

A NOVEL SURFACE ANTIGEN EXPRESSED BY A SUBSET  
OF HUMAN CD3<sup>-</sup>CD16<sup>+</sup> NATURAL KILLER CELLS  
Role in Cell Activation and Regulation of Cytolytic Function

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Peripheral blood large granular lymphocytes (LGL)<sup>1</sup> are known as cells able to spontaneously lyse certain tumor target cells through a non-MHC-restricted type of recognition (NK activity) (1-5). LGL are phenotypically distinguishable from T or B lymphocytes since they lack CD3/TCR complex or surface Ig (6, 7). In addition, they express cell surface markers such as CD16 and CD56 (NKH1, Leu-19) (8-10). The range of tumor cell recognition, as well as the intensity of killing, was shown to highly increase after culture of NK cells in IL-2-containing media (lymphokine-activated killer activity) (11-14). Recently, we also provided evidence that (at least some) CD3<sup>-</sup> cells mediate specific lysis of allogeneic cells after activation in mixed lymphocyte culture (15, 16).

Triggering of the lytic machinery in CD3<sup>-</sup>CD16<sup>+</sup> lymphocytes is mediated not only by interaction with tumor target cells but also by mitogens such as PHA (17). As recently shown, a similar effect can be induced by appropriate mAbs directed to cell surface molecules, including CD2 and CD16 (FcγR type III) (18-20). The study of surface molecules able to mediate CD3<sup>-</sup>CD16<sup>+</sup> lymphocyte activation (leading to signal transduction and triggering of the lytic machinery) may be important to the definition of receptor structures involved in tumor cell lysis (21, 22). In the present study, by the use of the GL183 mAb, we identified a novel 58 (55)-kD surface molecule that is expressed on a subset of resting or activated CD16<sup>+</sup> and CD56<sup>+</sup> cells. The GL183 mAb allows the unambiguous identification of two phenotypically distinct NK subsets, thus providing clear evidence for human NK cell heterogeneity. Moreover, interaction of CD3<sup>-</sup>CD16<sup>+</sup> cells with the GL183 mAb results

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<sup>1</sup> Abbreviations used in this paper: LGL, large granular lymphocytes; NEPHGE, nonequilibrium pH gradient electrophoresis.

in NK cell activation characterized by  $[Ca^{2+}]_i$  increments and induction of the cell's functional programs. Finally, the molecule defined by GL183 mAb appears to play a role in the regulation of the NK cell activation induced via CD2 or CD16 surface molecules.

### Materials and Methods

*Isolation and Culture of CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> 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 (15). To obtain purified GL183<sup>+</sup> cells from PBL, cells were stained with GL183 mAb followed by an isotype-specific (anti-IgG1) rabbit anti-mouse fluoresceinated antiserum (Southern Biotechnology Associates, Inc., Birmingham, AL) as a second reagent. Purified CD16<sup>+</sup> cells were sorted using the KD1 anti-CD16 mAb (19) and a fluoresceinated anti-IgG2a second reagent. The CD3<sup>-</sup>GL183<sup>-</sup> cell subset was obtained by treatment of PBL with anti-CD3 (OKT3) and anti-CD4 (CK79) mAbs followed by complement depletion. The resulting CD3<sup>-</sup>CD4<sup>-</sup> population was then stained with GL183 mAb as described above, and GL183<sup>-</sup> cells were isolated by sterile sorting as previously described (23). Cloning of the various cell subsets was performed under limiting dilution conditions in the presence of irradiated feeder cells and a source of exogenous IL-2 (rIL-2; Cetus Corp., Emeryville, CA) as previously described for both T and NK cells (15, 24). 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, Nurttinger, FRG).

*Production of GL183 mAb.* 5-wk-old male BALB/c mice were immunized with a cell clone termed E57 (surface phenotype: CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>) as previously described (25). The immunization schedule consisted of 2 wk of intravenous injections of  $10^7$  E57 cells. After eight injections, the mice were splenectomized and immune splenocytes were fused with P3U1 myeloma cells (25). The screening of hybridoma supernatants was based on the ability to modulate the cytolytic function of E57 against the human ovarian carcinoma cell line termed IGROV, used as  $^{51}Cr$ -labeled target cells in a 4-h  $^{51}Cr$  release assay. To this end,  $1.5 \times 10^4$  E57 cells were cultured together with  $5 \times 10^3$  IGROV target cells (for a final E/T ratio of 3:1) in the presence of 50  $\mu$ l of culture supernatants derived from the various hybridomas. The assay was performed in V-bottomed microtiter trays in a final volume of 200  $\mu$ l. After 4 h, 100  $\mu$ l 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 (23, 24). According to this screening procedure, a hybridoma, termed GL183, that was able to increase the cytolytic activity of E57 clone against IGROV 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 GL183 mAb (GL183 antigen), as compared with that of CD3, CD4, CD8, CD16, CD56, and CD2 antigens, was performed using two-color fluorescence cytofluorometric analysis, as previously described (9, 17). Cells were stained with GL183 mAb followed by FITC-conjugated (or PE-conjugated) goat anti-mouse IgG1 antibodies (Southern Biotechnology Associates, Inc.) and one of the following reagents: OKT3-PE (IgG2a), Leu-11-FITC (IgM), OKT8-PE (IgG2a), OKT4-PE (IgG2a), Leu-5-FITC (IgG2a), and NKH1 (IgM), followed by appropriate anti-IgM PE antibodies. In some experiments, FITC-conjugated GL183 mAb was used for double fluorescence analysis. OKT3, OKT8, and OKT4 mAbs were purchased from Ortho Pharmaceutical, Raritan, NJ, whereas Leu-11, Leu-5, and Leu-19 were purchased from Becton Dickinson & Co., Basel, Switzerland. The NKH1 mAb was kindly provided by Dr. Thierry Hercend, Institut Gustave Roussy, Villejuif, France. Other antibodies used for these analysis were MAR21 (anti-CD7) and MAR199 (anti-HLA-DR).

