STUDIES OF LYMPHOKINE-ACTIVATED KILLER (LAK) CELLS

I. Evidence Using Novel Monoclonal Antibodies That Most Human LAK Precursor Cells Share A Common Surface Marker

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It is generally accepted that the immune system comprises two classes of lymphoid cytotoxic effectors, (*a*) CTL that, upon prior sensitization to antigen, lyse antigen-bearing cells, such as virally infected cells or allogeneic cells and (*b*) NK cells that lyse, without prior sensitization, certain tumor and virally infected targets. The fundamental difference between NK and CTL is that the former's lytic process is not immunologically specific nor is it restricted by the MHC. The recent finding that non-MHC-restricted T cells ($CD3^+$) can mediate NK-like activity (1) has led investigators to reclassify these two effector populations to include MHC-restricted CTL, non-MHC-restricted CTL, and NK cells (2, 3). Within each of these classes of lymphocytes a variety of stimuli can augment lytic activity and/or broaden the spectrum of target killing, thereby giving rise to a myriad of effectors including: lectin-activated killer cells (4), anomalous killer cells (AK)[1] (generated under mixed lymphocyte tumor culture (MLTC) conditions) (5) and lymphokine-activated killer (LAK) cells (6–8). The involvement of the lymphokine IL-2 is central to all of these activation events (9–11). IL-2 promotes the growth and differentiation of both CTL and NK cells and under optimal conditions can even stimulate monocytes (12) and B cells (13). These activated killer cells, as defined by their ability to mediate cytotoxic effects on a broad spectrum of NK sensitive and NK-resistant targets, (including fresh autologous tumor targets), are thought to be a potential cancer therapy modality. Extensive study during the past five years has not revealed the precise relationship among these various cytotoxic cellular components of the immune system, and the heterogeneity contained within each, phenotypically and functionally, has no doubt complicated matters.

At present, there is some debate as to whether the majority of LAK cells are simply lymphokine-activated natural cells (LANK cells) (14) or whether they represent separate lineages of cytotoxic cells. Many investigators claim that only the Leu-11 (IgG Fc receptor) or NKH1 (Leu-19) positive lymphocytes (which include the resident

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[1] *Abbreviations used in this paper:* AK, anomalous killer cells; GaM, goat anti-mouse; HRP, horse radish peroxidase; LAK, lymphokine-activated killer cells; LAKp, LAK cell precursors; LGL, large granular lymphocyte; LU, lytic unit; MLTC, mixed lymphocyte tumor culture.

population of PBL that mediate true NK cytotoxicity) can give rise to LAK effectors in the presence of IL-2 (15–18). This is in contrast to the original work by Grimm et al. (19) stating that the LAK precursor (LAKp) cell(s) has no surface markers identifiable by currently available antisera against differentiation clusters (e.g. CD1, CD2, CD3, CD4, CD5, CD8, CD16, CD25). Although LAK activity can be generated from a cell population that morphologically belongs to the large granular lymphocyte (LGL) subset of PBL, a number of investigators have observed that rigorous treatment of PBL with anti-NK antibodies and rabbit complement (to remove the cells that mediate NK activity) has little effect on LAK generation (19–21). In addition LAK cells can be generated from lymph nodes, thymus, and the thoracic duct which have minimal, if any, NK activity (22).

The relationship between LAK cells and CTL is somewhat clearer. The generation of allospecific CTL can be totally abrogated by treating PBL with pan-T mAbs followed by negative FACS sorting or complement-mediated lysis (23). Such treatment, however, does not affect the development of LAK effector cells and may in fact augment their generation (24). This distinction between LAK cells and specific CTL, however, can be overcome at the effector level if high concentrations of IL-2 are administered during MLC (25), thereby, generating nonspecific CTL that functionally behave as LAK effectors. It has also been shown that a subpopulation of T cells ($CD3^+$) can mediate *non*-MHC-mediated cytotoxicity and that this population may contribute to the total LAK potential found in PBL (26). Further, cells of a more primitive lymphocyte lineage ($CD3^-$, $CD2^-$) can be induced to undergo differentiation into mature LAK effectors (27), illustrating the heterogeneity of cell types that are able to generate LAK-like activity.

In the present paper we have investigated the relationships between the LAK phenomenon and various other cytotoxic effector cell systems by generating mAbs directed against a cell population that can be induced to become LAK effectors in the presence of IL-2 and autologous serum. Depletion of the cells expressing determinants recognized by these antibodies reduces LAK generation appreciably, while leaving NK activity relatively intact. Further, these antibodies have little effect on the generation of either specific CTL or MLTC induced NK-like activity. These findings indicate that LAK cells are not necessarily derived only from mature NK or T cells, but can also be generated from a third population of cells that share a common surface marker. These cells may be identical to, or include, the NK precursor cell.

Materials and Methods

Preparation of PBL. Venous blood was collected from normal donors in heparinized Vacutainer tubes. Mononuclear cells were prepared by centrifugation over Ficoll-Isopaque (Pharmacia Fine Chemicals, Dorval, Quebec) and unless otherwise indicated, were depleted of monocytes by incubation on plastic for 1 h at 37°C. This nonadherent cell population is referred to simply as PBL.

Media and Reagents. Media used to culture cell lines consisted of RPMI 1640 containing 10% FCS and 10 mM L-glutamine. Media used for the generation of CTL, AK, or LAK cells consisted of culture media without FCS, supplemented with 10% autologous serum or 10% pooled human AB serum. Affinity-purified IL-2 was used at a concentration of 10% (Biotest, Fairfield, NJ) or rIL-2 at 30 U/ml (Amgen, Thousand Oaks, CA) or 1,000 U/ml (a kind gift from Cetus Corp., Emeryville, CA).

Cell Lines. PR-1, an EBV-lymphoblastoid cell line (LCL), was derived by infecting lym-

phocytes with virus preparations from the B95-8 cell line, as previously described (28). The maintenance and relative NK sensitivity of K562, an erythroleukemic cell line and Raji, a Burkitt's lymphoma cell line have been described previously (29). Cultures were fed three times weekly and periodically tested for mycoplasma contamination by DAPI (4,6-diamidino-2-phenylindole) (Sigma Chemical Co., St. Louis, MO) staining (30).

Monoclonal Antibodies. The following mAbs were used: Leu-11b (CD16), anti-IL-2R (CD25) (Becton Dickinson & Co., Mountain View, CA), OKT8 (CD8), OKT11 (CD2), and OKM1 (CR3) (Ortho Diagnostics, Raritan, NJ). Anti-Tac (IL-2R) was a gift from Dr. T. Waldmann, National Institutes of Health (Bethesda, MD). BBM1 (β_2-microglobulin), L243 (class II framework), W632 (class I framework), and OKT3 (pan-T) were grown as ascites from hybridomas obtained from the American Type Culture Collection (ATCC; Rockville, MD). The hybridomas DM-1 and DM-2 described in this report have been deposited with the ATCC (Numbers HB 9691 and HB 9692, respectively).

