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Antimicrobial Activity of Natural Killer Cells and Lymphokine-Activated Killer Cells against Mycobacterial Pathogens

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We studied the antimicrobial activity of natural killer（NK）cells and lymphokine-activated killer（LAK）cells against *Escherichia coli* and some nontuberculous mycobacteria（NTM）including *Mycobacterium fortuitum* and *Mycobacterium intracellulare*. When the antimicrobial activity of these cells was measured in terms of the number of residual colony forming units of test microorganisms, it was found that *E. coli* was rapidly killed by these effector cells during a 3-h coculture. NK cells and LAK cells exhibited comparable levels of bactericidal activity against *E. coli.* On the other hand, both the effector cells failed to exhibit microbicidal activity against *M. fortuitum* during 18 h of incubation. NK cells but not LAK cells displayed a mild inhibitory effect against *M. fortuitum.* Notably, LAK cells exhibited strong cytotoxicity against NK cell-susceptible YAC-1 cells compared to NK cells. Next, antimicrobial activity against mycobacteria was measured in terms of ^{3}H-uracil uptake after an 18-h coculture with the effector cells. NK cells displayed similar levels of activity against *M. fortuitum* and *M. intracellulare*. The antimicrobial action against *M. fortuitum* was blocked by NK cell-specific anti-asialo GM1 antibody. These findings indicate that NK cells but not LAK cells are capable of bacteriostatic activity against rapidly growing NTM（*M. fortuitum*）as well as slowly growing NTM（*M. intracellulare*）, although these NTM are much more resistant to NK cells than *E. coli.*

Key words: NK cells, lymphokine-activated killer cells, antimicrobial activity, *Mycobacterium fortuitum, Mycobacterium intracellulare, Escherichia coli*

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INTRODUCTION

Natural killer（NK）cells are principally defined phenotypically as large granular lymphocytes that express CD16（FcγRIII）with low-density expression of CD56（neural-cell adhesion molecule）and lack of expression of CD3, although they also contain minor populations showing a $CD56^{high}CD16^{low}$ or $CD56^{high}CD16^{-}$ phenotype［1, 2］. NK cells mediate non-MHC-restricted cytotoxicity against a variety of targets, including tumor cells, fetal cells, and virally infected cells［1, 3, 4］. Notably, activation of NK cells by IL-2, other cytokines including IL-12 and IL-15, and bacterial products such as staphylococcal enterotoxin B（SEB）not only potentiates their cytotoxic activity per cell but also increases the spectrum of target cells killed［5-8］. It has been demonstrated that NK cells are able to bind and kill the fungi *Cryptococcus neoformans* and *Histoplasma capsulatum*, as well as protozoal organisms such as *Toxoplasma gondii* and *Trypanosoma cruzi*［9-12］. In this context, Garcia-Penarrubia *et al*. reported that NK cells exhibited strong antimicrobial activity against Gram-negative rods including *Escherichia coli* and *Salmonella enterica* Typhi, and moderate activity against Gram-positive cocci such as *Staphylococcus epidermidis*［13］. They found that the bactericidal activity of NK cells is extracellular and is mediated by an antimicrobial factor released from NK cells［13, 14］.

The prevalence of chronic lung disease due to nontuberculous mycobacteria（NTM）is increasing in Japan and the United States, and exceeds that of tuberculosis［15-17］. Rapidly growing mycobacteria are environmental organisms belonging to NTM found in soil, bioaerosols, and natural and chlorinated water［18］. The most common rapidly