*Characterization of the GL183 Molecule.* Cloned GL183<sup>+</sup> cells were washed five times in cold RPMI 1640, twice in PBS, and their surface was 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 and precleared three times with 20  $\mu$ l of packed protein A-Sepharose beads for 2 h under rotation. Lysates were then incubated for 2 h with 200  $\mu$ l of GL183 culture supernatant. 20  $\mu$ l of packed protein A-Sepharose beads was then added, and samples were incubated overnight at 4°C under rotation. The immunoprecipitate was eluted from protein A-Sepharose by boiling for 5 min in buffer containing 1% SDS in the presence or absence of 5% 2-ME and analyzed on 11 or 8% discontinuous SDS-polyacrylamide gels (27). The nonequilibrium pH gradient electrophoresis (NEPHGE) was carried out using pH 3.5–10 ampholines, followed by 11% SDS-PAGE gels for size separation, as described (28). The iodinated sample was applied at the acidic end.

**Functional Analysis of CD3<sup>-</sup>CD16<sup>+</sup> Clones.** The cytolytic activity of the various clones was tested in a 4-h <sup>51</sup>Cr-release assay, as described above. In all instances, target cells were used at a concentration of 5 × 10<sup>4</sup>/ml and were represented by either human or murine tumor cell lines, as described in Results. The E/T ratios ranged from 10:1 to 0.5:1, as indicated. After titration, DEAE-Sephacel-purified GL183 mAb was used in most experiments at a concentration of 0.2 ng/ml resuspended in 50  $\mu$ l of medium. The other mAbs, including VD4 (anti-CD16), KD1 (anti-CD16), JT3A (anti-CD3), MAR206 (anti-CD2), and CK90 (anti-LFA1), were used at doses ranging from 2 to 0.2 ng/ml, depending upon preliminary titration experiments. In most cytolytic experiments, GL183 mAb and/or the above-mentioned mAbs were added at the onset of the culture together with effector and target cells. In other experiments, GL183 mAb was added at different intervals during the cytolytic test, as indicated in the figure. In this case, the other mAbs were added at the onset of the cytolytic test. The determination of TNF- $\alpha$  release by cloned cells was performed on cell-free supernatants obtained from clones stimulated for 24 h by either PMA alone (0.5 ng/ml) or in combination with either PHA (0.025% [vol/vol] of the stock solution), KD1 (anti-CD16), or GL183 mAb (1 ng/ml).

The enzyme immunoassay used for the quantitative determination of TNF- $\alpha$  was purchased from T Cell Sciences, Inc., Cambridge, MA. The sensitivity of the test ranged from 10 to 1,200 pg/ml.

**Determination of Free Cytoplasmic Ca<sup>2+</sup> Concentration.** Measurements of [Ca<sup>2+</sup>]<sub>i</sub> were performed as previously described (17). Briefly, GL183<sup>+</sup> cloned cells were loaded with acetoximethyl ester of Fura-2 (1  $\mu$ M final concentration), and the fluorescence of the cellular suspension (10<sup>6</sup>/ml) was monitored with a spectrofluorimeter (LS-5; Perkin-Elmer Corp., Pomona, CA) using a 2-ml quartz cuvette. The cell suspension was excited at 340–380 nm and fluorescence measured at 510 nm. 5-nm slit widths were used for both excitation and emission. All measurements were performed at 37°C using a thermostatically controlled cuvette holder and stirring apparatus. [Ca<sup>2+</sup>]<sub>i</sub> were calculated by the method of Grynkiewicz et al. (29). In the Ca<sup>2+</sup> depletion experiments, EGTA (final concentration, 10 mM) was added to the cellular suspension from a 500-mM stock (pH 7.4) 1–10 min before the addition of the stimulatory mAb.

**Preparation of F(ab)<sub>2</sub> Fragments of GL183 mAb.** 2 ml of DEAE-Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden) -purified IgG1 GL183 mAb (1 mg/ml) was dialyzed overnight against acetate buffer (0.1 M, pH 3.8). Next, the mAb was digested with 50  $\mu$ g of pepsin (Worthington Biochemical Corp., Freehold, NJ) for 6 h at 37°C, after which the digestion was stopped with 3 M Tris-HCl, pH 8.6, and by placing the reaction mixture on ice. This mixture was dialyzed overnight against 50 mM Tris-HCl, pH 8, and applied to a 10-ml DEAE-Sephacel column. 1-ml fractions were collected and assayed spectrophotometrically at 280 nm and by 7 and 11% SDS-PAGE under reducing and nonreducing conditions, respectively.

