bData shows means ± SD for four mice.

cSignificantly different from the value of mice treated with KLH + PBS (P < 0.01).

**Table 4.** Defensins induce tumor idiotype-specific resistance to syngeneic lymphoma in mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunogena</th>
<th>Dose of defensins (µg/day×7 days)b</th>
<th>Survival times (range in days)c</th>
<th>Log-rank P values versus Group 4</th>
<th>P values versus Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Id–KLH</td>
<td>1</td>
<td>18–&gt;90d</td>
<td>0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2</td>
<td>Id–KLH</td>
<td>0.1</td>
<td>15–47</td>
<td>0.47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>Id–KLH</td>
<td>0.01</td>
<td>18–27</td>
<td>0.14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>Id–KLH</td>
<td>0</td>
<td>18–19</td>
<td>–</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>5</td>
<td>Control IgM-KLH</td>
<td>0</td>
<td>13–18</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

aTen mice per group were given a single immunization with 50 µg of Id–KLH i.p. on day 0.

bDefensins were administered i.p. once per day, days −1 through +5.

cMice were challenged i.p. with 10⁸ 38C13 lymphoma cells two weeks after immunization with Id–KLH with or without defensins.

dSurvival times of individual mice (days): 18,18,18, 20, 20, 20, 22, 23, >90.
Defensins into murine skin does not cause any effects indicative of toxicity or tissue damage such as swelling, redness and ulcer formation (9). These observations indicate that the concentrations of defensins used in this study are not toxic for mice.

The tissue distribution of α-defensins is species dependent. They have been isolated from human, rabbit, guinea pig and rat neutrophils, but murine neutrophils lack detectable α-defensins (46). Nevertheless α-defensins (cryptdins) are expressed in mouse skin and intestinal Paneth cells (47,48). Apparently, our study shows that human defensins can act across a species barrier and stimulate Con A-induced proliferation and IFN-γ production by murine spleen cells in vitro.

The basis for the adjutant activity of defensins has been further elucidated by several recent findings. We have observed that human α-defensins are chemotactic for the subset of human CD45RA+ resting naive T cells and immature dendritic cells. Furthermore human β-defensin-1 and -2 chemotactically human CD45RO+ resting memory T cells and immature dendritic cells by activating the CCR6 chemokine receptor (49). Thus α- and β-defensins are capable of recruiting of antigen-presenting dendritic and T cells, and thus provide a basis for the immunoenhancing effects of defensins. We are currently engaged in identifying the receptor for α-defensin.

The potential value of novel immunological adjuvants can be assessed by their ability to enhance immune responses to inherently weak, syngeneic antigens. In this regard, lymphoma-derived Ig idiotype coupled to KLH can serve as a model of a weaker antigen since this antigen by itself induces low immune response to the Ig idiotype tumor antigen.

Our results clearly demonstrated the ability of human α-defensins to augment the antibody response to syngeneic murine idiotype by ~15-fold over baseline levels, compared with antigen alone. Furthermore, this enhanced humoral response was associated with significantly prolonged survival after challenge of mice with a dose of tumor representing 100 times the minimum lethal dose.

In conclusion, this study shows that α-defensins up-regulate antigen-specific Ig production in vivo, and suggested that defensins released by neutrophils during inflammation may contribute to the induction of antigen-specific cellular and humoral immune responses. These results provide more evidence that neutrophils not only engage in innate host defense but also utilize defensins as signals to mobilize subsequent adaptive immune responses.

Acknowledgements

The authors would like to thank Dr Arya Biragyn for help in ELISA assay, Ms Kathleen Bengali for her excellent technical assistance and Ms Cheryl Fogle for expert secretarial assistance. We are grateful for the critical reading of the paper by Drs Scott Durum and Michael Grimm. By acceptance of this article, the publisher or recipient acknowledges the right of the US Government to retain a non-exclusive, royalty-free license in and to any copyright covering the article. The experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare Publication 78-23 (National Institutes of Health, Bethesda, MD).

Abbreviations

Con A  concanavalin A
HNP  human neutrophil peptide
Ig  lymphoma-derived Ig
KLH  keyhole limpet hemocyanin
LPS  lipopolysaccharide

References

Defensins promote Ig responses in vivo.


