Possible Mechanism of Selective Killing of Myeloid Leukemic Blast Cells by Lymphokine-Activated Killer Cells


Major histocompatibility complex-unrestricted lymphokine-activated killer (LAK) cells have been proposed as therapy for a variety of hematologic malignancies. Because these cells recognize and kill their targets independently of their antigen specific CD3 receptor, it is unclear how they might discriminate between normal and malignant cells. We now propose one such mechanism for the selective killing of myeloid leukemia blasts. While both CD2+ and CD2- activated killer cells may inhibit the clonogenic growth of myeloid leukemia cells, only the CD2+ subset effectively inhibits the growth of normal myeloid (granulocyte-macrophage and granulocyte) progenitors. This difference appears to reflect differential requirements for cell adhesion molecule recognition between normal and malignant progenitor cells. Inhibition of the growth of normal granulocyte-macrophage colonies by CD2- LAK cells is blocked by antibodies to the CD2-lymphocyte function-associated antigen 3 (LFA-3) (CD58) cell adhesion system. In contrast, these antibodies have no effect on CD2+ LAK-mediated inhibition of malignant cell clonogenic growth. Instead, antibodies to the LFA-1 (CD11a/CD18)-intercellular adhesion molecule 1 (ICAM-1) (CD54) adhesion system reduce inhibition. These differences correspond to differential expression of the CD54 cell adhesion molecule by normal and malignant myeloid progenitor cells because less than 15% of normal CD34 positive cells are CD54+ while greater than 85% of CD34+ acute myeloid leukemia blasts express the CD54 antigen. LFA-3, the ligand for CD2, is strongly expressed by erythrocytes, and these cells competitively inhibit killing of normal but not malignant clonogenic cells in an analogous way to the effects of monoclonal antibody to the CD2-LFA-3 adhesion system. The operation of this effect in vivo may be a basis for selective cytotoxicity by CD2- LAK against clonogenic myeloid blast cells, and could be exploited further with infusion of appropriate monoclonal antibodies.

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ONE REQUIREMENT for the successful use of activated killer (AK) lymphocytes in cancer therapy is that these cells must selectively kill malignant target cells in preference to their normal equivalents. Functional analysis of AK lymphocytes generated by infusion of interleukin-2 (IL-2) shows that these cells kill autologous and allogeneic leukemic blast cells while having a limited effect on normal hematopoietic progenitor cells; however, the mechanism for this difference is unknown. The effector function of AK cells, unlike that of most cytotoxic T cells, is major histocompatibility complex (MHC)-unrestricted. These generally CD3+ cells also lack the only known antigen-specific receptor present on cytotoxic lymphocytes. Thus, even if unique antigens were to be expressed by malignant cells, it is not obvious how the apparent distinction between normal and malignant progenitor cells could be achieved. Without this discrimination, enhancement of AK cell function in vivo would be unlikely to produce overall benefit to the patient.

Preferential killing by AK cells could occur in either of two ways. First, normal and malignant progenitor cells may have different sensitivities to the cytotoxic cytokines released by AK cells, as suggested by in vitro studies showing that leukemic progenitor cells are more sensitive to growth inhibition by γ-interferon (γIFN) and tumor necrosis factor (TNF) than are normal progenitors. Second, normal and malignant cells may be recognized by different subpopulations of AK cells. Because AK cells are generally antigen-nonspecific, one mechanism by which this selectivity could occur would entail different cell adhesion molecule (CAM) systems for normal and malignant cell binding to effectors, which would be restricted to different AK cell subsets. We have now investigated this possibility, demonstrating phenotypic differences between AK subsets able to kill normal myeloid progenitors and malignant myeloid blasts and showing how differences in CAM binding may contribute to selective killing of clonogenic leukemia cells in vivo.

MATERIALS AND METHODS

Peripheral blood (PB) cell separation. Heparinized blood (10 U/mL) was collected from 12 normal volunteers and diluted 1:1 with RPMI 1640 medium (Flow Laboratories, McLean, VA) supplemented with L-glutamine (GIBCO, Oxbridge, UK) (2 mmol/L) and penicillin/streptomycin (GIBCO) (100 U/mL). Blood was layered in 10-mL aliquots on 10 mL Lymphoprep (Nyegaard & Co, Oslo, Norway). After centrifugation at 400g for 30 minutes at room temperature, interface PB mononuclear cells (PBMC) were collected and washed twice with RPMI. Contaminating red blood cells (RBCs) were removed from the pellet of isolated MC by hypotonic lysis with ammonium chloride on ice. Cells were washed twice again and resuspended at 2 × 10^6 cells/mL in RPMI containing 10% heat-inactivated fetal calf serum (FCS; Sera-Lab, Crawley, UK) screened for low mitogenicity.