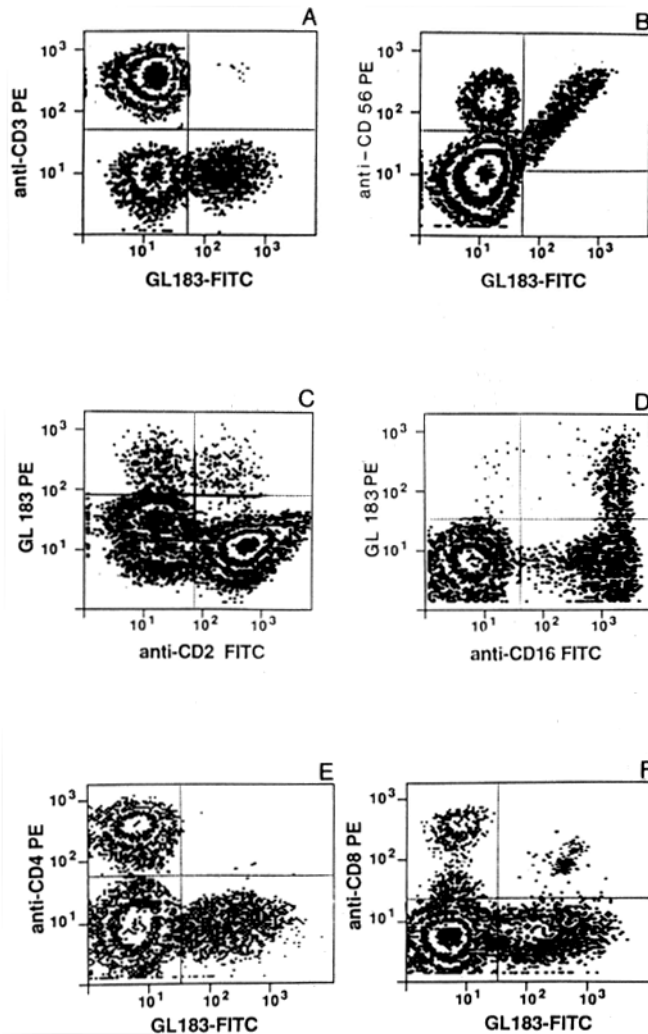
## Results

A lymphocyte clone termed E57 (surface phenotype CD3<sup>-</sup>, CD2<sup>+</sup>, CD7<sup>+</sup>, CD16<sup>+</sup>, CD56<sup>+</sup>) derived from peripheral blood CD3<sup>-</sup> lymphocytes was used for mice immunization. Clone E57, like most CD3<sup>-</sup> lymphocyte clones, displayed cytolytic activity against NK-sensitive (K562) and NK-resistant (IGROV, P815, Raji, M12, P3U1) target cells. The hybridoma supernatants were screened for their ability

to modulate the cytolytic activity of clone E57 against the human ovarian carcinoma target cells termed IGROV. A mAb (termed GL183) that displayed the ability to enhance the cytolytic activity of the immunizing clone was selected.

**Distribution of GL183 Antigen.** Immunofluorescence analysis on PBL revealed that cells reactive with GL183 mAb ranged between 2 and 12% (mean 6.5%) in 10 different donors analyzed.

To comparatively analyze the expression of GL183 antigen with that of other lymphocyte surface markers, further double fluorescence analysis was performed (Fig. 1). When PBL were stained simultaneously with GL183 and anti-CD56 mAbs (Fig. 1 *B*), all GL183<sup>+</sup> cells were also reactive with anti-CD56 (NKH1) mAb. However, only a fraction of the CD56<sup>+</sup> cell population reacted with GL183 mAb; in a series of five experiments, the percent of CD56<sup>+</sup> cells reactive with GL183 mAb ranged between 20 and 55%. Similar figures were obtained with anti-CD16 mAbs. Thus,



**FIGURE 1.** Distribution of GL183<sup>+</sup> cells in PBL. PBL were stained with PE-OKT3 (anti-CD3) and FITC-GL183 (*A*) or with PE-anti-CD56 and FITC-GL183 (*B*). (*C* and *D*) Cells were stained with PE-GL183 and FITC-Leu-5 (anti-CD2) or FITC-Leu-11 (anti-CD16), respectively. (*E* and *F*) Cells were stained with FITC-GL183 and PE-OKT4 (anti-CD4) or PE-Leu-2a (anti-CD8), respectively. 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 simultaneously stained in red and green in *A* were <0.3%, whereas in *B*, they represented 12.2%. In *C*, double fluorescent cells were 5.1%, whereas cells stained in red only (GL183<sup>+</sup>, CD2<sup>-</sup>) were 6.9%. In *D*, double fluorescent cells were 12%, whereas cells stained in green only (CD16<sup>+</sup>, GL183<sup>-</sup>) were 19%, and cells stained in red only (GL183<sup>+</sup>, CD16<sup>-</sup>) were <0.3%. In *E*, double fluorescent cells were <0.3%, whereas in *F*, they were 3.6%. This donor was selected for double fluorescence analysis because of the high numbers of CD16<sup>+</sup>, CD56<sup>+</sup> (~30%) cells present in peripheral blood.

GL183<sup>+</sup> cells were mostly confined into the CD16<sup>+</sup> population (Fig. 1 D), however, they represented only a fraction (ranging from 30 to 70% in four different individuals) of these cells. On the other hand, >95% of GL183<sup>+</sup> cells did not react with anti-CD3 mAbs (Fig. 1 A). It should be stressed, however, that small proportions were consistently CD3<sup>+</sup>. In addition, all GL183<sup>+</sup> cells expressed CD7 antigen (not shown), whereas they were heterogeneous with respect to CD2 antigen expression (Fig. 1 C). In Fig. 1, E and F, the reactivity of GL183 mAb is compared with that of anti-CD4 or anti-CD8 mAbs. While no GL183<sup>+</sup> cells coexpressing CD4 antigen could be detected (Fig. 1, E), a minor subset of GL183<sup>+</sup> cells consistently expressed low amounts of CD8 antigen (CD8 dim; Fig. 1 F). Therefore, expression of GL183 surface antigen appears to define a subset of CD56<sup>+</sup>CD16<sup>+</sup> cells. This concept was further substantiated by the analysis of different CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> polyclonal cell lines cultured in IL-2. Consistently, only a proportion of these cell populations reacted with GL183 mAb. GL183 mAb did not react with normal or EBV-transformed B lymphocytes, resting or activated monocytes, and granulocytes. In addition, all the tumor cell lines of T, B, or monocytic origin analyzed were GL183<sup>-</sup>. We can thus conclude that GL183 antigen expression defines a subset of CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> cells present in both fresh and cultured CD16<sup>+</sup>CD3<sup>-</sup> cell populations.

