Identification of Four Subsets of Human CD3$^-$CD16$^+$ Natural Killer (NK) Cells by the Expression of Clonally Distributed Functional Surface Molecules: Correlation between Subset Assignment of NK Clones and Ability to Mediate Specific Alloantigen Recognition

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Summary

In previous studies we identified a surface molecule (termed GL183) capable of mediating cell activation and selectively expressed by a subset of human CD3$^-$CD16$^+$ natural killer (NK) cells. In this study we analyzed whether other subset-specific functional molecules were expressed in GL183$^-$ NK cells. To this end, mice were immunized with the PE29 (CD3$^-$CD16$^+$GL183$^-$) NK clone. Monoclonal antibodies (mAbs) were selected by screening the hybridoma supernatants for their ability to trigger the cytolytic activity of clone PE29 against the human myelomonocytic leukemia U937. The EB6 mAb (IgG1) triggered the PE29 clone, but not a GL183$^+$ clone used as a control. EB6$^+$ cells ranged between 1 and 13% of peripheral blood lymphocytes and were largely included in the CD3$^-$CD16$^+$CD56$^+$ cell populations (only <2% of EB6$^+$ cells were CD3$^+$). Analysis of resting or activated CD3$^-$CD16$^+$ populations, or clones for the expression of EB6 or GL183 mAbs, allowed us to identify four distinct, phenotypically stable, NK subsets (EB6$^+$GL183$^-$; EB6$^+$GL183$^+$; EB6$^-$GL183$^+$; EB6$^-$GL183$^-$). Similar to GL183 mAb, the EB6 mAb selectively triggered the NK subset expressing the corresponding surface antigen to lyse human tumor cell lines including U937, IGROV-I, M14, and A549. In addition, EB6 mAb sharply inhibited the cytolytic activity of EB6$^+$ clones against P815, M12, and P3U1 murine target cells. In EB6$^+$GL183$^+$ ("double-positive") clones both EB6 and GL183 mAb inhibited the redirected killing of P815 cells induced by anti-CD16, anti-CD2 mAbs and phytohemagglutinin (PHA). Similar to GL183 molecules, molecules precipitated by EB6 mAb were represented by either single 58-kD chain or double chains of 55 and 58 kD (with no detectable differences in EB6$^+$GL183$^-$ or EB6$^+$GL183$^+$ clones). In sequential immunoprecipitation experiments using the double-positive clones CEG52 and CA25.50, preclearing of cell lysates with EB6 or GL183 mAb removed only EB6 or GL183 molecules, respectively, thus indicating that the two antigenic determinants are carried by two distinct molecules. Peptide map analysis indicated that EB6 (or GL183) molecules precipitated from double-positive clones were identical to the corresponding molecules isolated from single-positive ones. On the other hand, comparison of the EB6 and GL183 maps revealed peptides that were unique to each molecule, although most of the major peptides migrated to identical positions. We further investigated whether correlation existed between the phenotypic assignment of NK clones and their ability to mediate specific lysis of normal allogeneic cells. All mixed lymphocyte culture–derived NK clones displaying the previously defined "1 anti-A" specificity expressed the EB6$^+$GL183$^-$ surface phenotype. In addition, among peripheral blood–derived CD3$^-$CD16$^+$ clones (with PHA and IL-2), only those expressing the EB6$^+$GL183$^-$ phenotype specifically lysed PHA blasts susceptible to lysis by "1 anti-A" clones. Finally, in individuals susceptible to lysis by "1 anti-A" alloreactive clones, EB6$^+$GL183$^-$ NK cells (which would represent autoreactive cells) were virtually absent.

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Natural killer (NK) cells are represented by lymphocytes able to lyse tumor target cells through a non-MHC-restricted type of recognition (1–7). NK cells do not express surface molecules such as CD3/TCR or sIg, which function as receptors for antigens on T or B cells, respectively (8–10). Recently, attention has been focused on the mechanisms and surface structures involved in NK cell activation and function. In this context, it has been shown that triggering of $CD3^-CD16^+$ lymphocytes can be induced by mAbs directed to cell surface molecules, including CD2 and CD16 (11–15). In addition, another surface molecule, termed GL183, has been identified, different from CD16 and CD2 molecules, that is expressed on a subset of NK cells and selectively activates/regulates these cells (16, 17). Functional GL183 molecules are present on both resting and activated NK cells and their expression (or lack of expression) is not modified by cell activation, proliferation, or cloning (16).

Given the restricted distribution of GL183 molecules to a subset of $CD16^+$ cells, we investigated whether other triggering surface molecules were expressed in $CD16^+GL183^-$ NK cells. By immunizing mice with the PE29 ($GL183^-$) NK clone, we obtained a mAb (EB6) capable of triggering the immunizing clone, which selectively bound to a subset of resting or activated NK cells or clones. Both biochemical and functional analyses revealed that the EB6-reactive molecules shared most of their properties with the GL183 molecules. Although the two molecules appear to belong to the same family, they are clearly distinct as indicated by their cell distribution and by a number of biochemical evidences. The expression (or lack of expression) of EB6 or GL183 molecules allows us to identify four distinct, phenotypically stable subsets of NK cells. Importantly, a striking correlation could be established between one of these subsets and the ability to specifically recognize a given NK-defined alloantigen recently identified on normal lymphocytes (18–20).

Materials and Methods

Isolation and Cloning of $CD3^-CD16^+CD56^+$ Lymphocytes. PBL from normal volunteers were isolated by Ficoll-Hypaque gradients and subsequently separated into different subsets by cell sorting and/or treatment with mAbs followed by complement depletion (18). To obtain purified $GL183^+$ or $EB6^+$ cells from PBL, cells were stained with the appropriate mAb followed by an isotype-specific (anti-IgG1) rabbit anti-mouse fluoresceinated antiserum (Southern Biotechnology Associates, Birmingham, AL) as a second reagent (16). Purified $CD16^+$ cells were sorted using the KD1 anti-CD16 mAb and a fluoresceinated anti-IgG2a second reagent (16). The $CD3^-EB6^-$ cell subset was obtained by treatment of PBL with anti-CD3 (OKT3) and anti-CD4 (CK79) mAbs followed by complement depletion. The resulting $CD3^-CD4^-$ population was then stained with EB6 mAb as described above and $EB6^-$ cells were isolated by sterile sorting as previously described (21). Cloning of the various cell subsets was performed under limiting dilution conditions in the presence of irradiated feeder cells, 0.1% PHA (Gibco Limited, Paisley, Scotland), and a source of exogenous IL-2 (rIL-2; Cetus Corp., Emeryville, CA) as previously described for both T and NK cells (18, 22, 23). MLC-derived $CD3^-CD16^+$ clones were cultured as previously described in detail (18–20). Clones were maintained in culture for periods of time varying from 1 to 6 mo in the presence of 100 U/ml of rIL-2 at a cell concentration of $\sim 10^6$/ml in 96 round-bottomed microwells (Greiner Labor Technik, Nurtinger, FRG).

