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# *Mycoplasma arthritidis* Mitogen Up-Regulates Human NK Cell Activity

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**While the effects of superantigens on T lymphocytes are well characterized, how superantigens interact with other immune cells is less clear. This report examines the effects of *Mycoplasma arthritidis* mitogen (MAM) on human natural killer (NK) cell activity. Incubation of peripheral blood mononuclear cells (PBMC) with MAM for 16 to 20 h augmented NK cytotoxicity (against K562) in a dose-dependent manner ($P \leq 0.05$). Superantigen-dependent cellular cytotoxicity, an activity of superantigen-activated cytotoxic T cells, was not involved in lysis of K562 cells because the erythroleukemic tumor target cells expressed no class II major histocompatibility complex by fluorescence-activated cell sorter analysis. Kinetic experiments showed that the largest increase in NK activity induced by MAM occurred within 48 h. Incubation with MAM caused a portion of NK cells to become adherent to tissue culture flasks, a quality associated with activation, and augmented NK activity was found in both adherent and nonadherent subpopulations. Experiments using cytokine-specific neutralizing antibodies showed that interleukin-2 contributed to enhancement of the NK activity observed in superantigen-stimulated PBMC. Interestingly, MAM was able to augment NK lysis of highly purified NK ($CD56^+$) cells in the absence of other immune cells in 9 of 12 blood specimens, with the augmented lytic activity ranging from 110 to 170% of unstimulated NK activity. In summary, data presented in this report show for the first time that MAM affects human NK cells directly by increasing their lytic capacity and indirectly in PBMC as a consequence of cytokines produced by T cells. Results of this work suggest that, in vivo, one consequence of interaction with superantigen-secreting microorganisms may be up-regulation of NK lytic activity. These findings may have clinical application as a means of generating augmented NK effector cells useful in the immunotherapy of parasitic infections or of neoplasms.**

Exogenous superantigens are proteins secreted by various bacteria (including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Mycoplasma arthritidis*) and bind the major histocompatibility complex (MHC) class II surface molecules as well as the Vβ region of the T-cell receptor (29). By nature of their bifunctionality, superantigens bridge T cells bearing particular Vβ T-cell receptors with class II-bearing antigen-presenting cells and, as a result, activate T cells with specific Vβ T-cell receptors to up-regulate surface markers, secrete cytokines, and proliferate (25). Unlike presentation of conventional antigens to $CD4^+$ T cells by class II MHC or to $CD8^+$ T cells by class I MHC, superantigens may be presented in a class II MHC-dependent manner to both $CD4^+$ and $CD8^+$ T cells (35). Binding of MHC class II molecules typically requires no internalization and processing (40), and the interaction between superantigens and MHC molecules occurs along conserved regions outside the antigenic groove of class II MHC (22).

The *M. arthritidis* mitogen (MAM) superantigen was first described in 1981 as a cell-free supernatant of *M. arthritidis* cultures that supported proliferation of T lymphocytes from certain strains of mice (14). MAM is a heat- and protease-labile basic protein with a pI of 9.0 and an approximate molecular mass of 26 kDa (2). The ability of MAM to induce mitogenesis in T cells is dependent on the presence of $Ia^+$ (class II) radioresistant adherent accessory cells (18, 37). A role for MHC class II molecules in T-cell activation was defined when it was reported that antibodies specific for class II antigens inhibited MAM-induced proliferation of T lymphocytes (15). Presentation of MAM to murine T lymphocytes is known to be dependent upon class II MHC molecules (15, 18), and *H-2* Eα is used preferentially (13). However, unlike presentation of conventional antigens by class II MHC, intracellular processing is not required for presentation of MAM since *p*-formaldehyde-fixed accessory cells support proliferation (19). Like other superantigens, not all T cells proliferate in response to MAM. Recognition of MAM by murine T cells is associated primarily with $V\beta_8$ and $V\beta_6$ T-cell receptors (16, 17). Similarly, in the human system, MAM-induced T-cell responses, like those of superantigenic staphylococcal enterotoxins, are clonally expressed (42). Thus, MAM is a well-characterized protein with superantigenic properties (3, 12).

Superantigens can activate cytotoxic T cells to a higher lytic state through a process termed superantigen-dependent cellular cytotoxicity (SDCC). Cytotoxic T-cell-mediated lysis in the presence of superantigen typically uses the physiologic superantigen bridge to bring the T cell into physical proximity to the MHC class II-bearing target cell (4). Thus, any class II MHC bearing cell may be both a presenting cell and a target cell for the T-cell–superantigen interaction (36). SDCC is absolutely dependent on the presence of class II MHC on the surface of the potential cytotoxic target cell (23, 39) but is only one of the ways in which superantigens interact with T cells.

Although most superantigen studies have focused on interactions with T cells, superantigens can affect other immune cells as well. By binding to MHC class II-bearing cells, for example, superantigens up-regulated cytokine mRNA expression through signals that included transient increases in cytoplasmic calcium ion concentration (11). Both MAM (62) and staphylococcal enterotoxins (31) activate human B cells by bridging to T cells via class II MHC binding. Recently, it was

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reported that several microbial superantigens induced NFκB expression in THP-1, a human monocytic cell line (59), and that binding of superantigen to monocytes induced the production of tumor necrosis factor and interleukin-1 (IL-1) (45, 46, 60).

