MONOCLONAL ANTIBODY TO A TRIGGERING STRUCTURE EXPRESSED ON RAT NATURAL KILLER CELLS AND ADHERENT LYMPHOKINE-ACTIVATED KILLER CELLS

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The in vitro activation of splenocytes or PBL with rIL-2 generates a population of cytotoxic effector cells with broad antitumor reactivity (1-4). These lymphokine-activated killer (LAK) cells have been suggested to be useful for the adoptive immunotherapy of metastatic tumors in animal models and in man (5-7). LAK cells have the ability to bind and lyse virtually any tumor cell, but do not lyse normal cells (1, 4, 7-10). Although LAK cells have gained much attention due to this property, very little is known about the nature of the surface structures used by LAK cells to recognize and lyse tumors.

Recent evidence suggests that the majority of cells with LAK activity are derived from the NK/large granular lymphocyte (LGL) subset of lymphocytes. Many studies indicate that LAK progenitor cells from the mouse have a phenotype characteristic of NK cells, as they express asialo GM1, do not express L3T4 or Lyt-2, and as some express Thy-1 (11-14). Studies using human lymphocytes have suggested that LAK cell progenitors are mainly LGL with a CD3-, CD16+, NKH1+, phenotype (3, 9, 10, 15-17). In the rat, the majority of LAK progenitor cells have been described as being LGL that express an asialo GM1+, 0K8+, Lam+, 0X19-, 0X6-, W3/25-, Ig- phenotype (8, 18-20).

While the majority of evidence suggests that LAK progenitor cells are derived from LGL/NK cells, a number of groups have presented evidence that LAK cells can be generated from other lymphoid compartments. CD3+, NKH1+ lymphocytes have been demonstrated in human peripheral blood and have been associated with non-MHC-restricted cytotoxicity. Further, these cells have been used to produce clones with NK-like activity (21-23). Other studies suggest that LAK activity can be generated from a variety of subpopulations, including CD4+, CD8+, and B cells (24). Recent studies using cells from human thymus have shown that LAK activity can be generated from thymocytes of either a CD3+, CD4+, CD8- or CD3-, CD1-, CD2+ phenotype (18, 25-29).

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Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; A-LAK, adherent LAK; FDA, fluorescein diacetate; LAK, lymphokine-activated killer; LGL, large granular lymphocyte; PMN, polymorphonuclear leukocyte.
LAK effector cells have been shown to be very similar in phenotype to their progenitor cells. In human, they are mainly LGL that express a CD3−, NKH1+, CD16+ phenotype (3, 9, 10, 16, 17). Mouse LAK cells are characteristically asialo GM1+, L3T4−, Lyt-2− and may or may not express Thy-1 (11-14). Rat LAK cells are mainly LGL expressing an asialo GM1+, 0X8+, Lam−, 0X6−, 0X19−, W3/25−, Ig− phenotype (8, 18-20).

While there are several NK-specific markers available in the various species, none is without limitations for the study of the relationship of NK and LAK cells. In humans, CD16 (Leu-11) has been found on virtually all NK cells (30), but its expression decreases on LAK cells cultured 2-3 d in rIL-2 (unpublished data). NKH1 (Leu-19) is found on almost all NK cells, but it is also detected on a small percentage of CD3+ T cells (31, 32). In the mouse and rat, asialo GM1 is expressed on NK cells and LAK cells but it is also expressed on a variety of other subpopulations (14, 33-36). The marker 0X8 is expressed on rat NK and LAK cells, but it is also on ∼30% of rat T cells (18, 20, 37). Lam 1 is specific for rat NK cells, but not all NK cells (∼50%) express this marker (20). For these reasons, new markers that are specific for NK cells and that are expressed on LAK cells generated from NK cells, will be of great use for the characterization and enumeration of progenitor and effector populations of LAK cells.

Vujanovic et al. (38) recently developed a simple, reproducible technique for the isolation of large numbers of highly purified (>98%) populations of rat splenocytes with activated NK phenotype (0X6+, 0X8+, Lam−, Asialo GM1+, 0X19−, W3/25−, Ig−), LGL morphology, and LAK cytolytic activity (38). In isolating these cells, use is made of the observation that LGL selectively adhere to plastic surfaces after stimulation with rIL-2. Because of this property, these cells are termed adherent LAK (A-LAK) cells.

To explore the relationship of NK and LAK cells, and their function, we have generated a series of hybridomas secreting mAbs against A-LAK cells that affect the cytolytic activity or proliferation of A-LAK cells. In this report, we describe the production of a hybridoma that secretes an mAb (3.2.3; IgG) that recognizes a triggering structure expressed on rat LGL/NK cells and LAK cells generated from this subpopulation (A-LAK).

