A NOVEL 120-kD SURFACE ANTIGEN EXPRESSED BY A SUBSET OF HUMAN LYMPHOCYTES

Evidence that Lymphokine-activated Killer Cells Express this Molecule and Use it in their Effector Function

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Lymphokine-activated killer (LAK)[1] cells represent an operationally defined lymphoid population that can lyse NK-resistant fresh tumor cells upon culture in IL-2 (1, 2). The peripheral blood cell precursors (P) from which LAK cells arise are mostly confined within a lymphoid subset expressing the CD16 (Leu-11, Fcγ receptor) antigen, displaying large granular lymphocyte (LGL) morphology and expressing NK activity (3–5). However, according to recent data (6), at least some LAK cells and their precursors (LAK-P) belong to the T cell lineage and give rise to cell clones with mature T cell phenotype ($CD2^+$, $CD3^+$). Regarding the LAK effector mechanism, little is known about the surface molecules involved in cell-to-cell adhesion, target cell recognition, and lysis. mAbs to LFA-1 molecule have recently been shown (7) to inhibit LAK activity. However LFA-1 is a surface structure widely expressed on lymphoid and (some) non lymphoid cell populations and thus is involved in several cell functions. Here we describe a novel 120-kD surface antigen, termed LAK-1, that is expressed by virtually all LGL and includes the entire LAK-P pool. More importantly, unlike other LGL/NK cell markers, this antigen is expressed on the LAK effector cells as well. Finally, the LAK-1 molecule appears to be involved in LAK effector function, as indicated by the inhibition of target cell lysis by a specific mAb.

Materials and Methods

Production of the Anti-LAK-1 mAb. 6-wk-old male DBA/2 mice were immunized with the SF16 LAK clone as previously described (8). The immunization schedule consisted of three weekly intravenous injections of 10^7 SF16 cells. After 10 d, the mice received a booster injection of 1.5×10^7 cells followed by splenectomy 3 d later. Immune splenocytes were fused with P3-U1 myeloma cells (8). The initial screening of hybridoma supernatants (SN) was carried out using an indirect RIA: 10^5 SF16 cells were incubated with 100 μl of the various supernatants for 30 min in ice. ^{125}I-labeled rabbit anti-mouse antibody was then added for 1 h at 37°C. Subsequently, the cells were harvested and counted using a

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[1] *Abbreviations used in this paper:* LAK, lymphokine-activated killer cells; LGL, large granular lymphocytes; NCAE, naphthol-AS-D chloroacetate esterase; P, precursor.

γ-scintillation counter. The $F(ab')_2$ fragment was derived by digestion with pepsine from the purified mAb as described by Parham (9). The $F(ab')_2$ piece appeared in SDS-PAGE as an homogeneous band of ~100 kD. Other mAbs used in these studies were: Leu 7 (anti HNK-1) and Leu 11 (anti-CD16) (purchased from Becton Dickinson & Co, Sunnyvale, CA) and the previously described (10) MAR 21 (anti-CD7) and J90 (anti-LFA-1) mAbs.

Isolation of Subpopulations of Peripheral Blood. Human PBMC and granulocytes were isolated from healthy volunteer donors by Ficoll-Hypaque (F/H) density gradient centrifugation. E^+ and E^- cell fractions were obtained by rosetting PBMC with neuraminidase-treated sheep erythrocytes (11). The two fractions were separated by a F/H gradient centrifugation. Monocytes were isolated by adherence on plastic Petri dishes. After extensive washes to remove nonadherent cells, the adherent cells were detached by incubation in ice for 2 h. B cell–enriched population was obtained by removing E-rosetting cells from a spleen cell suspension. The residual population contained >95% of sIg^+ cells. Platelets were isolated after F/H density gradient centrifugation of heparinized blood. LAK-1^+ and LAK-1^- cells were sorted with a FACS (FACS II; Becton Dickinson Immunocytometry Systems, Mountain View, CA) after indirect immunofluorescence staining of PBMC with the LAK-1 mAb (12).