Percoll Gradients. Approximately 10^8 PBL were layered onto a seven-step Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) discontinuous gradient consisting of 1.5-ml layers of 42.5, 45.0, 47.5, 50.0, 52.5, 55.0, and 66.7% Percoll, adjusted to 285 mOsm/kg H_2O with 10× PBS, pH 7.3, before use. The conditions for gradient centrifugation were specifically defined so as to maximize the separation of LAKp and mature NK cells (see Results), similar to that described by Grimm et al. (6). Morphologic data were obtained from cytocentrifuged methanol-fixed Giemsa-stained cells. The cell-sorting experiments used a modified three-step gradient (45, 50, and 55% Percoll) to facilitate LAKp enrichment.

mAb Generation. 6-8-wk-old BALB/c female mice were injected with 1-2 × 10^7 Percoll enriched LAKp cells intraperitoneally four times at ~2-wk intervals. Fusion of spleen cells to the murine fusion partner SP2/0 Ag14 (non-Ig producer) took place after the fourth injection using standard hybridoma methodology (31, 32). Hybridoma supernatants were screened by ELISA on glutaraldehyde (Sigma Chemical Co.) fixed PBL or fixed Percoll enriched fraction cells, both of which were obtained from a donor HLA-unrelated to the donor whose cells were used for immunization. All positive hybridomas were grown as ascites in nude mice after they had been cloned at least three times. Isotyping of selected antibodies was done using an ELISA-based Isotyping kit (Boehringer Mannheim, Dorval, Quebec).

Complement-mediated Lysis. PBL were incubated with undiluted hybridoma supernatant or a 1:20 dilution of hybridoma ascites for 1 h at 4°C, washed once, and then incubated for 1.5 h at 37°C with a 1:5 dilution of Low-Tox rabbit complement (Cedarlane Laboratories Ltd., Hornby, Ontario) that had been screened for high lytic titer and low nonspecific toxicity. Dead cells were scored using the trypan blue exclusion test after the cells had been washed twice.

Panning. 35 × 10 mm petri plates were coated with 3 μg/ml goat anti-mouse (GAM) Ig (Cappel Laboratories, Malvern, PA) in Tris-HCl, pH 9.0, for 2 h at 4°C and then washed extensively. PBL (10^7 cells) that had previously been incubated with hybridoma supernatants for 1 h at 4°C were then incubated on the antibody-coated plates for 1 h in a 37°C, 5% CO_2 incubator. The nonaherent cells were collected by gently washing the plates with cold PBS, pH 7.3. The adherent cell population was collected by vigorous pipetting. The separated populations were washed twice with media before use and analyzed using flow cytometry to monitor the efficiency of the separation. In all cases the efficiency was >90%.

Indirect Immunofluorescence. 10^6 cells were incubated with hybridoma supernatant or a dilution of the corresponding ascitic fluid for 1 h at 4°C, washed twice and then further incubated with a 1:20 dilution of a FITC-conjugated GAM Ig (Boehringer Mannheim Biochemicals) or FITC-conjugated $(Fab')_2$ GAM Ig (Cappel Laboratories) for 1 h at 4°C. Cells were washed three times and layered on to 0.5 ml of FCS and centrifuged at 200 *g*. The FCS was aspirated off and the pellet was resuspended in 0.5 ml of RPMI 1640 containing 5% FCS and analyzed on a FACS IV (Becton Dickinson & Co., Mountain View, CA).

Cell Sorting. Cells (2 × 10^6/ml) labeled with DM-1 (indirect FITC label) were sorted into positive and negative populations at a flow rate of ~1,500 cells/s into collection tubes containing 1 ml of RPMI 1640, 10% autologous serum, and 100 μg/ml gentamycin on ice.

LAK Cell Generation. PBL isolated as described above were activated to generate LAK cells by in vitro incubation for 7 d in RPMI 1640, 10 mM L-glutamine, 10% autologous serum, and 30 U/ml rIL2 (Amgen), or 1,000 U/ml rIL-2 (Cetus Corp.) or 10% highly purified IL2

(Biotest). Cells were incubated at a concentration of 2×10^6/ml in T25-cm^2 tissue culture flasks (Corning Glass Works, Corning, NY) or in 13-ml tubes (Falcon Labware, Oxnard, CA) in a 5% CO_2, 37°C environment. The use of rIL-2 or highly purified IL-2 made little difference on proliferation or cytotoxic response.

MLC and AK Cell Generation. Before the addition of the responder lymphocytes, the stimulating lymphocytes (MLC assay) or tumor cell lines (AK assay) were x-irradiated at an air-dose rate of 4.7 Gy/min for 13.5 min using a superficial x-ray machine (model RT-100, Philips Electronic Instruments, Inc., Mahwah, NJ). The optimal stimulator-to-responder ratio was predetermined for each stimulator and was found to be 1:5 for the tumor stimulators and 1:2 for the allogeneic lymphocyte stimulators. The mixed cultures were then incubated in 24-well plates at a concentration of 2×10^6 responders/ml in RPMI 1640 containing 10% responder autologous serum or pooled AB+ serum. Cultures with responders and stimulators alone were set up as controls. To assess the proliferative response of responder lymphocytes during the co-culture period, 10^6 cells were removed on day 6 and seeded in 96-well flat-bottomed microtiter plates. 1 μCi of [^{3}H]thymidine (1 mCi/ml; New England Nuclear, Boston, MA) was added to each well, and the total volume was made up to 100 μl with RPMI 1640 containing 10% AB serum. Cultures were harvested 18 h later by means of a harvester (Skatron, Inc., Sterling, VA) on fiberglass filters and DNA isotope incorporation was measured using a liquid scintillation counter. All data are presented as mean cpm ± SD of incorporated [^{3}H]thymidine from 12 replicate wells.

Cytotoxicity Assays. NK and LAK activity were assessed in an 18-h ^{51}Cr-release assay against the targets K562 (NK-sensitive, LAK-sensitive) and RAJI (NK-resistant, LAK-sensitive) as described previously (29). Percent specific release was calculated as follows: percent specific release = 100 × {[cpm (test) − cpm (medium)]/[cpm (max) − cpm (medium)]}. All chromium release data were expressed in lytic units, defined as the number of effectors required to lyse 1,000 target cells (LU (20%)/10^6), calculated according to the equation $y = A(1\text{-}e^{-kx})$, where y is fraction chromium release, x is lymphocyte-target cell ratio, k is a constant proportional to effector cell number and equal to the negative slope of the target cell survival curve obtained by plotting $\ln(A\text{-}y)$ vs. x, and A is the maximal cell mediated lysis.

Western Blots. To characterize the moiety recognized by the mAb generated, Western blots were performed on unseparated PBL membrane preparations as described below. Briefly, 10^9 nonadherent cells were incubated on ice for 30 min in membrane buffer (0.1% BSA, 1 mM $MgCl_2$, 0.1 mM PMSF, 0.01 M Tris-HCl, pH 7.6, 1 mM iodoacetamide), homogenized in a dounce homogenizer (16 strokes), and centrifuged for 10 min at 4,000 *g* at 4°C. The supernatant was recovered and made up to 13 ml in membrane buffer and centrifuged for 1 h at 55,000 *g* at 4°C. The pellet was then resuspended in 200 μl of storage buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.02% NaN_3). 50 μl of this membrane-containing solution was added to 10 μl of electrophoretic sample buffer (0.0625 M Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 2% 2-ME, 0.001% bromophenol blue, 0.1 mM PMSF) and run on a 4.5%/12.0% SDS-PAGE according to the method of Laemmli (33). The lanes of interest were blotted on to nitrocellulose membranes using a Bio-Rad Laboratories (Richmond, CA) transmembrane blotting apparatus and carbonate buffer system (10 mM $NaHCO_3$, pH 9.9, 3 mM Na_2CO_3, in 20% methanol) after soaking the gel in 3% gelatin, Tris HCl, pH 7.2, for 1 h. After a 3-h transfer at 250 mA the membrane was blocked in 5% normal goat serum, 1% BSA, 3% gelatin in TBS (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) overnight with shaking at room temperature and then incubated with hybridoma supernatant or diluted ascites for 4 h at room temperature on a rocker platform. The membrane was washed three times for 10 min in TTBS (0.05% Tween 20 in TBS) and incubated with a 1:2,000 dilution of GAM Ig-horse radish peroxidase (HRP) (Boehringer Mannheim) for 2 hrs. After four 10-min washes in TTBS, the 4-CN (4-chloro-1-naphthol) (Sigma Chemical Co.) substrate was added and allowed to develop in the dark for 30 min. The reaction was stopped by rinsing the nitrocellulose membrane in distilled water.