growing mycobacteria causing human diseases, particularly lung diseases, are *Mycobacterium fortuitum, Mycobacterium abscessus,* and *Mycobacterium chelonae* [19]. Infections due to these mycobacterial pathogens, especially chronic pulmonary diseases and skin/soft-tissue infections, are emerging health concerns, especially in the United States [18, 19]. Rapidly growing mycobacteria as well as other NTM such as *Mycobacterium avium* complex can form biofilms on water-exposed surfaces, such as in hot tubs and water pipes, which make the NTM organisms resistant to common disinfectants, including chlorine [19]. As treatment success against infections due to rapidly growing mycobacteria is generally poor [18, 19], a greater understanding of the host immune response to these pathogens, particularly cellular mechanisms of bacterial elimination in hosts, is urgently needed. It is generally considered that host macrophages, which are activated by the helper function of mycobacterial antigen-sensitized Th1 lymphocytes, play a central role in the elimination of mycobacterial organisms at sites of infection in hosts [20]. In this context, it is of interest to know the roles of NK cells as effector cells in the host protective immunity against mycobacterial pathogens including rapidly growing mycobacteria. Indeed, we previously found that NK cells as well as macrophages play central roles in innate resistance to *M. fortuitum* by performing experimental *M. fortuitum* infections induced in NK cell-deficient beige mice and, moreover, A/J mice which had been treated with a NK cell-specific anti-asialo GM1 antibody to deplete NK cells [21]. In this study, we examined whether or not NK cells and lymphokine-activated killer (LAK) cells participate in bacterial elimination at sites of infection in hosts infected with *M. fortuitum*, by exhibiting antimycobacterial activity.

MATERIALS AND METHODS

Microorganisms

M. fortuitum strain 18367, *Mycobacterium intracellulare* strain N-260, and *E. coli* strain 81 were used. Mycobacterial organisms and *E. coli* were cultured at 37℃ in Middlebrook 7H9 broth and tryptic soy (TS) broth, respectively. Cultured organisms were harvested by centrifugation at 1,500 x g for 15 min, washed once with phosphate-buffered saline (PBS) by centrifugation, and the resulting bacterial pellet was re-suspended in TS broth followed by 5-min centrifugation at 150 x g. The upper layer (about 60% volume) was saved as an inoculum for each *in vitro* experiment.

Special agents

Special agents used in this study were as follows: Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich Corp., St. Louis, MO., USA), recombinant murine IL-2 (Genzyme Corp., Boston, MA., USA), anti-asialo GM1 Ab (Honen Corp., Tokyo, Japan), [^{3}H] thymidine (^{3}H-TdR) (New England Nuclear Corp., Boston, MA., USA), and [^{3}H]uracil (^{3}H-UR) (New England Nuclear Corp.).

Medium

RPMI1640 medium supplemented with 25 mM HEPES,2 mM glutamine, 100 μg/ml of streptomycin, 100 units/ml of penicillin G, 5 x 10^{-5} M 2-mercaptoethanol and 10% (v/v) heat-inactivated fetal bovine serum (FBS) was used for cell culture. This medium was designated "FBS-RPMI-medium".

Preparation of NK cells and LAK cells

NK cells were prepared according to the method of Garcia-Penarrubia *et al.* [8, 13] with slight modifications. Briefly, Splenic cells (3 x 10^7) prepared from normal male BALB/c mice were cultured in 30 ml of FBS-RPMI-medium with the addition of 10 μg/ml SEB at 37 ℃ in a CO_2 incubator (5% CO_2-95% humidified air) for 96 h. The resultant cells were harvested and washed once with FBS-RPMI by centrifugation and used as NK cells. Collecting the supernatant gently, adherent cells containing activated macrophage removed. For preparation of LAK cells, so prepared NK cells were further cultivated in 10 ml of FBS-RPMI medium containing murine IL-2 (200 units/ml) for 47 h, followed by subsequent washing with FBS-RPMI medium by centrifugation according to the usual methods [22, 23]. SEB-elicited splenic cells included 1 % or less $V\beta8^+V\alpha14^+$ NKT cells.

