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Inhibition of Intracellular *Histoplasma capsulatum* Replication by Murine Macrophages That Produce Human Defensin

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Although purified defensins are effective microbicides in vitro, their operation within intact phagocytes has not been established. To address this question, we inserted cDNA encoding human defensin HNP-1 into a pBabe/neo retroviral vector and transduced it into RAW 264.7 cells, a murine macrophage line that lacks endogenous defensins. We isolated five independent clones of HNP-1-transduced cells, all of which secreted prodefensin and contained small amounts of fully processed HNP-1. The two clones that produced the largest amounts of defensin (clones 5 and 14), together with wild-type RAW cells and pBabe/neo-transduced RAW cells (control), were used for the present study. All cells were grown in Dulbecco's modified Eagle's medium-F12 medium that contained 10% heat-inactivated fetal bovine serum and gentamicin. The medium used for the transduced cells contained aminoglycoside G418 in lieu of gentamicin. Both wild-type and transduced cells were placed in antibiotic-free medium 96 h prior to challenge with a yeast-phase strain of *Histoplasma capsulatum*. Phagocytosis of yeast cells was allowed to proceed for 90 min and was followed by washing and further incubation for 18.5 h. Whereas the phagocytic index did not differ significantly among the four cell populations under study, the mean level of intracellular growth of *H. capsulatum* in the defensin-transduced RAW cells was significantly lower than those observed for any other cell types ($P < 0.05$). These findings constitute the first instance of xenogeneic expression of an antimicrobial peptide by phagocytes and suggest that macrophages can be armed with defensins to enhance their ability to restrict certain intracellular pathogens.

Defensins are small (29- to 35-amino-acid-residue), arginine- and cysteine-rich cationic peptides that are abundant in many mammalian neutrophils, including those of humans, rabbits, guinea pigs, and rats (reviewed in reference 7). They are curiously absent from equine (1) and murine (2) neutrophils. Although purified defensins have been shown to exert potent antibacterial, antifungal, antiviral, and cytotoxic effects in vitro (7), their ability to exert similar effects intracellularly has not been demonstrated. To determine whether defensins could arm macrophages to inhibit ingested microorganisms, we challenged murine macrophages, which naturally lack defensins, with *Histoplasma capsulatum* before and after they had been engineered to produce human defensin HNP-1. *H. capsulatum*, a major fungal pathogen that can grow unrestricted within host macrophages, particularly in animals and humans with impaired cellular immunity, was found to be exquisitely sensitive to defensin HNP-1 in vitro (1a).

MATERIALS AND METHODS

Host cells and media. Wild-type murine macrophage-like cells, RAW 264.7 (ATCC TIB 71), were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco-BRL, Grand Island, N.Y.) that contained 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 50 μg of gentamicin sulfate per ml. Cells that had been transduced with either human defensin cDNA or control constructs were grown identically to RAW cells, except that 1 mg of the aminoglycoside G418 (Geneticin; Gibco-BRL) per ml replaced gentamicin in the culture medium.

Transduction and selection of host cells. The transduction of RAW 264.7 cells was done by the method described by Ganz et al. (4). Briefly, human defensin HNP-1 cDNA was amplified by the PCR with degenerate oligonucleotide primers. The PCR-generated fragment, containing the sequence encoding preproHNP-1, was digested with *Bam*HI, ligated into the retroviral vector pBabe/neo (9), and transformed into *Escherichia coli* XL-1 Blue (Promega, Madison, Wis.). The nucleotide sequences and orientations of the PCR-generated inserts were confirmed by dideoxynucleotide DNA sequencing. The pBabe/neo/defensin plasmid was transfected into an ecotropic packaging cell line, GP + E-86, whose retroviral progeny was, in turn, used to infect wild-type RAW 264.7 cells. Negative controls, consisting of both RAW cells transduced with a pBabe/neo construct that lacked the HNP-1 cDNA insert and RAW cells transduced with the pBabe/neo vector carrying the HNP-1 cDNA in an antisense orientation, were also assembled, selected for by growth in G418-containing medium, and examined for defensin expression by both immunoperoxidase staining of intact cells (5) and enzyme immunoassay (EIA) (10) of cell lysates (11). The release of defensin into the culture supernatant was assayed by EIA (10). We isolated five independent clones of HNP1-transduced cells, all of which secreted prodefensin and contained small amounts of fully processed HNP-1. The two clones that produced the largest amounts of defensin (clones 5 and 14), generated by two independent

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transduction events, together with wild-type RAW cells and pBabe/neo-transduced control RAW cells, were used in our fungal challenge experiments.