*Isolation and Cloning of GL183<sup>+</sup> Lymphocytes.* Given the bimodal distribution of GL183 antigen expression in PBL, GL183<sup>+</sup> cells could be easily separated by cell sorting and cloned under limiting dilution conditions (23). In parallel experiments, either CD16<sup>+</sup> lymphocytes (purified by cell sorting) or CD3<sup>-</sup>GL183<sup>-</sup> lymphocytes (obtained by treatment of PBL with anti-CD3 mAb and complement, followed by FACS sorting of GL183<sup>-</sup> cells) were cloned for comparison under the same culture conditions. Cloning efficiencies of CD16<sup>+</sup> or GL183<sup>+</sup> cell populations were similar in several experiments (and ranged between 1/3 and 1/10 cells). In addition, the majority of the clones yielded cell numbers sufficient for surface phenotype analysis and functional characterization. All the clones obtained from GL183<sup>+</sup>-sorted cells (>500 GL183<sup>+</sup> clones have been analyzed) maintained the GL183<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup> surface phenotype. The majority were CD3<sup>-</sup>CD2<sup>+</sup>4<sup>-</sup>8<sup>-</sup>; some clones, however, did not express detectable surface CD2 antigens (17). Clones expressing CD8 antigen represented ~20% of all GL183<sup>+</sup> clones, while only one (F6.1) reacted with anti-CD4 mAb. It should be stressed that the intensity of staining with anti-CD8 or anti-CD4 mAbs in these CD3<sup>-</sup>GL183<sup>+</sup> clones was rather weak in comparison with that of CD3<sup>+</sup>/TCR- $\alpha/\beta$ <sup>+</sup> clones. Unfrequently, (~1%) GL183<sup>+</sup> clones expressed surface CD3 molecules. Among five different GL183<sup>+</sup>CD3<sup>+</sup> clones analyzed so far, only one expressed TCR- $\gamma/\delta$ , while the remaining were TCR- $\alpha/\beta$ <sup>+</sup>, as suggested by the lack of reactivity with anti-TCR- $\gamma/\delta$  mAb (30) and by the reactivity with WT31 mAb. Interestingly, all these GL183<sup>+</sup>CD3<sup>+</sup>WT31<sup>+</sup> clones were CD8<sup>+</sup>, and also expressed antigens such as CD16, CD56, and CD11b, which are typical of NK cells (but infrequent in CD3<sup>+</sup> cells). Phenotypic analysis of clones derived from purified CD16<sup>+</sup> lymphocytes revealed that approximately one in three expressed GL183 surface antigens. In these experiments, the proportion of CD16<sup>+</sup> clones expressing GL183 antigen was similar to that of CD16<sup>+</sup> cells that were GL183<sup>+</sup> in the starting PBL population. Finally, clones derived from CD3<sup>-</sup>GL183<sup>-</sup> lymphocytes expressed the CD3<sup>-</sup>GL183<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup> surface phenotype, thus indicating that GL183 antigen is not acquired in culture by GL183<sup>-</sup> precursors.

The analysis of the distribution of GL183 antigen already suggested that it was distinct from other known surface antigens expressed by CD3<sup>-</sup> cells; still, the possibility existed that surface molecules carrying GL183 determinant could be associated, in some CD3<sup>-</sup> cells, to other known surface molecules. To analyze this point, we performed mAb-induced antigen capping experiments on GL183<sup>+</sup> clones. Thus, we analyzed whether capping of GL183 molecules resulted in cocapping of NK cell-related surface molecules recognized by anti-CD16, anti-CD56, NKH2, and Leu-7 mAbs. Since the binding of these mAbs was unaffected by capping of the GL183 molecules, we can conclude that the GL183 antigen is not physically associated to molecules recognized by anti-CD16, anti-CD56, NKH2, or Leu-7 mAbs (not shown).

**Biochemical Characterization of Proteins Recognized by GL183 mAb.** We next analyzed the biochemical characteristics of the surface antigen immunoprecipitated by GL183 mAb from a series of <sup>125</sup>I surface-labeled CD3<sup>-</sup>GL183<sup>+</sup> clones. Molecules immunoprecipitated by GL183 mAb were analyzed by SDS-PAGE under both reducing and nonreducing conditions (Fig. 2). It can be seen that the material precipitated by GL183 mAb in different clones was represented either by a 58-kD band or by a broad 55–58-kD band. In some experiments, the 55–58-kD component revealed the presence of two distinct bands. In 9 of 13 clones analyzed, surface molecules precipitated by GL183 mAb were represented by the 55–58-kD proteins, whereas four displayed the form with the 58-kD band only.