Unlike murine natural killer (NK) cells, a subpopulation of human NK cells expresses MHC class II molecules (7) and can function as presenting cells for conventional antigen (53). Therefore, we reasoned that MAM may directly activate human NK cells through engagement of surface MHC class II molecules. In mixed cell populations such as peripheral blood mononuclear cell (PBMC) populations, MAM may also indirectly affect the NK cell activity through cytokines produced by other MAM-activated cell types. Recently, Rink and coworkers described several cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, and gamma interferon [IFN-γ]) that were found in MAM-treated PBMC cultures (51). Ours is the first report describing the effects of MAM on human NK cells.

## MATERIALS AND METHODS

**Media.** Assays were done with complete medium, which consisted of RPMI 1640 medium supplemented with $5 \times 10^{-5}$ M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.), 10% normal human male AB serum (North American Biologicals, Miami, Fla.), 2 mM L-glutamine, 100 U of penicillin G and 100 μg of streptomycin per ml, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GIBCO, Gaithersburg, Md.). Cells were washed in Hanks balanced salt solution (HBSS; Sigma).

**Reagents and antibodies.** MAM was prepared by a modification of previously described methods (2, 3). In brief, $(NH_4)_2SO_4$ fractionation was followed by Sephadex G-50 chromatography and then by two cation-exchange columns, Sepharose-S and Mono-S (Pharmacia LKB). The resulting material was roughly ≤10% pure. Diluted MAM was stored at −70°C in aliquots in complete RPMI 1640 medium. Phycoerythrin-conjugated anti-Leu-19 (CD56; immunoglobulin G1 [IgG1]), fluorescein isothiocyanate (FITC)-conjugated anti-class II MHC (HLA-DR; IgG2a), and FITC-conjugated anti-keyhole limpet hemocyanin (IgG2a) were purchased from Becton Dickinson (Mountain View, Calif.). The OKT-3 (anti-CD3ε) hybridoma was purchased from the American Type Tissue Collection (Rockville, Md.); concentrated tissue culture supernatants of OKT-3 hybridoma were used at a concentration of 100 μg of IgG2a per ml per $10^8$ cells. Polyclonal rabbit anti-human IL-2 and mouse monoclonal anti-human IFN-γ were purchased from Genzyme (Cambridge, Mass.). Goat polyclonal anti-human IL-12 was a generous gift from M. Gately, Hoffmann-La Roche (Nutley, N.J.). Control goat IgG anti-*Pneumocystis carinii* was prepared by B. Amole, University of Miami School of Medicine; control polyclonal rabbit anti-rat lectin was a gift from P. Whitney, University of Miami School of Medicine; and mouse monoclonal anti-keyhole limpet hemocyanin (IgG2a) was purchased from Becton Dickinson.

**Cell lines.** K562 (38) and Raji (27) cells were maintained in complete medium with 10% fetal bovine serum (HyClone, Logan, Utah). Cell lines were free of mycoplasma as routinely determined by the Myco-Tect kit (GIBCO).

**Preparation of PBMC populations.** Leukopacks (buffy coats) were purchased from the American Red Cross (Miami, Fla.), and mononuclear cells were isolated within 24 h of collection as described previously (6). Briefly, contents were diluted 1:4 with phosphate-buffered saline, carefully layered on Ficoll-Hypaque cushions (Accurate Chemical, Westbury, N.Y.), and centrifuged (1,000 × *g*, 15 min, 25°C). Mononuclear cells were collected and washed three times with HBSS. The viability of the collected, washed mononuclear cells was determined by trypan blue vital dye exclusion and was routinely greater than 95%.

**Purification of NK cells.** Mononuclear cells were incubated for 2.5 h (37°C, under 5% $CO_2$ and air) at $2 \times 10^6$/ml of complete medium with 20% fetal bovine serum in tissue culture flasks (26). Plastic nonadherent cells were treated with monoclonal antibody OKT-3 and baby rabbit complement (brc′; Accurate Chemical) as described previously (5) to remove T lymphocytes, and viable cells were isolated by centrifugation on Ficoll-Hypaque cushions as described above. Cells were stained with 50 μl of undiluted phycoerythrin-conjugated Leu-19 (CD56) per $10^7$ cells for 30 min at 4°C, washed in HBSS, and subjected to fluorescence-activated cell sorting. Sorting was performed on a Becton Dickinson FACstar machine, and phycoerythrin-positive cells that fell within the lymphocyte gate on forward- and side-scatter parameters were collected. Sorts routinely yielded populations of 97 to 99% $CD56^+$ lymphocytes as determined by flow cytometry. Cells not identified as $CD56^+$ lymphocytes in the post-sort population represented cells that had died during the sorting process. Monocytes were restricted from the population by the lymphocyte gates used when sorting populations of NK cells, and no monocytes were present in the NK population as determined by Diff-Quick-modified Wright-Giemsa differential cell staining.