Materials and Methods

Reagents. mAbs for rat NK cells, T cells, macrophages, and B cells were obtained from Serotec Limited (Oxon, UK). R-phycoerythrin (RPE)- and FITC-conjugated goat anti-mouse Igs were obtained from Organon Teknika-Cappel (Malvern, PA). Human rIL-2 was the generous gift of Cetus Corp. (Emeryville, CA). Anti-HLA-DR (IgG1), used as an isotype control, was kindly provided by Dr. M. Trucco (Pittsburgh Cancer Institute). Na252CrO4 was purchased from New England Nuclear (Boston, MA). Tissue culture medium (RPMI 1640), FCS, and antibiotics (penicillin/streptomycin) were obtained from Gibco Laboratories (Grand Island, NY).

Animals, Cells from Selected Organs, and Cell Lines. Fischer 344 male rats (F344, 75-100 g) were obtained from Taconic Farms, Inc. (Germantown, NY) and maintained in a specific pathogen-free animal facility for at least 2 wk before use. BALB/c female mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free animal facility. CRNK-16, an F344 LGL leukemia; MADBi06, an F344 rat mammary adenocarcinoma; YAC-1, a mouse T cell lymphoma; PB15, a mouse mastocytoma; Daudi, a human B lymphoblast cell line; K562, a human erythroleukemia; and U937, a human histiocytic
lymphoma, were maintained as monolayers or stationary suspension cultures in RPMI 1640 medium containing 10% FCS, antibiotics, and L-glutamine (complete medium) at 37°C in a humidified 5% CO₂ atmosphere. Cell cultures were passaged as required to maintain the cultures in a log phase of growth. Lymphocytes from various organs were obtained by aseptic removal of the organs with subsequent isolation of cells by mincing (if required) followed by centrifugation on Ficoll or Percoll density gradient medium.

Preparation of NK and A-LAK Cells. NK and A-LAK cells were prepared as described (38). Briefly, splenic lymphocytes were isolated by density gradient centrifugation of splenocytes on Ficoll-Hypaque (Φ = 1.077) (Pharmacia Fine Chemicals, Piscataway, NJ) followed by passage over nylon wool columns. These cells were used as effector cells in NK assays. For A-LAK cells, nylon wool nonadherent lymphocytes were cultured at 2.0 x 10⁶/ml in complete medium containing 5 x 10⁻⁵ M 2-ME and 1,000 U/ml rIL-2 in 75-cm² tissue culture flasks (Corning Glass Works, Corning, NY). After stimulation with rIL-2, LGL selectively adhere to the plastic surface. After 48 h, the nonadherent cells were decanted and removed from the supernatant by centrifugation. The supernatant was then filtered and added back to the adherent cells (conditioned medium). The adherent cells were then cultured for an additional 3–4 d. Effector cells isolated in this fashion express markers and morphology characteristic of rat NK cells (18–20, 38).

mAbs. mAbs were produced as previously described (39). Supernatants of the fusion products of P3.653 and spleen cells of BALB/c mice, hyperimmunized with rat A-LAK cells, were screened for their effect on cytolytic activity of rat A-LAK cells. The supernatant from one well enhanced cytotoxicity against P815 target cells. These cells, which produce an IgG1 product, were cloned three times by limiting dilution and designated 3.2.3. Hybridoma supernatants and ascites fluid from hybridoma-bearing BALB/c mice were used in these studies.

Cytotoxicity Assays. Cytotoxicity was measured using a standard 4-h ⁵¹Cr release microcytotoxicity assay in 96-well, round-bottomed microtiter plates (Costar, Cambridge, MA) as previously described (40). YAC-1 or P815 target cells (2–3 x 10⁶) were labeled with 100 μCi of Na₂⁵¹CrO₄ for 60 min, washed three times, resuspended at 10⁶/ml in complete medium. Target cells were then seeded into 96-well plates at 5 x 10⁵/well in 50 μl. 10–20 μl of mAb supernatants were then added to each well. Suspensions of A-LAK effector cells were then added to triplicate wells to give various E/T ratios in a final volume of 200 μl. After incubation for 4 h at 37°C, the plates were centrifuged (100g) and 100 μl of supernatant was removed from each well and counted in a gamma counter to determine specific cytotoxicity. The percent cytotoxicity was determined by the formula: percent cytotoxicity = 100 x ((experimental release - spontaneous release)/(total release - spontaneous release)).

Assays of antibody-dependent cellular cytotoxicity (ADCC) were performed as described above, with the additional steps of pretreating the effector cells with F(ab')₂ fragments of mAb 3.2.3 and target cells with dilutions of specific antiserum for 30 min at 37°C followed by two washes.