To better characterize the molecules reactive with the GL183 mAb in the two different

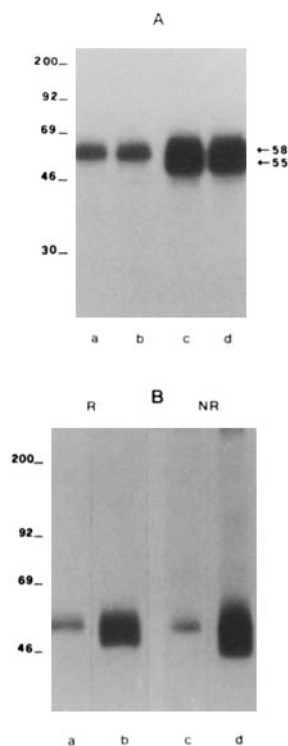


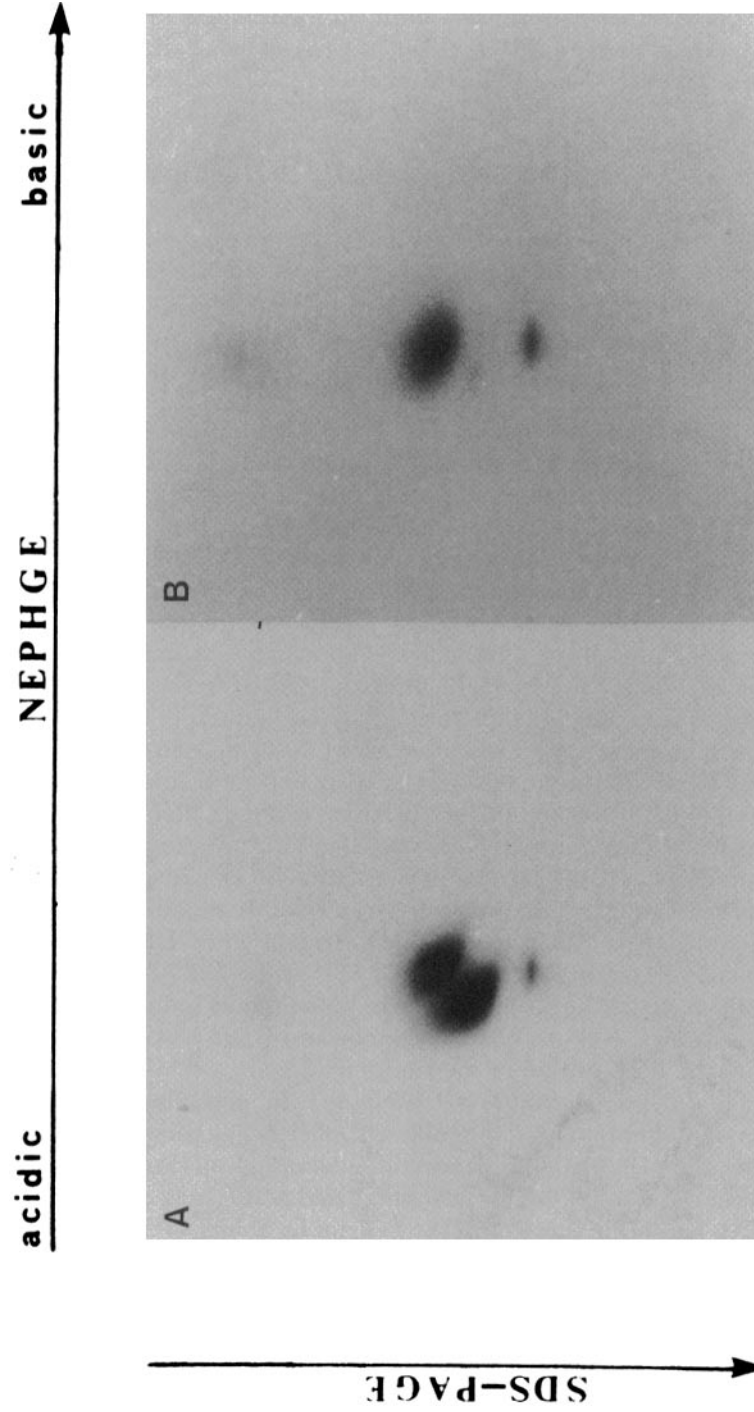
FIGURE 2. SDS-PAGE analysis of surface molecules immunoprecipitated by GL183 mAb from CD56<sup>+</sup>, CD16<sup>+</sup>, GL183<sup>+</sup> clones. (A) Cells ( $\sim 5 \times 10^6$ ) from clones F6.1 (lane a), CA9 (lane b), F25.58 (lane c), and F25.12 (lane d) were surface labeled with <sup>125</sup>I using the lactoperoxidase technique, and lysed in buffer containing 1% NP-40. Immunoprecipitation was performed by GL183 mAb coupled to protein A-Sepharose beads. SDS-PAGE analysis was performed under reducing conditions using 11% acrylamide gels. (B) Cells from clones F6.1 (lanes a and c) and F25.12 (lanes b and d) were treated as above and finally run under reducing (lanes a and b) or nonreducing (lanes c and d) conditions using 8% acrylamide gels.

types of clones, we further performed two-dimensional PAGE analysis. As shown in Fig. 3, the material precipitated by clone F6.1 (*B*) was characterized by a single acidic spot corresponding to the 58-kD band observed in SDS-PAGE. On the contrary, GL183 proteins immunoprecipitated by clone F25.58 (Fig. 3 *A*) were resolved in two distinct spots showing slightly different PI. The apparent molecular mass of these spots corresponded to the 55–58-kD bands detected in SDS-PAGE analysis. More importantly, in two-dimensional PAGE, the spot with higher molecular mass (58 kD) immunoprecipitated from clone F25.58 migrated at the same isoelectric point as the single spot immunoprecipitated from clone F6.1. These data suggest that the surface molecules immunoprecipitated by GL183 mAb are composed of a 58-kD molecule that can be associated (as in clone F25.58) to an additional molecule of 55 kD.