Antimicrobial activity of NK cells and LAK cells

NK cells or LAK cells (5 x 10^5) were sus-

pended in 0.2 ml FBS-RPMI medium containing test microorganisms (10^2 CFU to 10^4 CFU) in microculture wells and centrifuged at 600 x g for 5 min. The culture wells were incubated a at 37℃ in a CO_2 incubator for 18 h (mycobacteria) or 3 h (*E. coli*). The resultant cultures were mixed with 0.7 ml distilled water, sonicated for 12 s using a sonicator (Handy Sonic UR-20P, Tomy Seiko Co. Tokyo) and the resulting cell lysates were counted for the number of bacterial CFU on Middlebrook 7H11 (mycobacteria) or TS (*E. coli*) agar plates. Alternatively, the resultant cell lysates were cultivated at 37℃ for 24 h after the addition of ^{3}H-UR (1 μCi) and counted for the uptake of radioactivity by surviving bacteria as follows. The ^{3}H-UR-labelled bacteria were precipitated with 5% trichloroacetic acid and the resultant precipitate was harvested onto glass fiber filters and counted for radioactivity using a scintillation spectrometer.

Cytotoxic activity of NK cells and LAK cells

Cytotoxic activity of NK cells and LAK cells was measured in terms of growth inhibition against YAC-1 cells as target cells. YAC-1 cells (2.5 x 10^4) were cultured with 5 x 10^5 of NK cells or LAK cells, which had been treated with mitomycin C (50 μg/ml) at 37 ℃ for 1 h, in 0.2 ml FBS-RPMI in a culture well at 37℃ in a CO_2 incubator for 24 h and pulsed with 0.5 μCi of ^{3}H-TdR (2 Ci/mmol) for the final 6 h. Cells were harvested onto glass fiber filters and counted for radioactivity using a scintillation spectrometer.

RESULTS AND DISCUSSION

Fig. 1 shows the antimicrobial activity of NK cells and LAK cells against *E. coli* and *M. fortuitum* measured in terms of the number of residual bacterial CFU after co-cultivation with the effector cells, respectively. *E. coli* was rapidly killed by these effector cells added at 5 x 10^5 per well during a 3h-co-cultivation. Overall, LAK cells exhibited comparable levels of activity against *E. coli* added at 1.82 to 3.82 log-units (6.6 x 10^1 to 6.6 x 10^3) per well. On the other hand, the effector cells failed to exhibit microbicidal activity against *M. fortuitum* when added at 2.4 to 4.4 log-units (2.5 x 10^2 to 2.5 x 10^4) per well. In this case, only NK cells displayed mild inhibitory effect against *M. fortuitum*. These findings were confirmed by separate experiments repeated four times. In this context, we next examined the cytotoxic activity of the two effector cells against YAC-1 cells (NK cell-susceptible cell line). As shown in Fig. 2, LAK cells exhibited stronger cytotoxic activity than did NK cells. This tendency was confirmed by separate experiments repeated twice.

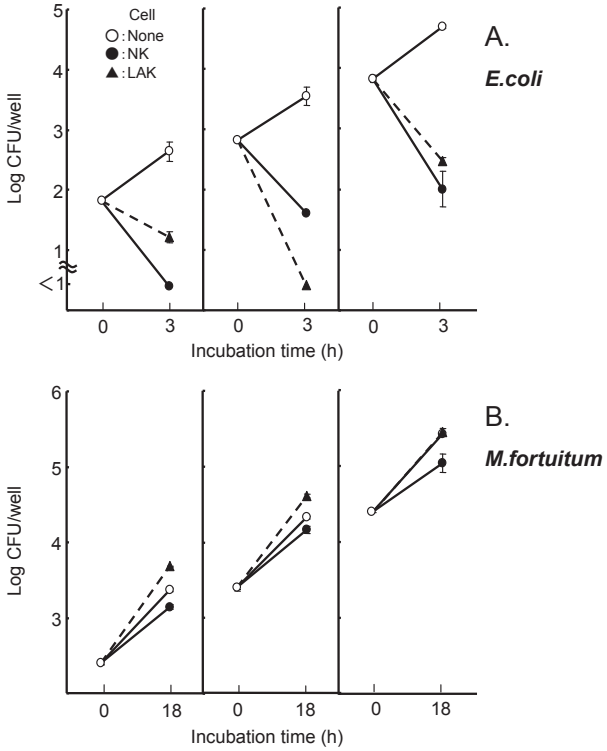

Fig. 1. Antimicrobial activity of NK cells and LAK cells against *E. coli* (A) and *M. fortuitum* (B). Test bacteria were incubated with the effector cells (5 x 10^5 /well) for 3 h (A) or 18 h (B). Each plot indicates the mean of two incubations.