Indirect Immunofluorescence and Flow Cytofluorometric Analysis. Cells were stained with hybridoma supernatants (25 μl/10^5 cells) followed by fluoresceinated goat $F(ab')_2$ anti-mouse Ig as previously described (12). The samples were analyzed on a flow cytometer gated to exclude nonviable cells. Results are expressed as arbitrarily normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity.

LAK Activation and Cytotoxicity Assay. PBMC at 10^6/ml concentration were cultured at 37°C for 3 d in complete medium (RPMI containing 20% FCS) supplemented with 50 U/ml of rIL-2 (Cetus Corp., Emeryville, CA). The cells were then harvested, washed twice, and used as effector cells for lysis of cryopreserved fresh melanoma tumor cells. Lysis was tested in a 4-h ^{51}Cr-release assay at the E/T ratio of 10:1. The results are expressed in percent specific lysis (13). In the inhibition experiments, effector cells were preincubated with graded amounts (0.01–100 μg/ml) of either purified anti LAK-1 mAb or of the $F(ab')_2$ fragment of the same antibody, for 1 h at room temperature (RT), washed twice, and used in the ^{51}Cr-release assay. As a control we used anti-CD7 or anti-LFA-1 mAbs.

Characterization of the LAK-1 Antigen. JA3 cells (8) were surface-labeled with ^{125}I using lactoperoxidase/glucose oxidase–catalyzed iodination (14). Cells were then washed five times in cold RPMI 1640, twice in PBS, and lysed in 10 mM Tris-buffered saline (pH 7.5) containing 1% NP-40, 1 mg/ml BSA, and 0.1 mM PMSF, for 20 min at 4°C. After spinning at 100,000 *g*, the supernatant was then immunoprecipitated as follows. Supernatants were dialyzed with PBS containing 0.05% NP-40 and 0.1 mM PMSF and precleared three times with 100 μl of packed protein A–Sepharose beads for 2 h under rotation. Aliquots (200 μl) were then incubated for 2 h with 20 μl of a 1:10 dilution of anti-LAK-1 mAb. 20 μl of packed protein A–Sepharose beads were then added and samples were incubated for 4 h at 4°C. The immunoprecipitate was eluted from protein A–Sepharose by boiling for 2 min in 5% SDS in the presence or absence of 5% 2-ME and analyzed on 8% discontinuous SDS-polyacrylamide gels (15).

Results and Discussion

To produce mAbs directed against surface molecules expressed by human LAK cells, DBA/2 mice were immunized with the clone SF16 derived as previously described (13), which displays a strong cytolytic activity against a variety of fresh or cultured human tumor cells (13). The surface phenotype of the SF-16 clone as assessed by immunofluorescence and FACS analysis was $CD3^-$, $CD28^-$ (Tp44), $CD4^-$, $CD8^-$, $CD2^+$, $CD7^+$, HLA-DR^+, HNK-1^-. This clone, which was derived from peripheral blood $CD16^+$ cells, was weakly stained by anti-CD16 (Leu-11) mAb. After fusion, hybridoma SN were first screened for

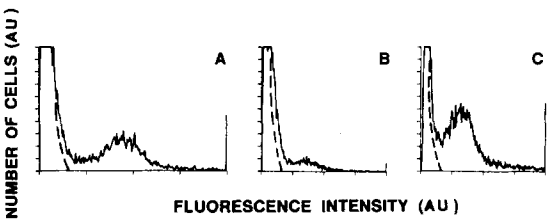

FIGURE 1. Fluorescence distribution of LAK-1 antigen on unfractionated PBMC (*A*) E-rosetting-positive (*B*) and E-rosetting-negative peripheral blood cell fractions (*C*). The various cell populations were stained with anti-LAK-1 mAb followed by fluoresceinated anti-mouse Ig. The dotted lines indicate the background fluorescence obtained using the second reagent alone. Samples were run on a FACS II gated to exclude nonviable cells.