Results

Separation of Mature NK Cells from LAK Precursor Cells. PBMC collected from normal individuals and depleted of adherent cells on plastic were fractionated on a seven-

step Percoll discontinuous gradient. To increase the concentration of LAKp cells relative to NK cells, a modification of the original method of Percoll fractionation described for NK enrichment by Timonen and Saksela (34) was undertaken in which the gradient was centrifuged for a longer time (45 min) and at a lower centrifugal force (300 *g*). The separation of mature NK cells from LAKp cells that can be achieved on such a gradient is illustrated in Fig. 1. By comparing NK activity at day 0 with LAK activity at day 7, in each respective fraction, it is apparent that these populations can be separated from each other according to densities. With regard to K562 cytotoxicity (Fig. 1 *A*), most of the NK activity was found in fraction 2 (45% Percoll), while most of the LAK potential was found in fractions 3 and 4 (47.5 and 50.0% Percoll, respectively). Similarly, when cytotoxicity against the NK resistant cell line RAJI (*B*) was examined at day 7 these same two fractions showed the highest LAK activity. The proliferation potential of the various fractions shown in Fig. 1 is an index relative to the number of cells in each fraction originally cultured in IL-2. From this figure it is evident that fraction 3 contained the highest proliferative potential. The percentage of *total* lytic units found in each fraction, (taking into account the cell yield of each fraction), both at Day 0 (NK) and at Day 7 (LAK) is given in Table I. It is interesting to note that the highest percentage of *total* lytic units of all six fractions at Day 0 is found in fraction 3 (47.5% Percoll) not in fraction 2, as it would have been if cell yield per fraction had not been taken into account. This indicates that although fraction 2 has a higher proportion of NK activity (either greater numbers of functional NK cells or simply more active NK cells), fraction 3 has greater total activity. After seven days in IL-2 culture, the Percoll fraction that exhibited the highest LAK effector activity in addition to the highest *total* lytic units of all cell fractions

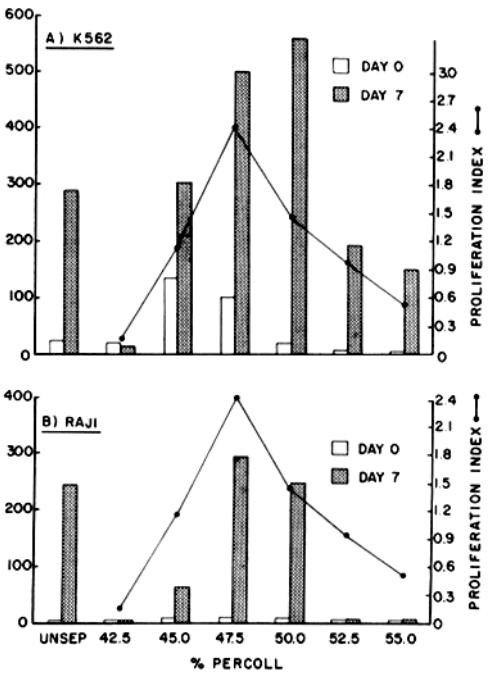

FIGURE 1. Percoll gradient separation of LAK progenitors from mature NK cells. ~10^8 cells were layered on a seven-step Percoll gradient (42.5–66.7%, fractions 1–6, respectively) and spun for 45 min at 300 *g*. Each fraction was then tested for NK activity and set up at 2 × 10^6 cells/ml in 5 ml of culture media containing 10% autologous serum and 30 U/ml rIL-2. Cytotoxicity vs. K562 (*A*) and Raji (*B*) of fractionated PBL at day 0 (□) and after incubation with IL-2 for 7 days (▦) is expressed in LU (20%)/10^6 cells. Cell yield on day 7 (—●—) is given as a proliferation index relative to the number of cells originally cultured. Data are representative of three separate experiments. The standard deviation of the lytic unit values did not exceed 15%.

TABLE I

Percent of Total Lytic Units of Individual Percoll Fractions at Day 0 (NK Activity) and at Day 7, after IL-2 Incubation (LAK Activity)

Day 0	LU(20%)/10^6	Total cells	LU (fraction)	Total LU
		%		%
PBL	22			
F1 (42.5%)	19	9.0	137	8.7
F2 (45%)	125	2.4	240	15.3
F3 (47.5%)	98	8.7	**682**	**43.4**
F4 (50%)	22	22.0	387	24.6
F5 (52.5%)	5	26.4	105	6.6
F6 (55%)	4	6.8	21	1.3
Day 7 (+IL-2)				
PBL	250			
F1 (42.5%)	20	9.0	144	0.8
F2 (45%)	290	2.4	557	3.0
F3 (47.5%)	500	8.7	3,480	18.7
F4 (50%)	550	22.0	**9,680**	**51.9**
F5 (52.5%)	190	26.4	4,013	21.5
F6 (55%)	140	6.8	762	4.0

Cells from Percoll fractions (generated as described in Fig. 1) and PBL were set up in IL-2 culture at a concentration of 2×10^6 cells/ml in T25-cm^2 flasks containing RPMI 1640, 10% autologous serum, and 30 U/ml rIL-2. The flasks were incubated for 7 d at 37°C in a 5% CO_2 incubator. Cells were removed, washed twice, counted for viability, and set up in a ^{51}Cr-release assay. Cell yield from the gradient was routinely 85% of the number of cells originally put in (including the cell pellet, fraction 7). Numbers in bold face indicate maximum NK activity (fraction 3) and maximum LAK activity (fraction 4).

was fraction 4. This indicates that at day 0, fraction 4 contained the highest relative proportion of LAKp cells as well as the highest absolute number of LAKp, while at the same time expressing relatively little NK activity.

Percoll Fraction Phenotype. To examine the phenotypic markers on the cells in each fraction, FACS analysis using a battery of mAbs was undertaken (Fig. 2). It was apparent that there is enrichment of Leu-11b$^+$ cells in fractions 2 and 3 representing most of the FcR$^+$ LGL in the upper fractions (Fig. 2 *A*). The staining pattern of OKT11 on PBL and the various Percoll fractions is shown (Fig. 2 *B*). PBL and fraction 6 contained ~80 and 87% OKT11$^+$ cells, respectively, whereas fractions 1 and 2, and 3 (combined) all contain <50%. OKT 3 showed a similar pattern to OKT11, staining 83% of PBL and 86% of fraction 6, while staining the less dense fractions only moderately. OKM1 stained 18% of PBL but showed increased staining in fractions 2 and 3 (47%).

