Title

Natural killer cells activated in a human mixed lymphocyte response culture identified by expression of Leu-11 and class II histocompatibility antigens.

Journal

Journal of Experimental Medicine, 159(4)

ISSN

0022-1007

Authors

Phillips, JH
Le, AM
Lanier, LL

Publication Date

1984-04-01

DOI

10.1084/jem.159.4.993

NATURAL KILLER CELLS ACTIVATED IN A HUMAN MIXED LYMPHOCYTE RESPONSE CULTURE IDENTIFIED BY EXPRESSION OF LEU-11 AND CLASS II HISTOCOMPATIBILITY ANTIGENS

By JOSEPH H. PHILLIPS, AN MY LE, and LEWIS L. LANIER

From the Becton Dickinson Monoclonal Center, Inc., Mountain View, California 94043

Lymphoid cells with the ability to lyse certain tumor cell lines in vitro without prior deliberate immunization collectively have been designated "natural killer" (NK)[1] cells. In man, these cells have been identified by morphological criteria (1–3) and by the presence of certain cell surface antigens (4–13). Recently, several investigators have reported that cells isolated from allogeneic or autologous mixed lymphocyte responses (MLR) also can demonstrate "NK-like" activity (14–21). These cells do not express certain antigens associated with cytotoxic T cells (e.g. Leu-4(T3)) and do not demonstrate antigen specificity against the immunizing cell population (14–21). Furthermore, they do not express certain antigens that previously have been used to identify resting peripheral blood NK cells, such as Leu-7 and OKM1 (14, 15). Thus, although cells with the functional properties of NK cells are present in MLR cultures, positive identification and isolation of these cells has proven difficult without a specific marker for these cells.

Recently, we have described a monoclonal antibody, anti–Leu-11, which reacts with essentially all NK cells in normal human peripheral blood (4, 5). In this study, we have examined whether or not the anti–Leu-11 antibody would react with NK cells present in an allogeneic MLR. Our findings indicated that it was possible to identify and isolate these NK-like cells using the anti–Leu-11 antibody. A detailed study of the MLR-activated Leu-11+ cells indicated that a subset of these NK cells demonstrated enhanced NK cytotoxic function and expressed "activation" related antigens, such as DR, DC, and transferrin receptor on their cell surface.

Materials and Methods

Preparation of Human Peripheral Blood Leukocytes. The cell fraction of plasmaphoresed human peripheral blood from normal donors was obtained from the American Red Cross, San Jose, CA. The mononuclear cells were isolated from Ficoll/Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) by standard methods. The isolated cells were washed twice in large volumes of sterile phosphate-buffered saline (PBS, 0.1 M phosphate, pH 7.3).

[1] *Abbreviations used in this paper*: ADCC, antibody-dependent cellular cytotoxicity; AMLR, autologous mixed lymphocyte response; C′, complement; E/T, effector/target; FITC, fluorescein isothiocynate; HBSS, Hank's balanced salt solution; LGL, large granular lymphocyte; MHC, major histocompatibility complex; NK, natural killer; PE, phycoerythrin; PBS, phosphate-buffered saline.

TABLE I
Specificity of Monoclonal Antibodies

Monoclonal antibody	Specificity
Leu-2a	Suppressor/cytotoxic T cell subset and NK subset
Leu-3a	Helper/inducer T cell subset and monocytes
Leu-4	T lymphocytes
Leu-7	HNK-1 antigen; large granular lymphocytes and subset of NK cells
Leu-10	HLA-DC, a polymorphic antigen absent on DR7 homozygous cells
Leu-11a & Leu-11b	Fc receptor of large granular lymphocytes, NK cells, and neutrophils
DR (clone L243)	HLA-DR, monomorphic determinant
Transferrin receptor (clone L01.1)	Human transferrin receptor

Reagents. All monoclonal antibodies used in these studies were prepared at the Becton Dickinson Monoclonal Center, Inc. The anti–Leu-11a monoclonal antibody is derived from the hybridoma NKP-15 (2, 4, 5) and the anti–Leu-11b monoclonal antibody from clone G022 (22). The anti-human transferrin receptor monoclonal antibody was produced by clone L01.1. This hybridoma was generated by immunizing BALB/c mice with pokeweed mitogen–activated human lymphoblasts and fusing the immune spleen cells with the P3X-Ag8-8463 myeloma line. The monoclonal antibody reacts with activated lymphocytes, monocytes, and tumor cell lines and immunoprecipitates a two-chain disulfide-linked molecule with a 90-kd (reduced) and 180-kd (unreduced) molecular weight. The antibody also immunoprecipitates complexes of ^{125}I labeled Fe-saturated transferrin/transferrin receptor from cells incubated in the presence of ^{125}I-labeled transferrin. The specificity of all monoclonal antibodies used in this study are presented in Table I.

Two-Color Immunofluorescence Staining. Immunofluorescence staining was performed as described in detail elsewhere (23). Isotype-matched fluorescein isothiocyanate (FITC) or phycoerythrin-conjugated monoclonal antibodies or myeloma proteins that did not specifically react with human cells were used to control for Fc-related bindings. All dilutions and cell washing were performed in cold PBS containing 0.1% sodium azide and all procedures were carried out at 4°C.

Two-Color Flow Cytometry. Two-color immunofluorescence experiments were performed as described previously (23). Fluorescein and phycoerythrin were excited using a single argon ion laser (488 nm, 200 mW). Dead cells, erythrocytes, and platelets were excluded from analysis by setting an appropriate threshold trigger on the low forward angle and 90° light scatter parameters. Low angle forward light scatter, 90° light scatter, green fluorescence, and red fluorescence signals were stored in list mode data files using a Consort 40 PDP/11 based computer system (Becton Dickinson FACS division, Sunnyvale, CA). Two-parameter data were collected into a 64 × 64 matrix and displayed as contour maps. "Contours" were drawn to indicate increasing numbers of cells in a defined area of the array. Areas within the contour map were integrated to determine the percentage of cells. These methods of analysis have been described previously (23).

Cytotoxicity Assay. Human tumor cells were labeled with ^{51}Cr and were used as targets in a 4-h radioisotope release cytotoxicity assay as described previously (23).

Sensitizer Cells. 10–20 × 10^6 CCRF-SB allogeneic tumor cells were incubated with mitomycin C (80 μg/ml) in serum-free RPMI-1640 media for 1 h at 37°C. After incubation the tumor cells were washed three times in Hank's balanced salt solution (HBSS) and resuspended at a concentration of 1 × 10^6 cell/ml in RPMI-1640 containing 5% heat-inactivated horse serum, 2 mM glutamine, and antibiotics.

Allogeneic MLR. Responder lymphocytes were suspended in RPMI-1640 media con-

taining 5% heat-inactivated horse serum, glutamine, and antibiotics at a concentration of 1×10^6 cells/ml. Responder cells (10×10^6) were mixed with stimulator cells (10×10^6) in a total volume of 10 ml media in T25 tissue culture flasks (Falcon Plastics, Oxnard, CA). The flasks were incubated in an upright position in a humidified 5% CO_2 atmosphere at 37°C for 5 d. On the fifth day the cells were harvested, washed in HBSS, and viable cells isolated by centrifugation on Ficoll/Hypaque (Pharmacia Fine Chemical).