their ability to bind to SF-16 cells. Selected mAbs were further analyzed by immunofluorescence for their reactivity with (*a*) unfractionated, E^+ and E^- peripheral blood mononuclear cells; (*b*) normal B cell–enriched spleen cells; (*c*) EBV-transformed B cell lines (including Raji, Daudi, BCLL, SKW6.9); (*d*) T cell lines; (*e*) monocytes, granulocytes, and platelets. Anti-LAK-1 mAb, like anti-CD16 and anti-HNK-1 mAbs, reacted with some T cell lines, including HSB-2, JA3, and Peer, whereas it did not react with either CEM or HPB-ALL T cell lines, nor with any of the B cell lines or normal spleen B cells analyzed. ~30% monocytes were LAK-1^+, whereas platelets did not react with the mAb. FACS analysis of isolated granulocytes showed that LAK-1 mAb was weakly reacting with the majority of these cells. Fig. 1 shows the pattern of reactivity of anti-LAK-1 mAb with different PBMC populations. The surface antigen recognized by anti-LAK-1 mAb displayed a clear bimodal distribution in all PBMC populations analyzed; thus, LAK-1^+ cells represented ~50 and 15% of E^- and E^+ cell fractions, respectively. In 10 different individuals, the percentage of LAK-1^+ cells in unfractionated PBMC populations ranged between 15 and 30%. Given the bimodal fluorescence distribution, LAK-1^+ and LAK-1^- cells could be separated by FACS sorting and further analyzed for morphology and functional capabilities. >90% of LAK-1^+ cells were LGL, as assessed by May-Grunwald Giemsa and naphthol-AS-D chloroacetate esterase (NCAE) staining (not shown) (16). The remaining LAK-1^+ cells (<10%) were represented by monocytes. The LAK-1^- cell fraction was represented by small lymphocytes, monocytes, and <2% LGL. As shown in Fig. 2, virtually all the NK activity exhibited by fresh PBMC was confined to the LAK-1^+ fraction. In addition, when LAK-1^+ and LAK-1^- cells were cultured with rIL-2 and analyzed at different time intervals for their ability to lyse fresh melanoma target cells (Fig. 2), LAK activity was clearly confined to the LAK-1^+ population. The cytolytic activity of this population was higher than that generated in unfractionated PBMC. Note also that unlike PBMC, LAK-1^+ cells maintained a strong LAK activity for a relatively long culture interval. These data indicate that the majority of the cells belonging to the LAK precursor pool express the LAK-1 surface antigen. Furthermore, >90% of the LAK-1^+ cells remained positive after 7 d of culture in rIL-2 (Fig. 3), whereas the LAK-1^- population did not express LAK-1 antigen after culture under the same conditions (not shown).

We next investigated whether LAK-1 was expressed on the LAK cells contained in unfractionated PBMC after culture in rIL-2. To this end, PBMC populations that had been cultured for 3 d in rIL-2 were separated by FACS

