

ANTIBACTERIAL ACTIVITY OF HUMAN NATURAL KILLER CELLS

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Natural killer (NK) cells are defined phenotypically as large granular lymphocytes (LGL)¹ that express both the antigen CD16 (Leu-11) and the NKH-1 (Leu-19) (1-7). NK cells may be defined functionally as cells that mediate non-histocompatibility-restricted cytotoxicity against a variety of cell targets, including tumor and normal undifferentiated cells (1, 8-10). Activation of NK cells by IL-2, other cytokines, and bacterial products not only enhances the degree of cytotoxic activity per cell but also increases the spectrum of target cells killed (11-13). The *in vivo* role of NK cells in defense against malignancy remains unclear.

The activity of NK cells against infectious agents has received less attention. Cytotoxic activity of NK cells has been shown against cells infected with herpes (14) and other viruses (15, 16). Cells infected with *Rickettsia rickettsii* are also susceptible to lysis by lymphokine-activated killer cells (17). NK cells are able to bind and kill the fungi *Cryptococcus neoformans* (18-20) and *Histoplasma capsulatum* (21), as well as the parasites *Toxoplasma gondii* (22) and *Trypanosoma cruzi* (23). Data on the antibacterial activity of NK cells are not conclusive. Nencioni et al. (24) found low levels of natural cytotoxic activity against *Salmonella typhimurium* among fresh monocyte-depleted nonimmune lymphocytes from murine spleen and intestinal lamina propria. In contrast, fresh NK cells were able to phagocytize but not kill *Staphylococcus aureus* (25). Bacteria and bacterial cell walls were able to activate NK cells to enhanced tumor target killing, but there was no antibacterial activity against the smooth and rough *S. typhimurium* strains used to activate the NK cells (13, 26). To resolve these conflicting reports, we studied the antibacterial activity of purified NK cells bearing the accepted phenotypic and functional characteristics. NK cells are able to kill both Gram-positive and Gram-negative bacteria, and this bactericidal activity is mediated, at least in part, by an extracellular mechanism.

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¹ Abbreviations used in this paper: LGL, large granular lymphocyte; MDC, monocyte-depleted cells; SEB, Staphylococcal Enterotoxin-B; SN, supernatants; TEM, transmission EM; TSB, tryptic soy broth.

Materials and Methods

Media and Reagents. Effector cells were prepared and incubated in a serum-free, protein-defined media HB104 (DuPont Co., Wilmington, DE). In a few experiments, cells were incubated in RPMI 1640, (Gibco Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated human AB serum (Irvine Scientific, Irvine, CA) and 2 mM L-Glutamine. No antibiotics were used. Both media yielded comparable results, and only experiments using HB104 are reported. All bacteria strains were cultured in tryptic soy broth (TSB, Difco Laboratories Inc., Detroit, MI). Staphylococcal Enterotoxin-B (SEB) was purchased from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated and -unconjugated mAbs Leu-2, Leu-3, Leu-4, Leu-11, Leu-19, and Leu-M3 (1 mg/ml) were purchased from Becton Dickinson & Co. (Mountain View, CA) and were used at 20 μ l in a 10^6 cell/ml suspension. Percoll and Ficoll-Hypaque were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Lidocaine was from Elkins-Sinn, Inc. (Cherry Hill, NJ).

Bacteria. The bacteria were: *S. typhi* strain ATCC 19142 (American Type Culture Collection, Rockville, MD); *S. epidermidis* strain UNM 301 isolated from wound infection; *E. coli* B/rA obtained from T. Kagoma (University of New Mexico, Albuquerque, NM); *E. coli* strain UNM 101 isolated from a case of pyelonephritis; *S. typhimurium* LT-2 obtained from J. K. Spitznagel (Emory Univ., Atlanta, GA). Stock cultures were stored at -70°C in 10% glycerol in TSB. Fresh inocula for each experiment was thawed in TSB and incubated overnight at 37°C .

Preparation of Effector Cells. To prepare monocyte-depleted cells (MDC), PBMC from the blood of normal healthy volunteers aged 20–40 yr were separated on a Ficoll-Hypaque cushion, followed by successive adherence for 60 min to a glass Petri plate and nylon wool column (27). The resulting preparation contained <0.2% monocytes determined by esterase stain (Table I).

Percoll-fractionated cells were prepared by using the discontinuous density Percoll gradient centrifugation method described previously (28, 29). LGL obtained from 42.5–45% Percoll layers, fraction numbers 1 and 2 with specific density 1.08 and 1.09 g/ml, respectively, contained >95% of the NK activity based on cytotoxicity of K562 targets, and comprised ~5–10% of the starting MDC. Fraction 1 and 2 contained 79–80% LGL by morphology in Wright-Giemsa stained cytopreparations, and the percentage of monocytes was <0.1% by staining with nonspecific esterase and direct FITC-conjugated Leu-M3 (Table I).

NK-enriched cells (Leu-11 enriched) were prepared by a negative panning technique (30). MDC were incubated with unconjugated mAbs Leu-2, Leu-3, and Leu-4 (20 μ l/ 10^6 cells) for 30 min at 4°C , then were adhered to polystyrene plates coated with goat anti-mouse IgG for 60 min at 4°C . For each experiment, the phenotype of nonadherent (NK-enriched) cells was tested by direct immunofluorescence staining with FITC conjugates Leu-11 and Leu-19, and enumerated by either fluorescence microscopy or by FACScan (Becton Dickinson & Co.). In some experiments, 100% Leu-19, Leu-11-enriched cells obtained by flow cytometric cells sorting (FACS) were used.