**Adherence of NK cells to plastic.** PBMCs were incubated (24 h, 37°C, under 5% $CO_2$ and air) at $10^6$ cells per ml in tissue-culture-treated polystyrene flasks (Costar, Cambridge, Mass.) with or without $10^{-5}$ MAM. To assess NK activation and adherence, the contents of the flasks were collected after 24 h of incubation. Nonadherent cells were collected by aspirating the contents of gently swirled flasks and pooling them with two washes of warm (37°C) medium. Cold medium (4°C) was then added to the flask, and adherent cells were collected with a cell scraper (Costar) and by aspiration. Cells were counted by trypan blue exclusion, and the percent adherent cells in the total population was calculated.

**Flow cytometry.** K562 or Raji cells were given fresh medium 1 day prior to use, washed with HBSS (three times), and counted by trypan blue dye exclusion and stained as described in the manufacturer's protocol with either FITC-conjugated anti-class II MHC or an FITC-labeled isotype-specific control. Ten thousand cells were analyzed on a Becton Dickinson FACstar flow cytometer, and data were analyzed with the Lysys II program.

**Chromium release assay.** Graded numbers of effector cells were incubated (16 to 20 h, 37°C, under 5% $CO_2$ and air) in quadruplicate in 96-well V-bottom microtiter plates (Costar) with or without $10^{-5}$ MAM. Plates were centrifuged (300 × *g*, 5 min, 25°C), supernatants were removed, and tumor target cells that were incubated (90 min, 37°C, under 5% $CO_2$ and air) with $Na_2{}^{51}CrO_4$ (100 μCi/$10^6$ cells per 200 μl) and washed (four times) with HBSS were added to each well in 200 μl of complete medium. Plates were again centrifuged (300 × *g*, 5 min, 25°C) to promote effector cell-target cell interaction and then were incubated for 4 h (37°C, under 5% $CO_2$ and air). After the incubation, 100 μl of supernatant was harvested from each well. Radioactivities of the supernatants were measured in a gamma counter, and percent specific lysis was calculated by the following formula: % specific lysis = $[(cpm_{exp} - cpm_{spont})/(cpm_{max} - cpm_{spont})] \times 100$, where cpm is counts per minute, exp is experimental release, spont is spontaneous release, and max is maximum release.

Differences in percent specific lysis values among the five wells for a particular condition were routinely less than 10%. Spontaneous release was determined from targets incubated without any effector cells, and maximum release was determined from 100 μl of well-suspended labeled target cells.

For the purposes of comparing experiments, percent specific lysis was converted to lytic units. Lytic units per $10^6$ cells ($LU_{25}$) represented the ability of an effector cell population to lyse 25% of target cells (8). Lytic unit calculation was done with the Expfit program determining exponential fit with user-chosen effector/target cell ratios (49). Standard deviations were also calculated by the Expfit program by linear regression analysis. The augmentation index (AI) represented the fold change in lytic activity of effector cells incubated with MAM compared with that of effector cells incubated without MAM and was calculated by the following formula: $AI = (LU_{25\ MAM})/(LU_{25\ Medium})$. The standardized decrease in augmentation (SDA) by neutralizing antibodies, which reflected the percent inhibition, was calculated by the following formula:

$$SDA = 100 \times [(LU_{25\ MAM} - LU_{25\ MAM + Antibody})/(LU_{25\ MAM} - LU_{25\ Medium})]$$

**Statistical analysis.** Data were analyzed by a one-tailed Student *t* test based on a directional hypothesis which postulated that incubation of effector cells with superantigen would increase cytotoxicity. Differences between $LU_{25}$ values observed in cultures incubated with or without MAM were considered statistically significant if $P$ was ≤0.05.

## RESULTS

**Effect of MAM on human NK cell cytotoxicity.** To investigate whether MAM affected human NK cells, several parameters of NK cell activity were studied. We tested the ability of the MAM superantigen to up-regulate NK cytotoxicity in a dose-dependent manner against K562 target cells and examined whether the effect was mediated directly on NK cells or mediated indirectly through cytokines produced by superantigen-activated cells in the peripheral blood.

To study the effect of MAM on the NK activity of human peripheral blood, PBMCs were incubated with or without MAM ($10^{-5}$ final dilution) and then used as effector cells in a 4-h chromium release assay against a conventional NK target. K562 is a well-described NK-sensitive human erythroleukemic cell line (38). As reported, and similar to results with the staphylococcal enterotoxin B (SEB) superantigen (24), baseline NK cytotoxicity levels varied among individuals tested, but in 13 of 13 samples, incubation with MAM yielded higher levels of killing of K562 than those recorded for PBMCs incubated without MAM (Table 1). The augmentation indices observed in PBMCs incubated with MAM ranged from 1.5 to 16.6. These studies were repeated with MAM prepared by additional purification steps such as hydrophobic interaction