Target Cell Binding Assays. mAbs were tested for their effects on conjugate formation of effector cells and target cells essentially as described (40, 41). A minor modification of this technique included first labeling of the A-LAK cells with fluorescein diacetate (FDA) so that these cells could be easily distinguished from the target cells under UV microscopy. Preliminary experiments suggest that this labeling does not interfere with A-LAK cytolytic function (data not shown). After washing, 5 x 10⁵ FDA-labeled A-LAK cells were incubated with antibody for 15 min at 4°C. 5 x 10⁵ target cells in 0.5 ml of complete medium were added to the mAb-treated A-LAK cells and the mixture centrifuged at 400 g for 5 min. Cells were then incubated 30 min to allow binding. The cell mixture was then suspended by gentle aspiration. A drop of the suspended cells was then added to glass slides and the number of conjugates determined microscopically. 200 A-LAK cells were counted and the percentage of cells bound to one or more target cells calculated using the formula: percent binding cells = 100 x (number of FDA labeled cells bound to one or more targets/total number of FDA labeled cells).

FACS Analysis and Cell Sorting. For surface marker analysis, 2–3 x 10⁵ cells were placed in 12 x 75-mm plastic tubes in 0.1 ml of staining buffer (PBS, pH 7.3, 0.1% sodium azide, 2% FCS). Antibody or serum was added (1:20–1:100 final dilution) for 30 min at 4°C. mAb
3.2.3 was tested (both intact and F(\text{ab}')\text{2} fragments) at dilutions up to 1:200,000. The cells were then washed twice and resuspended with RPE- or FITC-conjugated F(\text{ab}')\text{2} fragments of the appropriate second antibody (see figure legends; Cappel Laboratories, Malvern, PA). After 30 min of incubation at 4°C, the cells were washed twice and analyzed for fluorescent staining using a FACStar flow cytometer (Becton Dickinson & Co., Mountain View, CA). Two-color analysis was performed using directly labeled FITC-F(\text{ab}')\text{2} fragments of mAb 3.2.3 after staining with antibody to rat lymphocyte subsets and RPE-conjugated second antibody. FACScan analysis of highly purified blood LGL and polymorphonuclear leukocytes (PMN) (Fig. 2) were performed by Dr. Craig W. Reynolds (Biological ResponseModifiers Program, NCI, Frederick, MD). For sorting, fresh nylon wool nonadherent splenic lymphocytes were stained as above except that the staining buffer contained no sodium azide.

**Preparation of F(ab)\text{2} Fragments of mAb 3.2.3.** F(ab)\text{2} fragments of mAb 3.2.3 (ascites) were prepared as described (42). mAb 3.2.3 (1.75 mg/ml), in PBS with 0.1 M citrate, was incubated for 8 h (37°C) at pH 3.5 with 25 μg/ml pepsin (Sigma Chemical Co., St. Louis, MO). This preparation was dialyzed against HBSS to restore neutral pH and was analyzed by SDS-PAGE. F(ab)\text{2} fragments were the only large molecular mass product (>100 kD) and no further purification was done. FITC-F(ab)\text{2} fragments of the preparation were made using 0.06 mg FITC/mg protein in 0.2 M carbonate/bicarbonate buffer (pH 9.4). Unbound FITC was removed by passing the preparation over G-10 Sephadex.

**Assay of Exocytosis Induced by mAb 3.2.3.** Assays to determine BLT-esterase secretion (as a marker of granule exocytosis) were performed using a modification of the method described by Takayama et al. (43). Briefly, affinity-purified mAb 3.2.3 was immobilized in 96-well microtiter plates (MC2000; Dynatech Laboratories, Inc., Alexandria, VA) at various dilutions in 0.1 M carbonate/bicarbonate buffer (pH 8.0) (100 μl/ aliquot) by incubation at 4°C for 18 h. After incubation, the solution was removed and the wells were washed with RPMI 1640/10 mM Hepes/5% FCS. BLT-esterase secretion was measured using 10^6 A-LAK cells in 0.1 ml RPMI 1640/10 mM Hepes/5% FCS in the presence or absence of stimulus (mAb 3.2.3 in the wells). After 4 h of incubation at 37°C under 5% CO_2 tension, cells were resuspended by gentle pipetting and centrifuged at 100 g for 5 min. 50-μl aliquots of supernatant were used to assay enzyme activity. Total cellular content of BLT-esterase was determined using 0.1% Triton X-100 solubilized cells. Data are presented as a mean specific percentage of enzymatic activity released, which was calculated by the formula: percent release = \frac{100 \times [(E - S)/(T - S)]}{E}; where E equals the number of enzyme units in the supernatant of the experimental well, S equals the number of enzyme units in the supernatants of the wells with no stimuli, and T equals the total number of units of BLT-esterase in A-LAK cells per well. Culture medium (0.05 ml) was mixed with 200 μl of N\text{-}\alpha\text{-}benzoyloxycarbonyl-L-lysine thio- benzyl ester (BLT) solution (0.2 mM BLT, 0.22 mM 5,5'-dithio-bis-2-nitrobenzol acid [DTNB]) in PBS (pH 7.2). The mixture was incubated 120 min at 37°C and the reaction stopped by adding 5 μl of 0.1 M PMSF, which is dissolved in DMSO. Absorbance was measured in a microtiter ELISA reader (Dynatech Laboratories, Inc.) at 412 nm.