NK-depleted cells were obtained by detaching the cells adherent to plates coated with Leu-2, Leu-3, and Leu-4, using 1 ml of lidocaine for 10 min at 37°C .

NK Cells Activation by SEB. MDC, Percoll-fractionated and Leu-11-enriched cells (10^6 cells/ml), were mixed with 10 μ l of SEB (0.5 mg/ml), incubated 16 h at 37°C , and washed before using in bactericidal or cytotoxic assays.

Supernatants. Leu-11-enriched lymphocytes were adjusted to 2.5×10^6 cells/ml in HB104 medium and incubated overnight. After pelleting the cells by centrifugation at 300 g for 15 min, the supernatant was carefully removed, sterilized by passage through a 0.22- μ m filter (Millipore Corp., Bedford, MA), aliquoted, stored at -70°C , and used within 1 mo. No antibiotics were used in these cultures.

Bactericidal Assay. The bacteria from cultures grown in exponential phase for at least six generations immediately before the experiment were diluted in TSB to 10^5 CFU/ml. A 100 μ l aliquot of the bacterial suspension was centrifugated at 500 g for 5 min, then 100 μ l of a lymphoid cell suspension in concentrations varying from 10^3 – 10^7 cells/ml media was added to each tube and centrifugated at 500 g for 3 min. Finally, 100 μ l of media was added and the tubes were incubated at 37°C for 90 min. To enumerate surviving bacteria at the end of incubation, 0.7 ml sterile distilled water was added to lyse effector cells, and duplicate 0.1

ml samples of appropriate 10-fold dilutions were plated onto tryptic soy agar, incubated overnight at 37°C, and the number of CFU was determined. No broth enrichment for defective but surviving bacteria was performed. All experiments were performed in triplicate. Antibacterial activity was expressed as CFU as a percent of control at 90 min = $100 \times (\text{CFU of experimental cultures}/\text{CFU of control cultures})$ or antibacterial activity = $\log(\text{CFU of control cultures}/\text{CFU of experimental cultures})$.

Bacterial kinetics data was expressed by the function $\log_{10}N_t/N_0$, where N_0 represented the initial colony count and N_t represented the colony count after various times of incubation. Assay of bactericidal activity in supernatants (SN) was similar to that described for cells, except that 100 μl of serial two-fold dilutions of SN were substituted for the lymphoid cells. Results were plotted as percent bacterial survival against dilution, and activity is expressed as the reciprocal of the dilution at which survival is either 50 or 0.1%.

The t test was used to compare results between experiments at a given E/T ratio, considering $p < 0.05$ indicates a significant difference.

Cytotoxicity Assay. K562 cells were maintained in continuous culture (31). Between 0.5 and 1.5×10^6 target cells were labeled with 0.1 ml of $\text{Na}_2^{51}\text{CrO}_4$ (Amersham Corp, Arlington Heights, IL) for 1 h at 37°C. After two washes, K562 cells were resuspended at 10^5 cells/ml, and 0.1 ml was pipetted into conical-bottom microtiter plates. Effector cells in 0.1 ml of medium (RPMI 1640, 10% AB serum, 1% Glutamate, 1% penicillin-Streptomycin) were added to wells to provide E/T ratios of 25/1. After incubation at 37°C for 4 h, 0.1 ml of supernatant were collected from each well and counted for 1 min in a gamma counter. Percentage of cytotoxicity for each individual assay was determined by percent cytotoxicity = $[(\text{test cpm} - \text{spontaneous cpm})/(\text{total cpm} - \text{spontaneous cpm})] \times 100$.

Electron Microscopy. Three groups were tested: (a) purified NK cells prepared by negative selection (Table I); (b) NK cells after a 5-min incubation at 0°C with *E. coli* UNM 101 strain (E/T ratio 1/50); and (c) NK cells after 90 min of the antibacterial assay. Cells were fixed with 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, room temperature, 1 h). For studies of the antibacterial assay, 10 tubes were pooled before fixation. For postfixation, cells were rinsed in buffer (3–5 \times) and resuspended in 2.0% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. Cells were then placed in a centrifuge and pelleted (3,000 g), dehydrated through a graded series of ethanols, and embedded in Epon 812. Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined in an Hitachi H-600 transmission electron microscope.

Results

Kinetics of Antibacterial Activity by NK Cells. Unlike studies with tumor cell targets, bacterial targets continue to replicate during the assay incubation period. Thus, net bactericidal activity occurs only if the number of surviving bacteria (N_t) is less than

TABLE I
Surface Phenotype of Lymphocyte Populations

Group	Leu-19*	Leu-11	Leu-1	Leu-M3†
Leu-11 cells prepared				
by negative selection‡	99 \pm 1	99 \pm 1	0.2 \pm 0	0
LGL Percoll cells	78 \pm 1	79 \pm 4	22 \pm 2	0.1 \pm 0.1
MDC	15 \pm 1	15 \pm 0.4	85 \pm 1	0.1 \pm 0.1
Leu-11 ⁻ cells	0	0	72 \pm 2	0

The phenotype was studied by direct FITC mAbs.

* Leu-19 antibody was conjugated with phycoerythrin (red) for FACScan analysis.

† Leu-19, Leu-11, Leu-1, and Leu-M3 mAbs were from Becton Dickinson & Co., and were used at 20 $\mu\text{l}/\text{ml}$ in a 10^6 cell suspension.

‡ For obtaining the most purified population, MDC were panned with the mAbs Leu-2, Leu-3, and Leu-4.

the number at the onset of incubation (N_0). Since *E. coli* and *S. typhi* increase by 1.2 ± 0.1 logs and 0.85 ± 0.1 logs, respectively, during the 90-min incubation, net bactericidal activity was seen if percent surviving bacteria was $<94\%$ and 86% , respectively.