Production of mAbs to Pre-LAK Cells. Cells from Percoll fraction 4 were injected into 6–8-wk-old female Balb/c mice. Of the ~1,650 resultant hybridomas that reacted with fraction 4 lymphocytes, two hybridomas produced mAbs that reacted with cells that were required for LAK cell generation. These mAbs (DM-1 and DM-2) are both capable of depleting a cell population required for the generation of LAK effectors as judged by cytotoxic ability at day 7 after IL-2 incubation. These antibodies are described below.

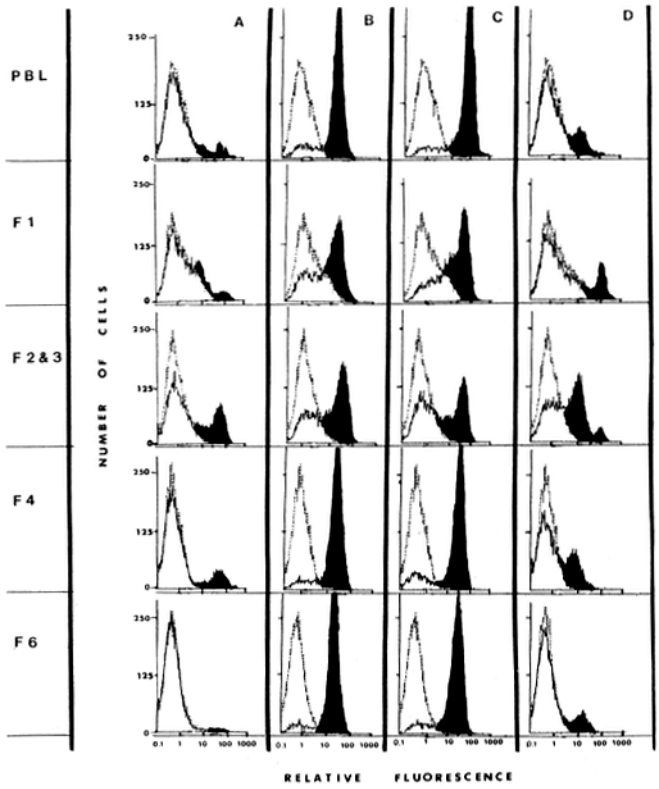

FIGURE 2. FACS analysis of PBL and Percoll gradient fractions 1, 2 and 3, 4, and 6 stained with the mAbs Leu-11 (CD16) (*A*), OKT11 (CD2) (*B*), OKT3 (CD3) (*C*), OKM1 (CR3) (*D*). 10^6 cells were incubated with antibody for 1 h at 4°C, washed twice, and then incubated further with FITC GAM Ig for 1 h at 4°C. Cells were washed three times, layered onto 0.5-ml FCS, and spun. The FCS was aspirated off and the cells were resuspended in 0.5 ml RPMI and analyzed on a FACS. The *y*-axis represents the number of cells and the *x*-axis represents relative fluorescent intensity. Background histograms (*dotted lines*) represent class-specific mAb controls and, to facilitate discrimination, any events above background have been shaded.

Functional Assays Using DM-1 and DM-2. To characterize the lineage and function of the cell type(s) recognized by these two antibodies (both IgM, k), experiments using PBL depleted of DM-1^+ or DM-2^+ cells were conducted. Figs. 3 and 4 illustrate the effect of using complement and antibody depletion (Fig. 3) and a panning separation technique (Fig. 4), on NK, LAK, and AK activity. The effect of Leu-11 (CD16), OKT11 (CD2), or OKT3 (CD3) depletion was also examined to monitor NK and T cell involvement. The results shown in Fig. 3 indicate that depletion of DM-1^+ or DM-2^+ cells had little effect on NK (K562) or AK (K562 and RAJI) activities, but markedly reduced LAK generation, whether measured using K562 or RAJI target cells. The parallel effects of DM-1 and DM-2 depletion on LAK generative potential indicate that the two markers are coexpressed on most of the LAK precursor cells. Conversely, Leu-11 treatment depleted NK activity and to an extent AK activity, but had little effect on LAK generation by day 7. Treatment with OKT11

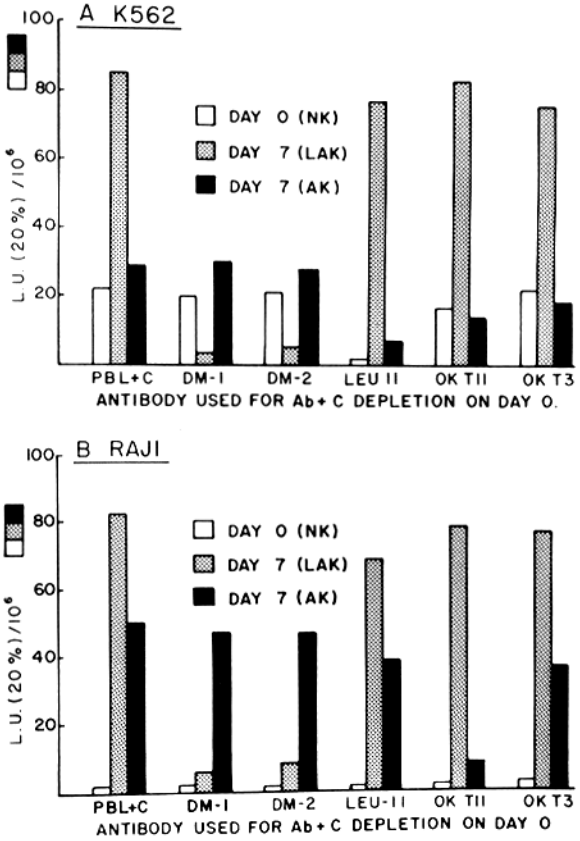

FIGURE 3. The effect of antibody plus rabbit complement on NK, LAK, and AK (co-culture with the lymphoblastoid cell line, PR-1) activities when PBL were treated on day 0. Briefly, 10^7 PBL were incubated with antibody for 1 h at 4°C, washed once, and resuspended in 100 μl prescreened rabbit complement and incubated for 1 h at 37°C. 1 h later an additional 100 μl of the complement was added, then washed twice before use. Data are representative of four separate experiments. The standard deviation in lytic unit values did not exceed 15%.

and OKT3 antibodies had minimal effect on NK (K562) and LAK (K562 and RAJI) lysis but reduced AK generation (K562 and RAJI). In all cases, the effect of cell depletion using the panning method (Fig. 4) was found to be similar to the complement plus antibody treatment of PBL.

It should be noted that when lytic activity is unchanged after depletion of cells with a particular marker, the proportion of killer cells with that marker is approximately equal to the proportion of marker-bearing cells in the original population, due to the fact that the treated cells are resuspended before the cytotoxicity assay at the same concentrations as the control cells, which enriches for the non-marker-bearing killer cells (see formula in reference 35). In the case of NK activity, the data in Figs. 3 and 4 are compatible with the NK effector cells being ~80% OKT11$^+$, 90% Leu-11$^+$, 5% DM-1$^+$, and 15% DM-2$^+$, based on the marker proportions observed in unfractionated PBL (see below) and the fact that Leu-11 treatment removed virtually all NK cytotoxicity. The lack of enhancement of NK activity with OKT3$^+$ cell depletion in this particular experiment is unexplained. The formula is not applicable to the calculation of the proportion of marker-bearing cytotoxic precursor cells, since there is not necessarily a direct relationship between precursor number at day 0 and effector number at day 7. The data do indicate, however, that a significant proportion of LAKp or LAKp accessory cells bear the DM-1 and DM-2 markers.