Complement-mediated Cytotoxic Depletions. Peripheral blood lymphocytes or MLR-generated cells were sensitized with primary antibodies (anti–Leu-1, anti-Leu-7, or anti–Leu-11b) at optimal concentrations for 45 min at room temperature. The cells were then centrifuged, the supernatant removed, and a pretitered optimal amount of neonatal rabbit complement added to the cells. The cells were then incubated at room temperature for 1 h with occasional agitation. After incubation the cells were washed twice in HBSS and incubated an additional 20 min at 37°C to allow for dead cells to break part. The viable cells were then isolated by centrifugation on Ficoll/Hypaque.

Morphology. Aliquots of cell suspensions were cytocentrifuged onto ethanol-cleaned glass slides. The slides were fixed in absolute methanol for 10 min and rapidly air dried. The fixed cells were then stained in a 10% aqueous solution of Giemsa for 10 min and washed in distilled water. Light microscopic analysis was performed by oil immersion microscopy according to standard morphological criteria.

Results

Phenotypic Analysis of Cells in Allogeneic MLR and Control Cultures. An allogeneic MLR was generated by co-culturing human peripheral blood mononuclear cells with a mitomycin C–treated allogeneic B lymphoblastoid cell line, CCRF-SB. The responder cells generated in 5-d allogeneic mixed lymphocyte cultures and control cultures (i.e., peripheral blood mononuclear cells cultured in the absence of a stimulator cell population) were examined by multi-parameter flow cytometry. Based on low forward angle and 90-degree light scatter profiles, ~60% of cells in the MLR culture were large lymphoblasts, whereas control cultures showed few lymphoblasts (<5%). The majority of the MLR-activated lymphoblasts expressed the Leu-4 and Leu-2 surface antigens, as would be expected in a vigorous T cell specific in vitro immune response. However, ~15% of the MLR-generated lymphoblasts expressed the Leu-11 antigen, a marker that is present on essentially all cytotoxic NK cells in normal human peripheral blood (4, 5, 23). Similar to the Leu-11+ lymphocytes present in normal peripheral blood and in control cultures, the Leu-11+ lymphoblasts did not co-express the pan T cell antigen, Leu-4, or the helper/inducer T cell subset marker Leu-3 (Fig. 1). However, ~25% of the Leu-11+ lymphoblasts did co-express Leu-2a in low cell surface density (Fig. 1). Leu-2a is expressed on the cytotoxic/suppressor T lymphocyte subset and on a subpopulation of normal resting Leu-11+ NK cells (23). Leu-11+ MLR-generated NK cells did not co-express Leu-M3 (monocyte antigen) or Leu-7, an antigen expressed on a large percentage of normal peripheral blood NK cells (13). A small percentage (~3%) of the MLR-generated lymphoblasts, however, did express the phenotype, Leu-4+,Leu-7+,Leu-11−.

The most striking difference between control and MLR-generated Leu-11+ cells was the co-expression of Leu-11 and Class II MHC antigens on the MLR-generated cells (Fig. 2). DR and DC(Leu-10) antigens were co-expressed on the MLR-generated Leu-11+ cells, but not on a significant percentage of Leu-11+ cells in freshly isolated human mononuclear cells or in control cultures (<2%). Since the SB cell line used as stimulator expressed both DR and DC antigens, it

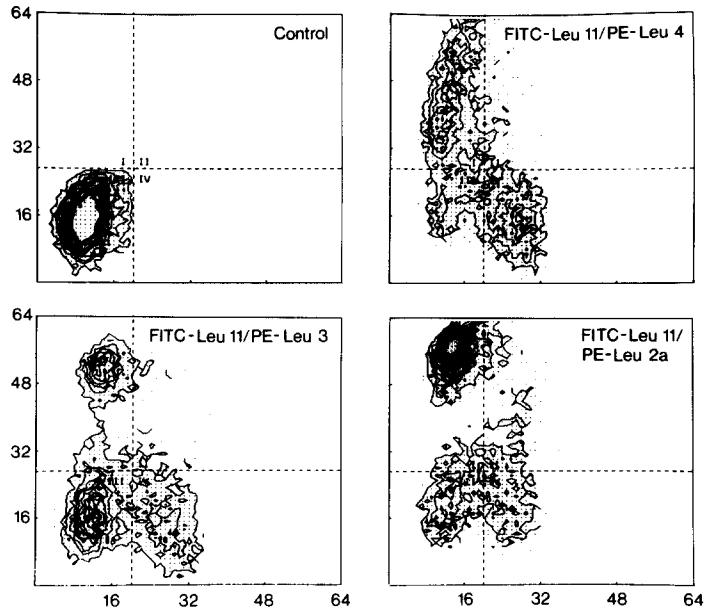

FIGURE 1. Two-color flow cytometric analysis of co-expression of Leu-11 with Leu-2, Leu-3, and Leu-4 on allogeneic MLR-activated lymphoblasts. Viable cells were isolated from a 5-d MLR culture generated by co-culture of human peripheral blood mononuclear cells and mitomycin C–treated CCRF-SB cells. Cells were stained with: FITC anti–Leu-11 and PE anti–Leu-4 (upper right); FITC anti–Leu-11 and PE anti–Leu-3 (lower left); FITC anti–Leu-11 and PE anti–Leu-2 (lower right); and FITC and PE conjugated isotype controls (upper left). Samples were analyzed using a FACS 440 system as described in Materials and Methods. Correlated green (*x* axis, log scale) and red fluorescence (*y* axis, log scale) on the lymphoblasts are presented as contour plots. Based on the control sample, the contour plots were divided into four quadrants: *I*, Cells with red fluorescence only; *II*, cells with both red and green fluorescence; *III*, unstained cells; and *IV*, cells with green fluorescence only. The number of cells within each of these areas was integrated and the percentage of the total number of cells analyzed, determined. The expression of Leu-11 with Leu-4 and Leu-3 was essentially nonoverlapping (e.g. Leu-4+,11+, <2% of lymphoblasts; Leu-3+,11+, <2% of lymphoblasts). However, 4% of lymphoblasts were phenotyped Leu-2+,11+ and 12% of lymphoblasts were Leu-2−,11+.

was possible that the Leu-11+ lymphoblasts passively acquired DR and DC antigens that had been shed from the B cell line. To exclude this possibility, MLR were generated using a DR7 homozygous B lymphoblastoid cell line, Mann, which was genetically negative for the Leu-10 DC antigen. Results from these experiments again showed that the Leu-11+ lymphoblasts co-expressed both DR and DC antigens. This conclusively demonstrated that the DC antigen was not passively acquired from the stimulator cell line. Further evidence for a qualitative change in the Leu-11+ cells was the presence of transferrin receptor on the surface membrane of the MLR-activated, but not resting, Leu-11+ cells (not shown).