Production of EB6 mAb. 5-wk-old male BALB/c mice were immunized with a cell clone termed PE29 (surface phenotype: $CD3^-CD16^+CD56^+GL183^-$) as previously described (24). The immunization schedule consisted of 2-wk intravenous injections of 10^7 PE29 cells. After six injections the mice were splenectomized and immune splenocytes were fused with P3U1 myeloma cells (24). The screening of hybridoma supernatants was based on the ability to modulate the cytolytic function of PE29 against the human cell line termed U937 used as ^{51}Cr-labeled target cells in a 4-h ^{51}Cr-release assay (18). To this end, 5×10^3 PE29 cells were cultured together with 5×10^3 U937 target cells (for a final effector/target ratio of 1:1) in the presence of 50 μl of culture supernatants derived from the various hybridomas. The assay was performed in V-bottomed microtiter trays in a final volume of 200 μl. After 4 h, 100 μl of supernatant was removed from each well and counted in a gamma counter for the assessment of ^{51}Cr release. Percent specific release was determined as previously described (21, 22). According to this screening procedure, a hybridoma, termed EB6, which was able to increase the cytolytic activity of PE29 clone against U937 target cells, was isolated and further subcloned in limiting dilution.

Two-color Flow Cytofluorometric Analysis. Analysis of PBL for the distribution of the surface antigen recognized by the EB6 mAb as compared with that of CD3, CD4, CD8, CD16, CD56, and CD2 antigens was performed using two-color fluorescence cytofluorometric analysis as previously described (25). Cells were stained with EB6 mAb followed by FITC-conjugated (or PE-conjugated) goat anti-mouse IgG1 antibodies (Southern Biotechnology Associates), and one of the following reagents: OKT3-PE (IgG2a), Leu-11-FITC (IgM), KD1-FITC (IgG2a), OKT8-PE (IgG2a), OKT4-PE (IgG2a), Leu-5-FITC (IgG2a), K218 (IgG1), and NKH1 (IgM) followed by appropriate anti-IgM PE antibodies. OKT3, OKT8, and OKT4 mAbs were purchased from Ortho Pharmaceutical (Raritan, NJ), whereas Leu-11 and Leu-5 were purchased from Becton Dickinson & Co. (Basel, Switzerland). The NKH1 mAb was kindly provided by Dr. Thierry Hercend, Institut Gustave Roussy, Villejuif, France.

Functional Analysis of $CD3^-CD16^+$ Clones. The cytolytic activity of the various clones was tested in a 4-h ^{51}Cr-release assay as described above. In all instances target cells were used at a concentration of 5×10^4/ml and were represented by either human or murine tumor cell lines or by PHA-induced lymphoblasts (18) as described in Results. PHA blasts derived from different individuals were obtained by culturing PBL for 4 d with 0.5% PHA (vol/vol) in the presence of rIL-2 (100 U/ml). The E/T ratios ranged from 5:1 to 0.5:1 as indicated. After titration, DEAE-Sephacel-purified EB6 mAb was used in most experiments at a concentration of 0.3 ng/ml resuspended in 50 μl of medium. The other mAbs including c288 (anti-CD16), KD1 (anti-CD16), MAR206 (anti-CD2), or GL183 were used at doses ranging from 10 to 0.2 ng/ml depending upon preliminary titration experiments. In cytolytic experiments, EB6 mAb and/or the above mentioned mAbs were added at the onset of the culture together with effector and target cells (16).

Biochemical Characterization of the EB6 Molecule. Cloned $EB6^+$ cells were washed five times in cold RPMI 1640, twice in PBS, and then surface labeled with ^{125}I using the lactoperoxidase/glucose oxidase–catalyzed iodination (26). After labeling, cells were washed once with PBS and resuspended (in ice) for 30 min in lysis

buffer containing 1% NP-40. After spinning, the supernatants were filtered and dialyzed with PBS. Lysates were then incubated overnight with 20 μl of packed Sepharose CNBr Beads (Pharmacia LKB, Uppsala, Sweden) previously coupled with purified EB6 mAb. The immunoprecipitate was eluted by boiling for 5 min in buffer containing 2.3% SDS in the presence or absence of 5% 2-ME and analyzed on 11% discontinuous SDS–polyacrylamide gels (27). For absorption studies, cell lysates derived from ^{125}I-labeled double-positive clones were immunoprecipitated three times with the first mAb and then the resulting precleared cell lysate was immunoprecipitated with the second mAb. After washing, the specific bound material was eluted and analyzed by SDS-PAGE as described above.

Western Blot Analysis. For Western blot analysis, (28) cell lysates were obtained after treatment of clones in buffer containing 1% NP-40 and 0.1% SDS for 15 min at room temperature. The supernatants were filtered and diluted 1:2 in buffer containing 2.3% SDS then separated by SDS-PAGE and transferred to Immobilon P Membranes (Millipore Continental Water Systems, Bedford, MA) in transfer buffer (0.025 M Tris, 0.19 M glycine, 20% methyl alcohol) at 50 V (constant voltage) overnight at 4°C. After transfer, membranes were blocked in TTBS (200 mM Tris/HCl, pH 7.5, 0.9% NaCl, 0.05% Tween) containing 2.3% nonfat milk.

Immunoblots were probed with purified mAbs diluted in TTBS-milk buffer for 2 h at room temperature, washed in the same buffer (four washes, 15 min each), further incubated with ^{125}I anti–mouse antiserum (100,000 cpm/ml in TTBS-milk) (Amersham, UK) followed by four washes in TTBS (15 min each), two washes in PBS (10 min each), and one wash in distilled water. Blots were then dried and subjected to autoradiography using Kodak XAR 5 film.

Peptide Mapping. Two-dimensional peptide mapping analysis was performed according to Accolla (29). Briefly, lysates derived from ^{125}I-labeled cells were immunoprecipitated with GL183 or EB6 mAbs, and the immunoprecipitated materials were separated by SDS-PAGE and subjected to autoradiography.

Bands corresponding to specific protein were cut from the dried gel, rehydrated overnight in PBS containing 0.1% SDS at 37°C, and the supernatants were filtered to remove residual pieces of acrylamide. 50 μg of albumin was added as a carrier. Reduction was performed by incubating for 30 min at room temperature with 20 mM dithiothreitol followed by alkylation with 60 mM of iodoacetamide for 1 h at room temperature in the dark.

Proteins were recovered by precipitation in 20% TCA at 4°C for 2 h and then washed three times with cold (−20°C) acetone. The dried protein pellet was resuspended in 100 μl 1% formic acid, 10% acetic acid buffer, and digestion with pepsin was carried out overnight at 37°C by adding 1 μg of enzyme. Samples were then dried under vacuum.