*Antitumor Cytolytic Activity and TNF- $\alpha$  Production by GL183<sup>+</sup> and GL183<sup>-</sup> Clones.* Next, we analyzed whether GL183<sup>+</sup> clones differed from GL183<sup>-</sup> ones with respect to functional capabilities such as antitumor cytolytic activity and TNF- $\alpha$  production (31). To this end, a large number of GL183<sup>+</sup> or GL183<sup>-</sup> clones were analyzed for their cytolytic activity against two different NK-resistant tumor target cells represented by the human ovarian carcinoma cell line IGROV and by the murine mastocytoma P815. Fig. 4 shows the cytolytic activity of 20 representative GL183<sup>+</sup> clones, as compared with that of 20 GL183<sup>-</sup> clones tested at an E/T cell ratio of 1:1. It is evident that, on average, GL183<sup>+</sup> clones lysed these tumor target cells less efficiently than GL183<sup>-</sup> ones. Although not shown, when analyzed at a higher E/T ratio (i.e., 5:1), most GL183<sup>+</sup> clones efficiently lysed both types of target cells. In most instances, approximately fivefold more effector cells were required for GL183<sup>+</sup> clones in order to obtain the same cytolytic effect detected with GL183<sup>-</sup> clones.

Next, the ability of GL183<sup>+</sup> or GL183<sup>-</sup> clones to produce TNF- $\alpha$  after cell stimulation was analyzed. Stimuli were represented by PHA, or by anti-CD16 or GL183 mAbs in the presence of 0.5% ng/ml of PMA. Cells ( $10^6$  cloned cells/ml) were cultured in the various culture conditions for 24 h, and culture supernatants were tested for the presence of TNF- $\alpha$ . As shown in Table I, PHA or anti-CD16 (in the presence of PMA) induced release of TNF by both GL183<sup>+</sup> and GL183<sup>-</sup> clones. More importantly, GL183 mAb (in the presence of PMA) consistently induced TNF production by GL183<sup>+</sup> clones. Neither PMA alone nor anti-CD16 or GL183 mAb alone induced significant TNF release in the clones analyzed.

*Enhancement of Cytolytic Activity by GL183 mAb.* In preliminary experiments, GL183<sup>+</sup> clones were shown to lyse (with different efficiency) a panel of human tumor target cells, including U937 (myelomonocytic leukemia), Raji (Burkitt lymphoma), IGROV (ovarian carcinoma), M14 (melanoma), and A549 (lung carcinoma). Since the GL183 mAb had been selected on the basis of its ability to modulate (i.e., increase) the cytolytic activity of the GL183<sup>+</sup> clone E57 against IGROV target cells, it was conceivable that a similar effect could be exerted also on the lysis of other target cells. In Fig. 5, the cytolytic activity of a representative GL183<sup>+</sup> clone (CA9), either in the presence or in the absence of the mAb, is shown. It can be seen that in the absence of GL183 mAb, the various target cells displayed different degrees of sensitivity to lysis. More importantly, in all instances, addition of GL183 mAb resulted in a marked enhancement of the cytolytic effect. Thus, for example, M14 and A549 target cells at an E/T ratio of 3:1 were poorly lysed by CA9 cells alone,



by NEPHGE using the second dimension 11% acrylamide gels for SDS-PAGE analysis.

FIGURE 3. NEPHGE analysis of molecules immunoprecipitated by GL183 mAb from two distinct CD56<sup>+</sup>, CD16<sup>+</sup>, GL183<sup>+</sup> clones. Clones F6.1 (B) and clone F25.12 (A) were treated as described in Fig. 2. Samples were analyzed



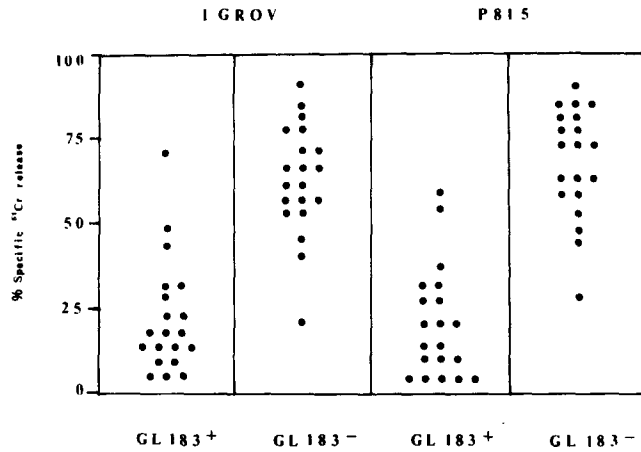


FIGURE 4. Cytolytic activity of GL183<sup>+</sup> and GL183<sup>-</sup> clones against human or murine NK-resistant tumor target cells. 20 GL183<sup>+</sup> clones and 20 GL183<sup>-</sup> clones were assessed for cytolytic activity at an E/T ratio of 1:1. <sup>51</sup>Cr-labeled target cells were represented by the human ovarian carcinoma cell line termed IGROV and by the murine mastocytoma cell line termed P815. Percent specific lysis was evaluated in a 4-h <sup>51</sup>Cr assay.

however, >50% lysis was achieved at the same E/T ratio upon addition of GL183 mAb. Also, in the case of U937 target cells that were, per se, more susceptible to lysis by CA9 effector cells, a marked increment of the cytolytic activity was detectable in the presence of the mAb (Fig. 5). Thus, in the presence of GL183 mAb, the same degree of cytolysis could be achieved with 10-fold less effector cells. Since none of the target cells expressed GL183 antigen, the mAb-induced enhancement of cytolytic activity was not consequent to an antibody-dependent cellular cytotoxicity mechanism, but it was likely to reflect the triggering of the effector cells by the antibody. Similar results were obtained with all (>20) GL183<sup>+</sup> clones analyzed against the same panel of target cells.