Cytotoxic Potential of Allogeneic MLR and Control Cultures. The cytotoxic capabilities and specificities of cells isolated from 5-d MLR and control cultures were examined using a 4-h ^{51}Cr release assay against the sensitizing tumor cell line, CCRF-SB, and the NK-sensitive target cell line, K562. Low levels of NK cell cytotoxicity against K562 were observed in control cultures, whereas cytotoxicity

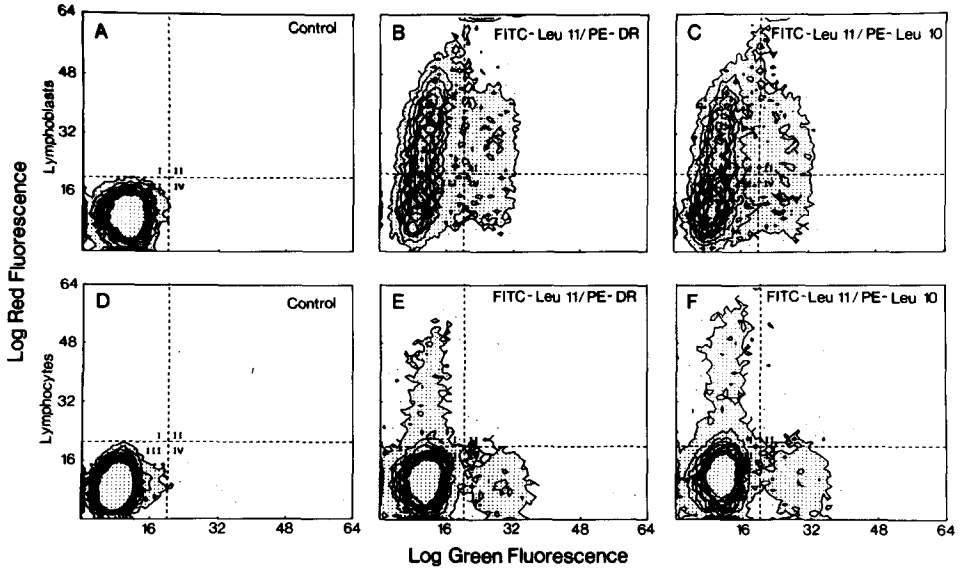

FIGURE 2. Two-color flow cytometric analysis of co-expression of Leu-11 with class II MHC antigens on MLR-activated lymphoblasts and control culture lymphocytes. Viable cells isolated from 5-d MLR (panels *A–C*) or control (panels *D–F*) cultures were stained with: FITC anti-Leu-11 and PE anti-DR (center panels); FITC anti-Leu-11 and PE anti-DC(Leu-10) (right panels); or FITC and PE isotype controls (left panels). Samples were analyzed with a FACS 440 system as described in Material and Methods. Data are presented as described in Fig. 1 legend. Note that a large percentage of the Leu-11+ MLR-generated lymphoblasts co-expressed DR and DC antigens (Leu-11+,DR+ 11% of lymphoblasts; Leu-11+,DR− 5% of lymphoblasts; Leu-11+,DC+ 10% of lymphoblasts; Leu-11+,DC− 6% of lymphoblasts; Leu-11−,DR+ 50% of lymphoblasts; and Leu-11−,DC+ 40% of lymphoblasts). In contrast, few Leu-11+ cells in the control cultures co-expressed DR or DC antigens (Leu-11+,DR+ <1% of lymphoid cells; Leu-11+,DC+ <1% of cells; Leu-11+,DC− 7% of cells; and Leu-11+,DR− 8% of cells).

against the NK-resistant cell line, CCRF-SB, was essentially nonexistent (Fig. 3). Analysis of the cell-mediated cytotoxicity potential of the MLR-generated cells showed substantially elevated levels of cytotoxicity against CCRF-SB, as well as augmented levels of cytotoxicity against the NK-sensitive tumor cell line K562 (Fig. 3). Treatment of MLR cultures with the pan T cell monoclonal antibody, anti-Leu-1, and complement essentially eliminated the cytotoxicity generated against the sensitizing tumor cell line, CCRF-SB, without diminishing cytotoxicity against K562 (Fig. 4). In striking contrast to the elimination of the specific T cell response with anti-Leu-1 and complement, treatment of MLR cultures with anti-Leu-11b and complement severely depleted cytotoxicity against K562, but not CCRF-SB. Thus, essentially all "NK-like" cytotoxicity activity was contained within the small population of cells expressing the Leu-11 antigen, but not the Leu-1 antigen.

Functional Analysis of the Leu-11+,DR+ Subpopulation. Functional studies of the MLR-generated effector cells clearly indicated that the cytotoxicity manifested against K562 was primarily mediated by effector cells expressing the NK cell-associated surface antigen Leu-11. Phenotypic analysis of the Leu-11+ cells in these MLR cultures showed that a majority of these cells also co-expressed

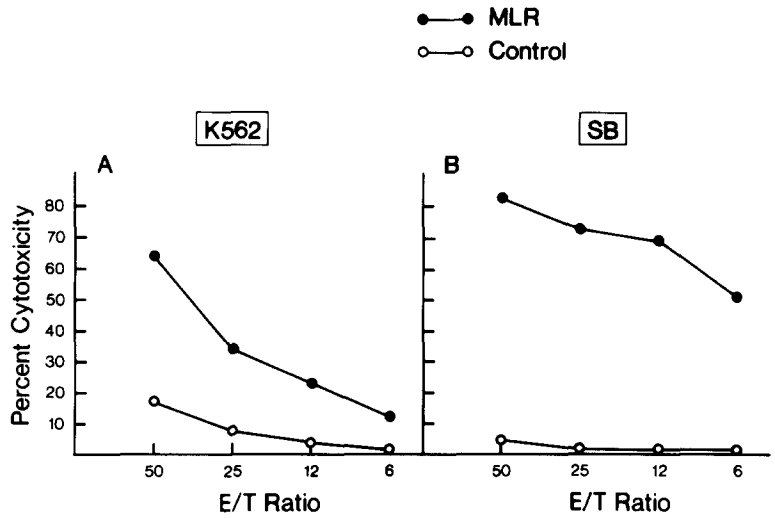

FIGURE 3. Cell-mediated cytotoxicity of MLR and control cultures. Lymphoid cells generated in a 5-d allogeneic MLR against CCRF-SB (●) and lymphocytes from control cultures (○) were assessed for cytotoxic activity in a 4-h ^{51}Cr release assay. K562 and CCRF-SB tumor cell lines were used as targets. The results are expressed as percent specific cytotoxicity vs. effector/target ratio (E/T).