First dimensions were obtained by spotting side by side two samples on silica gels. After electrophoresis in 10% acetic acid, 1% pyridin plates were cut in half and chromatography was performed at right angles in *n*-butanol/acetic acid/pyridine/water 75:15:50:40 (vol/vol). The dried plates were subjected to autoradiography.

Results

The lymphocyte clone PE29 used for mouse immunization displayed a strong cytolytic activity against several different histologic types of tumor cell lines. This clone, which expressed the CD3$^-$CD16$^+$CD56$^+$ phenotype, was selected because it was not stained by the GL183 mAb. The hybridoma supernatants were screened for their ability to increase the

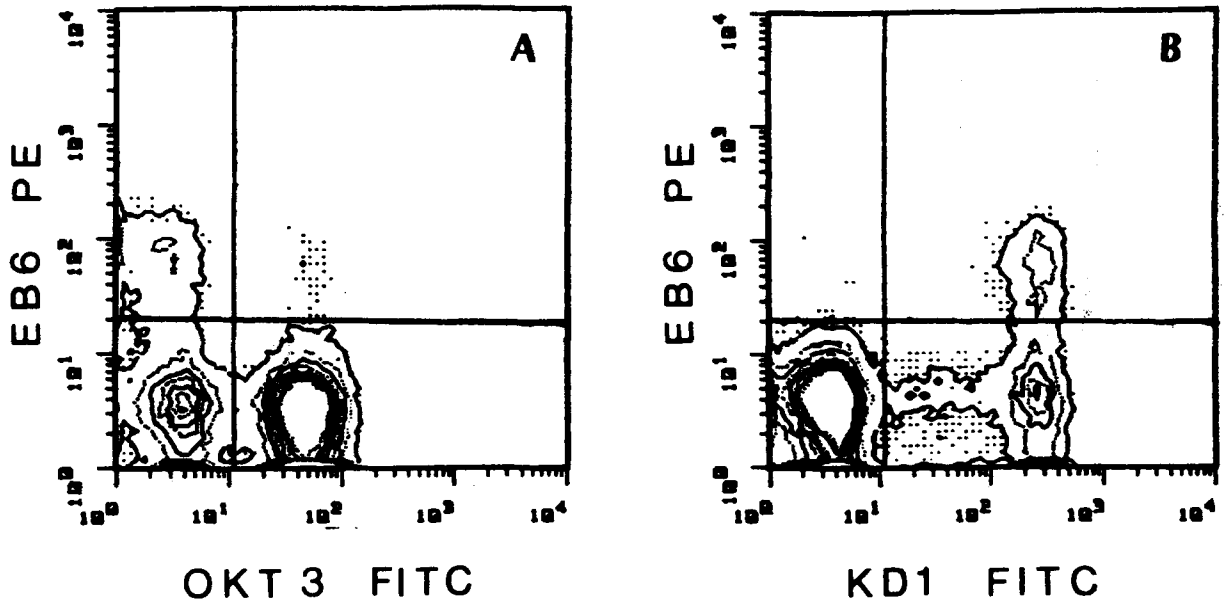

Figure 1. Distribution of EB6 antigen in PBL. In these experiments, the surface expression of EB6 antigen has been evaluated by double fluorescence and FACS analysis. Cells were stained with FITC-OKT3 (anti-CD3) (*A*) or KD1 (anti-CD16) mAb followed by FITC-conjugated goat anti–mouse IgG2a antiserum (*B*). Both cell suspensions were stained with EB6 mAb followed by PE-conjugated goat anti–mouse IgG1 antiserum. The contour plot was divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). Cells stained in red (EB6$^+$) in this donor were 9.6%, those stained by OKT3 mAb were 67%, and those stained by KD1 (CD16$^+$) were 22.2%. Cells simultaneously stained by OKT3 and EB6 mAbs were <0.3% whereas virtually all EB6$^+$ cells expressed CD16 antigen.

cytolytic activity of clone PE29 against the human myelomonocytic leukemic cell line U937. Previous studies have indicated that the cytolytic activity of GL183$^+$ clones against these target cells was highly augmented in the presence of GL183 mAb (16). The EB6 mAb increased the cytolytic activity of clone PE29 but not of a GL183$^+$ clone used as control.

Distribution of EB6 Antigen in CD3$^-$CD16$^+$ Populations and Clones. Analysis by indirect immunofluorescence revealed that cells reacting with EB6 mAb ranged between 1 and 13% in PBL derived from six different donors. Next, the expression of EB6 antigen was compared with that of other lymphocyte surface markers in PBL. These experiments, carried out by double immunofluorescence and FACS analysis, demonstrated that EB6 antigen is expressed on a cell subset expressing CD16 but lacking CD3 antigen. Only rare cells appeared to react simultaneously with CD3 and EB6 (<2% of the total EB6$^+$ population) (Fig. 1). Similar to GL183, the EB6 molecule was expressed in cells stained by anti-CD56 mAbs, while it was lacking on CD4$^+$ cells. In all instances, CD8 and CD2 antigens were expressed on a fraction of the EB6$^+$ cells (not shown). Thus, the distribution of EB6 molecule in PBL was reminiscent of that of the GL183 molecule (16, 17). However, FACS analysis by two-color fluorescence indicated that the percentages of the CD16$^+$GL183$^+$ or CD16$^+$EB6$^+$ cells (expressed as a fraction of CD16$^+$ cells) greatly varied in different individuals (Fig. 2). Moreover, when cells were stained by a mixture of the two antibodies, the percentages of positive cells were always less than the sum of the percentages of EB6$^+$ and GL183$^+$ cells. Taken together, the above data suggest that GL183 and EB6 mAbs recognize two distinct, but partially overlapping, subsets of human CD16$^+$ NK cells. To better analyze the distribution of the two molecules in CD16$^+$ cells, we studied a large panel of NK clones (a direct comparison of the distribution of the two molecules by double fluorescence and FACS analysis was impaired by the fact that both mAbs belong to the IgG1 subclass). In experiments in which CD3$^-$CD16$^+$ populations were fractionated into EB6$^+$ and EB6$^-$ subsets and cloned under limiting dilution, the resulting clones maintained the original EB6$^+$ or EB6$^-$ surface phenotype. Thus, similar to GL183 antigen (16), the expression or lack of expression of EB6 antigen appears to be a stable phenotypic property of two distinct NK cell subsets. Subsequently, clones were derived from purified CD3$^-$ lymphocytes obtained from different individuals and analyzed for the surface expression of GL183 and EB6 molecules. Four distinct phenotypic groups of CD3$^-$CD16$^+$ clones could be identified: GL183$^+$EB6$^-$; GL183$^+$EB6$^+$; EB6$^+$GL183$^-$; and EB6$^-$GL183$^-$. Clones representative of each group are shown in Figure 3. Although in the double-positive clone CA 25.50, the antigen density of EB6 and GL183 appears to be higher than antigen density in single-positive clones (PE 29 and CEG 5), this finding could not be generalized to other double-positive clones. The percentages of clones belonging to each group were found to be extremely variable among different individuals. Thus, for example, out of 162 clones derived from individual C.E., 49 were EB6$^+$GL183$^-$, whereas only 3 were EB6$^-$GL183$^+$. 78 clones stained simultaneously by the two mAbs (double positive), and the remaining 32 were EB6-GL183$^-$ (double negative). On the other hand, the majority of clones derived