**Cell Surface Iodination and Immunoprecipitation.** A-LAK cells (5.0 x 10^6) were washed three times in PBS and pelleted by centrifugation (400 g for 5 min). 100 μl of lactoperoxidase and glucose oxidase-coupled enzymobeads (Bio-Rad Laboratories, Richmond, CA) were then added to the pellet. Subsequently, 20 μl of lactoperoxidase (1 mg/ml in PBS) and 100 μl of 2 × PBS were added to the pellet, followed by 20 μl of 125I (50 mCi/ml; New England Nuclear). The peroxide-forming reaction was then begun by the addition of 20 μl of a 1% solution of β-ν-glucose in PBS. The cells were then gently resuspended and incubated at ambient temperature for 15 min with periodic mixing. The peroxide-forming step was then repeated a second time. After washing, the cells were lysed at 4°C in PBS containing 1% Triton X-100, 0.1% SDS, and a cocktail of protease inhibitors including PMSF (1 mM), leupeptin (1 μg/ml), pepstatin A (5 μg/ml), aprotonin (5 μg/ml), and 5 mM EDTA at pH 7.4. Cell debris was removed by centrifugation at 100,000 g for 30 min.

Before immunoprecipitation, the cell extract was precleared with Sephadex G-10 (vol/vol) with lysis buffer followed by three passages over BSA-coupled Sephadex beads. Total counts were then determined in cold ethanol precipitates. Immunoprecipitation was carried out using 25 μl of mAb 3.2.3-coupled beads incubated...
with 10^7 cold ethanol precipitate counts at 4°C for 4 h on a rotating wheel. As controls, 10^7 cold ethanol precipitable counts were also incubated, as above, with 25 μl of beads coupled to BSA or irrelevant mAb. Antigen was stripped from the affinity matrix by treatment with buffer containing 0.25 M Tris, 2% SDS, 1% glycerol, and 1% 2-ME for the reduced sample. For nonreduced samples, the buffer was the same except for a lack of 2-ME. Samples were then subjected to electrophoresis on 3–20% gradient SDS-PAGE slab gels at 20 mA/slab. The proteins were fixed in the gels with a solution of TCA/methanol followed by a solution of ethanol/acetic acid/H2O. The gels were then washed in deionized water and placed in a solution of Fluorohance (Research Products International Corp., Mount Prospect, IL) supplemented with 10% glycerol to prevent cracking. Gels were then dried and placed under preflashed x-ray film for 2 wk.

Results

Tissue and Cellular Distribution of the Epitope Identified by mAb 3.2.3. The expression of the epitope identified by mAb 3.2.3 on various normal tissues of F344 rats, as well as a panel of tumors of mouse and rat origin, was determined by flow cytometry. The epitope identified by mAb 3.2.3 was found to be expressed on 94% of LGL isolated from peripheral blood (95% LGL) (Fig. 1 a) and 93% of the LGL isolated from spleen (data not shown). The staining pattern on LGL was not homogeneous (two peaks consistently apparent). This marker was also expressed in an homogeneous staining pattern on 96% of A-LAK cells (activated NK); on 83% of F344 PMN; and on 84% of CRNK-16 (an NK-like, LGL leukemia in F344 rats) (Fig. 1 b and Table I). The marker was also expressed on ~10% of PBL, spleen cells, and peritoneal cells, and its expression correlated with the percentage of LGL present in these preparations (Table I). The epitope identified by mAb 3.2.3 was present on 2% of bone marrow and lymph node cells but was totally absent on thymocytes. Finally, a small population of peritoneal exudate cells (PECs) (~10%) stained positively with mAb 3.2.3 and this corresponded to the percentage of LGL in this preparation (Table I). Nevertheless, we performed two-color analyses on purified populations of rat macrophages using the rat macrophage specific marker 0X41 and mAb

![Figure 1](https://jem.rupress.org/content/137/11/1377/F1.large.jpg)  
**Figure 1.** Expression of the epitope recognized by mAb 3.2.3 on F344 rat LGL and PMN. F344 rat LGL were isolated from peripheral blood on a discontinuous Percoll gradient to a purity of >95% as measured by Giemsa staining. PMN were also isolated from F344 rat blood on Percoll, and were >85% neutrophils.
3.2.3 and found that macrophages did not coexpress OX41 and 3.2.3 (data not shown). Tumor cells, including YAC-1, P815, and MADB106, were negative for the expression of 3.2.3.