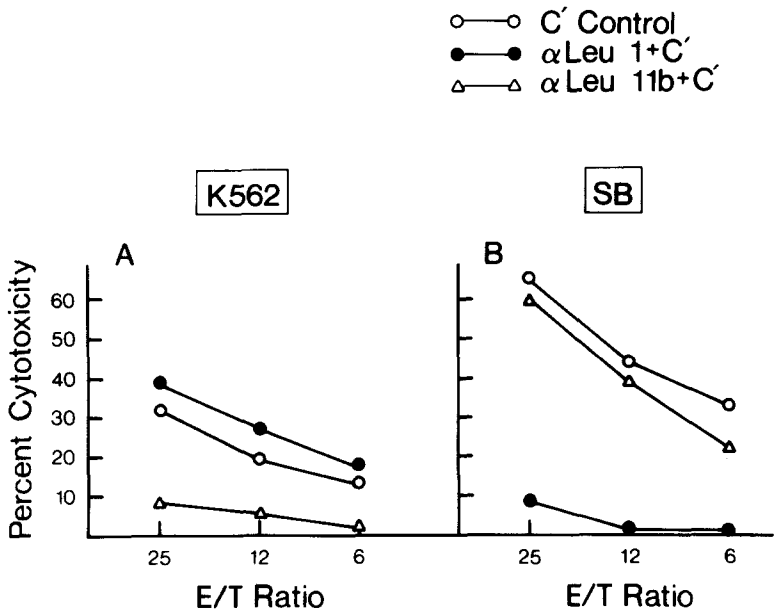

FIGURE 4. Cell-mediated cytotoxicity of MLR cultures depleted of Leu-1+ or Leu-11+ effector cells. Lymphoid cells generated in a 5-d MLR against CCRF-SB were treated with complement (C′) alone (○), with anti-Leu-1 and C′ (●), or anti-Leu-11b and C′ (△) before being assayed for cytotoxic activity in a 4-h ^{51}Cr release assay against K562 (*A*) and CCRF-SB (*B*) at varied E/T ratios.

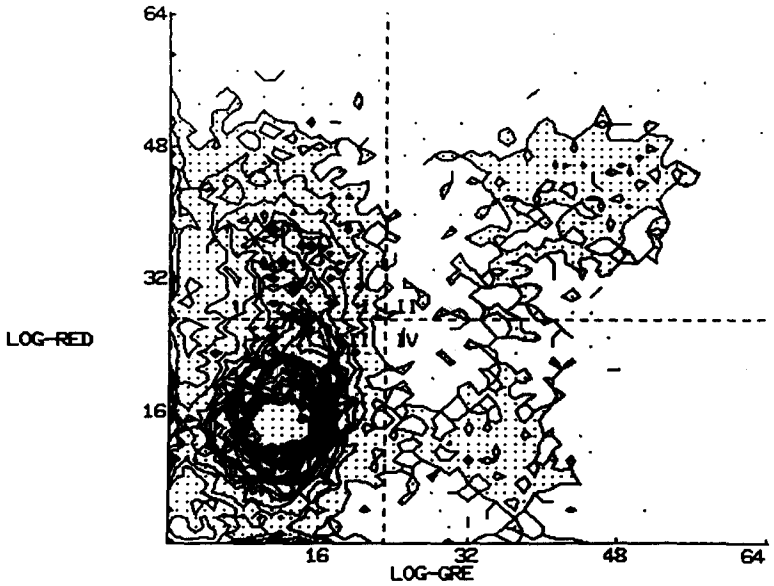

FIGURE 5. Two-color cell sorting of Leu-11+,DR+ and Leu-11+,DR− MLR-generated lymphoblasts. Viable lymphoid cells isolated from a 5-d MLR culture against CCRF-SB tumor cells were stained with: FITC anti-Leu-11 and PE anti-DR monoclonal antibodies. Cells were analyzed using a FACS 440 system as described in Materials and Methods. The profiles of Leu-11 (green) and DR (red) fluorescence are presented. The gain controls on the log amplifiers were adjusted to optimize separation of the four populations. Control cells, stained with isotype-matched FITC and PE myeloma proteins, were all present in quadrant *I* (not shown). Sorting windows were set on the Leu-11+,DR+ cells (quadrant *II*) and Leu-11+,DR− cells (quadrant *IV*). Re-analysis of the sorted populations indicated that each population was >95% pure.

DR and DC antigens. Since the expression of class II MHC gene products on Leu-11+ NK cells appears to be a direct result of the MLR conditions, experiments were performed to determine whether this phenotypic alteration was accompanied by change in functional capacity. Cells from a 5-d MLR culture were stained simultaneously with FITC anti-Leu-11a and PE anti-DR monoclonal antibodies for two-color immunofluorescence analysis. As indicated in Fig. 5, the Leu-11+,DR+ and Leu-11+,DR− subpopulations were isolated to 95% purity by using two-color cell sorting with a fluorescence-activated cell sorter. The cytotoxic activities of the sorted subpopulations against K562 are presented in Fig. 6. The results of these experiments demonstrated that on a per-cell basis the Leu-11+,DR+ cells were more efficient cytotoxic effectors than the Leu-11+,DR− cells, although both mediated significant cytotoxicity against K562.

Examination of Giemsa-stained preparations of the sorted subpopulations by light microscopy indicated that the Leu-11+,DR− cells were a relatively homogeneous population of large granular lymphocytes with prominent azurophilic granules (Fig. 7). The morphology of the Leu-11+,DR− cells was essentially identical to the morphology of large granular lymphocytes (LGL) isolated from fresh peripheral blood. The Leu-11+,DR+ cells, however, were morphologically heterogeneous. ~70% of the Leu-11+,DR+ cells were large agranular lymphoid cells with prominent vacuoles and lobular euchromatic nuclei, 20% of the cells

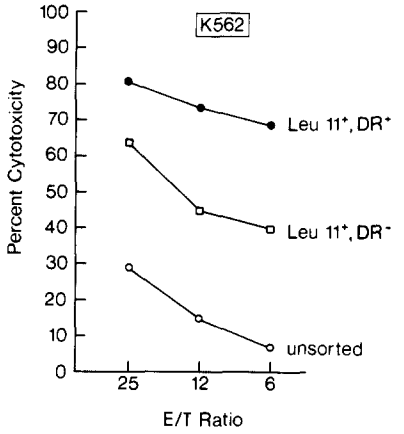

FIGURE 6. Cell-mediated cytotoxicity of Leu-11+,DR+ and Leu-11+,DR− cells. The Leu-11+,DR+ and Leu-11+,DR− cells were purified from a 5-d MLR culture against CCRF-SB tumor by two-color FACS cell sorting as described in Fig. 5. Cell-mediated cytotoxicity was assessed in a 4-h ^{51}Cr release assay against K562 tumor cells. Note that the cytotoxic potential of the Leu-11+,DR+ cells was substantially greater than the Leu-11+,DR− cells, particularly at the lower E/T ratios, and that both subsets demonstrated more cytotoxic activity than the antibody-stained, unsorted control cells.