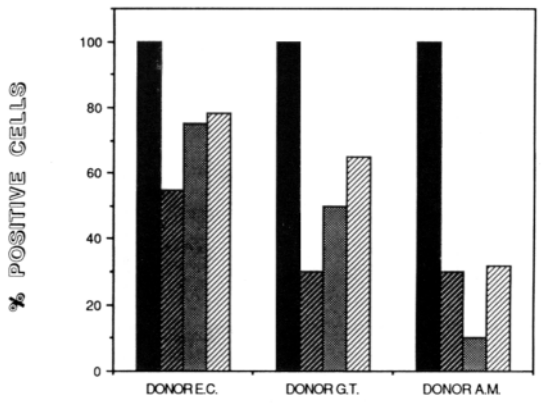

Figure 2. Expression of EB6 and GL183 antigens in peripheral blood–derived CD16$^+$ lymphocytes. PBL derived from three representative individuals were analyzed for the expression of EB6 and GL183 antigens in relationship with the expression of CD16 antigen by double fluorescence and FACS analysis. Cells were stained with KD1 (anti-CD16, IgG2a) mAb followed by goat anti–mouse IgG2a antiserum (*green fluorescence*) followed by either GL183 or EB6 mAbs or a mixture of the two mAbs followed by FITC-conjugated goat anti–mouse IgG1 antiserum (*red fluorescence*). It should be stressed that, in all instances, cells stained by GL183 or EB6 mAbs (or by a mixture of the two mAbs) were simultaneously CD16$^+$. Therefore, in each individual, data are expressed as percentages of CD16$^+$ lymphocytes reacting with GL183 or EB6 mAbs or with a mixture of the two mAbs. It is evident that these various percentages greatly differ in various individuals. (■) CD16$^+$ cells; (▨) GL183$^+$ cells; (▩) EB6$^+$ cells, and (▨) cells reacting with a mixture of GL183 and EB6 mAbs.

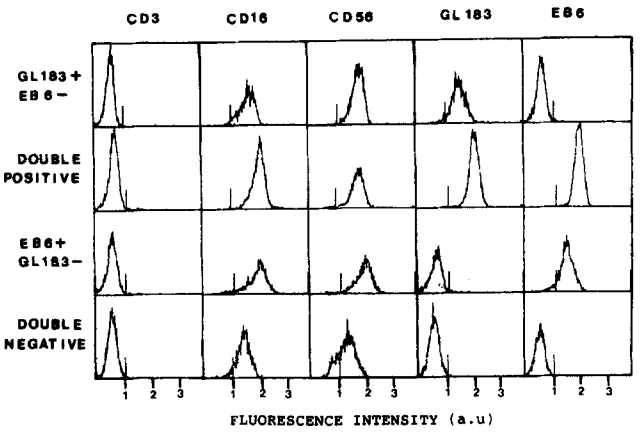

Figure 3. Flow cytofluorometric analysis of surface antigens expressed by CD3$^-$CD16$^+$ clones. Cells were stained with either one of the following mAbs: anti-CD3 (OKT3), anti-CD16 (KD1), anti-CD56 (K218), GL183, or EB6, as described in Materials and Methods. Fluorescein-conjugated goat anti–mouse Ig was used as second reagent. Note that on the basis of GL183 and EB6 antigen expression four distinct types of NK clones can be distinguished. The four representative clones shown are CEG5 (GL183$^+$EB6$^-$), CA.25.50 (double positive), PE29 (EB6$^+$ GL183$^-$), and CES9 (double negative).

from individual A.M. (97/128) were double negative, whereas 21 were double positive, 9 were GL183$^+$EB6$^-$ and only 1 was EB6$^+$GL183$^-$. Thus, clonal analysis confirmed that the subsets identified by EB6 and GL183 mAbs are partially overlapping and that the two mAbs allow us to identify four distinct subsets of human CD16$^+$ NK cells.

EB6 mAb Selectively Triggers the NK Subset Expressing the Corresponding Surface Antigen. EB6 mAb has been selected according to its ability to trigger the cytolytic activity of the immunizing PE29 clone (CD3$^-$CD16$^+$GL183$^-$). Since the combined use of GL183 and EB6 mAbs allows us to identify four distinct NK subsets, we investigated the ability of the two mAbs to trigger the cytolytic activity of clones belonging to these subsets. A large panel of clones (>200 clones) has been analyzed for the ability to lyse the U937 cell line either in the absence or presence of either mAb. Four representative clones are shown in Fig. 4. It is evident that EB6 mAb stimulated not only the EB6$^+$GL183$^-$ clone (belonging to the same subset as the PE29 clone) but also clones expressing both EB6 and GL183 antigens. Similarly, GL183 mAb triggered both GL183$^+$EB6$^-$ and double-positive clones. The two mAbs failed to induce GL183$^-$EB6$^-$ (double-negative) clones. Since similar results were obtained in all clones analyzed, it appears that the surface expression of GL183 or EB6 antigen predicts the susceptibility of a given clone to stimuli delivered by the corresponding mAbs.

We previously reported that GL183 mAb, and its F(ab)$_2$ fragment, could enhance the cytolytic activity of GL183$^+$ clones against a panel of human tumor cell lines of different histotypes (16). These included both the FcγR$^+$ U937 and A549 (lung carcinoma) cell lines, and the FcγR$^-$ IGROV (ovarian carcinoma) and M14 (melanoma) lines. Although not shown, the EB6 mAb had a similar effect when EB6$^+$ clones were tested against the same panel of target cells. Therefore, lysis of human tumor cells of different origin can be efficiently enhanced by triggering of effector cells with either EB6 or GL183 mAbs.

Regulatory Role of EB6 Surface Molecules. As previously shown, in cytolytic assays against murine target cells, GL183 mAb resulted in effects that were opposite to those observed with human target cells (i.e., inhibitory effects) (16). This inhibition required the presence of FcR on target cells and could not be mediated by the F(ab)$_2$ fragments of the GL183 mAb. Identical results were obtained with EB6 mAb when EB6$^+$ clones were tested against murine target cells (including the mastocytoma P815, the B cell lymphoma M12, and the myeloma P3U1; (not shown).