The kinetics of the antibacterial activity of Leu-11-enriched cells, analyzed as CFU of *E. coli* B/rA strain (Fig. 1 A), and *S. typhi* (Fig. 1 B), after varying incubation periods, revealed that the activity was dependent on both the bacterial target and the dose of effector cells. Studies with *E. coli*, at a relatively high effector/bacteria ratio (1:1), resulted in immediate bacterial killing. In contrast, at a lower ratio (1:10), viable cell counts increased at a rate similar to that of control cultures for 30 min, after which viability of the culture was lost at an exponential rate. *E. coli* were more sensitive to killing than *S. typhi*. After 30 min, the percent of survival of *E. coli* was $<1\%$ at 1:1 E/T ratio, while *S. typhi* showed a 64% survival at a E/T ratio of 15:1. When effector cells caused $<50\%$ inhibition of bacterial growth, maximum inhibition was seen at 90 min (data not shown), and this time was selected for subsequent studies.

Effect of the Purification of NK Cells on Salmonella typhi Viability. Successive purification of Leu-11/Leu-19 cells resulted in incremental increases in bactericidal activity against *S. typhi* (Fig. 2). Although antibacterial activity of monocyte-depleted cells against *S. typhi* is dose-dependent, the maximum activity was never >0.22 logs inhibition (60% survival) for the highest E/T ratio tested, 200:1. The fractionation of the lymphocytes in Percoll gradients resulted in an increase in the bactericidal activity (Fig. 2). The inhibitory activity at 15:1 E/T ratio was greater in all three studies of Percoll-concentrated cells than in six studies of monocyte-depleted PBMC at the same ratio.

Leu-11-enriched cells obtained by panning showed the most potent antibacterial activity (Fig. 2). Inhibitory activity was always >1.0 log ($<10\%$ survival) at an E/T ratio of 1:1. There was no surviving bacteria at an E/T ratio of 20:1. This finding was confirmed using the highly purified Leu-11 cell populations, obtained by FACS (Fig. 2). In this experiment, bactericidal activity was >3 logs inhibition ($<0.1\%$ survival) at 1:1 cell/bacteria ratio. Conversely, the antibacterial activity of the cell popu-

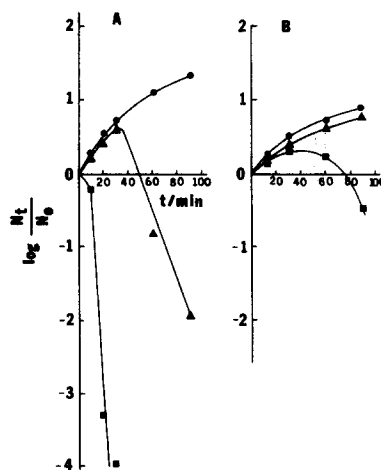


FIGURE 1. Bacterial kinetics of *E. coli* B/rA strain (A) and *S. typhi* (B), (2.5×10^5 CFU *E. coli* or 1.7×10^4 CFU *S. typhi*). Bacteria in exponential growth were incubated with Leu-11-enriched cells (see Table I) at the NK cell/bacteria ratios: (A); (■) 1:1, (▲) 1:10; (B) (■) 15:1, (▲) 1.5:1. All four experiments used cells from the same donor analyzed concomitantly. At the indicated times, samples were diluted by distilled water to lyse eukaryotic cells, and colony counts were performed to evaluate bacterial survival. (●) Control bacteria incubated without cells. Bacterial number at indicated times t are expressed as the $\log_{10} N_t/N_0$, where N_0 is the number at the onset of incubation.

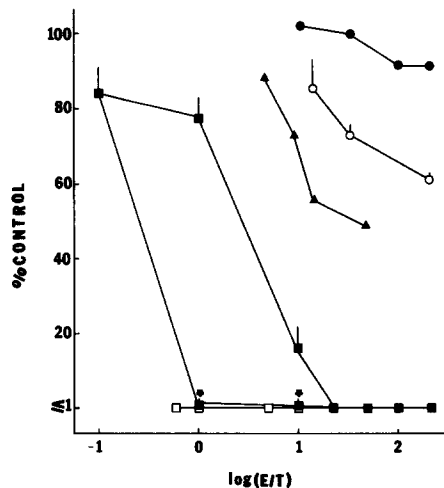


FIGURE 2. Augmentation of antibacterial activity on NK cells against *S. typhi*, by successive enrichment of NK cells at different E/T ratios. Leu-11-depleted cells (●), MDC (○), Percoll-enriched (Percoll fractions 1+2 of MDC) (▲), Leu-11/19 cells purified by panning (■), and Leu-11/19 cells purified by cell sorting (□), were studied over a range of E/T 1:10–200:1. The most active Leu-11-enriched population (*) is shown separately from the other three experiments. Each point with SD bars represents the mean of three separate experiments; other points represent one experiment.

lation specifically depleted of Leu-11-enriched cells was similar to controls (log inhibition <0.05 ; $>90\%$ survival). In control experiments, addition of mAb (Leu-2, -3, and -4; 20 $\mu\text{g/ml}$ each) to bacteria *E. coli* B/rA suspended in HB104 media had no effect on bacterial growth. Identical treatment of MDC and *E. coli* with the mAbs did not enhance the antibacterial activity of NK cells (Table II). In fact, addition of mAb at this concentration slightly increased bacterial survival. In combination, these results demonstrated that the observed antibacterial activity is associated with NK cells and not immune complex-induced degranulation or direct antibacterial activity of these reagents.