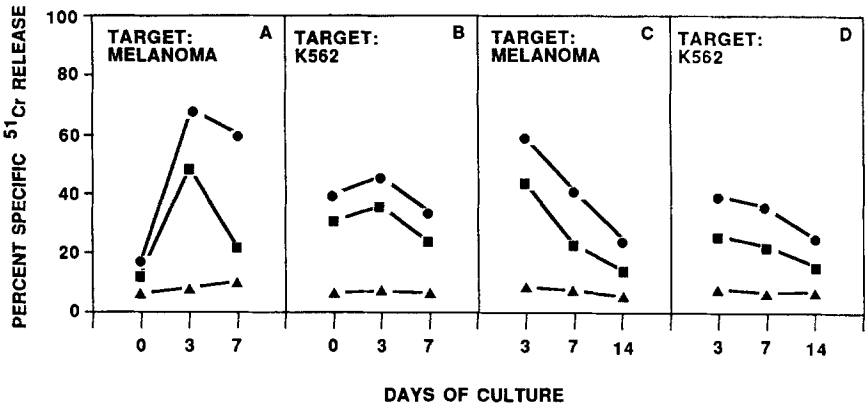

FIGURE 2. Cytolytic activity of LAK-1$^+$ (●) and LAK-1$^-$ (▲) cell populations fractionated either before (*A* and *B*) or after (*C* and *D*) culture in rIL-2. The cytolytic activity of unfractionated PBMC (■) is shown for comparison. PBMC were sorted into LAK-1$^+$ and LAK-1$^-$ cells and cultured in 50 U/ml rIL-2 (*A* and *B*). The cytolytic activity against the NK-resistant fresh melanoma cells and against the NK-sensitive K562 cell line was tested either 12 h after separation (day 0) or at days 3 and 7. Experiments reported in *C* and *D* were performed after culturing unfractionated PBMC in 50 U/ml rIL-2 followed by sorting of LAK-1$^+$ and LAK-1$^-$ populations. The two subsets, as well as the unfractionated populations, were recultured in rIL-2 and then assessed for cytolytic activity either 12 h after separation (day 3) or at day 7 and 14.

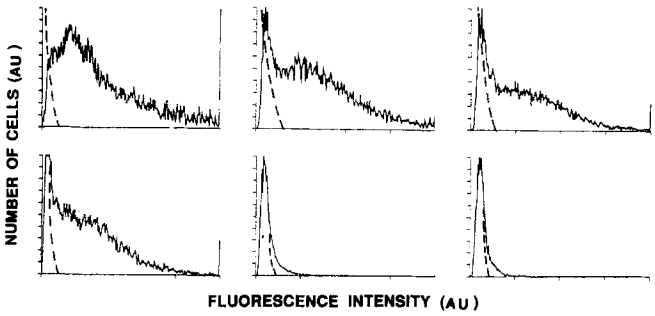

FIGURE 3. Stability of LAK-1 antigen expression. The expression of LAK-1, and CD16 antigens was analyzed on the purified LAK-1$^+$ population after different culture intervals in rIL-2. Cells were stained either with anti-LAK-1 mAb (*top panels*) and the fluoresceinated second reagent or with the fluoresceinated anti-CD16 (Leu-11) mAb (*bottom panels*) and analyzed at the FACS. (*Left panels*) Fluorescence distribution of the different antigens on a purified LAK-1$^+$ population 12 h after cell sorting. (*Middle* and *right panels*) Expression of the two antigens after 10 and 20 d of culture in rIL-2.

into LAK-1$^+$ and LAK-1$^-$ subsets (FACS analysis showed a clear bimodal distribution of LAK-1 expression on these cells) and were analyzed for their cytolytic activity against melanoma cells. Sorted cells were extensively washed and cultured for ~12 h before performing the cytolytic assay. Fig. 2, *C* and *D*, show that cytolytic cells were confined to the LAK-1$^+$ cell fraction. Taken together, these data suggest that the expression of LAK-1 antigen represents a stable characteristic of LAK cells. In another group of experiments, FACS-sorted peripheral blood LAK-1$^+$ cells were cultured for up to 3 wk in rIL-2 and analyzed at different culture intervals for the expression of LAK-1 antigen and other surface antigens expressed by LGL, such as CD16 and HNK-1. Whereas the expression of CD16 (Fig. 3) and HNK-1 (not shown) decreased progressively during culture, LAK-1 antigen was still brightly expressed on the majority of cells even after 20 d of culture (Fig. 3).

TABLE I
Inhibition of LAK Activity by anti-LAK-1 mAb Compared with Anti-LFA-1 and anti-CD7 mAbs

Exp.	Percent specific lysis with mAbs added			
	None	Anti-LFA-1	Anti-CD7	Anti-LAK-1
1	58	16	49	15
2	62	24	58	36
3	68	35	73	35
4	60	40	55	42
5	57	27	58	25
6	70	25	66	39

PBMC were cultured for 3–5 d in rIL-2, then incubated with the various mAbs for 1 h at room temperature and used as effector cells in a ^{51}Cr-release assay against fresh melanoma tumor cells (E/T ratio 10:1).