GL183 mAb was shown to inhibit both spontaneous and redirected killing of P815 target cells by GL183$^+$ clones (16). These experiments demonstrated that GL183 mAb is able to downregulate cytolytic responses of human NK cell clones to stimuli such as PHA, anti-CD16, and anti-CD2 mAbs. To compare the ability of EB6 and GL183 mAbs to inhibit the redirected killing of P815 target cells, we analyzed a series of EB6$^+$ clones (>100 clones). Fig. 5 shows a representative experiment using the double-positive clone T49. It can be seen that both mAbs inhibited, in a dose-dependent fashion, the redirected killing of P815 cells induced by different stimuli. No inhibition was observed with K218 (anti-CD56) or MAR21 (anti-CD7) mAbs, which belong to the same IgG1 subclass as GL183 and EB6 mAbs (not shown). Therefore, the effects of EB6 mAb on the cytolytic function of EB6$^+$ clones appear to be indistinguishable from that of GL183 mAb on GL183$^+$ clones.

Biochemical Characterization of Proteins Precipitated by EB6 mAb. The biochemical characteristics of the surface antigen recognized by EB6 mAb were studied in a series of ^{125}I surface-labeled CD16$^+$EB6$^+$ clones. The molecules immunoprecipitated by EB6 mAb and analyzed by SDS-PAGE displayed an apparent molecular mass of ~58 kD under both reducing and nonreducing conditions (Fig. 6). In some clones

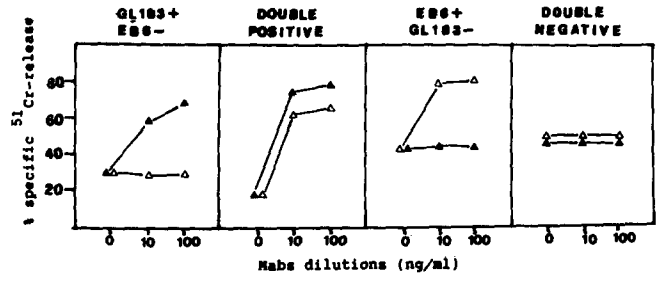

Figure 4. Enhancement of cytolytic activity mediated by EB6 or GL183 mAbs in NK clones belonging to different subsets. Clones CEG5 (GL183$^+$EB6$^-$), EG2.50 (double positive), CEG76 (EB6$^+$GL183$^-$), and CES9 (double negative) were tested for cytolytic activity against ^{51}Cr-labeled U937 cells in the presence of graded amounts of either GL183 (▲) or EB6 (△) mAbs as indicated. The E/T ratios were 3:1 for clones CEG5 and EG2.50 and 1:1 for clones CEG76 and CES9.

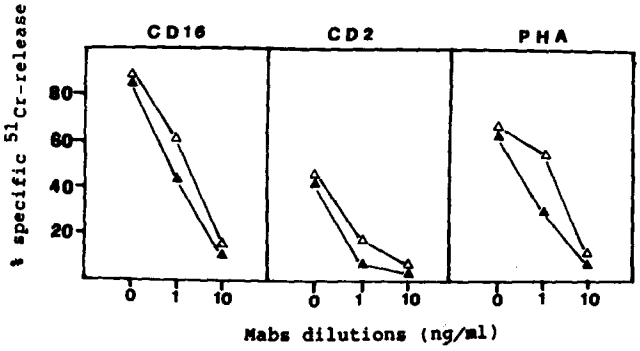

Figure 5. Inhibitory effect of EB6 or GL183 mAbs on the PHA or mAb-induced redirected killing of P815 target cells. Clone T49 (double positive) was tested for cytolytic activity against ^{51}Cr-labeled P815 target cells in a 4-h ^{51}Cr-release assay at an E/T ratio of 3:1. It should be noted that clone T49 at an effector/target ratio of 3:1 induced 18% specific lysis of P815 target cells in the absence of added stimuli. PHA or mAbs directed to CD16 or CD2 (both mAbs used at a concentration of 10 ng/ml) were added at the onset of the cytolytic test either in the absence or in the presence of graded amounts of purified EB6 (△) or GL183 (▲) mAbs.

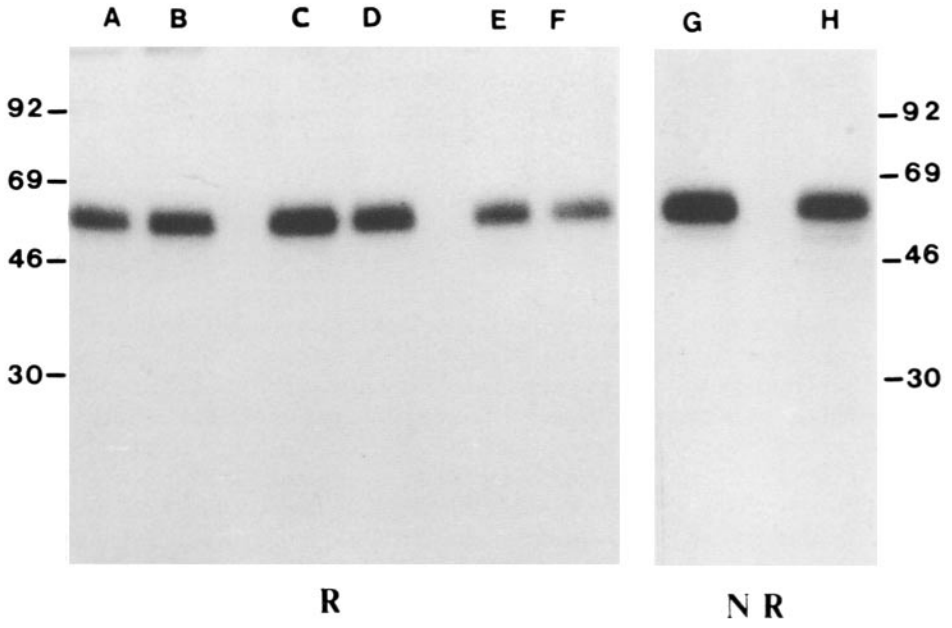

Figure 6. SDS-PAGE analysis of surface molecules precipitated by EB6 or GL183 mAbs from CD3$^-$CD16$^+$ clones. 5 × 10^6 cells from clone CEG76 (EB6$^+$GL183$^-$; lane *A*), clone F25 (EB6$^+$GL183$^-$; lane *B*), clone CIK52 (double positive; lanes *C* and *D*) and clone T49 ("double positive"; lanes *E* and *F*) were surface labeled with ^{125}I using the lactoperoxidase technique and lysed in buffer containing 1% NP-40. Immunoprecipitations were performed by using GL183 (lanes *C* and *E*) or EB6 (lanes *A*, *B*, *D*, and *F*) mAbs coupled to protein A–Sepharose beads. SDS-PAGE analysis was performed under reducing conditions using 11% acrylamide gels. In lanes *G* and *H*, immunoprecipitates from the double-positive clone CA25.50 were analyzed under non-reducing conditions (lane *G*, GL183; lane *H*, EB6).