Lymphokine-activated killer (LAK) cell culture. LAK cells were prepared by culturing PBMC, or CD2+ cells from PB/marrow (see text) or CD2+ cells from PB with 500 U of recombinant human IL-2/mL for 72 hours in RPMI + 10% FCS in round bottom tissue culture tubes (Nunclon, Paisley, Scotland).
Bone marrow (BM) cell preparation and culture. BM samples from 10 normal donors and from nine patients off therapy in complete remission of acute myeloid leukemia (AML) were collected into preservative-free heparin, and the MC were separated on Lymphoprep. After adherence on plastic, cells were resuspended at 3 x 10^9 cells/mL in supplemented McCoy's 5A medium containing 15% FCS. For isolation of CD2+ cells, 1 mL of a 10% solution of neuraminidase-treated sheep RBCs was added to 3 mL of BM cell suspension. After 10 minutes of incubation at 37°C, and 30 minutes at 4°C, cells were again separated on Lymphoprep and collected from the interface as the CD2- cell population. The CD2+ enriched pellet (>96% CD2+ cells) was washed twice, and contaminating RBC were removed by ammonium chloride lysis. After washing, CD2+ cells were suspended at 2 x 10^6 cells/mL in RPMI/10% FCS, while CD2- marrow MC were suspended in McCoy's 5A medium/15% FCS. These cells were kept in liquid culture for up to 3 days, until used for cytotoxicity or colony-forming unit granulocyte-macrophage (CFU-GM) assay with autologous or allogeneic CD2- LAK cells.

BM cryopreservation. BM cell fractions, isolated as described above, were resuspended at a concentration of 10 to 40 x 10^6 nucleated cells/mL. A solution containing 20% dimethyl sulfoxide (DMSO) plus 40% FCS in McCoy's 5A medium was added in equal amounts to the cell suspensions. The vials containing the mixture of cells and solution were placed in a freezing rack above the liquid nitrogen level for 2 hours of slow initial freezing and then stored in a liquid nitrogen freezer.

Leukemic blast cells. AML blasts were isolated from nine leukemia patients at presentation or relapse by Ficoll Hypaque followed by plastic adherence to deplete contaminating monocytes. Three patients were French-American-British (FAB) type M2, one M3, two M4, and one M5. The blasts were cryopreserved as above.

CD2+ and CD2- LAK cell generation. CD2+ cells from BM and PB were cultured at 2 x 10^6 cells/mL in RPMI/10% FCS with 500 U IL-2/mL for 72 hours in tissue culture tubes (Nuncelon). CD2+ LAK from BM contained 78% ± 2% CD2+, 8% ± 2% CD16+, 6% ± 2% CD56+, and 64% ± 12% CD3+ cells. CD2+ LAK from PB contained 78% ± 7% CD2+, 6% ± 0.5% CD16+, 4% ± 4% CD56+, and 87% ± 2% CD3+ cells. CD2+ LAK contained 5% ± 2% CD2+, 7% ± 3% CD16+, 6% ± 2% CD56+, and 2% ± 1% CD3+ cells. The remaining cells in the CD2+ PB LAK population were B cells and monocytes.

CFU-GM assay. BM cells were plated (1 x 10^4/mL) in 0.3% agar in supplemented McCoy's 5A medium and 15% FCS in multiwell tissue culture plates (Linbro, Flow Laboratories, VA). Recombinant human granulocyte-macrophage colony-stimulating factor (rHGM-CSF; Glaxo, Greenford, UK) was used as a source of colony-stimulating activity in a final concentration of 1,000 pmoI. Cultures were incubated at 37°C in a humidified incubator in 5% CO2. Normal myeloid (G and GM) colonies and leukemia colonies (> 40 cells) were counted on day 7 and day 14 using an inverted microscope. Colony morphology was examined by transferring intact cultures onto glass slides, fixing them in methanol, and staining with May-Grünwald-Giemsa.