TABLE 1. Effect of MAM on NK activity of PBMC[a]

| Donor no. | Mean $LU_{25} \pm SD$[b] | | AI[c] |
|---|---|---|---|
| | Medium | MAM | |
| 1 | 2.5 ± 0.5 | 6.8 ± 0.2 | 2.7 |
| 2 | 0.7 ± 0.2 | 1.5 ± 0.2 | 2.1 |
| 3 | 4.3 ± 0.2 | 8.8 ± 0.5 | 2.0 |
| 4 | 2.2 ± 0.5 | 9.0 ± 0.7 | 4.1 |
| 5 | 3.6 ± 1.0 | 13.6 ± 2.4 | 3.8 |
| 6 | 2.8 ± 0.3 | 8.3 ± 0.9 | 3.0 |
| 7 | 5.8 ± 0.3 | 12.2 ± 2.8 | 2.1 |
| 8 | 8.6 ± 3.1 | 16.5 ± 3.1 | 1.9 |
| 9 | 1.4 ± 0.3 | 7.9 ± 1.2 | 5.6 |
| 10 | 4.8 ± 1.6 | 12.9 ± 2.1 | 2.7 |
| 11 | 1.6 ± 0.3 | 3.7 ± 0.5 | 2.3 |
| 12 | 0.8 ± 0.5 | 13.3 ± 1.2 | 16.6 |
| 13 | 3.9 ± 0.9 | 5.8 ± 0.7 | 1.5 |

[a] PBMCs were harvested from leukopacks from the American Red Cross. Various numbers of effector cells from individual donors ($10^6$ cells per ml) were incubated in the absence (Medium) or presence (MAM) of MAM ($10^{-5}$ final dilution). After the incubation, $^{51}Cr$-labeled K562 cells were added, and cytotoxicity was calculated as described in Materials and Methods.

[b] $LU_{25}$ (lytic units per 25% target cell lysis) were calculated from percent specific lysis values from three effector/target cell ratios (50:1, 25:1, and 12.5:1) as described in Materials and Methods.

[c] Augmentation index (AI) = $LU_{25\ MAM}/LU_{25\ medium}$; values greater than 1.0 indicate increase in lytic potential.

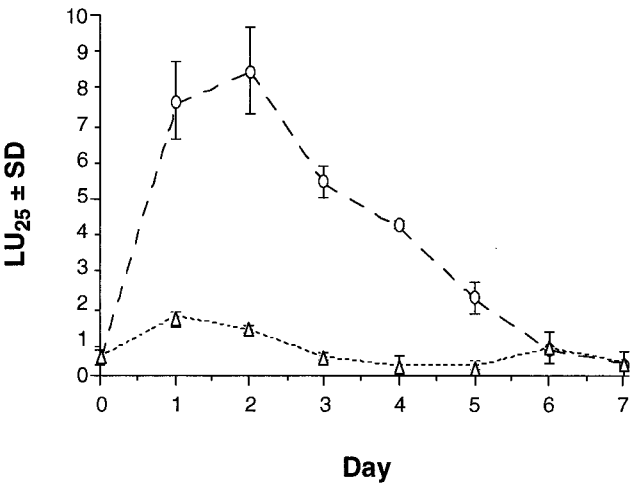


FIG. 2. Kinetics of MAM-mediated NK augmentation in PBMCs. PBMCs ($10^6$/ml) were incubated (37°C, under 5% $CO_2$ and air) for the indicated times in 96-well V-bottom microtiter plates with a $10^{-5}$ final dilution of MAM (open ovals) or with no superantigen (open triangles). NK cytotoxicity was then determined by the ability of PBMCs to lyse $^{51}Cr$-labeled K562 target cells as described in Materials and Methods. This experiment was repeated three times with similar results.

chromatography (3), and similar results were obtained (data not shown). These data show that MAM consistently augmented the NK activity of human PBMCs in a statistically significant manner. The factors contributing to the variation in the degree of augmentation observed between individuals, however, remain uncharacterized. We next found that MAM-induced NK augmentation was dose dependent by observing that the magnitude of superantigen-activated killer (SAK) activity was relative to the concentration of superantigen present in the incubation mixture (Fig. 1).

Having shown that MAM routinely augmented the NK cytotoxicity in overnight PBMC cultures, we were interested in the kinetics of the MAM-induced SAK response. The optimal incubation time for PBMC SAK induction was determined by testing the cytotoxic potential of PBMCs cultured with MAM daily for 7 days against K562 target cells. Kinetic experiments showed that the greatest change in NK activity occurred within the first 24 h, and peak levels of SAK activity were reached by 48 h (Fig. 2).