**Coexpression of 3.2.3 with Lymphocyte Subsets Identified by Other mAbs.** To determine the coexpression of 3.2.3 with mAbs identifying other rat lymphocyte subsets, two-color analyses were performed with fresh, nonadherent splenocytes using markers associated with rat T cells (OX19 and OX8), NK cells (OX8), B cells (OX6), and macrophages (OX6 and OX41) (Fig. 2). As shown in Fig. 2A, OX6 (Ia-like) and 3.2.3 stain discrete populations of fresh splenic lymphocytes. The 3.2.3+ cells constitute 9.6% of the total population (Fig. 2A, quadrant 4), and none of these cells were OX6+ (quadrant 2). Staining these cells with OX8 (cytotoxic T cell and NK cell specific) and 3.2.3 (Fig. 2B) demonstrated that 25.9% of the cells were OX8+ (quadrants 1 and 2). Virtually all of the 3.2.3+ cells were in the double-stained population, i.e., coexpressed OX8 (Fig. 2B, quadrant 2). The OX8+ cells could clearly be divided into the T cell compartment (Fig. 2B, quadrant 1, 17.2%) and NK cell compartment (quadrant 2, 8.2%) by double staining with 3.2.3. Confirmatory results were demonstrated by double staining with OX19 (pan T marker) and 3.2.3 (Fig. 2C). OX19 staining was demonstrated on 68.3% of these cells (quadrant 1). None of these cells coexpressed OX19 and 3.2.3 (quadrant 2). Staining of spleen cells with OX41 (macrophage and endothelial cell specific) and 3.2.3 also showed that these two regions were not coexpressed on any cells (Fig. 2D).

**Correlation of NK Activity and Staining with mAb 3.2.3 by Positive Cell Sorting.** NK cell lytic function was assayed in unsorted and in positive and negative cell sorts after staining of nonadherent splenocytes with mAb 3.2.3 (Fig. 3). The unsorted cells were 35% positive for expression of the 3.2.3 epitope and contained NK cells as defined by cytotoxicity against YAC-1 target cells. The 3.2.3+ cells (96% pure) were highly cytolytically active against YAC-1 target cells, and the positive sort resulted in a doubling of cytotoxic activity expressed in the unsorted cells, on a cell to cell

| Cell tissue    | Percent LGL | mAb 3.2.3 |
|---------------|-------------|-----------|
| Fresh LGL     | 95          | 95*       |
| A-LAK         | 96          | 96        |
| Bone marrow   | <1          | 1         |
| Thymus        | <1          | 0         |
| Blood/PBL     | 8           | 11        |
| Spleen        | 8           | 11        |
| Lymph node    | 2           | 2         |
| Peritoneal cells | 10       | 12        |
| CRNK-16       | 88          | 84        |
| YAC-1         | 0           | <1        |
| P815          | 0           | <1        |
| MADB106       | 0           | 6         |
| PMN           | 0           | 83        |

* Percent positive staining cells.

TABLE I

*Correlation of the Expression of the Epitope Identified by mAb 3.2.3 and Percentage of LGL in Various Tissues and Cell Lines*
basis. The cells in the 3.2.3\textsuperscript{-} sort (99.0\% pure) were not cytotoxic against YAC-1 target cells at any E/T ratio assayed.

**Expression of the Epitope Recognized by mAb 3.2.3 on F344 Rat A-LAK Cells at Various Time Points After Stimulation with rIL-2.** Expression by A-LAK cells of the epitope
identified by mAb 3.2.3 was analyzed by flow cytometry at various time points after stimulation with rIL-2 (Fig. 4). In Fig. 4, peak a represents the intensity of staining of A-LAK cells by mAb 3.2.3 after 24 h in culture with rIL-2. More than 93% of the cells expressed the epitope at this time point, with a mean fluorescence of 43.7. After 48 h of culture (peak b), 92.8% of the A-LAK cells were positive for the expression of this marker, at a mean fluorescence of 147. After 120 h of culture (peak c), 94.1% of the A-LAK cells expressed this marker with a mean fluorescence of 871. A-LAK populations were homogeneous (stained as a single peak) at each of the time points tested.
TABLE II

Effects of mAb 3.2.3 on NK Cell and A-LAK Cell Cytotoxicity Against Selected Target Cells

Percentspecificcytotoxicity as determinedina 4-h ⁵¹Cr release assay. NK assayswere performedusinga 100:1 E/T ratio and A-LAK assayswere per-
formed usinga 3:1 E/T ratio.

| Effector        | P815   | YC-1   | MADBI06 | P815   | YAC-1   | MADBI06 |
|-----------------|--------|--------|---------|--------|---------|---------|
| NK cells        |        |        |         |        |         |         |
| Control         | 1*     | 40     | 6       | 18     | 43      | 28      |
| mAb dilutions¹ |        |        |         |        |         |         |
| 10⁻¹            | 4      | 46     | 6       | 56     | 43      | 24      |
| 10⁻²            | 13     | 49     | 4       | 59     | 45      | 29      |
| 10⁻³            | 12     | 41     | 7       | 63     | 44      | 26      |
| 10⁻⁴            | 8      | 29     | 6       | 65     | 43      | 22      |
| 10⁻⁵            | 2      | 34     | 4       | 43     | 45      | 29      |
| 10⁻⁶            | 3      | 33     | 4       | 32     | 51      | 31      |
| 10⁻⁷            | 0      | 33     | 5       | 22     | 44      | 30      |

* Percent specific cytotoxicity as determined in a 4-h ⁵¹Cr release assay. NK assays were performed using a 100:1 E/T ratio and A-LAK assays were performed using a 3:1 E/T ratio.