Cot-culture of marrow with LAK effectors. BMMC depleted of CD2+ lymphocytes by 2.5 x 10^6/mL were added to autologous or allogeneic CD2+ or CD2- LAK at 1:10 ratio. To examine the influence of lymphocyte function-associated antigen 3 (LFA-3) bearing RBC on the inhibition of CFU-GM/G formation by CD2- LAK cells, autologous RBC were added to the cultures at 50:1 erythrocyte to BM cell ratio. The cells suspended in supplemented McCoy's 5A medium with 15% FCS were spun down at 4g for 5 minutes for closer cell-cell contact and incubated for 4 hours at 37°C. After the incubation, cells were agitated and plated for the colony assay.

Antigbodies. Normal marrow or malignant blast cells were incubated with 20% normal rabbit serum for 10 minutes at 4°C before washing and staining. Monoclonal antibodies (MoAbs) used with the microplate method29 or flow cytometric analysis of lymphocytes and marrow were: CD3 (OKT3; Ortho, Westwood, MA), CD2 (RFT11; a gift of Prof G. Janossy, Department of Immunology, Royal Free Hospital), CD56 (Leu9; Becton Dickinson, Mountain View, CA), CD34 (ICH3-63; Dr F. Katz), CD54 (intercellular adhesion molecule 1 [ICAM-1]; Serotec, Oxford, UK), CD11a (LFA-1; Immunotech), CD58/LFA3 (TS2/9.1; a gift of Dr C. Gregory), and NKI-L16 (activated LFA-1, chain; gift of Dr C.G. Figdor). Second-layer fluorescent antimouse antibodies were provided by Southern Biotechnology (Birmingham, AL). Cells were analyzed under a Nikon epi-immunofluorescent microscope and by a fluorescence-activating cell analyzer (FACSCAN; Becton Dickinson). Fluorescence analysis was performed on 5,000 to 10,000 cells with and without second-layer antibodies and with isotype-matched control mouse Ig to determine the fluorescence cutoff.

Measurement of cytotoxicity. A 4-hour 51Cr-release assay was used to measure the cytotoxicity of effector cell populations. Target cells were labeled with 400 µCi of Na251CrO4 (Amersham, Aylesbury, UK) for 90 minutes in 0.3 to 0.4 mL medium. The cells were then washed once and incubated for 30 minutes in RPMI/10% FCS at room temperature. After washing, 5 x 104 target cells were added to each well containing effectors to produce multiple effector to target ratios. After 4 hours of incubation at 37°C the culture supernatants were harvested and counted in a gamma counter. Maximal release of the incorporated 51Cr into targets was measured by lysis of the targets by detergent Triton X-100 (Boehringer Mannheim, Germany). Spontaneous release was measured by counting supernatants of targets incubated with medium alone. The spontaneous release from the target cells was 5% to 20%. The percentage of specific lysis was calculated as: (experimental cpm - spontaneous cpm)/(maximal release cpm - spontaneous cpm). Determinations were done in triplicate.

Blocking assays. Adhesion molecules on effector cells and 51Cr-labeled targets were blocked with ICAM-1, LFA-1, and LFA-3 antibodies. Cells were incubated for 30 minutes at 4°C with 100 µg of the appropriate antibody per milliliter of solution. After washing twice, the cells were used in the cytotoxicity assay.

Statistics. Paired or unpaired t tests were used to compare untransformed or log-transformed data for significant differences using a BBC Master Series computer and a Rubycrere Beebstab analysis package (London, UK).

RESULTS

Although AK cells may be derived from either CD3+ CD16+ T cells or CD3+ CD16- natural killer (NK) lymphocytes,25,26,28 the CD2+ subpopulation in both phenotypic groups has the greatest cytotoxicity against myeloid progenitor cells.27,28 This effect is illustrated in Fig 1A with IL-2-treated PBMC from normal donors cultured with autologous normal marrow MC. The direct cytotoxic effects of PB CD2+ AK cells and their capacity to inhibit normal myeloid clonogenic growth in a colony assay are readily apparent. The CD2+ population produced significantly higher direct cytotoxicity and greater inhibition of colony formation than did PB CD2- cells. By contrast, both CD2+ and CD2- AK cells can inhibit the growth of autologous

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Clonogenic leukemia cells (Fig 1B). Allogeneic co-cultures produced the same pattern of response (data not shown).