Donor Variability of NK Cells Antibacterial Activity. Purified NK cells from eight healthy donors displayed marked variability in antibacterial activity against *E. coli* B/rA strain (Fig. 3). Cells from all donors achieved 100% killing at the E/T ratio of 1:1. NK cells from some donors showed 10–100-fold greater activity than less potent donor cell populations. The ability of some NK cells preparations to kill bacteria at cell/bacteria ratios of 1:100 or greater was seen only with *E. coli* targets. Furthermore, addition of pooled human serum (type AB) to cell-culture media did not significantly influence the antibacterial activity of NK cell populations. The mean value (four donors) of E/T ratios required to produce 50% growth inhibition of *E. coli* B/rA cultures in the presence of 10% serum was 1:75 (range 1:14–1:200), which is not significantly different from incubations in serum-free media (mean 1:89, Fig. 3)

TABLE II
Effects of mAb and SEB on NK Cell Antibacterial Activity

Exp.	E/T ratio		
	10/1	5/1	1/1
MDC	<0.1	<0.1	44
MDC + mAb	<0.1	<0.1	66
MDC + mAb + SEB	<0.1	<0.1	71
SEB-activated MDC	<0.1	<0.1	<0.1

Percent of control at 90 min is measured.

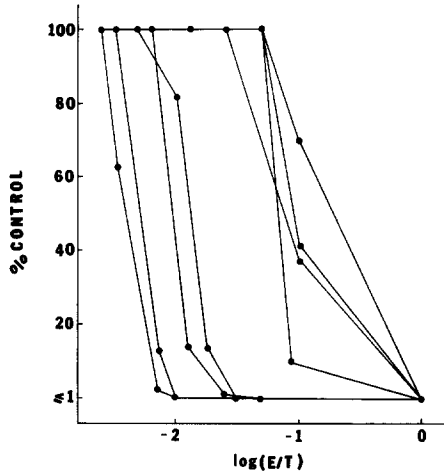


FIGURE 3. Antibacterial activity of Leu-11-enriched cells obtained from eight different donors against *E. coli* B/rA strain growing in exponential phase. For every donor serial, twofold dilutions of NK cell suspensions were incubated with $\sim 2.5 \times 10^5$ CFU of *E. coli*. The effector/bacteria ratios ranged between 1:1 and 1:320. Bacterial survival was determined by colony counting and referred as percent-viable bacteria of control group (without NK cells) at 90 min.

Comparison of Fresh and Pre-incubated NK Cells. The experiments described above studied NK cells that had been incubated for 16 h in HB104 media, due to the duration of cell purification procedures during the preceding day. To study the effect of 16-h incubation without additional lymphokine stimulation, MDC and Leu-11-enriched populations were purified from a single donor, and their antibacterial activity was assessed either immediately or after 16-h (overnight) incubation at 37°C in HB104 media. Fresh MDC demonstrated insignificant antibacterial activity against all three bacterial targets (Fig. 4 A), while Leu-11-enriched cells displayed substantial antibacterial activity, particularly against *E. coli*. UNM 101 strain. After a 16-h incubation (Fig. 4 B), the antibacterial activity of both cell population was significantly enhanced against all bacterial targets.

Antibacterial Activity of NK Cells Activated with SEB. SEB is a potent activator of

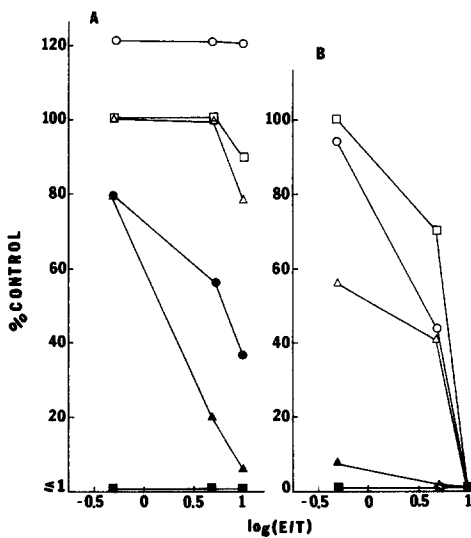


FIGURE 4. Effect of in vitro overnight incubation in HB104 on antibacterial activity of MDC (open symbols) and Leu-11-enriched cells (filled symbols) against *S. typhi* (○, ●), *E. coli* (UNM 101 strain) (□, ■), and *S. epidermidis* (Δ, ▲) at three different E/T ratios. (A) The antibacterial activity of fresh cells tested for 4-5 h after the blood was drawn. (B) The results obtained with the same cell population 24 h later. The cells were washed in HB104 before mixing with bacteria. These results show one representative experiment. Every point was done in triplicate.

cytotoxic activity of purified NK cells against K562 and other tumor targets (32). After activation of MDC, Percoll-fractionated, and Leu-11-enriched cells with SEB, the cells were washed and tested for both antibacterial activity against *S. typhi*, (Fig. 5) and cytotoxic activity against K562 (Fig. 5). For each cell preparation, the antibacterial activity was significantly enhanced by SEB activation ($p < 0.05$). Likewise, the tumoricidal activity of the different effector cell preparations (Fig. 5 B) was enhanced by SEB activation, as previously shown (32). Fig. 5 shows that the lysis or inhibition of both K562 cells and *S. typhi* increased with purification of the NK cell population.