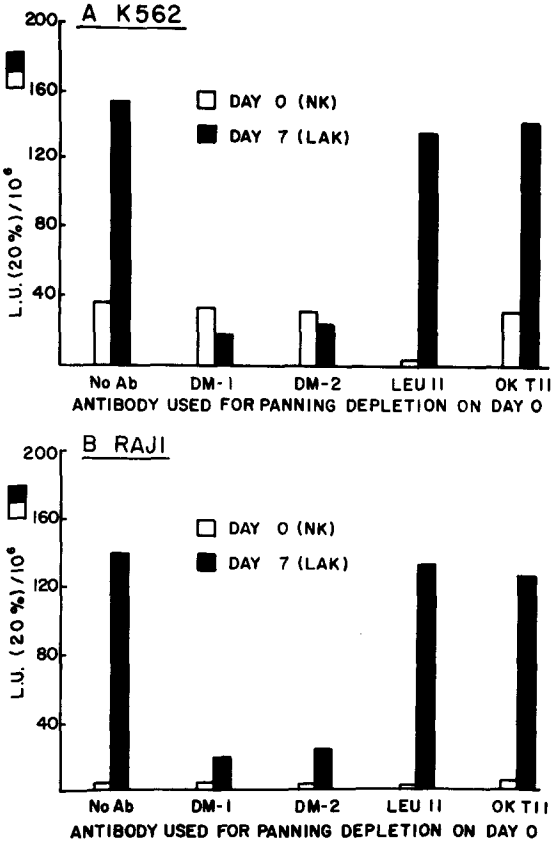

FIGURE 4. Effect of panning depletion of PBL pretreated with the indicated antibodies on NK and LAK activities. Petri plates were coated with 3 μg/ml GAM Ig for 2 h and then washed six times. 10^7 PBL that had previously been incubated with antibody for 1 h were then incubated with the plates for 2 h at 37°C. The nonadherent population was recovered via gentle washing and the adherent population was removed by vigorous pipetting. Data are representative of three separate experiments. The standard deviation in lytic unit values did not exceed 15%.

When DM-1$^+$ or DM-2$^+$ cells were depleted from PBL and the resulting population set up in an AxB_x MLC, little reduction in cytotoxicity generated against the stimulator cell target (B), K562 or RAJI occurred (Table II). However, when OKT8$^+$ (CD8) or OKT3$^+$ (CD3) cells were depleted from PBL and then set up in an identical culture, a marked decrease in cytotoxicity against the stimulator target, as well as K562 and RAJI, was observed. The proliferative response generated during the MLC, as measured by [^{3}H]thymidine uptake, is shown in Table III. Only pretreatment with the OKT3 and OKT8 mAb affected the proliferative response. Pretreatment with DM-1 or DM-2 did not have any appreciable effect.

Cell Sorting. Positive selection of DM-1$^+$ cells was undertaken to provide evidence that DM-1 reacts with LAKp cells and not with an accessory population required for LAK cell generation. Fig. 5 represents the flow cytometric histograms of PBL and modified (three-step) Percoll gradient fractions 1 (45%), 2 (50%), and 3 (55%). The cells found in Percoll fraction 1 provided an enriched DM-1$^+$ population for cell sorting into positive and negative populations. Unstained and irrelevant mAb control-treated cells were used to establish fluorescence parameters. The low fluorescent staining of DM-1 (Fig. 5) made the inclusion of highly autofluorescent cells within the positive sort window unavoidable. Upon reanalysis, 60% DM-1$^+$ cells and 40%

TABLE II

Cytotoxicity Generated during an A × B_x MLC

Responder	Stimulator	Targets			
		K562	RAJI	A	B
A + Control Ab	B	148 ± 62	210 ± 62	11 ± 7	44 ± 19
A + DM-1	B	135 ± 44	191 ± 54	9 ± 2	39 ± 22
A + DM-2	B	140 ± 34	170 ± 50	12 ± 3	41 ± 19
A + OKT8	B	21 ± 7	14 ± 3	8 ± 1	2 ± 1
A + OKT3	B	53 ± 11	31 ± 9	6 ± 1	5 ± 1
A + Leu-11	B	115 ± 31	185 ± 49	9 ± 2	53 ± 29
A + Control Ab	-	12 ± 6	3 ± 1	-	-
—	B	-	-	-	-

Responder lymphocytes (8×10^6 cells) that had been treated with antibody plus complement were mixed with irradiated (x) stimulator lymphocytes (4×10^6 cells) in 24-well plates that contained 2 ml RPMI plus 10% responder autologous serum. The plates were incubated for 7 d in a 5% CO_2 incubator at 37°C. On day 7 the cells were washed twice, counted for viability, and set up in a ^{51}Cr-release assay (LU[20%]/10^6 ± SD). Cultures containing responders and stimulators alone were set up as controls. The reverse B × A_x results were similar.

low fluorescent or negative contaminating events were found in the positive sorted population.

The sorted populations were then evaluated for NK and LAK cytotoxic potential using a ^{51}Cr-release assay (Table IV). There was a slight increase in lytic unit values when the NK activity of PBL was compared with the unsorted Percoll fraction 1 (73 ± 1 to 83 ± 2, respectively). However, substantial NK augmentation occurred when fraction 1 was depleted of DM-1$^+$ cells (114 ± 5 LU). Conversely, the DM-1$^+$ population harbored relatively low NK activity (17 ± 1 LU). The LAK activity generated after 5 d of IL-2 incubation was highest in the DM-1$^+$ population (2,556 ± 260 LU) as compared with the DM-1$^-$ population (387 ± 48 LU). The DM-1$^+$ population exhibited more LAK activity than did unfractionated PBL or the unsorted fraction 1 population. The results of these positive sort experiments indicate

TABLE III

Proliferation Generated during A × B_x MLC as Measured by [^{3}H]Thymidine Uptake

Responder	Stimulator	cpm
A + Control Ab	B	8,031 ± 1,630
A + DM-1	B	7,586 ± 1,787
A + DM-2	B	6,369 ± 977
A + OKT8	B	4,386 ± 697
A + OKT3	B	1,413 ± 335
A + Leu-11	B	9,749 ± 1,814
A + Control Ab	B	600 ± 128
—	B	41 ± 27

Cells were removed from the MLC cultures (see Table II legend) on day 6 and seeded into 96-well flat-bottomed microtiter plates (10^6 cells). 1 μC [^{3}H]thymidine was added to each well and the total volume was made up to 0.1 ml. Cultures were harvested 18 h later on fiberglass filters and DNA isotope incorporation measured. (Data presented as mean cpm ± SD of 12 replicate wells.)