One explanation for this link between CD2 positivity and an overall increase in inhibitory/cytotoxic effector function against normal myeloid cells is that the CD2 molecule and its ligand, LFA-3 (CD58), are important in the binding of effector cells to normal marrow myeloid progenitors and/or in their subsequent killing of these cells. Therefore, we added CD2+ effector cells from normal marrow to autologous or allogeneic normal marrow, with or without anti-LFA-3 MoAb. This addition largely abrogated the inhibition of colony formation mediated by CD2+ AK cells (Fig 2A). CD2+ AK cells were also potentially inhibitory of the growth of malignant myeloid progenitors (Fig 1B), but this effect did not depend on CD2–LFA-3 interaction, as shown by the failure of LFA-3 antibody to alter inhibition of leukemic colony growth by CD2+ LAK cells (Fig 2B). These results imply that binding of LAK cells to normal myeloid progenitors depends largely on CD2–LFA-3 interaction, while binding to leukemic targets relies on the interactions of other CAMs, allowing killing of leukemic blasts by both CD2+ and CD2– AK cells.

We next investigated the importance of an alternate cell adhesion pathway in leukemic cell killing. Pretreatment of CD2+ effectors with anti-LFA-1 α-chain antibody, directed against a component of the LFA-1 (CD11/18)–ICAM-1 (CD54) cell adhesion system, does not abrogate their ability to inhibit the growth of autologous or allogeneic normal myeloid colonies, but abolishes any inhibition of both

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**Fig 1.** Comparison of cytotoxicity and growth inhibitory activity of PB-derived CD2+ and PB-derived CD2– LAK cells in autologous co-cultures with normal marrow (A) or cryopreserved blast cells (B). The cytotoxicity results are given as the mean ± SD percentage of 51Cr released (in five experiments) at an effector:target ratio of 100:1 (left side of figure). Colony inhibition is expressed as a percentage of myeloid/leukemic colony growth in control cultures (no lymphocytes added) after co-culture with PBMC-LAK, with CD2+ LAK or with CD2– LAK, all added at a 10:1 effector:target ratio (right side of figure). Control cultures of normal marrow produced 64 ± 7 colonies, while control cultures of malignant blasts produced 29 ± 5 colonies. *P* values were derived from paired t-test analysis, which showed that CD2+ effectors are significantly more effective than CD2– effectors in both direct cytotoxicity (*P* < .001) and colony inhibition (*P* < .01) for normal myeloid colonies, but were not significantly different for inhibition of leukemic colonies.

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**Fig 2.** The effects of antibodies to CAMs on inhibition of clonogenic growth of normal (A) and malignant (B) cells. Lymphocyte-depleted remission marrow or malignant blast cells were cultured alone, with CD2+ AK cells at 10:1 or with CD2+ AK cells and antibodies to LFA-1 or LFA-3 (see Materials and Methods). Colonies produced in the presence of AK cells are quoted as a percentage of control colonies in each experiment produced by marrow (mean number of colonies, 73 ± 11) or by blasts (mean number of colonies, 26 ± 5) cultured alone. Data shown are the means of triplicate or quadruplicate measurements in each culture condition in seven experiments for normal marrow and patient blasts. Experiments 1 through 3 were with autologous cells. Experiments 4 through 7 were with allogeneic co-cultures. *T* testing showed: (1) Significant inhibition of normal (*P* = .04) and malignant colony formation (*P* < .001) by CD2+ LAK cells. (2) Greater inhibition of malignant than normal cells by CD2+ AK effectors (*P* = .01). (3) Abrogation of significant inhibition of normal colony growth only by anti-LFA-3, while significant inhibition of malignant colony cell growth was abrogated only by anti-LFA-1. Antibodies to CAMs alone did not modify the growth of normal or malignant clonogenic cells.
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autologous and allogeneic leukemia blast cell colony formation (Fig 2A and B).

LFA-3 is strongly expressed by normal RBCs and their progenitors. Hence, when CD2-LFA-3 interaction is an important component of the cytotoxic process, free RBCs should competitively inhibit killing. This property allowed us to investigate whether differential CAM usage by normal and malignant cells would allow selective killing of myeloblasts by CD2+ AK cells in vivo. Figure 3 shows reduced inhibition of myeloid blast colony growth by CD2+ effectors was entirely unaffected by free autologous RBCs so that mean colony inhibition in the absence of erythrocytes was 64% ± 9%, while in the presence of erythrocytes it was 61% ± 11% (±SD, n = 5). These data suggest that, in vivo, in the presence of competition from LFA-3-expressing RBCs and their progenitors, CD2+ AK cells will preferentially bind to, hence preferentially kill, leukemic clonogenic cells rather than normal myeloid progenitor cells.