however, a second 55-kD band could be observed together with the 58-kD band (not shown). In addition, EB6 molecules immunoprecipitated either from EB6$^+$GL183$^-$ or from double-positive clones displayed similar characteristics under the above conditions. It is of note that no correlation existed between the presence or the absence of the additional 55-kD band and the coexpression of GL183 antigen on EB6$^+$ cells (Fig. 6). Further comparative analysis of GL183 and EB6 mAb–reactive molecules was performed by two-dimensional PAGE. In these studies, spots corresponding to EB6 molecules were found to migrate to identical positions as those corresponding to GL183 molecules (16). These results were obtained in both double-positive (EB6$^+$GL183$^+$) and single-positive (EB6$^+$GL183$^-$ or EB6$^-$GL183$^+$) clones (not shown). Since preliminary experiments showed that both mAbs reacted in Western blot analysis, cell lysates derived from NK clones were analyzed for reactivity in Western blot with either one of the two mAbs. Cell lysates derived from single-positive clones were reactive only with the appropriate mAb whereas the double-positive clone EG2.50 reacted with both mAbs (Fig. 7). These data further confirmed the lack of reactivity of EB6$^+$GL183$^-$ clones with GL183 mAb (and vice versa for EB6$^-$GL183$^+$ clones with EB6 mAb). Since in double-positive clones molecules recognized by EB6 or GL183 mAbs were indistinguishable, we further investigated whether the antigenic determinants defined by the two mAbs were expressed on the same or on different molecules. To define this point, sequential immunoprecipitation experiments were performed on cell lysates derived from surface-iodinated double-positive clones CEG52 and CA25.50 (Fig. 8). Thus, preclearing of GL183-reactive molecules completely eliminated the reactivity of cell lysates with this antibody but did not affect the reactivity with EB6 mAb. In addition, preclearing of EB6-reactive molecules greatly reduced the reactivity of cell lysates with this antibody, but not with GL183 mAb. These experiments suggest that the antigenic determinants recognized by EB6 mAb or GL183 mAbs are expressed on distinct molecules also in double-positive clones.

Peptide Map Analysis of EB6- and GL183-reactive Molecules. To determine whether EB6- and GL183-reactive chains were different with regard to their peptide structure, comparative peptide maps of the two chains were performed after digestion with proteolytic enzymes. As shown in Fig. 9, the peptide maps of EB6- and GL183-reactive chains isolated from the double-positive CEG52 clone, appear very similar when run together in order to allow a direct comparison of the peptides. It can be seen that most major peptides migrate to identical positions. In contrast, at least one peptide (*arrow*) was found in the GL183-reactive chain but not in the EB6-reactive chain. In addition, the EB6 chain showed at least two peptides with minimal mobility in the chromatographic dimension that were absent in the GL183 chain. Thus, these data confirm that GL183 and EB6 molecules are similar but not identical.

In Fig. 9 *C*, we also analyzed clone CEG5 derived from the same donor, but which expresses the GL183$^+$EB6$^-$ surface phenotype. In this clone it has been possible to obtain a similar number of cpm as in the double-positive clone. It is evident that the molecules immunoprecipitated by GL183 mAb are resolved into a series of spots which are identical to those present in Fig. 9 *B*. Although not shown, the EB6-specific peptides derived from the EB6$^+$GL183$^-$ clone CEG76 were identical to those of Fig. 9 *A*. Thus, the peptide map comparison provides unequivocal evidence that the EB6- and GL183-reactive chains are analogous to one another, since they share several peptide fragments in common. How-

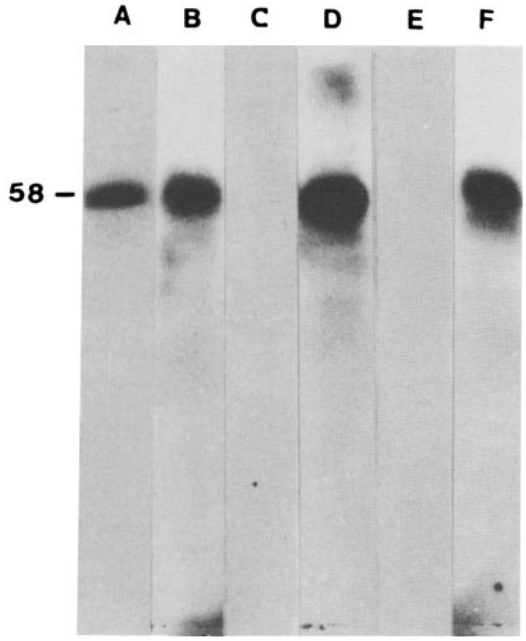

Figure 7. Immunoblotting analysis of EB6 and GL183 molecules in NK clones belonging to different subsets. Total cell lysates (5×10^6 cell equivalents per lane) derived from the double-positive clone EG2.50 (lanes *A* and *D*), the GL183$^+$EB6$^-$ clone CEG5 (lanes *B* and *E*), or the EB6$^+$GL183$^-$ clone CEG76 (lanes *C* and *F*) were separated on SDS–polyacrylamide gels in the absence of 2-ME. After transfer to Immobilon P membranes, blots were probed with GL183 (Lanes *A–C*) or EB6 (lanes *D–F*) mAbs as described in Materials and Methods.

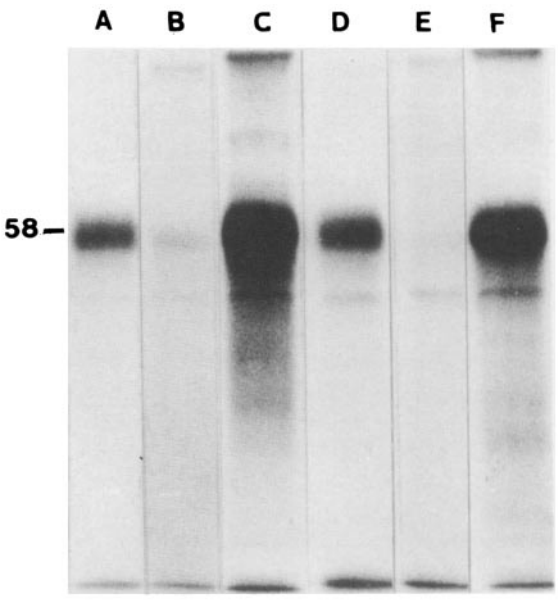

Figure 8. Sequential immunoprecipitation of EB6 and GL183 antigens from a double-positive NK clone. 20×10^6 cells from clone CA25.50 were surface labeled and lysed as described in Materials and Methods. Cell lysates were divided into two aliquots and immunoprecipitated with either EB6 (lane *A*) or GL183 (lane *D*) mAbs coupled to protein A–Sepharose. The preclearing procedure with a given mAb was repeated four times. EB6-precleared and GL183-precleared lysates were then immunoprecipitated with either one of the two mAbs and analyzed in an 11% polyacrylamide slab gel system. EB6-precleared cell lysate immunoprecipitated with EB6 (lane *B*) or GL183 mAb (lane *C*). GL183-precleared cell lysate immunoprecipitated with either GL183 (lane *E*) or EB6 mAb (lane *F*). In lanes *B*, *C*, *E*, and *F* twice as much cell lysate was used as compared with lanes *A* and *D*.