Addition of SEB (12.5 $\mu\text{g/ml}$) to bacteria suspended in HB104 media or directly to MDC, did not affect either bacterial growth or the antibacterial activity of NK cells. The addition of SEB and mAb (Leu-2, -3, and -4) to MDC did not enhance the antibacterial activity of NK cells (Table II). The effect of SEB activation on purified Leu-11-enriched cells was examined in greater detail for *S. typhi* targets (Fig. 6). Activation produced an almost 100-fold increase in antisalmonella activity. Thus, at an E/T ratio of 1:1, activated cells resulted in 1% survival of *S. typhi* as compared with 87% survival for *S. typhi* incubated with nonactivated NK cells.

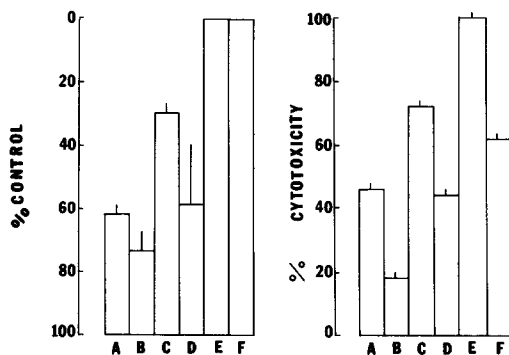


FIGURE 5. Antibacterial activity against *S. typhi*, (left bars) and antitumor killing against K562 cells (right bars) for different effector cell populations. Effector/bacteria ratio was 25:1 for both bacteria and K562 cells. Effector cells: SEB-stimulated MDC (A); MDC (B); SEB-Percoll fraction 1+2 (C). Percoll fraction 1+2 (D); SEB-Leu-11-enriched cells (E); Leu-11-enriched cells (F). SEB stimulation was performed by incubation of the cells populations with 10 μl of SEB (0.5 mg/ml), 16 h at 37°C. The cells were washed just before they were used. Bars represent mean of 2-3 experiments \pm SD.

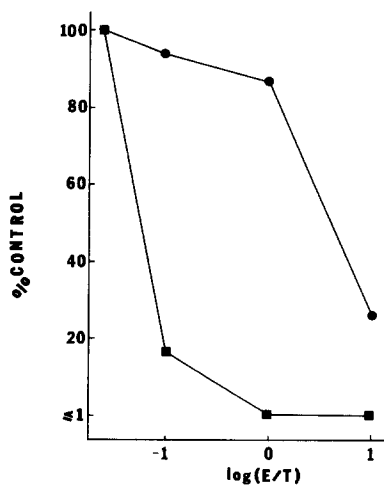


FIGURE 6. The survival of *S. typhi* after 90-min incubation with Leu-11-enriched cells preincubated 16 h with (■), or without (●) SEB 0.5 mg/ml. This figure is a representative experiment with cells from a single donor.

Ultrastructure of Bactericidal Assay with NK Cells and E. coli. Morphological features of the relationship between NK cells and bacteria were studied by transmission EM (TEM). A representative micrograph of NK cells enriched by panning (Fig. 7) showed the typical morphology of LGL. Sections through pseudopodial regions reveal numerous electron-dense granules and vesicles. A section through the nuclear region of a cell is also present. Cells often showed eccentric nuclei and cytoplasm containing prominent mitochondria, numerous free ribosomes, and well-developed Golgi complexes. Small cytoplasmic vesicles were plentiful and, on occasion, were seen to fuse with the cell membrane. In contrast, rough endoplasmic reticulum is sparse. The interaction of purified NK cells and bacteria *E. coli* UNM 101 strain is illustrated in Figs. 8 and 9. The cell surface is composed of numerous processes that contact bacteria (after 5-min contact at 0°C; Fig. 8), and bacterial ghosts (after 90-min incubation at 37°C; Fig. 9). The processes observed are typical of filopodia that contain cytoskeletal elements but that lack membranous organelles in their interior. Bacteria were not observed to be incorporated into the cytoplasm of Leu-11-enriched cells. In addition, cytoplasmic ultrastructure lacks evidence of lysosomes or other organelles that might digest incorporated bacteria. Mere contact with bacteria at 0°C was insufficient to cause degranulation (Fig. 8), but in cells bearing bacterial ghosts, cytoplasmic granules were sparse (Fig. 9). These observations suggest that bacteriolysis is contact mediated, but the role of degranulation remains unclear.

Antibacterial Activity of NK Cell Supernatant. Cell-free supernatants harvested from 18 h cultures of purified Leu-11-enriched cells demonstrated significant bactericidal activity against both Gram-negative and Gram-positive bacteria. In a representative experiment, a significant loss of viability of 10^4 *S. typhi* was apparent by 3 min of culture, and no viable bacteria remained at 15 min (Fig. 10). SN obtained from

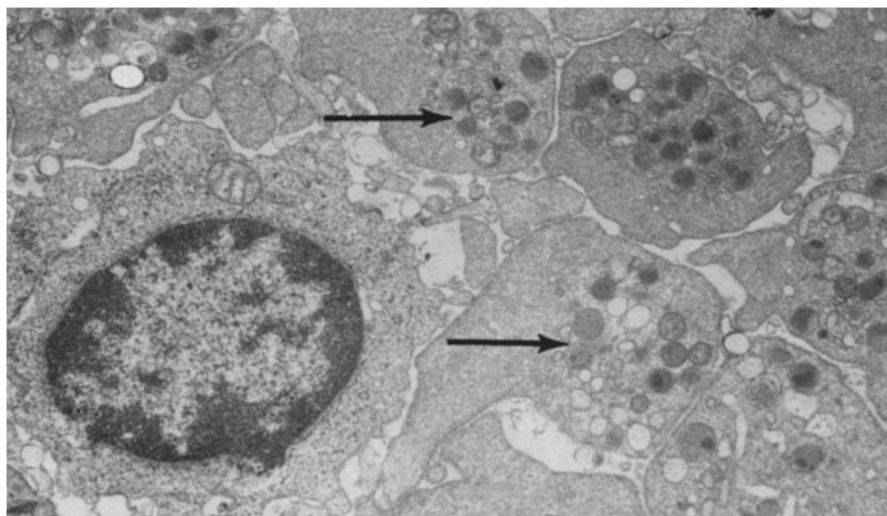


FIGURE 7. TEM of Leu-11-positive NK cells enriched by mAbs panning. This micrograph shows sections of both the nuclear and pseudopodial regions of the cell. Electron-dense granules (arrows), vesicles and mitochondria typical of NK cells are present ($\times 8,000$).