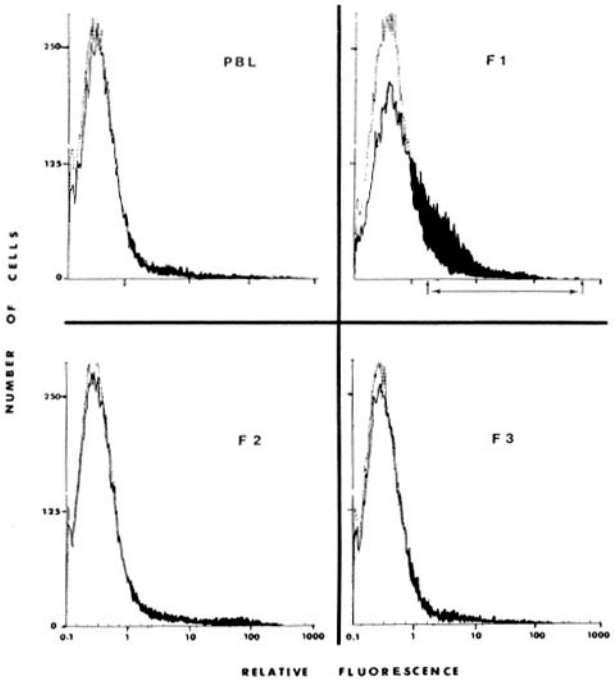

FIGURE 5. FACS analysis of PBL and modified (three-step) Percoll gradient fractions 1, 2, and 3 (45, 50, and 55% Percoll, respectively). The cells and data were treated as they were in Fig. 2. The gates used to separate DM-1$^+$ from DM-1$^-$ cells are indicated by the arrows on the *x*-axis of the fraction 1 histogram.

that DM-1$^+$ cells are LAK precursor cells and are not simply accessory cells necessary for LAK generation.

DM-1$^+$ and DM-2$^+$ Cells during LAK Generation. The frequency with which DM-1 and DM-2 stain fresh PBL can be seen in Fig. 6. DM-1 stains 2–15%, and DM-2 15–30% of PBL. These numbers closely parallel the percent dead cells observed after DM-1 and DM-2 depletion using the respective antibody and complement. When expression of DM-1 and DM-2 during LAK activation was examined (Fig. 6), both antibodies recognized cells present at day 0 but over the 8-d IL-2 activation period there was a gradual loss of these markers. In contrast, the proportion of Leu-11$^+$ cells was moderately increased, as expected. The expression of OKT3 and OKT11 was essentially unchanged during the activation period.

Western Blot Analysis. To characterize the antigens recognized by these two antibodies, Western blot analysis of membrane preparations of PBL were undertaken. DM-1 recognizes a 38,000 dalton protein and DM-2, a 44,000 dalton protein on reducing SDS-PAGE (Fig. 7).

Discussion

Normal human PBL are capable of several cytotoxic functions mediated by a diverse array of effectors, namely the functionally defined NK cells (36), MHC-restricted CTL that recognize targets via a CD3/Ti antigen receptor structure (2), non-MHC-restricted CTL that recognize MHC$^-$ targets also via CD3/Ti (3), non-MHC-restricted CTL that recognize targets via a CD3-associated molecule different from the Ti molecule (37), lectin-activated killer cells (4), killer cells generated under MLC

TABLE IV

Cytotoxicity Exhibited by DM-1$^+$ and DM-1$^-$ Cell Populations after Cell Sorting

Cell population	DM-1$^+$	LU(20%)/10^6 NK	LU(20%)/10^6 LAK
	%		
PBL – Unfractionated	4	73 ± 2	1,310 ± 104
Fraction 1 – Unsorted	21	83 ± 6	1,807 ± 190
Fraction 1 – DM-1$^+$ (sorted)	62	17 ± 1	**2,556 ± 260**
Fraction 1 – DM-1$^-$ (sorted)	1	**114 ± 5**	387 ± 48
Fraction 2 – Unsorted	7	72 ± 3	702 ± 88
Fraction 3 – Unsorted	2	12 ± 1	266 ± 54

Fraction 1 cells obtained from a modified three-step Percoll gradient (45, 50, and 55% Percoll) were sorted into DM-1$^+$ and DM-1$^-$ populations by flow cytometry (2×10^6 cells/ml at a flow rate of 1,500 cells/s). The resultant populations were tested immediately for NK activity and reanalyzed for percent DM-1$^+$ cells. LAK activity was assessed after 5 d incubation with 1,000 U/ml rIL-2 (Cetus Corp.). Cytotoxicity was measured against the K562 target in a ^{51}Cr-release assay for 18 h (LU[20%]/10^6 ± SD). Data are representative of two separate experiments.

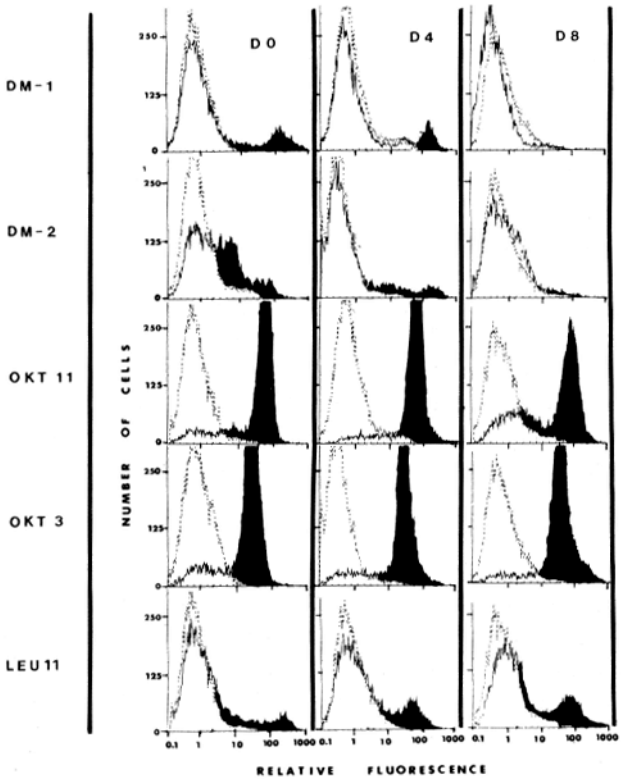

FIGURE 6. FACS analysis of PBL stained with the mAbs DM-1, DM-2, OKT11, OKT3, and Leu-11 at day 0, 4, and 8 of incubation with IL-2. The cells and data were treated as in Fig. 2.

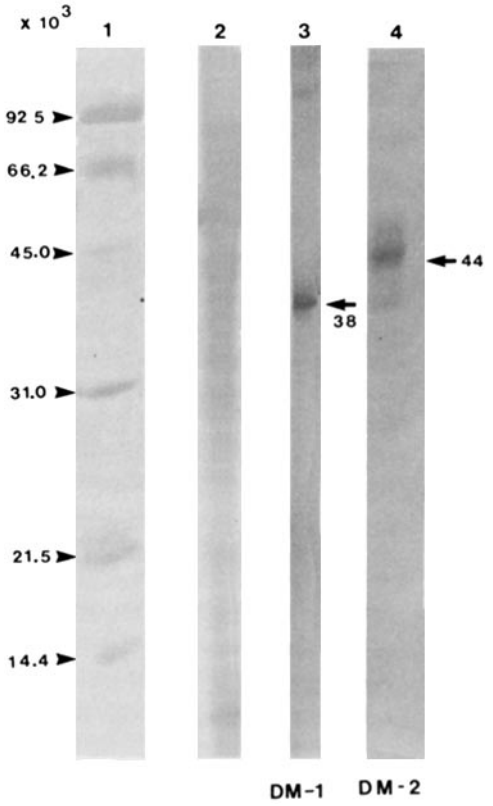

FIGURE 7. Western blot analysis of DM-1 and DM-2 antigens from PBL. Membrane-containing preparations were run on SDS-12% polyacrylamide gels (33) and transferred to nitrocellulose. DM-1$^+$ and DM-2$^+$ bands were visualized using a HRP-4CN system (as outlined in the Materials and Methods). (Lane *1*) Molecular weight markers; (lane *2*) Coomassie-Blue-stained gel of PBL membrane preparation; (lanes *3* and *4*) DM-1 and DM-2-stained transfers of lane *2*, respectively.

or MLTC conditions, i.e., AK cells (5), and finally, LAK cells (8). The relationships among these effector cells remain elusive despite considerable research (38). In the present study we have investigated the relationship between NK, LAK, AK, and CTL at the level of their precursors. The experiments described were undertaken to isolate a cell population that was neither a mature NK cell nor T cell, and could be induced by IL-2 alone to become mature LAK cells. This was done in two ways: (*a*) by the Percoll gradient separation of mature NK cells from a population of cells that generates maximal LAK potential upon exposure to IL-2 and (*b*) by the production of mAbs that react with these Percoll-enriched LAKp but not with mature NK cells or other cytotoxic lymphocytes. The argument that mature NK cells are the sole progenitors of LAK effectors is weakened by the finding that treatment of PBL with several anti-NK antibodies has only a moderate influence on LAK effectors or their precursors (20, 21). Further, LAK activity can be generated from various lymphoid tissues that are devoid of NK activity (22).