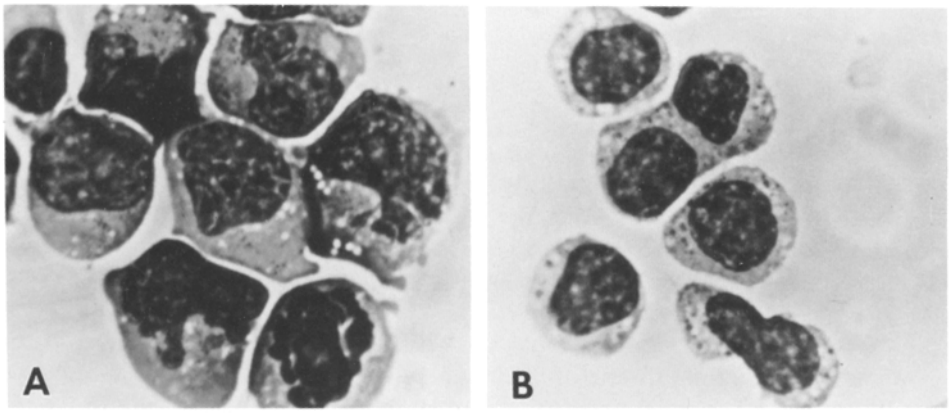

FIGURE 7. Light microscopic morphology of Leu-11+,DR+ and Leu-11+,DR cells. The Leu-11+,DR+ (*A*) and Leu-11+,DR− (*B*) subsets purified by two-color FACS cell sorting as described in Fig. 5 were stained with Giemsa. Note that the Leu-11+,DR− cells are a homogeneous population of typical LGL, whereas the Leu-11+,DR+ cells are more heterogeneous with numerous agranular blasts, and mitotic configurations. Giemsa stain, × 4,000.

displayed typical LGL morphology, and 10% of the Leu-11+,DR+ cells were typical mitotic configurations.

Analysis of the Peripheral Blood Precursor of the MLR-generated NK Cells. Studies to determine the precursor of the Leu-11+ NK cell in the MLR culture were undertaken using depletion of Leu-7+ or Leu-11+ cells before the initiation of the MLR cultures. Treatment of the responder cell population with anti-Leu-7 and complement had no effect on the subsequent MLR-induced generation of Leu-11+,DR+ NK cells (Fig. 8). However, depletion of Leu-11+ cells from the responder population with anti-Leu-11b and complement before MLR culture

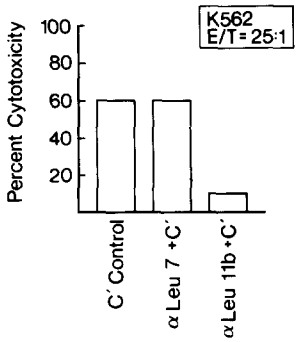

FIGURE 8. Cell-mediated cytotoxicity of MLR cultures depleted of Leu-7 or Leu-11 positive lymphocytes before MLR. Peripheral blood mononuclear cells were treated with complement alone, anti–Leu-7 and complement, or anti–Leu-11b and complement before the MLR culture. Viable cells from 5-d MLR cultures against CCRF-SB were assessed for cell-mediated cytotoxicity in a 4-h ^{51}Cr release assay against K562 at an E/T of 25:1. Note that only depletion with anti–Leu-11b before the MLR culture significantly diminished the generation of cytotoxic cells for K562 target cells.

prevented the generation of Leu-11+ cells and appreciable levels of cytotoxicity against K562 (Fig. 8). The results clearly indicated that the MLR-generated Leu-11+,DR+ subset originated from Leu-11+ precursors, which lacked the Leu-7 antigen.

Discussion

Co-culture of human peripheral blood mononuclear cells with an allogeneic tumor cell can result in the proliferation of certain T lymphocytes and the activation of T lymphocytes with the capacity to lyse the tumor. Many of these cytotoxic T lymphocytes demonstrate exquisite specificity for the immunizing tumor cell line. Although exceptions have been noted, in general, cytotoxic T cells generated against a histoincompatible class I major histocompatibility complex (MHC) target express the pan T cell antigen, Leu-4 (T3), and the Leu-2 (T8) subset marker (24–28). It has been observed that other lymphoid cells in the MLR culture are cytotoxic, yet are not specific for the immunizing cell. Since some of these cells demonstrate functional properties similar to natural killer cells, they have been deemed "NK-like" cells (14–21). These cells occur in low frequency in the MLR culture, and there have been few markers that permit isolation of these cells for phenotypic and functional studies.

Recently, we have described a monoclonal antibody, anti–Leu-11, which reacts with NK cells in human peripheral blood (4, 5). This antibody recognizes an antigen, associated with the Fc receptor for IgG, that is present on LGL and neutrophils (4, 5). Leu-11 antigen is not present on resting monocytes, eosinophils, B lymphocytes, and most mature T lymphocytes expressing the Leu-4 antigen (4, 5). Similar antibodies have been described by other investigators (7–9). Essentially all cytotoxic NK cells and cells capable of mediating antibody-dependent cellular cytotoxicity (ADCC) in human peripheral blood can be isolated using anti–Leu-11 antibody (4, 5, 23). Preliminary reports by Thompson and co-workers (22) indicated that NK cells present in a MLR culture could be eliminated by treatment with the anti–Leu-11b and complement.

In these studies, we have addressed the question of whether or not Leu-11 antigen is present on the "NK-like" cells present in a MLR culture generated by co-culture of human peripheral blood mononuclear cells and an allogeneic B lymphoblastoid cell line (CCRF-SB). Whereas CCRF-SB specific cytotoxic T cells generated in these cultures did not express the Leu-11 antigen, anti–Leu-11 and complement treatment removed most "non-specific" cytotoxicity against the NK-sensitive target cell K562. In contrast, anti–Leu-1 and complement eliminated CCRF-SB specific cytotoxic T cells, but not K562 "NK-like" cytotoxic effectors. Hence, Leu-11 provided the specific probe needed to isolate these unique cytotoxic cells for study of their function, phenotype, and morphology.

How do these MLR-activated "NK-like" cells relate to NK cells freshly isolated from peripheral blood? In a series of experiments using multicolor immunofluorescence and flow cytometric analysis, the cell surface antigen profile of NK cells was examined in detail (5, 23, 29). Studies of both Percoll gradient–purified LGL (29) and Ficoll-Hypaque–isolated peripheral blood mononuclear cells (23) indicated that within the population of Leu-11–positive cells, considerable heterogeneity exists with regards to expression of other surface antigens. For example, using a combination of both anti–Leu-2 and anti–Leu-11 for two-color immunofluorescence, four populations can be defined in some individuals, i.e. Leu-2+,11+; Leu-2+,11−; Leu-2−11+; and Leu-2−,11− (23). Functionally, the Leu-2+,11+ and Leu-2−,11+ populations were equally efficient and potent mediators of cytotoxicity against K562. Leu-2+,11+, NK cells differed from Leu-2+ T cells, in that the Leu-2+,11+ cells did not co-express the pan T cell markers, Leu-1 and Leu-4 (23). Additionally, the quantity of Leu-2 antigen on the NK cell population was significantly less than on the Leu-2+,4+ T cell subset (23). Perussia and co-workers (30) also have demonstrated the existence of a low density Leu-2+,4− NK cell subset.