ever, the presence of unique peptides after proteolysis of EB6- or GL183-reactive chains further indicates that the two molecules are not identical.

Correlation Between EB6/GL183 mAb-defined NK Subsets and Specific Lysis of Allogeneic Cells. As previously shown, MLC-stimulated human CD3$^-$CD16$^+$ NK cells display the ability to specifically lyse normal allogeneic cells (PHA-blasts) derived from the stimulating donor (18). Although several NK-defined allospecificities appear to exist (reference 20; Moretta, A., E. Ciccone, O. Viale, D. Pende, G. Tripodi, C. Bottino, and L. Moretta, manuscript in preparation), only one has been analyzed in detail so far (20). This allospecificity

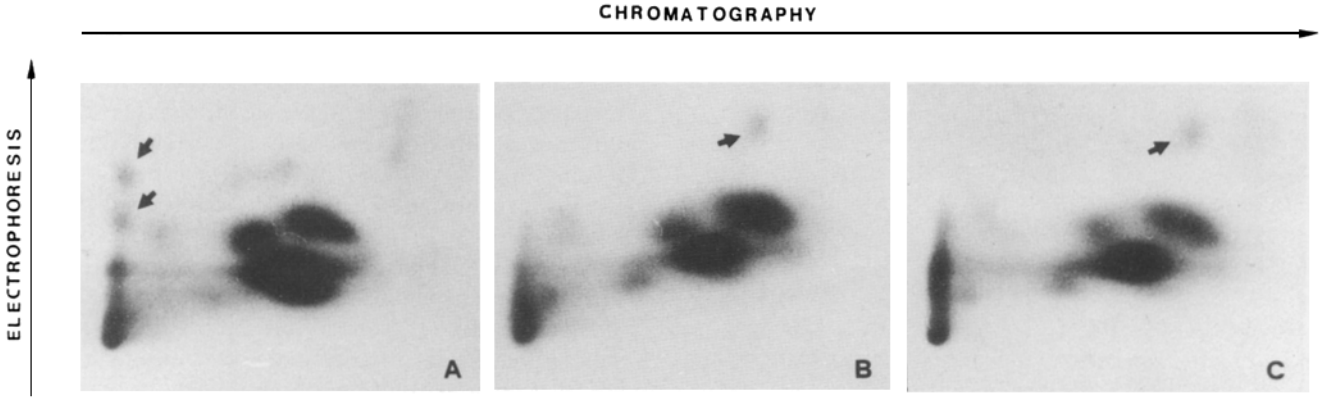

Figure 9. Comparative analysis of the two-dimensional peptide maps of EB6 and GL183 molecules. EB6 (*A*) and GL183 (*B*) molecules purified from the double-positive clone CEG52 were digested with pepsin. In *C*, the GL183 molecule isolated from GL183$^+$EB6$^-$ clone CEG5 is shown for comparison. Each sample was spotted on a silica gel plate and peptides were separated by electrophoresis (*vertical dimension*) and chromatography (*horizontal dimension*). Note that 6,000 cpm were applied per individual sample and that *A* and *B* were run in parallel. Arrows indicate specific peptides in the EB6 or GL183 molecules.

has been defined by the use of NK clones termed "1 anti-A." 1 anti-A clones were found to lyse one out of four normal random donors (20). In this study, we selected a series of $CD3^-$ $CD16^+$ clones displaying 1 anti-A specificity (i.e., capable of specifically lysing appropriate normal allogeneic PHA blasts). These clones were derived from three different individuals resistant to lysis by 1 anti-A clones. We found that all (over 100 clones) were characterized by the $EB6^+$ $GL183^-$ surface phenotype. In another series of experiments, clones were obtained directly from PBL without preselection in MLC (19). Thus, purified $CD3^-CD16^+$ cells were cultured under limiting dilution in the presence of PHA, feeder cells, and an exogeneous source of IL-2. The clones obtained, representative of the four different EB6/GL183 mAb–defined NK subsets, were assessed for specific cytolytic activity against ^{51}Cr-labeled PHA-blasts derived from donors that were either susceptible or resistant to lysis by 1 anti-A clones (20). As shown in Fig. 10, only clones expressing the $EB6^+$ $GL183^-$ phenotype were able to specifically lyse 1 anti-A–susceptible target cells. More importantly, only 1 of 18 clones expressing the $EB6^+GL183^-$ surface phenotype failed to lyse these target cells. Similar results were obtained by the analysis of clones derived from two additional donors. In agreement with previous results (18), these clones did not lyse autologous or allogeneic PHA blasts previously characterized as resistant to lysis by 1 anti-A clones.

The above data suggest that, in different donors, most cells belonging to the $EB6^+GL183^-$ NK subset may be capable of lysing 1 anti-A–susceptible target cells. If this holds true, in donors carrying the 1 anti-A specificity, $EB6^+GL183^-$ cells would represent a potentially autoreactive subset. To explore this possibility, we analyzed the EB6/GL183 surface phenotype of clones derived from such donors. In all of these individuals, clones expressing the $EB6^+GL183^-$ phenotype were extremely rare or absent, whereas they represent 20–40% of $CD3^-CD16^+$ clones derived from 1 anti-A–resistant donors. For example, they represented only 1 out of 128 $CD3^-CD16^+$ clones derived from donor A.M. In addition, this clone failed to lyse 1 anti-A–susceptible target cells. It should be noted that these results at the clonal level are consistent with data reported in Fig. 2 (donor A.M.). Indeed in this donor, the proportion of peripheral blood $CD16^+$ cells expressing GL183 antigen was similar to that of cells stained by a mixture of GL183 and EB6 mAbs. These data already suggested that the small proportion of $CD16^+EB6^+$ cells was almost entirely represented by double-positive ($GL183^+EB6^+$) cells.