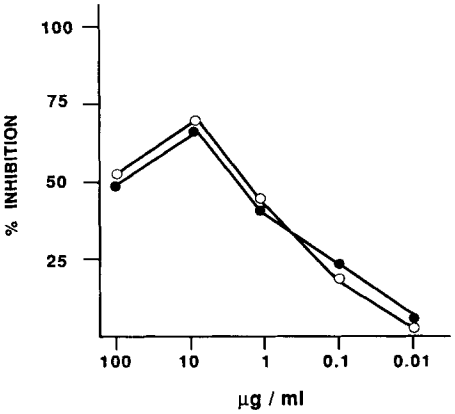

FIGURE 4. Inhibition of LAK activity mediated by either purified anti-LAK-1 mAb (○) or the F(ab')$_2$ fragment (●). Results are expressed as percent inhibition of LAK activity. LAK effector cells were obtained by culturing PBMC for 3–5 d in rIL-2. LAK cells were then incubated with the mAb for 1 h at room temperature and used as effector cells in a ^{51}Cr-release assay against fresh melanoma tumor cells (E/T ratio 10:1). The specific lysis of the target by LAK cells in the absence of the mAb was ~50%.

In another series of experiments we studied whether LAK-1 was involved in the cytolytic activity of LAK effector cells. As shown in Table I, the LAK activity of PBMC cultured for 3 d in rIL-2 was inhibited by preincubation of the effector cells with anti-LAK-1 or anti-LFA-1 mAb. On the other hand, an mAb directed to the CD7 antigen (which is expressed also by all LAK cell clones studied so far) (12) had no inhibitory activity. Similarly, LAK activity was not inhibited by mAbs directed to HNK-1 or CD16 antigens (data not shown). Unlike anti-LFA-1 mAb, however, anti-LAK-1 mAb did not inhibit the cytolytic activity of alloreactive T cell populations derived from mixed lymphocyte cultures (data not shown). To determine the concentration of anti-LAK-1 mAb needed for inhibition of lysis of fresh tumor targets, graded amounts either of purified mAb or of the F(ab')$_2$ fragment were used in the inhibition assay. As shown in Fig. 4, maximal inhibition was observed with 10 μg/ml of both inhibitors.

Given the inhibitory activity of anti-LAK-1 mAb on LAK effector function, we next investigated whether the mAb also inhibited the NK activity. Anti-LAK-1 mAb did not significantly block lysis of the NK-sensitive K562 target cells either by freshly derived PBMC or by lymphocytes cultured for 3 d in rIL-2 (<20% inhibition). It is noteworthy, however, that in parallel experiments, anti-LFA-1 mAb was also in most instances unable to efficiently block lysis of K562 target cells (not shown).

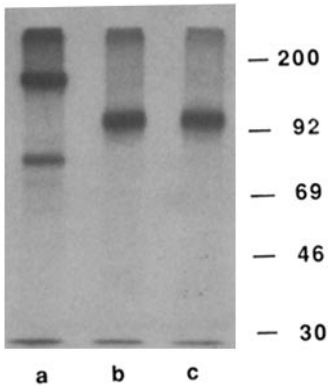

FIGURE 5. Analysis of LAK-1 molecule by SDS-PAGE under reducing (*b*) or nonreducing (*c*) conditions. LFA-1 molecule (*a*) is shown for comparison. JA3 cells were surface-labeled with ^{125}I and lysed as described in Materials and Methods. Cell lysates were immunoprecipitated with either one of the two mAbs and protein A–Sepharose. SDS-PAGE was performed in an 8% polyacrylamide gel followed by autoradiography.

To investigate the molecular nature of the surface antigen recognized by anti-LAK-1 mAb, cells were labeled with ^{125}I and the immunoprecipitated molecules were analyzed in SDS-PAGE. As shown in Fig. 5, under both reducing and nonreducing conditions, LAK-1 precipitated as a band of ~120 kD. The molecules immunoprecipitated from the same labeled cells by an anti-LFA-1 mAb are shown for comparison.