H. capsulatum. *H. capsulatum* 505 was obtained from Dexter H. Howard (Department of Microbiology and Immunology, University of California at Los Angeles) and grown in a blood-cysteine-agar slant for 48 h at 37°C in 5% CO_2–95% air. The surface of the *H. capsulatum* slant culture was scraped with a sterile wire loop and transferred into 12 ml of warm RPMI 1640 medium (Gibco-BRL), vigorously mixed, and then allowed to settle for 30 min at 37°C in a 5% CO_2 incubator. The top 1 ml of the yeast suspension, which contained >85% single yeast cells, was then carefully aspirated with a sterile pipette and transferred to a fresh sterile tube. After the mixture was vortexed briefly a 10-μl aliquot was removed, thoroughly mixed with 10 μl of 0.05% Trypan blue in phosphate-buffered saline (PBS; pH 7.4; Gibco), and counted in a modified Neubauer hemacytometer. Viability of the yeast suspensions, by Trypan blue exclusion, was consistently >97%. In vitro sensitivity of *H. capsulatum* to HNP-1 was evaluated by incubating a yeast-phase suspension (10^6 cells per ml) in 10 mM Na phosphate buffer (NaPB, pH 7.4) with 0, 5.0, or 50 μg of HNP-1 per ml in NaPB for 2 h in a 37°C shaking water bath. The incubation mixtures were assessed for fungal growth by a slide germination assay (6) on glucose-peptone agar followed by fluorescence microscopy after the preparations were stained with a 0.01% solution of the fluorochrome Uvitex 2B (Fungiqual A; Ciba-Corning, Medfield, Mass.) in PBS.

Fungal challenge to RAW cells. *H. capsulatum* was prepared as described above, and its concentration was adjusted to 1.25×10^6 yeast cells per ml in RPMI 1640 medium (Gibco-BRL) containing 10% FBS. Adherent wild-type and transduced RAW cells were grown to confluency, washed with PBS (pH 7.4), and placed in aminoglycoside-free medium 96 h prior to fungal challenge. Cells were harvested by the addition of PBS–0.02% EDTA followed by gentle scraping, pelleted by centrifugation at 160 × *g* for 10 min at 20°C, and resuspended in antibiotic-free medium. A 10-μl aliquot of each cell suspension was removed, thoroughly mixed with 10 μl of 0.05% Trypan blue in PBS, and counted in a hemacytometer. Each cell type, whose typical viability ranged between 87 and 100%, was suspended in DMEM-F12 containing 10% FBS to a final concentration of 5×10^5 viable macrophages per ml. The identity of each cell suspension was blinded until the completion of the experiments. A 200-μl aliquot of each cell suspension was gently deposited on round 13-mm-diameter plastic coverslips (Thermanox; Nunc, Naperville, Ill.) previously placed in 24-well tissue culture plates (Costar, Cambridge, Mass.). Macrophages were allowed to adhere to the coverslips for 2.5 h at 37°C in room air with 5% CO_2. Coverslips were washed twice with warm PBS to eliminate nonadherent cells and debris and then carefully overlaid with 200 μl of either yeast suspension in RPMI–10% FBS or yeast-free medium. Following a 90-min incubation, the coverslips were washed twice with warm PBS to remove noningested *H. capsulatum*. At this point, half of the coverslips were fixed in buffered methanol and stained with Diff-Quik (Baxter, Irvine, Calif.) to measure the phagocytic index, as described below. The remaining coverslips were overlaid with medium and incubated for an additional 18.5 h. At the end of this period, all coverslips were removed, fixed, and stained with Diff-Quik.

Interpretation of results and data analysis. The phagocytic index was defined in this study as the mean number of yeast cells per macrophage after 90 min. A minimum of 100 yeast-containing macrophages was counted on duplicate coverslips in an oil immersion (magnification, ×1,000). The yeast mean growth index was defined as the ratio of the mean number of yeast cells per macrophage after 20 h to the mean number of yeast cells per macrophage after 90 min. The growth index was calculated after counting >150 yeast-containing macrophages on duplicate coverslips. Two and three identically designed experiments, for clones 5 and 14, respectively, were done with duplicate coverslips for each cell type and for each time point. Differences between the means, compared by use of nonpaired *t* tests for RAW/HNP-1 cells versus wild-type RAW 264.7 and paired *t* tests for RAW/HNP-1 cells versus each one of the transduced control cell populations, were deemed statistically significant when P was <0.05.