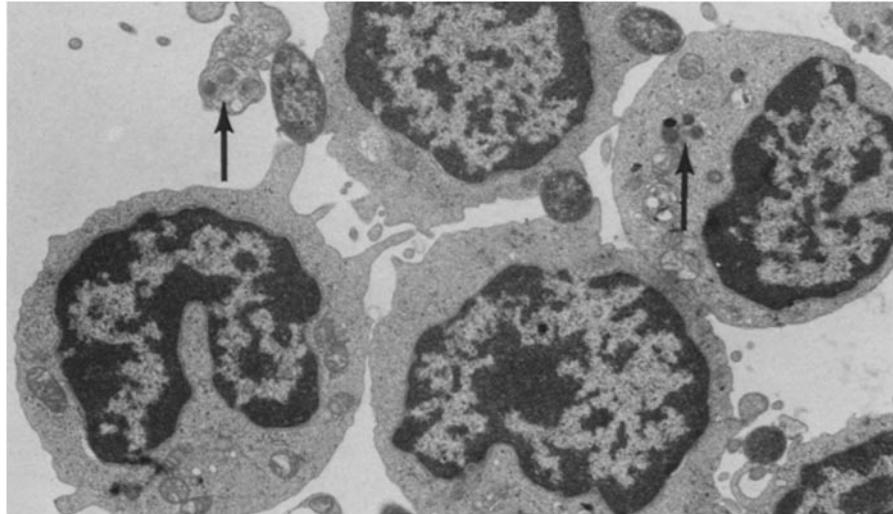


FIGURE 8. TEM of Leu-11-enriched NK cells mixed with *E. coli* UNMH strain 101 at 0°C. Intact bacteria in close association with the cytoplasmic membrane on NK cells are shown. It should be noted that in contrast to cultures incubated at 37°C (Fig. 9) no bacterial ghosts were observed. Persistent granules are shown by arrows ($\times 8,000$).

18-h cultures of MDC or Leu-11-depleted cells (data not shown), or media alone (Fig. 10) showed insignificant antibacterial activity.

If extracellular NK products found in SN participate in the antibacterial activity of NK cells, the target specificities of cells and SN should be similar. Both purified Leu-11-enriched cells and their SN exhibited antibacterial activity against all the bacterial targets tested (Table III). The efficiency (dilution to loss of activity) of SN to inhibit (50% survival) and kill (0.1% survival) was approximately the same for all bacterial strains tested. The efficiency of NK cells to inhibit and kill was also comparable for all strains, with the exception of both *E. coli* strains, which were 300-fold more susceptible than the other bacteria. Thus the relationship between cell-mediated killing and SN-mediated killing may be complex.

It is notable that in six experiments, neither high E/T ratios of purified NK cells, nor undiluted potent supernatants of these cells, were able to reduce the viability of *S. epidermidis* to 0.1% of control (Table III), yet both cells and SN were able to kill at least some of the *S. epidermidis* inoculum. This observation is consistent with a significant role for NK cell products in cell-mediated antibacterial activity.

Discussion

This work is the first specific examination of the antibacterial activity of NK cells. We have clearly demonstrated NK cell antibacterial activity against a variety of bacterial targets using NK cell populations enriched by mAb panning and characterized phenotypically as bearing the CD16 (Leu-11) and HNK-1 (Leu-19) markers. Previous studies, which found little or no antibacterial activity among NK cells, used either unpurified murine lymphocyte populations (24) or freshly prepared monocyte-depleted Percoll-fractionated cells (26). The study (25) finding 19% inhi-