Similarly to the freshly isolated Leu-11+ cells, MLR-activated Leu-11+ cells did not co-express T-associated antigens, Leu-1, Leu-3 or Leu-4, and did not co-express Leu-M3, a marker of mature monocytes. A proportion of MLR-derived NK cells were observed to co-express low cell surface density of the Leu-2 antigen. Similar studies by Lopez-Botet et al. (16) have indicated that the NK-like cells within a mixed lymphocyte response culture lacked the T cell–associated antigens, T4 (Leu-3) and T8 (Leu-2). Strassman et al. (14) also have demonstrated that MLR-activated NK cells do not express T3 (Leu-4) or T8 (Leu-2).

Recently, we have reported that in freshly isolated peripheral blood four discrete subpopulations can be identified using the anti–Leu-7 and anti–Leu-11 monoclonal antibodies in two-color immunofluorescence (i.e., Leu-7−,11−; Leu-7−,11+; Leu-7+,11+; and Leu-7−,11−) (23). Functionally, the Leu7−,11+ cell demonstrated that most cytotoxicity against K562 on a per-cell basis, whereas the Leu-7+,11+ and Leu-7+,11− generally were less efficient (23). The majority of the Leu-7+,11− cells, which demonstrate weak cytotoxic activity, co-expressed the pan T cell antigen Leu-4 and high cell surface density of Leu-2 antigen (23). Abo and co-workers (31) similarly have shown that the Leu-7 subset that co-expressed Leu 4 (T3) is an inefficient cytotoxic effector. Based on ontogeny studies, these investigators suggested that this phenotype may reflect "pre-NK"

cells (32). In the MLR cultures, few Leu-11+ cells co-expressed Leu-7, however, a small population of Leu-4+,7+ cells were present.

The most significant difference between freshly isolated Leu-11+ cells and the MLR− activated Leu-11+ cells was the presence of class II MHC gene products on a significant proportion of the MLR-derived Leu-11+ cells. The majority of freshly isolated Leu-11+ cells did not express DR or DC (Leu-10) antigens, as determined by two-color immunofluorescence. However, after MLR activation, Leu-11+ cells that co-express DR and DC were evident. The Leu-11+,DC+ cells did not passively acquire DC antigens from the stimulating B lymphoblastoid cells, since Leu-11+,DC+ cells were present in MLR cultures generated using a genetically DC-negative B cell line. Furthermore, co-culture of peripheral blood mononuclear cells depleted of the DR+,DC+ monocytes and B cells with a genetically DC-negative B cell line did not prevent generation of Leu-11+ cells that co-expressed DR and DC (unpublished observation). These experiments suggest that synthesis of the class II molecules was induced as a consequence of the MLR. Biosynthetic labeling studies will be required to definitively confirm this. It should be noted that Ortaldo and co-workers (6, 33) have reported that a proportion of Percoll gradient–isolated LGL react with an anti-DR monoclonal antibody. Although we have occasionally observed individuals with Leu-11+,DR+ cells, this was not the general situation. Perhaps this discrepancy is due to differences in blood preparation or the specificity of the anti-DR antibodies. Nonetheless, we have clearly shown that a significant increase in the proportion of Leu-11+ cells that co-express DR and DC is a consequence of MLR culture. Whether this represents de novo synthesis of class II MHC gene products or expansion of a small Leu-11+,DR+ cell present in effector cell population before generation of the MLR is under study. Further evidence for phenotypic alteration resulting from MLR activation was the appearance of Leu-11+, transferrin receptor+ cells. Resting Leu-11+ cells did not express detectable levels of transferrin receptor. The induction of increased levels of transferrin receptor has been shown to be an indicator of cell activation (34).

What is the significance of the presence of DR and DC antigens on MLR-activated NK cells? Two-color cell-sorting experiments definitively showed that on a per-cell basis that MLR-activated Leu-11+ lymphoid cells that co-expressed DR antigens were more efficient killers of K562 targets than the DR-negative Leu-11+ population. Therefore, the phenotypic change in the MLR-activated cells appears to correlate directly with a change in cell function. In a study of NK cells present in an autologous MLR culture, Goto and Zvaifler (15) showed that treatment with anti-DR and complement partially reduced the cytotoxic activity of AMLR cells against the NK-sensitive target K562, indicating that a subset of the NK cells must express this marker.

The induction of class II MHC gene products as a consequence of cell activation has been observed for both T cells and monocytes. Human peripheral blood T lymphocytes, which do not express DR or DC antigens, acquire these glycoproteins during MLR culture (34, 35). Studies by Basham and co-workers (36) have shown increased levels of class II gene products on human monocytes after co-culture with interferon. In the mouse, biosynthesis and expression of Ia antigens can be induced on macrophages (37, 38) and macrophage tumor cell lines (39–

41). Recent studies have indicated that gamma interferon mediates the induction of Ia protein synthesis in macrophages (42). Whether or not gamma interferon is responsible for induction of DR and DC on MLR-activated NK cells is under investigation. The induction of Ia antigen expression on murine macrophage cell lines was shown to be associated with the ability of these cells to efficiently function in antigen presentation (39, 40). Similarly, expression of class II MHC molecules on human monocytes is thought to influence antigen presentation (43). Since human NK cells share many common properties with monocytes, the ability of DR-positive NK cells to present antigen should be considered.

The morphological characteristics of human NK cells have become a useful and widely accepted marker of this cell population. Light microscopic studies of human NK cells purified from peripheral blood on Percoll gradients have shown that these cells are "large granular lymphocytes" (1, 2). Likewise, Leu-11+ lymphocytes isolated from freshly isolated peripheral blood mononuclear cells demonstrated typical LGL characteristics (4). The morphology of the Leu-11+,DR− cells from MLR cultures was consistent with the LGL morphological classification. The Leu-11+,DR+ cells were more heterogeneous, containing typical LGL, numerous agranular lymphoblasts, and a significant proportion of mitotic configurations. The existence of lymphoblastoid cells, as well as mitotic figures in the Leu-11+,DR+ subset clearly demonstrates MLR-induced activation of this NK cell population. Thus, the morphological heterogeneity of the Leu-11+,DR+ subset may reflect the various phases of the activation/mitotic cycle of the Leu-11+ NK cells.

If the MLR-activated Leu-11+,DR+ cells are a member of the NK lineage(s?), what is their direct precursor in the peripheral blood? Depletion studies indicated that removal of Leu-11+ cells from the peripheral blood mononuclear cells before co-culture with the SB target cell prevented generation of the Leu-11+,DR+ subset. In contrast, removal of Leu-7 cells before establishment of the MLR culture did not effect the subsequent generation of cytotoxic Leu-11+,DR+ cells. Furthermore, elimination of the Leu-7 cells present in the MLR culture neither enhanced nor diminished specific cytotoxic activity against the B lymphoblastoid cells used as the stimulator. These data suggest that the Leu-7 cells in the MLR do not serve as specific cytotoxic cells or act as suppressors of the cytotoxic T cells. These studies are in agreement with Abo and co-workers (44) who have reported that NK-like cells in a MLR did not arise from Leu-7–positive precursors. Thus, these cells must arise from a Leu-7−,11+ precursor. Further studies will be required to establish whether or not the Leu-7−,11+ cell develops from the Leu-7+,11+ or Leu-7+,11− populations or rather arises from a distinct precursor.