TABLE 1. Phagocytosis and intracellular replication of *H. capsulatum* in wild-type and transduced RAW 264.7 cells

Cell type	Mean ± SD	
	Phagocytic index[a]	Growth index[b]
RAW/HNP-1 clone 5	1.65 ± 0.13	1.20 ± 0.12
RAW/HNP-1 clone 14	1.62 ± 0.22	1.97 ± 0.28
RAW wild type	1.50 ± 0.14	3.70 ± 0.21
RAW/antisense	1.40 ± 0.12	2.69 ± 0.38
RAW/pBabe	1.55 ± 0.25	2.52 ± 0.22

[a] Phagocytic indices were similar among the five cell populations ($P > 0.1$).
[b] Growth indices for RAW/HNP-1 (clones 5 and 14) were significantly lower than those of RAW wild-type ($P < 0.01$) or transduced control ($P < 0.05$) cells.

RESULTS

In vitro sensitivity of *H. capsulatum* to HNP-1. In our slide germination assays, 50 and 5 μg of HNP-1 per ml inhibited *Histoplasma* growth by 100 and 98%, respectively, compared with that of the control.

Defensin content in wild-type and transduced RAW cells. The secreted form of HNP-1 proved to be a 75-amino-acid prodefensin whereas the cellular form consisted mostly of the mature, 29- to 30-amino-acid-residue forms (4). We selected the two clones that produced the largest amounts of defensin (of five) for the present study. By EIA, the mean immunoreactive defensin content (± standard deviation) in RAW/HNP-1 cells was 1.3 ± 0.38 ng of HNP-1 per 10^7 cells (clone 14). The concentration of prodefensin in the medium of 10^7 RAW/HNP-1 cells after 3 to 5 days of incubation ranged between 40 and 200 ng/ml for clones 5 and 14. We did not detect any defensin in the cells or supernatant fluids of either the wild type or the two transduced RAW cell controls, which were also negative for HNP-1 by immunoperoxidase staining.

Fungal challenge experiments. Comparable numbers of *H. capsulatum* yeast cells were ingested by defensin HNP-1-transduced and control RAW cell populations (Table 1). HNP-1-transduced cells, however, restricted the intracellular growth of the fungus significantly more than did wild-type ($P < 0.01$) or control ($P < 0.05$) cells (Table 1). While many infected wild-type macrophages contained four to eight yeast-phase organisms per cell after 20 h of incubation, most of the phagocytic macrophages that expressed HNP-1 contained one or two yeast cells (Fig. 1A and B). Degraded "ghost" forms of *H. capsulatum* with altered staining properties were common in the HNP-producing macrophages (Fig. 1C) but were rarely seen in the wild-type or control cells. Although the RAW/pBabe and RAW/HNP-1-antisense control cells also restricted the intracellular replication of *H. capsulatum* relative to that of the wild-type cells, they caused significantly less inhibition ($P < 0.05$) than either of the HNP-transduced clones (Fig. 2).