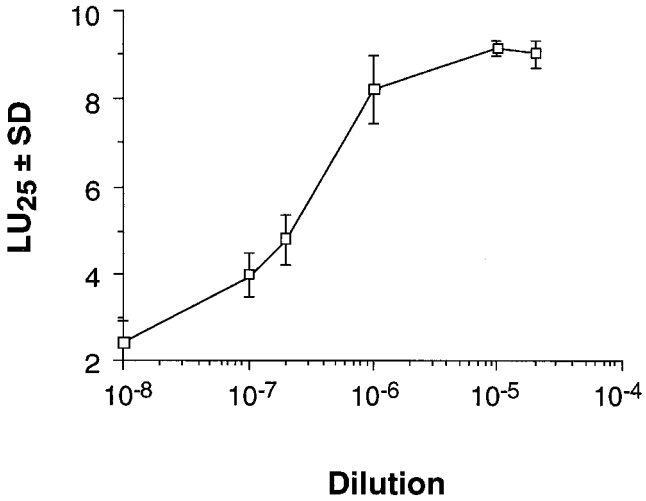


FIG. 1. Dose-dependent up-regulation of NK activity by the MAM superantigen. PBMCs ($10^6$/ml) were incubated (16 to 20 h, 37°C, under 5% $CO_2$ and air) in 96-well V-bottom microtiter plates with the indicated concentration of MAM. NK cytotoxicity was then determined by the ability of the cells to lyse $^{51}Cr$-labeled K562 target cells as described in Materials and Methods. This experiment was repeated four times with similar results.

**Effect of MAM on adherence of NK cells to plastic.** Experiments described thus far show that MAM activated human NK cells to a higher cytotoxic state. Another parameter of activation of NK cells is the induction of plastic adherence, which may be related to a higher expression of cell surface adhesion molecules and/or an increased binding affinity of preexisting adhesion molecules on the cell surface (10). Induction of plastic adherence of NK cells is associated with exposure to NK-activating cytokines (33, 43, 47). Therefore, to assess the effects of MAM on the adhesion characteristics of human NK cells, PBMCs were incubated with or without MAM for 24 h. Nonadherent and adherent cells were collected, quantified, and tested for their ability to lyse K562 target cells. Incubation of PBMCs with MAM for 24 h induced approximately 17% of the total cells to become adherent to plastic (data not shown). In contrast, cultures of PBMCs incubated in the absence of MAM contained no adherent cells at 24 h. Although the expression of adhesion molecules is a consequence of the activation process, this adhesion is not a marker for SAK activity, which is defined by enhanced NK cytotoxicity. SAK activity was detected in both adherent and nonadherent populations incubated with MAM (Table 2). These data showed that some MAM-activated NK cells became plastic adherent, implying that in MAM-treated cultures, NK cells were being acted upon by locally produced cytokines.

To assess the potential for T-cell-mediated, rather than NK-cell-mediated, lysis of K562 cells, K562 cells or Raji cells (positive control) were stained with fluorochrome-conjugated anti-class II MHC antibodies and analyzed by flow cytometry (Fig. 3). Whereas Raji, a human B-cell lymphoma line known to express class II molecules (21), was strongly positive for HLA-DR, K562 cells did not stain positively for MHC class II,

TABLE 2. NK activity of adherent and nonadherent populations in MAM-treated PBMCs[a]

| Donor no. | Mean $LU_{25}$ ± SD[b] | | | AI[c] | |
|---|---|---|---|---|---|
| | Medium, nonadherent | MAM, nonadherent | MAM, adherent | MAM, nonadherent | MAM, adherent |
| 13 | 10.7 ± 1.6 | 17.0 ± 1.4 | 14.0 ± 2.6 | 1.6 | 1.3 |
| 14 | 9.9 ± 0.5 | 14.2 ± 1.4 | 13.9 ± 0.2 | 1.4 | 1.4 |
| 15 | 4.1 ± 0.9 | 9.6 ± 0.2 | 8.4 ± 2.1 | 2.3 | 2.0 |

[a] PBMCs were harvested from leukopacks from the American Red Cross; adherent and nonadherent populations were obtained as described in Materials and Methods. Cells ($10^6$ cells per ml) from each donor were incubated (24 h) in the presence (MAM) or absence (medium) of MAM prior to their use as effector cells in 4-h cytotoxicity assays against K562 target cells.
[b] $LU_{25}$ (lytic units) were calculated as described in Table 1, footnote *b*.
[c] The augmentation index (AI) was calculated as described in Table 1, footnote *c*.

TABLE 3. Effect of MAM on NK activity of sorted $CD56^+$ cells[a]

| Donor no. | Mean $LU_{25}$ ± SD[b] | | AI[c] |
|---|---|---|---|
| | Medium | MAM | |
| 1 | 11.4 ± 0.9 | 14.8 ± 1.0 | 1.3 |
| 2 | 31.5 ± 1.0 | 34.3 ± 1.9 | 1.1 |
| 3 | 243.2 ± 2.8 | 203.2 ± 47.3 | 0.8 |
| 4 | 50.4 ± 4.5 | 57.5 ± 2.9 | 1.1 |
| 5 | 184.9 ± 11.8 | 220.5 ± 13.7 | 1.2 |
| 6 | 51.4 ± 2.8 | 70.2 ± 4.3 | 1.4 |
| 7 | 252.6 ± 13.3 | 316.1 ± 59.6 | 1.3 |
| 8 | 166.3 ± 28.9 | 195.6 ± 39.5 | 1.2 |
| 9 | 96.3 ± 9.5 | 141.3 ± 3.6 | 1.5 |
| 10[d] | 81.3 ± 5.2 | 85.9 ± 4.7 | 1.1 |
| 11[d] | 81.2 ± 5.9 | 87.6 ± 6.2 | 1.1 |
| 12 | 30.5 ± 2.9 | 50.4 ± 5.4 | 1.7 |
| 13 | 16.8 ± 1.7 | 12.6 ± 1.6 | 0.5 |