In the past, using standard Percoll separation techniques, most LAK activity has been generated from those LGL populations that also contained peak NK activity. We have shown that by varying centrifugation conditions from those used for standard LGL enrichment (34), the separation of cells mediating NK activity (fractions 2 and 3) from cells that exhibit maximal LAK activation (fraction 4) can be achieved (Fig. 1). It is of interest to note that the proliferative response to IL-2 did not peak

in fraction 4, where maximal LAK potential resided, but peaked in the less dense fraction 3. Two possible explanations may account for this. First, other cells found in fraction 3, which are not cytotoxic to the targets K562 and RAJI, are proliferating in response to IL-2, thus resulting in increased proliferation without increased cytotoxicity. A second possibility is that the cells in fraction 3, although proliferating to a greater extent, are less cytotoxic on a per cell basis than are cells in fraction 4, thus suggesting that heterogeneity exists among those cells that become cytotoxic upon exposure to IL-2.

This partial separation of LAKp and NK effectors on Percoll gradients has allowed us to examine a population of IL-2-reactive lymphocytes in isolation from the majority of cells mediating NK activity. mAbs were generated after immunization with these cells to further characterize the relationship of fraction 4 cells and LAKp cells. Two antibodies, DM-1 and DM-2 (both IgM,k), were chosen from those antibodies that react with fraction 4 lymphocytes for further study because of their specificity for a cell population that generated LAK effectors upon incubation with IL-2. The incubation of PBL with either of these two antibodies followed by complement-mediated lysis or panning, decreased LAK effector generation markedly, and formed the basic strategy for the preliminary functional screening.

The possibility that these mAbs react with an accessory population required for LAK effector generation, and not LAKp cells, cannot be excluded using negative selection techniques. Therefore, positive cells were sorted using flow cytometry to provide evidence that the DM-1 mAb does react directly with LAKp cells and not with an accessory cell population. To enrich the number of DM-1$^+$ cells in the population to be sorted, a three-step Percoll gradient was used (Fig. 5). Fraction 1 of the gradient was routinely enriched 10-fold over unseparated PBL. After incubating the sorted populations in Il-2 for 5 d, the DM-1$^+$ sorted population exhibited almost a sevenfold increase in cytotoxicity over the DM-1$^-$ fraction. The enrichment for LAKp cells found in this fraction was also demonstrated by the 1.4-fold increase in cytotoxicity over unsorted fraction 1 cells. When the NK activity of these sorted populations was examined the DM-1$^+$ fraction exhibited minimal NK activity compared with the DM-1$^-$ fraction (7-fold higher) and unsorted Percoll fraction 1 (1.5-fold higher). Based on the enrichment of DM-1$^+$ cells, in association with markedly increased LAK generative potential, it is unlikely that the effects seen are due to contaminating DM-1$^-$ cells. This apparent contamination could have been due to modulation of the DM-1 antigen, ungated cell debris resulting from the sorting procedure, and/or the choice of gates necessary to include relatively low fluorescent DM-1$^+$ cells allowing inclusion of some highly autofluorescent events (Fig. 5).

The knowledge that a variety of lymphocytes (CD4$^+$ T cells, CD8$^+$ T cells, CD20$^+$ B cells, CD2$^-$ CD16$^-$ CD20$^-$ null cells and CD16$^+$ Leu-19$^+$ NK cells) can give rise to LAK activity (10), coupled with the fact that DM-1 and DM-2 only react with ~2–15 and 15–30% of PBL, respectively, raises obvious questions. If all the above cell types can give rise to LAK effectors then an antibody that reacts with the majority of LAK precursors must react with some or all of these cell types. Two-color double-marker analysis of DM-1$^+$ and DM-2$^+$ cells is currently in progress using HPLC-purified, FITC-labeled DM-1 and phycoerythrin-labeled Leu-11b, Leu-4, and Leu-19 antibodies (Morris, D. G., J. R. Ortaldo, J. Johnson, and H. F. Pross, manuscript in preparation). Initial experiments with one donor's PBL (3% DM-

1^+) showed that the DM-1^+ cells were 33% Leu-11b$^+$ and 50% Leu-4^+, with no Leu-19^+ DM-1^+ cells being detectable. Conversely, Leu-11b$^+$ cells and Leu-4^+ cells were 25% DM-1^+ and 2% DM-1^+, respectively. These preliminary findings indicate that DM-1 is coexpressed on subsets of CD16^+ and CD3^+ cells. It is probable that only a subset of each distinct population is able to respond in a cytotoxic manner to IL-2 and that DM-1 and DM-2 are unique markers for most of the cellular populations that go on to become LAK effectors. We have, however, observed some heterogeneity among different donors with regard to the correlation between DM-1 expression and LAK potential. In the cell depletion studies, lymphocytes from one individual that showed only a moderate (53%) reduction in LAK activity after depletion with DM-1, exhibited high NK activity, suggesting that the reduced effect of the mAb on LAK generation may be explained by residual IL-2 augmentable mature NK cells.

DM-1^+ and DM-2^+ cells were also examined by FACS analysis to determine possible heterogeneity of the staining population and to monitor epitope expression throughout the course of activation. The FACS histograms indicate that >85% of cells stained by DM-1 and DM-2 express low levels of their respective epitopes, i.e., most cells stained with low fluorescent intensity. Further, the decrease of surface expression of these epitopes during IL-2 activation (Fig. 6) indicates that IL-2 exposure downregulates the structures recognized by DM-1 and DM-2. Whether high or low affinity IL-2 receptors (39) are involved in this regulation (directly or indirectly) is unknown at this time. Thus, unlike the activation markers T9 (transferrin receptor, recognized by OKT9) (40) and TLiSA1 (recognized by LeoA1) (41) that become expressed during activation, the DM-1 and DM-2 determinants are gradually lost.

The relationship between the determinants recognized by DM-1 and DM-2 appears intimate: both mAbs are capable of depleting a population of cells that, in part, mediate NK generative potential, both stain cells with a low intensity, and both recognize determinants expressed at the precursor but not at the effector level. Hence, it would be logical to conclude that both antibodies recognize a similar, if not the same determinant. FACS profiles (Fig. 6) of DM-1 and DM-2 stained PBL, however, appear different and, upon further investigation using Western blot methodology of PBL membrane preparations, DM-1 and DM-2 were found to recognize 38,000 and 44,000 dalton antigens, respectively. These molecular weight data rule out the possibility that the antibodies are directed against determinants such as the low affinity IL-2 receptor (mol wt 75,000) or the complement (iC3b) CR3 receptor (two chains, mol wt 165,000 and 95,000), which have been shown to be present on LAK precursor cells (42–44). The fact that DM-1 and DM-2 antigen expression was decreased during the incubation period also argues against this possibility. As with Leu-11 (FcR)-positive cell depletion, removal of CR3^+ cells has a significant effect on NK activity, which is in direct contrast with DM-1^+ and DM-2^+ cell depletion.