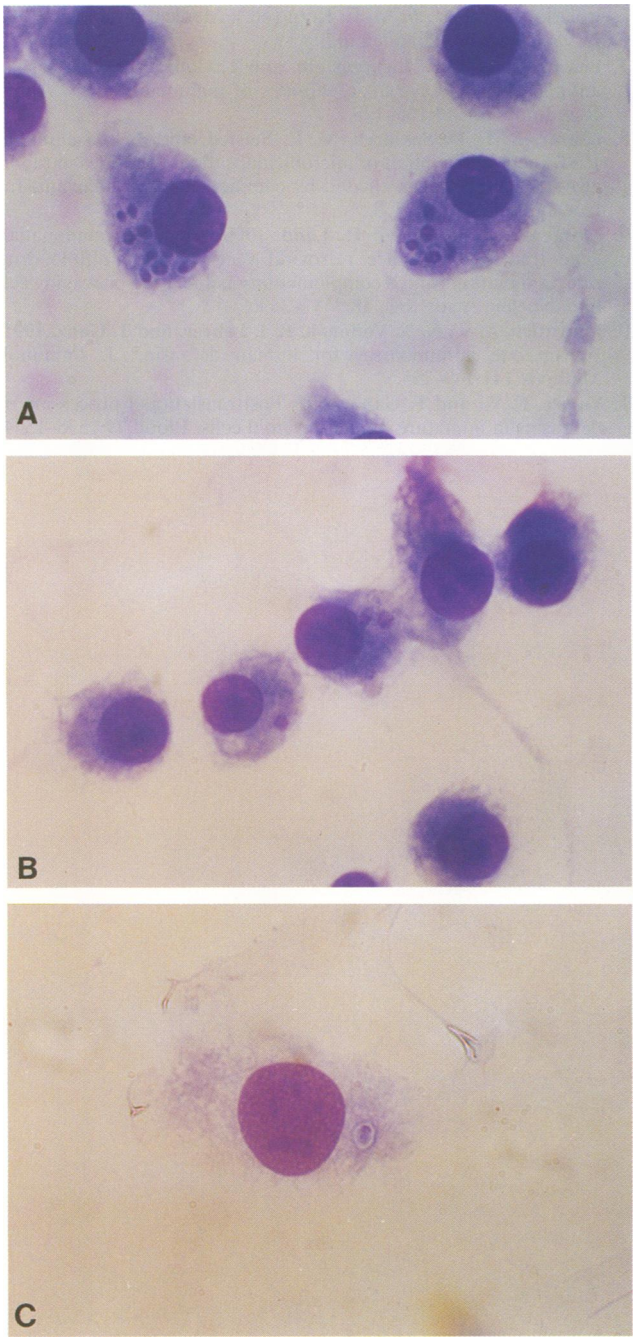

FIG. 1. Photomicrographs of RAW cells 18 h after challenge with *H. capsulatum* (Diff-Quik stain; original magnification, ×1,000). (A) Wild-type RAW 264.7 macrophages. The two infected cells contain seven or nine intracellular yeast cells each. (B) HNP1-transduced RAW cells. The two infected cells contain one or two intracellular yeast cells each. (C) Degraded ghost forms of *H. capsulatum* were frequent among defensin-transduced macrophages.

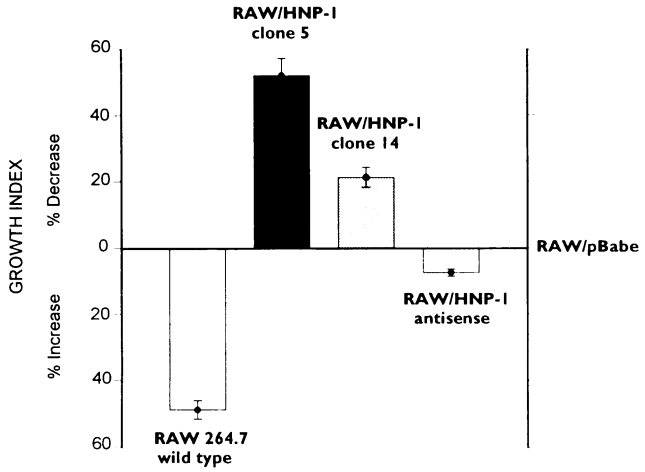

FIG. 2. Inhibition of intracellular growth of *H. capsulatum* in transfected RAW cells. The growth indices were normalized by allowing yeast replication in the RAW/pBabe-transduced cells to serve as the baseline control for the other cell types.

DISCUSSION

This study sought to determine whether defensins could enhance the ability of murine macrophages to inhibit the replication of ingested *H. capsulatum* yeast cells. This fungus, which was markedly sensitive to defensin HNP-1 in vitro, was significantly inhibited also in the intracellular compartment by murine macrophages that had been transduced with human defensin HNP-1. The mild antifungal activity displayed by the transduced control cells in some of the experiments possibly reflected a residual effect of aminoglycoside G418, despite our use of a 96-h G418 washout period before the addition of *H. capsulatum*. The RAW/proHNP-1-transduced macrophages processed prodefensin rapidly (1 h) to the 3.5-kDa mature peptide and released relatively large amounts of prodefensin into the supernatant (4). The antifungal effects seen in these experiments, however, are unlikely to be attributable to the secreted HNP-1 precursor, because (i) *H. capsulatum* was introduced to the adhered RAW cells immediately after each well had been replenished with fresh antibiotic-free medium and (ii) virtually all uningested organisms were removed after 90 min. Even though the intracellular content of immunoreactive defensin in RAW/HNP-1 cells was over 4 orders of magnitude lower than the total defensin content of mature human neutrophils (≈50 $\mu g/10^7$ cells) or rabbit alveolar macrophages (6.5 to 20 $\mu g/10^7$ cells) (3, 8), the estimated intracellular concentration of defensin in RAW/HNP-1 cells (5 to 10 μg/ml) was well within the in vitro antimicrobial range of HNP-1 against *H. capsulatum*, as determined in our preliminary studies. This estimate was based on the assumption that the volume of each RAW cell is ≈500 μm^3 and that defensins are distributed in an intracellular compartment occupying approximately 2 to 5% of the total cell volume.

The present findings indicate that even low levels of human defensins within macrophages can exert antifungal activity. Future efforts to explore ways to obtain higher concentrations of intracellular defensins (e.g., by increasing the rate of transcription of preproHNP-1 via the use of stronger promoters, by diminishing the extracellular secretion of prodefensin and/or enhancing its processing to the mature form, by reducing proteolytic degradation of the mature peptide, or by liposome-mediated uptake of defensins) are planned. Therapeutic strategies that augment the defensin content of macrophages could enhance host resistance to infections caused by defensin-sensitive microorganisms that replicate within these phagocytes.

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