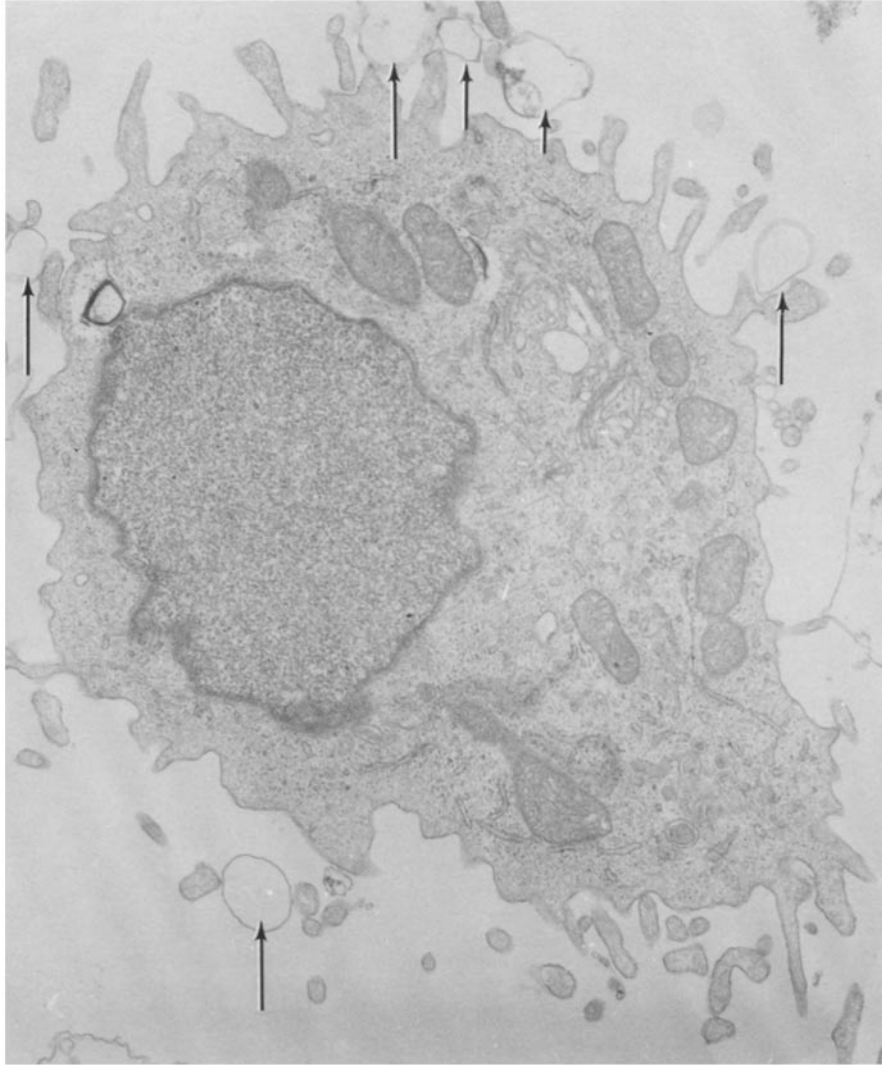


FIGURE 9. EM of Leu-11-enriched cells after 90 min of culture with *E. coli*. Bacterial ghosts (arrows) are prevalent associated with numerous cytoplasmic projections at the cell surface. The cell contains prominent mitochondria, free ribosomes, small vesicles, and a Golgi complex, whereas rough endoplasmic reticulum and dense core vesicles are sparse. ($\times 14,000$).

bition of *S. aureus* by Percoll-concentrated cells agrees with our results using the same cells and *S. typhi* targets (Fig. 2). Effector cells were contaminated by $<0.5\%$ monocytes identified as nonspecific esterase-positive cells; while these cells may have contributed to the bactericidal activity observed, their activity was likely minimal. Cell populations lacking Leu-11⁺ cells did not inhibit bacterial growth. Since T cells were removed in the panning procedure, HNK-1, CD3⁺ cells were not considered for antibacterial activity. Freshly prepared monocyte-depleted cells showed weak or no inhibition, in agreement with prior reports (25, 26). The striking increase in an-

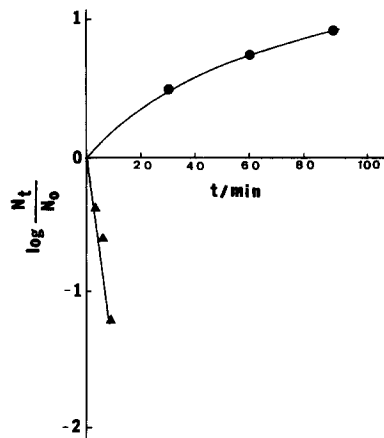


FIGURE 10. Bacterial kinetics of *S. typhi* (10^4 CFU), incubated without (●) or with (▲) undiluted supernatant harvested from a 16-h incubation of Leu-11-enriched cells in HB104 media. Bacterial number at indicated times t are expressed as the $\log_{10}N_t/N_0$ where N_0 is the number at the onset of incubation.

tibacterial activity seen between Percoll-fractionated cells (70% LGL) and panned cells (98% LGL) suggests that cells not bearing the Leu-11/19 phenotype may inhibit the expression of NK cell antibacterial activity. In fact, cocubation of suppressor cells bearing the CD8 (Leu-2) surface antigen and purified NK cells at a ratio of 1:3 demonstrated a >90% reduction of NK cell-associated antibacterial activity (unpublished data, Garcia-Peñarrubia et al.).

NK cell preparations from different donors showed wide variability in their antibacterial activity (Fig. 3, Table III). This variability may explain the inability of some authors to identify antibacterial activity if small numbers of donors are used. Variability may be explained in part by genetic differences or unreported stimulants among our volunteer blood donors. A number of NK cell-activating agents have been identified, including viral infections, aspirin, and other cytokines (33). The relationship of NK cell stimulation in the induction of antibacterial activity was studied further. Activation of NK cells was studied in two ways: pharmacologic stimulation by SEB, and simple incubation for 16–24 h at 37°C, and both induced substantial

TABLE III
Comparison of the Sensitivity of Bacterial Strains to Inhibition by NK Cells and their Supernatants

Bacteria	Leu-11-enriched cells			Supernatants	
	Log inhibition at E/T ratio of 5/1	Mean bacteria/NK cell ratio at two levels of bacteria inhibition		Dilution (reciprocal) at percent viables	
		50% [§]	0.1% [§]	50% [§]	0.1% [§]
	mean ± SD	mean		mean	
<i>E. coli</i> B/rA	>4 (8)*	89(12–250;8)†	34(5–100;8)	77.5(59–96;2)	24(20–28;2)
<i>E. coli</i> UNM 101	>4 (6)	ND	ND	192(128–256;2)	85(40–150;3)
<i>S. typhi</i>	1.1 ± 0.7 (4)	0.24(0.1–5;3)	0.08(0.06–0.1;2)	85(74–96;3)	32(32–32;4)
<i>S. typhimurium</i> LT-2	0.4 ± 0.3 (4)	0.26(0.1–5;3)	0.11(0.05–0.5;3)	ND	ND
<i>S. epidermidis</i>	0.9 ± 0.1 (4)	0.32(0.2–2;3)	<0.3(0.01–0.03;3)‡	63(3–128;2)	<1‡

* Number of experiments.