Strassmann and co-workers (14) have observed that the peripheral blood precursor of the MLR-activated NK cells reacted with the OKM1 and B73.1 monoclonal antibodies. However, the activated NK cell generated after the 5-d MLR culture did not express the OKM1 or B73.1 antigens. This is noteworthy since the anti–Leu-11 and B73.1 monoclonal antibodies both react with essentially all NK cells in freshly isolated peripheral blood and give essentially identical immunofluorescence staining patterns with large granular lymphocytes, when analyzed using quantitative flow cytometric analysis (Lanier, unpublished obser-

vation). Similarly, both antibodies inhibited the binding of IgG complexes to large granular lymphocytes, suggesting that these antibodies react with a determinant near the Fc receptor of large granular lymphocytes (4, 5, 7, 8, 23). However, B73.1 differs from Leu-11 in that B73.1 is unreactive with MLR-activated NK cells (14) and is only weakly reactive with neutrophils (7, 8). In contrast, Leu-11 is present on the majority of MLR-activated NK cells and is present in high cell surface density on neutrophils (4, 5, 23). The structural difference in the Leu-11 and B73.1 antigens is currently under investigation.

An important question is whether or not the Leu-11+,DR+ cells play a role in vivo in response to infectious disease or malignancy. As a practical sequence of these studies, the combined use of anti–Leu-11 and anti–class II monoclonal antibodies provides a convenient way to assess the role of these "activated" NK cells in the clinical research laboratory. Study of the presence and function of these cells in transplant recipients and patients undergoing therapy with immunomodulators known to affect NK cells such as interferon and interleukin 2 may allow us to better understand the physiological role of these cells.

Summary

Lymphoid cells with natural killer (NK)-like function, morphology, and antigenic phenotype have been identified in a mixed lymphocyte culture generated by co-culture of human peripheral blood mononuclear cells with an allogeneic human B lymphoblastoid cell line CCRF-SB. The majority of these mixed lymphocyte (MLR)-response activated NK cells express the Leu-11 surface antigen, but do not express certain T cell–associated antigens (Leu-1, Leu-3, and Leu-4) or the mature monocyte specific antigen, Leu-M3. Unlike most freshly isolated Leu-11+ human NK cells, the MLR-activated Leu-11+ cells expressed class II major histocompatability antigens, DR and DC. Concomitant with expression of class II gene products, the Leu-11+,DR+ NK cells demonstrate enhanced cytotoxicity against the NK-sensitive tumor cell line K562. The presence of mitotic cells in the Leu-11+,DR+ population and the acquisition of increased levels of transferrin receptor on the cell surface were further indicators of activation of these cells. The direct precursors of the MLR-activated Leu-11+,DR+ cell were Leu-11+ cells that lacked expression of another NK-associated antigen Leu-7, i.e., Leu-7−,11+. These studies provided a definitive identification of the "NK-like" cell in MLR cultures and thus allow quantitation and isolation of these cells for further study.

We thank Dr. Frances Brodsky for invaluable discussion and advice in the studies involving HLA-DC expression. We also thank Mr. Bryan Woodhouse for expert assistance with the flow cytometry studies, and Mr. Mark Siragusa for expert assistance with the photography.

Received for publication 7 November 1983.

References

1. Babcock, G. F., and J. H. Phillips. 1983. NK cells: light and electron microscope characteristics. *Surv. Immunol. Res.* 2:88.
2. Timonen, T., J. R. Ortaldo, and R. B. Herberman. 1981. Characterization of human

large granular lymphocytes and relationship to natural killer and K cells. *J. Exp. Med.* 153:569.

3. Huhn, D., C. Huber, and J. Gastl. 1982. Large granular lymphocytes: morphological studies. *Eur. J. Immunol.* 12:985.
4. Phillips, J. H., and G. F. Babcock. 1983. NKP-15: a monoclonal antibody reactive against purified human natural killer cells and granulocytes. *Immunol. Lett.* 6:143.
5. Lanier, L. L., J. H. Phillips, N. L. Warner, and G. F. Babcock. 1984. A human natural killer cell-associated antigen defined by monoclonal antibody anti-Leu 11 (NKP-15): functional and two-color flow cytometry analysis. *J. Leukocyte Biol.* In press.
6. Ortaldo, J. R., S. O. Sharrow, T. Timonen, and R. B. Herberman. 1981. Determination of surface antigens on highly purified human NK cells by flow cytometry with monoclonal antibodies. *J. Immunol.* 127:2401.
7. Perussia, B., S. Starr, S. Abraham, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. I. Characterization of the lymphocyte subset reactive with B73.1. *J. Immunol.* 130:2133.
8. Perussia, B., O. Acuto, C. Terhorst, J. Faust, R. Lazarus, V. Fanning, and G. Trinchieri. 1983. Human natural killer cells analyzed by B73.1, a monoclonal antibody blocking Fc receptor functions. II. Studies of B73.1 antibody-antigen interaction on the lymphocyte membrane. *J. Immunol.* 130:2142.
9. Rumpold, H., D. Kraft, G. Obexer, G. Bock, and W. Gebhart. 1982. A monoclonal antibody against a surface antigen shared by human large granular lymphocytes and granulocytes. *J. Immunol.* 129:1458.
10. Griffin, J. D., T. Hercend, R. Beveridge, and S. F. Schlossman. 1983. Characterization of an antigen expressed by human natural killer cells. *J. Immunol.* 130:2947.
11. Lohmeyer, J., P. Rieber, H. Feucht, J. Johnson, M. Hadam, and G. Riethmuller. 1981. A subset of human natural killer cells isolated and characterized by monoclonal antibodies. *Eur. J. Immunol.* 11:997.
12. Nieminen, P., R. Paasivuo, and E. Saksela. 1982. Effect of a monoclonal anti-large granular lymphocyte antibody on the human NK activity. *J. Immunol.* 128:1097.
13. Abo, T., and C. M. Balch. 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antibody (HNK-1). *J. Immunol.* 127:1024.
14. Strassman, G., F. H. Bach, and J. M. Zarling. 1983. Depletion of human NK cells with monoclonal antibodies allows the generation of cytotoxic T lymphocytes without NK-like cells in mixed cultures. *J. Immunol.* 130:1556.
15. Goto, M., and N. J. Zvaifler. 1983. Characterization of the killer cell generated in the autologous mixed leukocyte reaction. *J. Exp. Med.* 157:1309.
16. Lopez-Botet, M., A. Silva, J. Rodriguez, and M. O. deLandazuri. 1982. Generation of T cell blasts with NK-like activity in human MLC: cellular precuresors, IL 2 responsiveness, and phenotype expression. *J. Immunol.* 129:1109.
17. Santoli, D., B. Perussia, and G. Trinchieri. 1981. Activation of natural killer cells by interferon and by allogeneic stimulation. *In* Mediation of Cellular Immunity in Cancer by Immune Modifiers. M. A. Chirigos, M. Mitchell, M. J. Mastrangelo, and M. Krim. editors. Raven Press, New York. p. 143.
18. Seeley, J. K., G. Masussi, A. Poros, E. Klein, and S. H. Golub. 1979. Studies on cytotoxicity generated in human mixed lymphocyte cultures. II. Anti-K562 effectors are distinct from allospecific CTL and can be generated from NK-depleted T cells. *J. Immunol.* 123:1303.
19. Svedmyr, E., H. Wigzell, and M. Jondal. 1974. Sensitization of human lymphocytes against autologous or allogeneic lymphoblastoid cell lines: characteristics of the reactive cells. *Scand. J. Immunol.* 3:499.