Taken together, our data indicate that LAK-1 is a novel surface marker that is expressed on human LGL and includes LAK precursor cells. More importantly, unlike other LGL/NK cell markers, LAK-1 displays a noticeable stability of expression since effector cells with LAK activity were found to be LAK-1$^+$ as well and the expression of LAK-1 was maintained for >3 wk of culture in rIL-2. The fact that in resting PBMC the precursors of LAK cells were LAK-1$^+$ indicates that the expression of this antigen is not induced by rIL-2 during the process of LAK cell induction. The LAK-1 molecule is clearly distinct from other LGL/NK-specific antigens. Thus, CD16, OKM1, NKH1, and NKH2 have different molecular weights (17–19). HNK-1 is expressed by only a fraction of LGL, moreover, the SF-16 immunizing clone was HNK-1$^-$. In addition, the surface expression of at least some of the above NK cell markers often decreases after cell activation (HNK-1, OKM1, and CD16) or culture in rIL-2. Thus, on the basis of LAK-1 expression it is now possible to isolate in PBMC a cell population containing LAK precursors. Moreover, it is also possible to isolate LAK effector cells from PBMC after culture in rIL-2. An interesting property of LAK-1 molecule is its involvement in LAK effector function. Thus, mAb to LAK-1 molecule, similar to anti-LFA-1 antibody (13), inhibited lysis of fresh tumor target cells, but, unlike anti-LFA-1 mAb, did not inhibit alloreactive CTL. Both mAbs were shown to reduce LAK activity when added to the effector (but not to the target) cells; moreover, the tumor target cells were found to be negative for both LFA-1 and LAK-1 antigens (not shown). Although both LFA-1 and LAK-1 are involved in LAK function, the two molecules are clearly distinct with respect to their molecular structure. Moreover, unlike LFA-1, LAK-1 appears to be restricted to a functionally and phenotypically defined lymphocyte subset. The finding that a minor proportion of T lymphocytes were LAK-1$^+$ is not surprising since T cells contribute to the LAK-P pool and may display an LGL morphology (6).

Summary

A human cell clone (SF-16) displaying strong cytolytic activity against fresh tumor target cells was used for production of murine mAbs against surface antigens expressed by lymphokine-activated killer (LAK) cells and their peripheral blood precursors. The preliminary screening of hybridoma supernatants was performed according to the ability to bind SF-16 cells. Selected mAbs were further analyzed for their reactivity with several T and B cell lines and with peripheral blood T and non–T cell populations. A selected mAb, termed anti-LAK-1, only reacted with some T cell lines and with 15–30% of PBMC. ~10–15% E-rosetting (T) cells and 40–50% E-rosette-negative cells were LAK-1$^+$, as determined by cytofluorometric analysis. As the fluorescence distribution of LAK-1 antigen was clearly bimodal, LAK-1$^+$ and LAK-1$^-$ cells could be separated by FACS. Positive cells were composed of large granular lymphocytes (LGL), whereas negative cells were mostly small lymphocytes and monocytes without LGL. After culture in rIL-2, purified LAK-1$^+$ (but not LAK-1$^-$) cells acquired the ability to lyse NK-resistant fresh melanoma target cells. In addition, only the LAK-1$^+$ fraction of PBMC cultured for 5 d in rIL-2 lysed fresh tumor targets, thus indicating that the LAK-1 antigen is expressed also on LAK effector cells. Unlike some other LGL/NK cell markers, LAK-1 antigen is characterized by a stable expression: thus, LAK-1$^+$ cell populations cultured for up to 20 d in rIL-2 maintained the LAK-1 antigen expression, whereas HNK-1 and, partially, CD16 were lost. Finally the cytolytic activity of LAK effector cells generated from PBMC cultured for 3 d in rIL-2 was susceptible to inhibition by the anti-LAK-1 mAb.

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