¹ Dilutions of mAb 3.2.3 (hybridoma supernatant) were present throughout the assay. Anti-HLA-DR (IgG1, isotype control) did not modulate cytotoxicity against any target tested. Further, numerous other isotype-matched, anti-A-LAK cell-specific mAbs also did not modulate cytotoxicity against any target tested.

Selective Enhancement of Cytotoxicity Induced by mAb 3.2.3. mAb 3.2.3 was screened for its effect on cytolytic activity of NK and A-LAK cells against various tumors. As indicated in Table II, mAb 3.2.3 selectively enhanced the cytolytic activity of NK and A-LAK cells against P815 but not YAC-1 or MADBI06 target cells. The enhancement of lysis of P815 by mAb 3.2.3 was dose dependent over a wide range of dilutions. Cytotoxicity by NK cells was enhanced against P815 target cells at dilutions up to 10⁻⁴. A-LAK cell cytotoxicity against P815 target cells was boosted at dilutions as high as 10⁻⁶.

Effects of mAb 3.2.3 on Formation of A-LAK Cell/Tumor Target Cell Conjugates. To explore the feasible basis for the selective augmentation of cytotoxicity against P815 target cells, the effects of mAb 3.2.3 on conjugate formation between A-LAK cells and P815 or YAC-1 tumor target cells were determined (Fig. 5). Effector and target cells (1:1 E/T ratio) were incubated in the presence of various dilutions of mAb 3.2.3. Dilutions of 10⁻¹ and 10⁻² caused a 100% and 120% increase in conjugates formed between A-LAK cells and P815 target cells, respectively. A dilution of 10⁻³ caused an increase of 38% in conjugates formed. In contrast, the number of conjugates formed between A-LAK cells and YAC-1 target cells was not affected by the presence of the mAb. While the results suggest that mAb 3.2.3 facilitates conjugate formation, the dilutions that enhance cytotoxicity are several logs (up to 10⁻⁶) greater than those that induce an increase in conjugate formation.

Comparison of Induction of Lysis of Intact and F(ab')₂ Fragments of mAb 3.2.3 Against a Panel of Target Cells. Preparations of intact and F(ab')₂ fragments of mAb 3.2.3 were compared for their ability to cause an enhancement of cytotoxicity of A-LAK cells against a panel of target cells expressing (P815, Daudi, and U937) or lacking expression (YAC-1, MADBI06, and K562) of a receptor for the Fc portion of Ig (Fig. 6). Intact mAb 3.2.3 caused an increase in A-LAK cell cytotoxicity against FeR⁺ target
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**Figure 5.** Effects of mAb 3.2.3 on conjugate formation of A-LAK cells and P815 or YAC-1 target cells. FITC-labeled A-LAK cells and unlabeled target cells (1:1 ratio) were incubated with various dilutions of 3.2.3 for 30 min at 4°C. 200 A-LAK cells were counted and the percentage of cells bound to one or more target cells was calculated. Anti-HLA-DR (IgG1, isotype control) had no effect on conjugate formation.

**Figure 6.** Induction of lysis (reverse ADCC) by 3.2.3 IgG and F(ab')2 against a panel of target cells. F344 A-LAK cells were mixed with the indicated target cells (10:1 E/T ratio) in the presence or absence of 100 ng/ml purified 3.2.3 IgG or F(ab')2. Cytotoxicity was determined after a 4-h assay. Anti-HLA-DR (IgG1, isotype control) had no effect only on YAC-1 on P815. Further, numerous (IgG) isotype-matched anti-A-LAK cell-specific mAbs also had no modulating effect on cytotoxicity.
Immobilized mAb 3.2.3 Induces Exocytosis of BLT-Esterase. Affinity-purified mAb 3.2.3 was incubated at various dilutions for 18 h at 4°C in microtiter plates (model II; Immulon; Dynatech Laboratories, Inc.; Chantilly, VA). A-LAK cells (100 µl at 10^7/ml) were then added to each well and incubated 4 h. BLT-esterase activity was determined in 50-µl aliquots of those supernatants as described (43). Maximum releasable BLT-esterase was determined in 0.1% Triton X-100-solubilized cells. Background release was determined in supernatants of A-LAK cells incubated in the absence of mAb 3.2.3.