Fig. 3 shows the antimicrobial activity of NK cells against *M. fortuitum* and *M. intracellulare* when measured in terms of ^{3}H-UR uptake by the microorganisms after 18-h co-cultivation with NK cells. In this experiment, the antimycobacterial activity of NK cells was potentiated by cultivating a further 48 h in FBS-RPMI medium containing 60% (v/v) culture fluid of Con A-stimulated splenocytes. The resultant NK cells reduced the replicating ability of test bacteria after 18 h-coculture. *M. fortuitum* and *M. intracellulare* showed similar levels of susceptibility to the NK cell-mediated antimicrobial

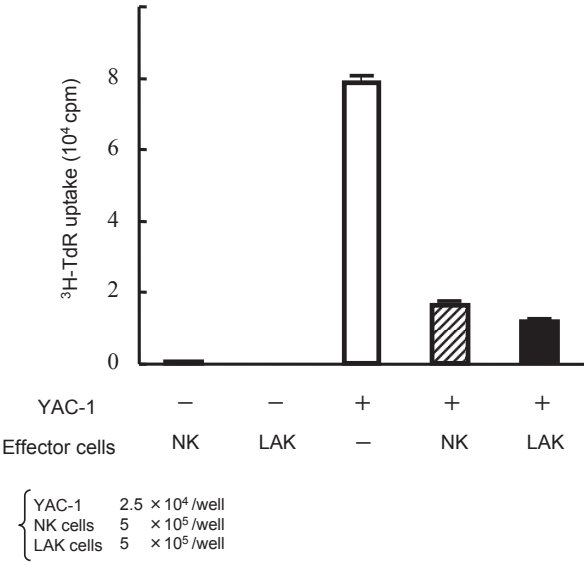

Fig. 2. Cytotoxicity of NK cells and LAK cells against YAC-1 cells. YAC-1 cells (2.5 x 10^4/well) were co-cultivated with the effector cells (5 x 10^5/well) for 24 h and pulsed with ^{3}H-TdR for the final 6 h. Each bar indicates the mean of two or three incubations.

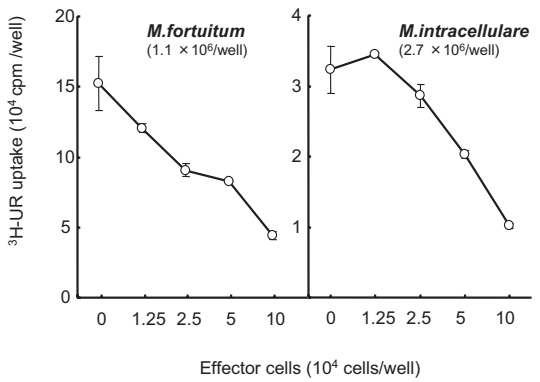

Fig. 3. Antimicrobial activity of NK cells against *M. fortuitum* and *M. intracellulare*. Test bacteria, *M. fortuitum* (1.1 x 10^6/well) or *M. intracellulare* (2.7 x 10^6/well) were incubated with the effector cells at indicated cell densities for 18 h. After cell lysis, released bacteria were collected by centrifugation and pulsed with ^{3}H-UR for 8 h (*M. fortuitum*) or 24 h (*M. intracellulare*). Each plot indicates the mean of two or three incubations.

action. As shown in Table 1, in this experiment, the majority of the antimicrobial activity of the NK cell preparation appears to be mediated by the NK cells themselves, since the anti-asialo GM1 antibody, which is specific to NK cells, blocked the NK cells' antimicrobial action by 80%.