To determine whether these differences in CAM usage reflect differences in CAM expression on normal and malignant progenitors, we examined ICAM-1, LFA-1, and LFA-3 expression on CD34+ marrow progenitor cells and CD34+ malignant blasts. Results illustrated in Fig 4 indicate similar expression of LFA-1 and LFA-3 (CD58) molecules by normal and malignant “progenitor” cells, but a reduced level of expression of ICAM-1 (CD54) by normal compared with malignant CD34+ cells. In six normal donors 12% ± 2% (SEM) of CD34+ marrow cells were ICAM-1 (CD54) positive while in six patients with AML 87% ± 8% CD34 positive blasts were also CD54 positive. There was no difference in the expression of the activated form of LFA-1 α-chain between normal and malignant CD34+ cells, as determined by binding of NKI-L16 (data not shown).29

DISCUSSION

We have shown that the CD2 molecule plays a central role in determining whether AK cells can discriminate between normal and malignant myeloid progenitor cells in both autologous and allogeneic settings. Only CD2+ LAK cells from PB or marrow inhibit normal myeloid progenitors, and killing can be blocked by antibody to LFA-3, the CD2 ligand. However, killing of malignant blasts does not depend on CD2-LFA-3 interaction. Because the LFA-3 molecule is present in high concentration on erythrocytes and erythroid progenitors, LAK binding of normal myeloid progenitors may consequently be subject to competitive inhibition in marrow.

IL-2-generated AK cells are MHC-unrestricted and apparently are antigen-nonspecific cytotoxic effector cells. Although some of these cells are CD3+, killing does not involve target cell recognition by this antigen-specific T-cell receptor.8 Despite this lack of antigen specificity, different populations of AK cells may kill different types of targets.54-56 Our data demonstrate one mechanism by which such discrimination may occur—differences in requirements for CAM recognition between normal and malignant myeloid progenitor cells. It has been shown that alterations in CAM expression can modify the tissue localization of tumors, while the absence of these molecules on the surface of malignant cells impedes their killing by MHC-restricted antigen-specific cytotoxic T cells.51-53 The results presented here show that differences in CAM recognition by AK cells in their interaction with normal and malignant cells may also underlie the preferential killing of malignant cells by antigen-independent cytotoxic effector mechanisms.

Binding to and/or killing of normal myeloid progenitor cells appears largely dependent on interaction between CD2 and LFA-3 and can, therefore, be blocked by pretreatment of target cells with anti-LFA-3. By contrast, inhibition of the growth of malignant blast cells does not depend on CD2-LFA-3 interaction, because anti-LFA-3 has no effect. Instead, inhibition of leukemic blast cell growth by CD2+ effector cells is abrogated by antibodies to the LFA-1/ICAM-1 system, which have no effect on CD2+ lymphocyte-mediated inhibition of normal myeloid progenitor cell growth.

These results are associated with detectable differences in expression of the adhesion molecule ICAM-1 (CD54) between normal and malignant progenitor cells (Fig 4). No other differences were detected in the LFA-1–ICAM adhesion system. In particular, there was no evidence that LFA-1 was expressed in an inactive state on normal myeloid progenitor cells.29

Whatever the mechanism for the disparity in the importance of CD2–LFA-3 for killing normal and malignant cells, the strong expression of LFA-3 by normal RBCs and their progenitors12,15 means that this disparity may be particularly important in vivo where LFA-3 expressing RBCs may compete with normal progenitors for the binding of CD2+ AK effectors. Differences in CAM expression may therefore mean not only that LAK cells bind less strongly to
normal than to malignant cells, but that binding of normal progenitors is further impeded in the presence of erythrocytes, while binding of malignant cells is unimpaired.

Thus, although AK cells lack MHC-restricted antigen-specific effector function, they can still discriminate between normal and malignant progenitor cells. This discrimination may depend in part on differential target cell sensitivity to the cytotoxic cytokines the AK cells secrete. But, as shown here, it may depend also on differential usage of CAMs when normal or malignant cells are engaged. Infusion of appropriate MoAbs to CAMs and their ligands might allow exploitation of these differences to further increase selective killing of malignant cells. For example, in the context described here, infusion of IL-2 in combination with anti-CD2 or anti-LFA-3 MoAb would reduce still further the binding of AK cells to normal myeloid progenitor cells while leaving the killing of leukemic progenitor cells unimpaired.

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