[a] $CD56^+$ cells were enriched from leukopacks from the American Red Cross; the purity of sorted cells was 97 to 99% $CD56^+$ lymphocytes. Donor numbers are the same as those in Table 1. Incubation procedures correspond to those described in Table 1, footnote *a*.
[b] $LU_{25}$ (lytic units) were calculated as described in Table 1, footnote *b*.
[c] The augmentation index (AI) was calculated as described in Table 1, footnote *c*.
[d] No increase in $LU_{25}$ was observed when overlapping standard deviations were considered.

thereby ruling out the contribution of T cells (SDCC) in MAM-augmented cytotoxicity observed in our experiments.

**Effect of MAM on highly purified NK cells.** Because human NK cells express class II molecules (7, 44), it was reasonable to postulate that MAM could activate NK cells via binding and signaling through MHC class II surface molecules. To study the direct effect of MAM on the cytotoxicity of human NK cells, $CD56^+$ cells were purified from PBMCs by cell sorting, incubated overnight in the presence or absence of MAM, and then evaluated for their cytotoxicity against K562 target cells. MAM-induced augmentation indices of the cytotoxic activity of sorted $CD56^+$ cells ranged from 0.8 to 1.7, and compared with resting activity were increased above 1.0 in 9 of 13 specimens (Table 3). Thus, incubation of highly purified $CD56^+$ cells with MAM resulted in statistically significant augmented NK cytotoxicity. Together, the results in Tables 1 and 3 support the hypothesis that MAM can induce a signal to increase lytic activity in NK lymphocytes in the absence of other immune cells. The fact that the presence of other immune cells appears to support the highest levels of MAM-induced NK augmentation suggests that optimal activation requires more than one signal.

**Effect of neutralizing antibodies to cytokines on SAK activation.** Since MAM-mediated SAK induction was greater in the presence of other immune cells (PBMCs) than with sorted NK cells alone, we were interested in the mechanism of SAK activation in mixed PBMC cultures. Induction of SAK activity in PBMCs could be mediated by secretion of soluble factors (i.e., cytokines) from superantigen-activated cells or by direct cell-cell contact between cells. To determine whether soluble factors were involved in MAM-induced NK activation, PBMCs were incubated with MAM in the presence or absence of neutralizing antibodies to various cytokines known to up-regulate NK activity (Table 4). Antibodies to IL-2 and IFN-γ each inhibited SAK activity in five of seven experiments, and antibodies to IL-12 inhibited SAK induction in five of six experiments. In determining whether anticytokine antibodies affected MAM-induced NK augmentation, lytic unit values of PBMCs incubated with MAM alone or with MAM plus an anticytokine antibody were compared by a paired Student *t* test. Therefore, the collective differences in lytic activity between MAM-treated PMBCs incubated with or without the specific anticytokine antibody were analyzed statistically, and differences were considered significant if $P$ was $<0.05$. Overall, anti-IL-2 antibodies significantly inhibited SAK induction, while anti-IFN-γ or anti-IL-12 antibodies alone did not significantly affect MAM-mediated SAK activity. Isotype and species-specific control antibodies were used at similar concentra-

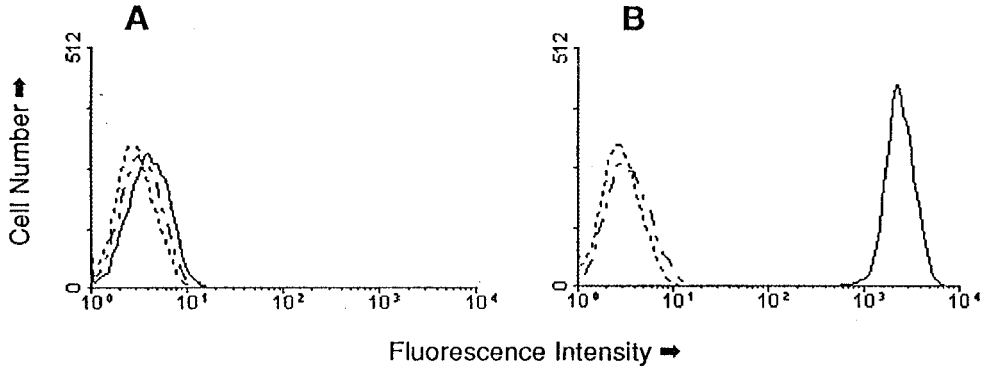


FIG. 3. Expression of class II MHC molecules by tumor target cells. K562 cells (A) or Raji cells (B) were stained with either no antibody (dotted line), an FITC-conjugated isotope-specific control antibody (anti-keyhole limpet hemocyanin; dashed line), or FITC-labeled anti-MHC class II antibody (anti-HLA-DR; solid line) as described in the manufacturer's instructions. Cells were analyzed by flow cytometry on the fluorescence intensity axis as shown.