Unlike other target cells, K562 were highly susceptible to cytolysis by the CA9 clone (and by all of the GL183<sup>+</sup> clones analyzed). Addition of GL183 mAb did not augment the cytolytic activity against these target cells, but rather, it had a minor

TABLE I  
TNF Production by GL183<sup>+</sup> and GL183<sup>-</sup> Clones

Clone	GL183 antigen expression	Stimuli				
		None	PMA*	PMA + PHA	Anti-CD16 + PMA	GL183 + PMA
		<i>pg/ml</i>				
134T5	-	10	20	350	270	15
134T53	-	20	40	140	150	40
72F4	-	20	40	320	220	40
134T49	+	30	40	300	900	450
78F2	+	40	70	>1,200	>1,200	390
74F32	+	<10	15	350	300	200

2 × 10<sup>5</sup> cloned CD3<sup>-</sup>CD16<sup>+</sup> cells (either GL183<sup>+</sup> or GL183<sup>-</sup>) were cultured in triplicate microwells (final volume of 200 μl/well) for 24 h at 37°C in the presence of one or another of the various stimuli. Supernatant fluids were collected and tested for TNF content using an enzyme immunoassay as described.

\* PMA was used at 0.5 ng/ml in all instances. PHA was used at a final dilution of 1:400 of the stock solution; anti-CD16 and GL183 mAbs were used at 1 ng/ml.

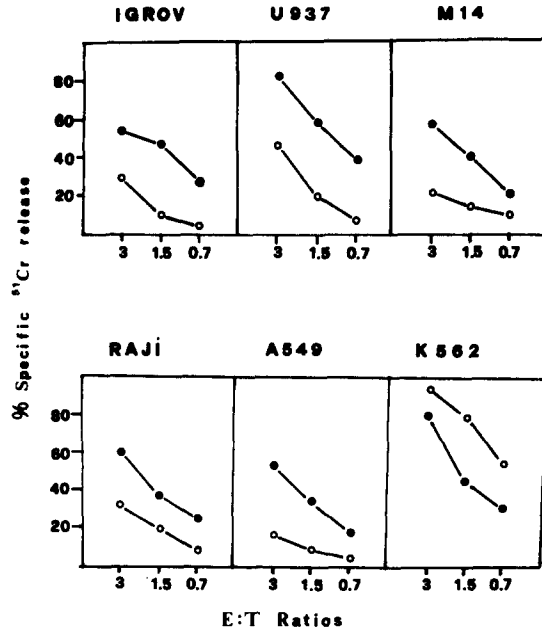


FIGURE 5. Enhancement of cytolytic activity by GL183 mAb. Clone CA9 (surface phenotype: CD16<sup>+</sup>, CD3<sup>-</sup>, CD56<sup>+</sup>, GL183<sup>+</sup>, CD2<sup>+</sup>) was tested for cytolytic activity at different E/T cells as indicated. <sup>51</sup>Cr-labeled target cells were represented by human NK-resistant cell lines termed IGROV (ovarian carcinoma), Raji (Burkitt lymphoma), A549 (lung carcinoma), M14 (melanoma), U937 (myelomonocytic leukemia), and by the NK-sensitive cell line K562. GL183 mAb (5 ng/ml) was added to the cytolytic test at the onset of the 4-h <sup>51</sup>Cr release assay. Cytolytic activity in the presence (●) or in the absence (○) of GL183 mAb.

inhibitory effect. On the other hand, it should be noted that, similar to GL183 mAb, other stimuli, such as PHA or anti-CD16 mAb, were unable to enhance the lysis of K562 cells mediated by CD3<sup>-</sup>CD16<sup>+</sup> clones (not shown).

*GL183 mAb Inhibits the Cytolytic Activity of CD3<sup>-</sup>GL183<sup>+</sup> Clones against Certain Murine Target Cells.* The Fcγ receptor-positive murine mastocytoma P815 has been extensively used in previous studies for mAb-dependent redirected killing assays using either CD3<sup>+</sup> or CD3<sup>-</sup> effector cells and mAbs capable of triggering their cytolytic functions (19, 30). Therefore, this cytolytic assay was used to analyze whether GL183 mAb could enhance the cytolytic activity of GL183<sup>+</sup> clones. The data obtained with the clone CA9, representative of 16 CD3<sup>-</sup>GL183<sup>+</sup> clones, are shown in Fig. 6. It is evident that GL183<sup>+</sup> cells were able to lyse (to some extent) P815 cells in the absence of added stimuli. Addition of GL183 mAb, in this case, resulted in a strong