The present findings indicate that NK cell lineages are capable of exerting antimicrobial activity against not only *E. coli,* as reported by Garcia-

Table 1. Blocking of anti-*M. fortuitum* activity of NK cells by anti-asialo GM1 Ab[a]

NK cells	^{3}H-UR uptake (10^3 cpm/ well ± SEM)[b]		
	- Ab	Control Ab	Anti-asialo GM1 Ab
−	157.0 ± 11.2	132.7 ± 26.2	110.0 ± 28.6
+	84.0 ± 8.2	82.7 ± 4.5	95.2 ± 5.1
	(54 ± 5%)[c]	(62 ± 3%)[c]	(87± 5%)[c]

[a] *M. fortuitum* organisms (5.7 x 10^7/well) were co-cultured with NK cells (5 x 10^5/well) in the presence or absence of control Ab or anti-asialo GM1 Ab at 1:10 dilution for 18 h, and then were allowed to incorporate ^{3}H-UR for 8 h.
[b] The mean of three incubations is indicated.
[c] Percentages of inhibition of ^{3}H-UR uptake by NK cells are indicated.

Penarrubia *et al*., but also *M. fortuitum* and *M. intracellulare* [13], although these mycobacteria were much more resistant to the bactericidal action of NK cells. Because NK cells exhibited mild inhibitory effect but not bactericidal activity against *M. fortuitum* (Fig. 1), it is thought that the NK cell lineages play only minor roles in the host defense mechanisms against mycobacterial infections, if any.

It is of interest to note that LAK cells did not exhibit stronger activity than NK cells in killing *E. coli* and in inhibiting *M. fortuitum*, although LAK cells exhibited greater ability than NK cells in terms of the exhibition of cytotoxic effects against NK cell-susceptible YAC-1 cells. NK cells and T cells can differentiate into LAK cell subsets under appropriate conditions, such as in the presence of IL-2, IL-12 or IL-15 at high concentrations [5-7, 24]. Differing from NK cells, LAK cells recognize and kill a broad spectrum of tumor targets, regardless of tissue origin or MHC expression [25], although the activity and specificity of LAK cells differ depending on their source [22]. Nevertheless, despite apparent differences in target recognition between NK cells and LAK cells, they use the same two killing mechanisms to destroy target cells as follows: The first mechanism is the granzyme/perforin-mediated lysis of target cells and the second is the Fas-ligand and Fas-mediated apoptosis of target cells [26]. It has been demonstrated that the bactericidal activity of NK cells is at least partly mediated by humoral effectors extracellularly released from NK cells [13, 14] and that granzyme (NK-lysin in the

case of porcine and chicken) plays a central role in the NK cell-mediated killing of certain bacteria, including *E. coli, Bacillus megaterium*, *Acinetobacter calcoaceticus*, and *Streptococcus pyogenes* [27, 28]. This may explain why, in the present study, LAK cells did not exhibit stronger antimicrobial activity against *E. coli* and *M. fortuitum* compared to NK cells. It is likely that both NK cells and LAK cells use the same mechanisms to recognize these bacteria. In addition, it appears that NK cells and LAK cells produce similar amounts of antibacterial effectors, such as granzyme, in response to stimulation with target microorganisms.

This is the first report demonstrating *in vitro* antimycobacterial activity of NK cells. It is of interest that the mycobacterial organisms were much more resistant to NK cell-mediated bactericidal action than common bacteria including *E. coli*, *S. epidermidis*, and *S.* Typhi, which were easily killed by NK cells during overnight co-cultivation, although the susceptibility of these bacteria to the NK-mediated bactericidal action differed considerably in the order *E. coli, S. epidermidis*, and S. Typhi [13]. Studies are currently underway to elucidate the precise mechanisms of recognition of target mycobacteria by NK cells.

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