To investigate the role of DM-1^+ and DM-2^+ cells in coculture and under MLC conditions, complement-mediated and panning depletion were undertaken. As mentioned above, there are certain pitfalls encountered when forming conclusions about an antigen-positive population based on data from studies on the corresponding negative populations. For example, the depleted cell may be an essential accessory cell and not the precursor cell itself. Also, the antigen under study may be modulated

off the cell surface giving the impression that the cells have been removed from the population and are therefore not involved in other types of cytotoxicity being examined. Finally, control depletions using other markers may actually be invalid because of antigen modulation instead of cell depletion. In our experiments, depletion of cells bearing the CD16 marker, a known NK cell marker, removed the majority of NK activity. Furthermore, in the case of OKT3 (CD3) depletion, reanalysis by FACS for residual $CD3^+$ cells with OKT3 combined with monitoring of $CD2^+$ cells found within this population showed that >90% of the CD3 and CD2 cells had been removed. These results indicate that the depletion technique was efficient and that down-modulation had not occurred to any appreciable extent. Since there are no known mAbs directed to unrelated antigens exclusively on DM-1 and/or DM-2 cell subsets, an independent assessment of $DM\text{-}1^+$ cell depletion versus modulation, based on markers, is impossible. However, the results from the functional assays support the finding that the depletions were efficient and that modulation was not a factor.

Functional studies using DM-1 or DM-2 depleted populations in coculture (Fig. 3) and MLC (Table II) indicate that these mAbs are not involved in the generation of the cytotoxic cells produced under these conditions. Treatment with Leu-11 or OKT11 does affect AK generation, however, and this suggests that the cells generated during co-culture with PR-1 stem from the LGL morphologic NK pool (5).

To summarize the relationships among these various effector systems, we propose a model that shows the possible activation lineages between NK, CTL, and LAK (Fig. 8). It is well documented that a minor subset of T lymphocytes ($CD3^+$) can, in the presence of exogenous IL-2, become LAK effectors (25, 26). Similarly, mature NK cells upon exposure to IL-2 can be induced to lyse many formerly NK-resistant targets (15, 16, 36). The delineation of the relative contribution of T cells, NK cells, and their precursors in the generation of a maximal LAK response from

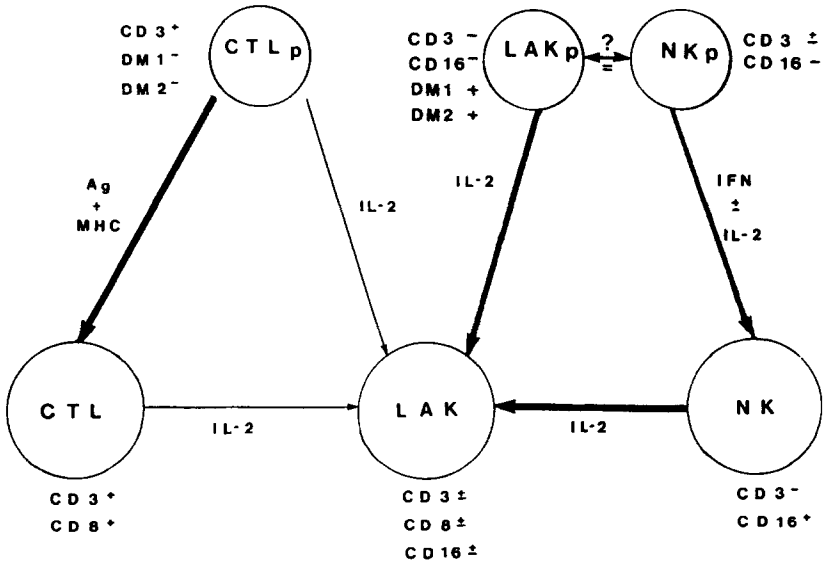

FIGURE 8. Proposed model of the relationship among T cells, NK cells, and LAK cells and their precursors. Bold face arrows represent major pathways of activation.

PBL was a major goal of this study. From our data we conclude that the contribution of $CD3^+$ cells represents an insignificant component of LAK generation, in agreement with others (7, 45–47). The contribution of NK cells, however, is significant. Most investigators agree that NK cells, defined by morphologic, phenotypic and cytotoxic parameters, are capable of becoming LAK cell effectors. However, few investigators have looked at NK precursor cells (48, 49). Our data presented here and our unpublished data support the idea that although the response to IL-2 by mature NK cells may account for the early cytotoxicity exhibited during activation, the cytotoxicity observed after 3–7 d of culture may be the result of the activation of NK precursors into LAK effectors. As suggested in Fig. 8, the $CD16^-$ NK precursor and the DM-1^+ LAK precursor may be identical, and the data in this report are compatible with this possibility.

This study clearly indicates that LAK cells can be generated from a population of cells that are not mature NK cells nor of a T cell phenotype. By producing mAbs to Percoll-fractionated lymphocytes (in which maximal LAK activity can be generated in populations containing little NK activity) we have shown that LAK effector generation can be markedly reduced without effect on the cells responsible for mediating CTL, NK, and AK activity. The further characterization of this isolated population of cells with regard to the LAK effectors generated from the separated populations is now currently under investigation and will form the basis for further study.

Summary

Separation of LAK precursor (LAKp) cells (as defined by LAK effector generation after incubation with IL-2 for 7 d) from cells with NK activity/LGL morphology was achieved on Percoll gradients using a longer, slower centrifugation than that used for optimal NK enrichment. mAb were generated using the various Percoll fractions as the immunizing cells and used for separation and depletion studies. Two mAbs DM-1 (IgM,k) and DM-2 (IgM,k) recognizing 2–15% and 15–30% of PBL, respectively, abrogated a large proportion of LAK generative potential after complement depletion, but had little effect on NK or LAK effector activity. Cell sorting experiments indicated that the majority of LAKp cells are found within the DM-1^+ population and that DM-1^+ cells are not simply an accessory cell required for LAKp generation. Further, these two mAbs do not recognize cells that are responsible for generating cytotoxicity during MLC or co-culture with the PR-1 EBV lymphoblastoid cell line. Western blot analysis indicated that DM-1 and DM-2 recognize a 38,000 and 44,000 dalton moiety, respectively. The frequency of cells bearing these antigens and the intensity of cell surface staining decreased during the 7-d culture period, suggesting that these antibodies recognize determinants found only at the precursor level. These findings indicate that cells other than NK effectors or mature T cells are capable of generating a LAK cell response. These LAK precursor cells share a common differentiation surface antigen and are different from AK or antigen-specific CTL precursors. The possibility exists that these cells are identical to, or include, the NK precursor cell.

We would like to thank Mrs. Pam Bandy-Dafoe and Mrs. Gail Lawrance for expert technical assistance and Mr. Bill Longhurst and Dr. Jim Johnson for FACS analysis.

Received for publication 23 November 1987 and in revised form 9 November 1988.

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