TABLE 4. Neutralization of MAM-mediated NK augmentation in PBMCs by anticytokine antibodies[a]

| Donor no. | Mean $LU_{25} \pm SD$[b] | | | | |
|---|---|---|---|---|---|
| | Medium | MAM | MAM + anti-IL-2 | MAM + anti-IFN-γ | MAM + anti-IL-12 |
| 16 | 4.5 ± 1.4 | 19.3 ± 4.3 | 8.0 ± 1.4 (76.4)[c] | 10.0 ± 2.4 (62.8) | 18.1 ± 2.6 (8.1) |
| 17 | 7.2 ± 0.5 | 16.0 ± 0.9 | 12.1 ± 0.7 (44.3) | 10.1 ± 2.2 (67.0) | 12.1 ± 1.0 (44.3) |
| 18 | 2.1 ± 0.5 | 5.1 ± 1.0 | 5.9 ± 0.9 (0.0) | 3.9 ± 1.0 (40.0) | 4.0 ± 0.3 (36.7) |
| 19 | 0.8 ± 0.2 | 2.1 ± 0.2 | 1.6 ± 0.2 (38.5) | 3.2 ± 0.5 (0.0) | 1.8 ± 0.2 (23.1) |
| 23 | 2.1 ± 0.3 | 9.5 ± 0.7 | 7.5 ± 2.1 (27.0) | 12.2 ± 1.7 (0.0) | ND[d] |
| 37 | 4.7 ± 0.7 | 14.0 ± 0.5 | 10.9 ± 1.9 (33.3) | 13.8 ± 2.3 (2.2) | 16.1 ± 2.4 (0.0) |

[a] PBMCs were incubated (16 to 20 h, 37°C, under 5% $CO_2$ and air) with medium alone, MAM ($10^{-5}$ dilution), MAM plus polyclonal rabbit anti-IL-2 (1:40), MAM plus mouse monoclonal anti-human IFN-γ (1:100), or MAM plus polyclonal goat anti-human IL-12 (10 μg/ml). Control isotype and species-specific antibodies used at similar concentrations had no effect on SAK activity.

[b] $LU_{25}$ (lytic units) were calculated as described in Table 1, footnote *b*.

[c] Values in parentheses indicate the standardized decrease of augmentation (SDA), which is calculated as follows: $SDA = 100 \times [(LU_{25\ MAM} - LU_{25\ MAM + Antibody})/(LU_{25\ MAM} - LU_{25\ medium})]$.

[d] ND, not determined.

tions and did not affect SAK induction ($P > 0.05$). We concluded from these experiments that cytokine products of superantigen-stimulated T lymphocytes contribute to MAM-mediated SAK induction in PBMCs and that the degree of involvement of specific cytokines in SAK induction varies between individuals.

## DISCUSSION

Effects of superantigens on T-cell functions have been well studied, but how superantigens interact with and affect NK cells is less clear. This report shows that the MAM superantigen activates NK lymphocytes isolated from human peripheral blood to a state of increased adhesion and heightened lytic potential. The novel findings reported in this manuscript are as follows: (i) incubation of human PBMCs with MAM enhances NK cytotoxic activity; (ii) MAM increases the lytic activity of purified NK cells in the absence of accessory cells; and (iii) optimal enhancement of NK cytotoxicity by MAM is mediated in part through cytokines.

MAM, a 26-kDa protein produced by the murine pathogen *M. arthritidis*, is implicated in causing rheumatoid arthritis in rodents. Besides being active in the murine system, MAM also affects human T cells by virtue of its superantigenic properties (12). Interestingly, when MAM was tested for its effects on human NK cells in PBMCs, we found that MAM augmented NK cytotoxicity against K562 cells. An important caveat of these experiments is that superantigen (bound to T cells and/or class $II^+$ cells in the stimulated PBMC cultures) may have been present during the cytotoxicity assays (SDCC) against K562. While this would influence T-cell-mediated cytotoxicity against class II-bearing target cells, there is no evidence that the presence of superantigen per se affects interactions between NK cells and class II $MHC^-$ target cells such as K562.