FIGURE 7. Immobilized mAb 3.2.3 induces exocytosis of BLT-esterase. Affinity-purified mAb 3.2.3 was incubated at various dilutions for 18 h at 4°C in microtiter plates (model II; Immulon; Dynatech Laboratories, Inc.; Chantilly, VA). A-LAK cells (100 µl at 10^7/ml) were then added to each well and incubated 4 h. BLT-esterase activity was determined in 50-µl aliquots of those supernatants as described (43). Maximum releasable BLT-esterase was determined in 0.1% Triton X-100-solubilized cells. Background release was determined in supernatants of A-LAK cells incubated in the absence of mAb 3.2.3.

cells P815, Daudi, and U937 but had no effect on cytotoxicity against YAC-1, MADB106, or K562. F(ab')2 fragments of mAb 3.2.3 did not affect cytotoxicity against FcR+ or FcR- target cells.

Immobilized mAb 3.2.3 Induces Exocytosis of BLT-Esterase by A-LAK Cells. Using a modification of the technique described by Takayama et al. (43), mAb 3.2.3 was assayed for its ability to trigger exocytosis of the granule-associated enzyme BLT-esterase (Fig. 7). Various concentrations of affinity-purified mAb 3.2.3 were incubated in microtiter plates (model II; Immulon; Dynatech Laboratories, Chantilly, VA) for 18 h at 4°C. The wells were then washed and 100 µl of a suspension of A-LAK cells (10^7/ml) added to wells. The plates were then incubated 4 h. BLT-esterase was
demonstrated in supernatants of these cultures with peak release (~40%) being induced at 12.5 ng/ml. Both lower (3.13 ng/ml) and higher (100 ng/ml) concentrations of antibody induced less enzyme release.

**SDS-PAGE Analysis of mAb 3.2.3 Immunoprecipitates.** SDS-PAGE analysis of 125I-labeled 3.2.3 immunoprecipitates yielded a single band of ~30 kD molecular mass under reducing conditions (Fig. 8). When analyzed under nonreducing conditions, there was a single band of ~60 kD. These results suggest that the structure recognized by mAb 3.2.3 is a 60-kD dimer composed of two 30-kD chains.

**Discussion**

In this report, we describe an mAb of mouse origin (mAb 3.2.3; IgGlk) that recognizes a triggering structure expressed on rat NK cells and A-LAK cells. This epitope is also expressed on PMN. We have defined the specificity of mAb 3.2.3 for the NK cell subset of mononuclear cells by a variety of criteria, including its pattern of tissue distribution, a comparison of its expression with other markers for subpopulations of lymphoid cells and monocytes, a correlation of expression of the epitope recognized by this antibody and LGL morphology, and by positive cell sorting for NK cell cytolytic function. The epitope recognized by mAb 3.2.3 is expressed on >95% of blood and splenic LGL; on >80% of blood PMN; on ~10% of PBL splenocytes, and peritoneal cells; and on 1-2% of bone marrow and lymph node cells. Thymocytes were <1% positive for this marker. CRNK-16, an F344 rat LGL leukemia expressing NK activity, was also positive for mAb 3.2.3 (>84%). In all cell preparations and tissues examined, the expression of the epitope identified by mAb 3.2.3 correlated with LGL morphology, with the exception of PMN. By two-color FACS analysis of fresh splenic lymphocytes, the cells stained by mAb 3.2.3 were identified as NK cells. Cells positive for mAb 3.2.3 were also positive for 0X8 (NK, CTL), but were negative for 0X6 (Ia-like; macrophages and B cells), 0X19 (pan T marker) and 0X41 (macrophages and endothelial cells). Cell sorting of nonadherent splenic lymphocytes into mAb 3.2.3+ and 3.2.3- populations demonstrated that the NK cytolytic activity was totally contained in the 3.2.3+ sort. These data suggest that mAb 3.2.3 represents the best LGL/NK cell marker available in the rat system, as other markers, such as asialo GM1 and 0X8, are also expressed on other subpopulations including T cells (14, 18, 20, 33, 37). Monoclonal and polyclonal antibodies to laminin have been shown to identify a subset of LGL/NK cells in mice and rats (20, 44, 45). The mAb Lam-1, which reacts with the B2 subunit of laminin, identifies ~50% of peripheral blood LGL/NK cells and has been shown to inhibit cytolytic function of NK cells. Although the cell and tissue distribution of the epitopes recognized by mAbs 3.2.3 and Lam-1 are similar (i.e., LGL/NK cells), several features distinguish these antibodies. These include: (a) the expression of the epitope recognized by mAb 3.2.3 on LGL/NK cells is at a much higher intensity than that recognized by mAb Lam-1 (350 relative fluorescence intensity units [R. F. I.] vs. 60 R. F. I.; reference 20); (b) the inhibitory effects on cytotoxicity of mAb Lam-1 vs. the boosting effects of mAb 3.2.3; and (c) the expression of the Lam-1 epitope on ~50% of LGL/NK cells while ~100% express the epitope recognized by mAb 3.2.3. Since virtually all LGL/NK cells react with mAb 3.2.3, this antibody will be useful as a pan-LGL/NK cell marker in rats, and the heterogeneity of staining by Lam-1 and 0X8 suggest that those antibodies may be useful for the subsetting of LGL/NK cells.