† Range; number of experiments.

§ Viable bacteria as percent of control at 90 min.

‡ 99.9% inhibition was never achieved despite high E/T ratios; figure given is an extrapolation from experiments in which *S. epidermidis* survival was between 0.1% and 1.0%.

antibacterial activity. Although there was a modest increase in the number of Leu-11/19 cells during the 16-h incubation, this alone could not account for the increase in antibacterial activity. Fresh Leu-11/19 cells from healthy donors do not express IL-2 and transferrin receptors markers of activation, whereas, after the 16-h incubation, 25–30% express both receptors (A. Bankhurst, unpublished data).

Stimulation of cells during a 16-h incubation with SEB induced significant increases in antibacterial activity. SEB produces its effect on NK cells by an IL-2-dependent mechanism (32), and is a potent agent for differentiation and proliferation of eukaryotic cells (34). The enhancement of antibacterial activity was reflected in the enhancement of tumoricidal activity against K562 cells (Fig. 5). Whether all NK cells or only a subpopulation can be stimulated to express antibacterial activity is not known.

Several characteristics of the mechanism of antibacterial activity can be deduced from the present data. Cell contact between bacteria and NK cells is possibly an important step. Transmission EM studies have shown that bacteria and bacterial cell wall ghosts are bound to the NK cell membrane (Figs. 8, 9). Second, *S. typhi* is less susceptible than *E. coli* to NK cell-mediated killing, while both *E. coli* and *S. typhi* are equally susceptible to killing by cell-free SN. One interpretation of this observation may be heterogeneity in bacterial-NK cell binding. This is supported by the difference in susceptibility of rough and smooth strains of *S. typhimurium* to killing by purified Leu-11 cells (unpublished data). The identification of the NK cell receptor(s) or bacterial ligand(s) is not yet known, but the considerable knowledge of bacterial cell wall molecular structure will aid in the future studies of both receptor and ligand.

The mechanism of antibacterial activity of NK cells appears to be, at least in part, extracellular as supported by TEM studies and the presence of potent bactericidal activity in NK cell supernatants. In transmission EM studies with highly purified Leu-11 cells, no phagocytosis was visualized, and all bacterial ghosts were bound to the NK cell membrane. The mechanism appears to be different ultrastructurally from the cytolytic process against tumor cells, since the Golgi apparatus does not become polarized to one end of the cell. Since bacteria are scattered over the surface of the NK cell, polarization of organelles would not be possible.

The antibacterial activity in NK cell SN reflects several characteristics of the cell-mediated activity. Both cells and their SN can induce marked loss of bacterial viability during 5 min of incubation. Both cells and their SN kill a variety of bacterial targets. Neither cells nor their SN were able to decrease the viability of *S. epidermidis* to 0.1% of control. Finally, MDC populations that lack antibacterial activity also lack activity in their SN.

Preliminary studies suggest that the activity in SN is due to substance or substances unique from previously described cytolytic substances produced by NK cells. Activity migrates as a small molecule on gel filtration chromatography, and is resistant to heat, acid, base, and proteolysis (unpublished data). These characteristics differentiate antibacterial activity from the known cytokines such as IL-2 (35), IFN- γ (36), TNF- α or - β (37), perforin (38), NK cytotoxic factor (39), bacterial permeability increasing factor (40), cationic proteins (CAP 57 and CAP 37) (41), and defensins (42). The identity of this factor will aid significantly in the definition of the bactericidal mechanism(s) involved. The mechanism of NK antibacterial activity

appears to be different from mechanisms described for other professional phagocytes. No conclusive evidence has been presented demonstrating oxygen-dependent killing mechanisms in purified NK cells (43). Moreover, phorbol esters and bacterial products have failed to stimulate chemiluminescence in purified NK cells (D. van Epps and A. Bankhurst, unpublished data). Also, no EM evidence for bacterial phagocytosis was found in experiments with purified Leu-11 cell populations, although differences in culture conditions and bacterial targets may yet reveal evidence for phagocytosis (25). Studies using animal models are needed to define the role of NK cells in nonimmune antibacterial host defense, particularly in the granulocytopenic host.

Summary

The *in vitro* effects of human NK cells on viability of Gram-negative and Gram-positive bacteria was investigated. PBLs depleted of glass-adherent cells showed a significant antibacterial activity that was increased as the concentration of NK cells became higher. Leu-11-enriched cells exhibited the most efficient bactericidal activity. Stimulation of NK cells with staphylococcal enterotoxin B for 16 h produced a significant increase in the antibacterial activity of all NK cells tested. The antibacterial activity of monocyte-depleted cells and Leu-11-enriched cells was also enhanced after culturing *in vitro* for 16–24 h without exogenous cytokines. Dependence of the antibacterial activity on the presence of serum in the culture medium was not found. Ultrastructural studies revealed close contact between NK cell membranes and bacteria, no evidence of phagocytosis, and extracellular bacterial ghosts, after incubation at 37°C. Supernatants from purified NK cells exhibited potent bactericidal activity with kinetics and target specificity similar to that of effector cells. These results document the potent antibacterial activity of purified NK cells and suggest an extracellular mechanism of killing.

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