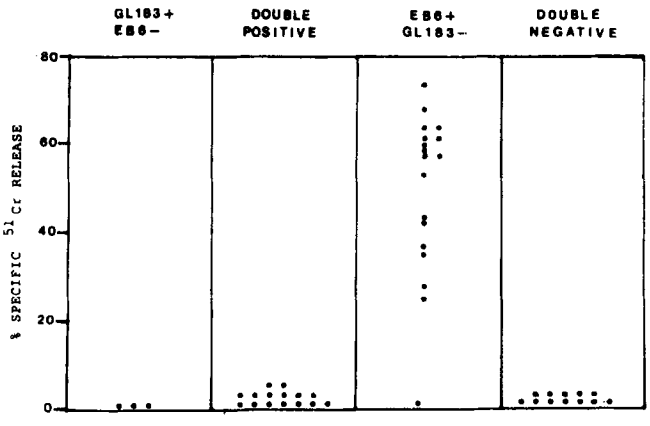

Figure 10. Specific lysis of normal allogeneic cells by $EB6^+GL183^-$ clones. Clones derived from peripheral blood $CD3^-$ lymphocytes of donor 1 (E.C.) were tested for 1 anti-A cytolytic activity against PHA-induced lymphoblasts derived from donor A.M. Each point corresponds to the cytolytic activity mediated by a single clone. Clones belonging to different subsets are represented in different quadrants. Note that only $EB6^+$ $GL183^-$ clones displayed 1 anti-A specificity.

Discussion

In the present study we describe a novel surface molecule (EB6) expressed by a subset of human NK cells that mediates cell triggering. The EB6 molecule is functionally and structurally related to the previously described GL183 molecule (16, 17). At least four different NK cell subsets could be identified on the basis of the expression of EB6 and/or GL183 molecules. More importantly, a direct correlation has been found between a given phenotypically defined NK cell subset and its ability to specifically recognize normal allogeneic cells.

Our previous studies revealed the existence, at the NK cell surface, of a functional molecule (GL183) that, different from other molecules involved in $CD3^-CD16^+$ cell activation, (i.e., CD16 and CD2), defined a subset of NK cells. The expression or lack of expression of GL183 surface molecules represents a stable phenotypic property of NK cells, as the $GL183^+$ or $GL183^-$ phenotype of freshly derived $CD3^-$ $CD16^+$ cells was maintained after cell culture, exposure to lymphokines, and clonal expansion (16). Another important feature of GL183 surface antigen was its ability to exert a regulatory role in the control of cell activation induced by different stimuli, including anti-CD16 mAb, anti-CD2, and PHA. The newly identified EB6 molecule shares most of the structural and functional properties of GL183 molecules. However, a series of experimental evidences clearly indicated that the two molecules are distinct and define NK cell subsets that are only partially overlapping. First, it should be noted that the EB6 mAb was obtained against the PE29 clone that did not express detectable GL183 molecules. This clone was subsequently found to be representative of an NK subset expressing the $EB6^+GL183^-$ surface phenotype. Clonal analysis also demonstrated that the EB6 molecule is expressed in some $GL183^+$ cells, thus defining a subset coexpressing EB6 and GL183 molecules (double positive). In addition, GL183 molecules are also present in cells that lack surface expression of EB6 ($GL183^+EB6^-$ subset). Finally, a fourth subset of $CD3^-CD16^+$ cells could be defined by the simultaneous lack of reactivity with the two mAbs. The finding that double-positive clones reacted with the two mAbs raised the question of whether the antigenic determinants recognized by EB6 or GL183 mAbs represented two different epitopes expressed by a single (polymorphic) molecule or rather by distinct surface molecules. Preclearing of cell lysates de-

rived from double-positive clones with either one of the two mAbs did not remove molecules recognized by the other mAb, thus indicating that the two mAbs react with distinct surface molecules. In addition, peptide map analysis of the molecules precipitated by GL183 or EB6 mAbs from double-positive or $GL183^+EB6^-$ or $EB6^+GL183^-$ clones, revealed the existence of unique peptides in each molecule, thus confirming that, in spite of the biochemical and functional similarities, the two molecules are different. However, peptide map analysis also shows that GL183 and EB6 molecules share the majority of the peptides, thus suggesting that they belong to the same family. Since double-positive clones simultaneously express EB6 and GL183 molecules, one may speculate that NK clones presently defined as single positive also may actually express an additional chain ("x chains") belonging to the same molecular family. According to this hypothesis, at least some double-negative NK cells may express two still-undefined members of this molecular species.

Having recently demonstrated that NK cells can specifically recognize allogeneic cells (18–20), we investigated whether a relationship existed between this function and the expression of EB6 and/or GL183 molecules. We found that the NK-defined allospecificity that had been termed 1 anti-A (20) displayed a striking correlation with one of these subsets. Thus, all the MLC-derived $CD3^-CD16^+$ clones displaying 1 anti-A specificity were characterized by the $EB6^+GL183^-$ surface phenotype. In addition, virtually all $EB6^+GL183^-$ clones derived directly from PBL (without preselection in MLC) were found to lyse 1 anti-A–susceptible target cells. These data are reminiscent of a recent report in mice in which a mAb-defined subset of NK cells mediated the rejection of incompatible Hh-1-*d* bone marrow graft (30).

A remarkable finding was that in donors susceptible to lysis by 1 anti-A NK clones, $EB6^+GL183^-$ cells were virtually absent. In addition, a single $EB6^+GL183^-$ clone derived from individual A.M. failed to lyse autologous cells (susceptible to lysis by all $EB6^+GL183^-$ clones derived from allogeneic donors). These findings are in agreement with our previous reports (18–20) and are in line with the hypothesis that NK cells are also capable of self/nonself discrimination. It is also of note that other (at least 4) NK-defined specificities have been recently identified. For example, in the case of the specificity termed "A anti-1" (reference 20, our unpublished results), preliminary experiments indicate that all clones displaying this specificity belong to the double-positive NK subset.

Although the receptor molecules mediating specific recognition by NK cells have not been identified, our present demonstration that the expression of $EB6^+GL183^-$ surface phenotype precisely correlates with a specific function may suggest the involvement of EB6 molecules in specific NK cell recognition. Along this line, preliminary experiments showed a partial inhibitory effect (~30%) of EB6 mAb on the lysis of 1 anti-A–susceptible targets mediated by $EB6^+GL183^-$ clones. An interesting question is why double-positive clones, in spite of the surface expression of EB6 molecules, did not lyse 1 anti-A–susceptible targets. If we assume that this family of molecules is somehow involved in NK receptor function and that the concerted action of the two chains is required to confer a given specificity, the 1 anti-A specificity of $EB6^+GL183^-$ clones would be determined by both EB6 and "x" chains. In double-positive clones specificity would be conferred by the combinatory effect of EB6 and GL183 molecules and would be different from 1 anti-A.

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