mAb 3.2.3 was produced by immunizing mice with rat A-LAK cells (rIL-2-activated
NK cells) and then screening hybridomas for their effects on A-LAK cell cytotoxicity. Interestingly, mAb 3.2.3 induces a selective increase in cytotoxicity of both NK and A-LAK cells against certain tumor target cells. Using a panel of tumor target cells to screen for this effect, it was determined that FcR+ target cells including P815, Daudi, and U937 were lysed more efficiently in the presence of mAb 3.2.3. However, FcR- target cells including YAC-1, MADBI06, and K562 were not. This effect was not observed with either FcR+ or FcR- target cells when using F(ab’)2 fragments of the antibody. Assays of conjugate formation demonstrated a selective increase in conjugation of A-LAK cells with P815 target cells, but this occurred only at high concentrations of antibody, while boosting of cytotoxicity occurred at several logs greater dilution of the antibody.

The selective boosting of cytolytic activity by intact mAb 3.2.3 and the tissue distribution (LGL and PMN) of the epitope recognized by mAb 3.2.3 are similar to data reported for antibodies to CD16 (Leu-11a/NKP-15, Leu-11b/3G8, Leu-11c/B73.1) in humans (30, 46-48). As with mAb 3.2.3, levels of cytotoxicity and conjugate formation by NK cells could be increased by antibody to CD16 using FcR+ target cells (P815) if the mAb was of the appropriate isotype for the target cell FcR (e.g., IgG1) (49). The increase in cytotoxicity is suggested to be, in effect, a reverse ADCC reaction and is termed “induction” (49). Induction has also been demonstrated using antibodies to other triggering structures including CD2 and CD3 (49-53).

While some of our data suggest that the triggering structure identified by mAb 3.2.3 may be analogous to CD16, there is also evidence that it may recognize a different structure. First, unlike Leu-11a staining of purified human A-LAK cells, which decreases after their culture with rIL-2 (Whiteside, T. L. and R. B. Herberman, personal communication), the epitope identified by this antibody increased on rat A-LAK cells after culture in rIL-2. Secondly, the antigen immunoprecipitated by mAb 3.2.3 from 125I-labeled A-LAK cells appeared as a 60-kD band in nonreduced gels and as a 30-kD band in reduced gels. This is different than the size reported for CD16, which appeared as a broad band at a molecular mass of 45-70 kD (54-56). Preliminary data (not shown) suggest that mab 3.2.3 does not block functions mediated through the FcR, such as ADCC. A further examination of this and the ability of mAb 3.2.3 to block binding of aggregated Ig and EA rosette formation are currently under way.

In this report, we describe the first mAb produced against a purified population of cells with LAK activity. It has previously been shown that the cells with LAK cytolytic function, produced by the selective adherence technique, develop from the NK compartment (38), and the fact that this antibody was reactive against both A-LAK and NK cells further confirms this observation. The boosting of cytolytic function of A-LAK and NK cells by mAb 3.2.3, and the induction of BLT esterase release by immobilized mAb 3.2.3, are strong evidence that this antibody recognized a triggering structure. Further, this antibody recognized a dimeric surface structure on NK and A-LAK cells that has characteristics unlike CD2 or CD16, and may, in fact, be a unique triggering structure.

**Summary**

To study the cellular structures involved in NK and lymphokine-activated killer (LAK) cell function, we have produced a panel of mAbs that modulate the cytolytic function of a population of cells with LAK activity that derive from large granular
lymphocyte (LGL)/NK cells (adherent LAK [A-LAK] cells). In this report, we describe an mAb (3.2.3; IgGlk) that recognizes a triggering structure that is expressed on rat LGL/NK cells and A-LAK cells. This epitope is also expressed on polymorphonuclear leukocytes (PMN). The expression of the epitope identified by mAb 3.2.3 increased progressively on A-LAK cells after culture in the presence of rIL-2. mAb 3.2.3 enhanced the cytolytic activity of NK and A-LAK cells against FcR⁺ target cells, but not FcR⁻ target cells. However, this effect was not induced by F(ab')₂ fragments of 3.2.3. This antibody also induced the release of N-α-benzyloxy carbonyl-L-lysine thiobenzy ester esterase by A-LAK cells. These data suggest that the epitope identified by mAb 3.2.3 is on a triggering structure expressed on rat NK cells and A-LAK cells. The expression of the epitope recognized by mAb 3.2.3 on LGL/NK cells and PMN suggests that this structure may be analogous to that identified by the anti-CD16 (-FcR) mAbs. However, the molecule immunoprecipitated by mAb 3.2.3 was a 60-kD dimer composed of two 30-kD chains. These data suggest that mAb 3.2.3 recognizes a unique triggering structure. As mAb 3.2.3 is the first antibody recognizing a determinant with functional significance, selectively expressed on both rat NK cells and A-LAK cells, it will be a useful tool for the study of NK cell ontogeny and function, and the development of cells with LAK activity from the NK cell compartment.

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