We also studied the effects of another superantigen, SEB, on human NK cells (24). Using PBMCs isolated from the same donor on the same day, we compared the abilities of MAM and SEB to upregulate NK cytotoxicity against K562 target cells. In 13 of 13 donors tested, SEB up-regulated NK cytotoxicity to a greater extent than MAM, and therefore we concluded that SEB was a more potent stimulator of human NK cells than MAM in mixed cell (PBMC) populations (24). We postulate that SEB is stronger than MAM because *S. aureus* and its products evolved to interact with the human immune system, whereas *M. arthritidis* evolved to interact with the murine system. If this postulate is true, then differences in the response of human NK cells to MAM and SEB may be due to host species preferences of the two superantigens. T-cell activation studies by other laboratories support this hypothesis: (i) MAM is a more potent superantigen in the murine system than staphylococcal enterotoxins (9), and (ii) staphylococcal superantigens evoke greater T-cell proliferation in the human system than MAM does (30). An alternative hypothesis is that differences in potency may reflect differences in the three-dimensional structures between the two superantigens. All superantigens may not conform to one general folding motif, and different superantigens are thought to interact with T-cell receptors or MHC class II molecules differently. Although the three-dimensional structure of MAM has not yet been determined, X-ray crystallographic studies showed that two other superantigens, toxic shock syndrome toxin 1 and SEB, exhibited structural differences (1, 58).

The hypothesis that superantigen would directly activate NK cells was raised because a subpopulation of resting human NK cells expresses class II MHC, and class II MHC-mediated cell activation (either through ligation by monoclonal antibody or superantigen) was demonstrated in other cell types (11, 31, 56, 59–61). We propose that superantigens bind to MHC class II on the surface of human NK cells and that this binding stimulates NK cells. In support of this hypothesis, we showed that the addition of anti-HLA-DR antibodies abrogates the ability of NK cells to function as superantigen-presenting cells (24a). Therefore, we conclude that superantigens interact with NK cells via class II MHC molecules.

Both MAM and SEB (24) were used to test the hypothesis that superantigen directly augments NK cytotoxicity. Surprisingly, the results differed between MAM and SEB. When sorted $CD56^+$ cells were incubated with superantigen, MAM, but not SEB, induced a slight but statistically significant increase in NK activity (24). The reason why MAM, but not SEB, significantly up-regulated NK activity in cultures of only $CD56^+$ cells is not known and was surprising since SEB was more potent than MAM in PBMC cultures. An intriguing but untested possibility is that human NK cells express a costimulatory receptor in addition to MHC class II that is bound and activated by MAM but not SEB. Human NK cells may have evolved so that this receptor (which recognizes superantigen from a murine pathogen but not that from a human pathogen) fails to bind superantigen from frequently acquired infections. In this model, activation of NK cells by superantigen might interfere with the orderly sequence of events in conventional immune responses to microorganisms possibly by inappropriate secretion of cytokines (52). Thus, it seems evolutionarily

advantageous to have NK cells not recognize frequently encountered superantigens such as the staphylococcal exotoxins.

The observation that the NK cytotoxic augmentation induced by MAM was greater in PBMC cultures than in purified populations of NK cells suggests that a significant effect of superantigen on NK cells was mediated through the participation of other cells. Thus, for the optimal effect of MAM on human NK cells, a second signal is required, and we hypothesize that cytokines secreted by MAM-activated cells in the peripheral blood may be able to provide the cosignal. Previous workers showed that the supernatant of MAM-treated human PBMCs contained cytokines known to increase NK activity (51). In these studies, we show that IL-2 played an important role in MAM-induced NK activation, and we cannot rule out the possibility that this process might involve other NK-augmenting cytokines as well.

Work presented in this report may have therapeutic implications for a variety of patients who would benefit from enhanced NK cytotoxic activity. Removing immune cells from cancer patients, activating them in vitro with cytokines, and reinoculating them into the patient in hopes of promoting immune clearance of tumors have been well studied (34, 54, 55). In particular, Rosenberg and others have investigated such lymphokine-activated killer (LAK) cells and their effect on patients with established cancers. Their procedure for generating LAK cells involved high-dose stimulation with IL-2 (48, 63), and in certain types of cancer (e.g., renal cell carcinoma and melanoma), LAK therapy provided some benefit in certain patients (28, 64). NK cells were present among IL-2-generated LAK cells, and they mediated significant antitumor cytotoxicity (50). Our data show that similar cytokines up-regulate NK cell cytotoxicity in response to superantigen exposure. In particular, IL-2 was involved in SAK activation, and we do not rule out the possibility that other cytokines participate in SAK induction. It follows, then, that treatment of immune cells with superantigens generates a potent cytotoxic activity involving IL-2 and other unidentified cytokines as well as direct interaction.

A novel idea generated from our studies is to use MAM in vivo as well as in vitro as immunotherapy against infectious agents in immunocompromised individuals. It is known that NK cells have antibacterial (32), antimycoplasmal (41), and antiparasitic (20, 57) activity and that their cytotoxicity against microorganisms is enhanced in the presence of cytokine activators (20). Thus, we and others have suggested that therapy with activated NK cells might benefit immunocompromised individuals in the treatment of infections (32, 41). SAK activation may be an effective therapy in the defense against a variety of bacterial, viral, and parasitic infections. By use of a superantigen such as MAM, which has fewer effects on human T cells than staphylococcal superantigens but adequately upregulates human NK cytotoxicity, this method of generating SAK cells may incur fewer side effects than staphylococcal superantigens (since products of activated T cells cause shock-like effects in vivo). Therefore, less-toxic superantigens could possibly be coinjected with the SAK cells in situ in hopes of maximizing SAK activation.

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