20. Poros, A., and E. Klein. 1979. Distinction of anti-K562 and anti-allocytotoxicity in vitro stimulated populations of human lymphocytes. *Cell. Immunol.* 46:57.
21. Jondal, M., and J. Targan. 1978. In vitro induction of cytotoxic effector cells with spontaneous killer cell specificity. *J. Exp. Med.* 147:1621.
22. Thompson, J. S., N. E. Goeken, S. A. Brown, and J. L. Rhoades. 1982. Phenotypic definition of human monocyte/macrophage subpopulations by monoclonal antibodies. II. Distinction of non-T, non-B natural killer cells from antigen-presenting stimulator cells in the mixed lymphocyte response. Ann. Assoc. Clinical Histocompatibility Testing Annual Meeting. p. *a* 23 (abstract).
23. Lanier, L. L., A. M. Le, J. H. Phillips, N. L. Warner, and G. F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu 7 (HNK-1) and Leu 11 (NKP-15) antigens. *J. Immunol.* 131:1789.
24. Reinherz, E., and S. Schlossman. 1980. The differentiation and function of human T lymphocytes. *Cell.* 19:821.
25. Engleman, E. G., C. Benike, F. C. Grumet, and R. L. Evans. 1981. Activation of human T lymphocyte subsets: helper and suppressor/cytotoxic T cells recognize and respond to distinct histocompatibility antigens. *J. Immunol.* 127:2124.
26. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector T cells recognize products of major histocompatibility complex regions. *Proc. Natl. Acad. Sci. USA.* 79:4395.
27. Spits, H., H. Ijssel, A. Thompson, and J. E. de Vries. 1983. Human T4+ and T8+ cytotoxic T lymphocyte clones directed at products of different class II major histocompatibility complex loci. *J. Immunol.* 131:678.
28. Biddison, W. E., P. E. Rao, G. Talle, G. Goldstein, and S. J. Shaw. 1982. Possible involvement of the OKT4 molecule in T cell recognition of class II antigens. Evidence from studies of cytotoxic T lymphocytes specific for SB antigen. *J. Exp. Med.* 156:1065.
29. Phillips, J. H., N. L. Warner, and L. L. Lanier. 1984. Correlation of biophysical and cell surface antifenic profile of percoll gradient separated human natural killer cells. *Nature Immunity and Cell Growth Regulation.* In press.
30. Perussia, B., V. Fanning, and G. Trinchieri. 1983. A human NK and K cell subset shares with cytotoxic T cells expression of the antigen recognized by antibody OKT8. *J. Immunol.* 131:223.
31. Abo, T., M. D. Cooper, and C. M. Balch. 1982. Characterization of HNK-1 (Leu-7) human lymphocytes I. Two distinct phenotypes of human NK cells with different cytotoxic capacities. *J. Immunol.* 129:1752.
32. Abo, T., C. A. Miller, G. L. Gartland, and C. M. Balch. 1983. Differentiation stages of human natural killer cells in lymphoid tissues from fetal to adult life. *J. Exp. Med.* 157:273.
33. Herberman, R. B., T. Timonen, and J. R. Ortaldo. 1981. Characteristics of NK cells and their possible role in vivo. *In* Mediation of Cellular Immunity in Cancer by Immune Modifiers. M. A. Chirigos, M. Mitchell, M. J. Mastrangelo, and M. Krim. editors. Raven Press, New York. p. 131.
34. Cotner, T., J. M. Williams, L. Christenson, H. M. Shiparo, T. B. Strom, and J. Strominger. 1983. Simultaneous flow cytometric analysis of human T cell activation antigen expression and DNA content. *J. Exp. Med.* 157:461.
35. Evans, R. L., T. J. Faldetta, R. E. Humphries, D. M. Pratt, E. J. Yunis, and S. F. Schlossman. 1978. Peripheral human T cells sensitized in mixed leukocyte culture synthesize and express Ia-like antigens. *J. Exp. Med.* 148:1440.
36. Basham, T. Y., and T. C. Merigan. 1983. Recombinant interferon-gamma increases HLA-DR synthesis and expression. *J. Immunol.* 130:1492.

37. Steinman, R. M., N. Nogueira, M. D. Witmer, J. D. Tydines, and I. S. Mellman. 1980. Lymphokine enhances the expression and synthesis of Ia antigens on cultured mouse peritoneal macrophages. *J. Exp. Med.* 152:1248.
38. Scher, M. G., D. I. Beller, and E. R. Unanue. 1980. Demonstration of a soluble mediator that induces exudates rich in Ia-positive macrophages. *J. Exp. Med.* 152:1684.
39. Walker, E. B., L. L. Lanier, and N. L. Warner. 1982. Concomitant induction of the cell surface expression of Ia determinants and accessory cell function by a murine macrophage tumor cell line. *J. Exp. Med.* 155:629.
40. Birmingham, J. R., R. W. Chestnut, J. W. Kappler, P. Marrack, R. Kubo, and H. M. Grey. 1982. Antigen presentation to T cell hybridomas by a macrophage cell line. *J. Immunol.* 128:1491.
41. McNicholas, J. M., D. P. King, and P. P. Jones. 1983. Biosynthesis and expression of Ia and H-2 antigens on a macrophage cell line are stimulated by products of activated spleen cells. *J. Immunol.* 130:449.
42. Steeg, P. S., H. M. Johnson, and J. J. Oppenheim. 1982. Regulation of murine macrophage Ia antigen expression by an immune interferon-like lymphokine: inhibitory effect of endotoxin. *J. Immunol.* 129:2402.
43. Gonwa, T. A., L. J. Picker, H. V. Raff, S. M. Goyert, J. Silver and J. D. Stobo. 1983. Antigen-presentation capabilities of human monocytes correlates with their expression of HLA-DS, an Ia determinant distinct from HLA-DR. *J. Immunol.* 130:706.
44. Abo, T., and C. M. Balch. 1982. Characterization of HNK-1 (Leu-7) human lymphocytes. II. Distinguishing phenotypic and functional properties of natural killer cells from activated NK-like cells. *J. Immunol.* 129:1758.