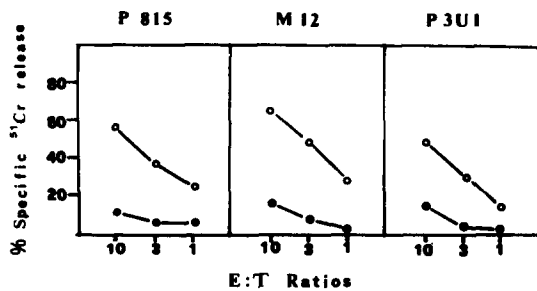


FIGURE 6. Cytolytic activity of GL183<sup>+</sup> clones against murine target cells is inhibited by GL183 mAb. The same clone CA9 used in the experiments reported in Fig. 5 was tested for cytolytic activity against <sup>51</sup>Cr-labeled target cells in a 4-h <sup>51</sup>Cr release assay. Target cells were represented by the murine mastocytoma cell line termed P815, by the murine B lymphoma M12, and by the murine myeloma P3U1. GL183 mAb (0.2 ng/ml) was added at the onset of the cytolytic test. Cytolytic activity in the presence (●) or in the absence (○) of GL183 mAb.

inhibition of cytolysis. An inhibitory activity of GL183 mAb on all (>50) GL183<sup>+</sup> clones tested could also be detected in cytolytic assays in which target cells were represented by the murine B lymphoma M12 or the murine myeloma P3U1 (Fig. 6). Thus, it appears that, in cytolytic assays against these murine target cells, GL183 mAb results in effects that are opposite those observed with human target cells. Perhaps more importantly, the effect of GL183 mAb was antithetical to that previously described for other stimuli, including PHA, anti-CD16 mAb, and anti-CD2 mAb (18, 19). Thus, as shown in Table II, PHA, anti-CD16 mAb, and anti-CD2 mAb strongly enhanced the cytolytic activity against P815 target cells mediated by CD3<sup>-</sup>GL183<sup>+</sup> or CD3<sup>-</sup>GL183<sup>-</sup> clones. Note that, in agreement with previous data from our and other laboratories (18, 19), single stimulatory anti-CD2 mAbs could trigger the lytic machinery of CD3<sup>-</sup>CD16<sup>+</sup> cells (Table II). It is evident that GL183 mAb had, instead, a marked inhibitory effect on all five representative GL183<sup>+</sup> clones reported. On the other hand, GL183 mAb had no inhibitory effect on the two CD3<sup>-</sup>GL183<sup>-</sup> clones used as control. Note also that the GL183<sup>+</sup> clones reported in Table II were representative of the various phenotypic groups described above for the CD3<sup>-</sup>GL183<sup>+</sup> peripheral blood cells. Thus, the expression or lack of expression of surface antigens such as CD2, CD8, or CD4 does not appear to influence the susceptibility to inhibition by GL183 mAb.

*Inhibitory Effect of GL183 mAb on the Redirected Killing Induced by PHA, Anti-CD16, or Anti-CD2 mAbs.* Since the effect of GL183 mAb on the cytolytic activity against P815 target cells was opposite that induced by other stimuli (including PHA, anti-CD2 mAb, and anti-CD16 mAb), this cytolytic assay was applied to investigate whether a functional relationship existed among different stimuli acting on CD3<sup>-</sup>GL183<sup>+</sup> cells. To this end, GL183 mAb has been added to the cytolytic assay in combination with one or another stimulus. Table III shows the cytolytic activity of four represen-

TABLE II  
Effect of mAbs on the Cytolytic Activity of CD3<sup>-</sup> CD16<sup>+</sup> CD56<sup>+</sup>  
Clones against P815 Target Cells

Clone*	GL183 antigen expression	Stimuli added to the cytolytic test					
		None	PHA	Anti- CD16	Anti- CD2	GL183	Anti- LFA-1
F6.1 <sup>†</sup>	+	48 <sup>§</sup>	79	88	67	6	14
F25.58	+	54	85	100	71	11	19
F25.12	+	33	72	78	35	4	8
F12.8	+	29	64	73	29	5	7
F25.52	+	61	92	98	84	16	27
3M30.2	-	46	85	99	ND	48	12
3M12.5	-	57	88	100	80	55	15

\* The first five clones were GL183<sup>+</sup>, whereas the two remaining clones were GL183<sup>-</sup>. All the clones were CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>. Clone F6.1 was CD4<sup>+</sup>, clones F25.58 and F25.52 were CD8<sup>+</sup>, whereas the remaining were CD4<sup>-</sup>CD8<sup>-</sup>. All the clones expressed CD2 surface antigens, with the exception of F25.12 and F12.8.

<sup>†</sup> GL183<sup>+</sup> clones were tested at an E/T ratio of 5:1. The two GL183<sup>-</sup> clones, 3M30.2 and 3M12.5, were tested at an E/T ratio of 1:1 in view of their higher "spontaneous" cytolytic activity against P815.

<sup>§</sup> Results are expressed as percent 51Cr release.

TABLE III  
*GL183 mAbs Inhibit Spontaneous and Redirected Killing Induced by PHA or Anti-CD16 and Anti-CD2 mAbs*

Clone <sup>§</sup>	GL183 antigen expression	Stimuli added to the cytolytic test			
		None	PHA	Anti-CD16	Anti-CD2
F25.52	+	13 (54) <sup>§</sup>	26 (89)	28 (98)	15 (79)
F25.58	+	6 (46)	11 (82)	21 (94)	10 (73)
F6.1	+	9 (41)	14 (75)	16 (92)	8 (71)
F12.8	+	5 (23)	8 (68)	15 (72)	8 (23)
3M12.5	-	57 (60)	92 (91)	103 (100)	74 (86)

\* The different stimuli and GL183 mAbs were added at the onset of the 4-h <sup>51</sup>Cr release assay. The anti-CD16 mAb was represented by the VD4 mAb (IgG1), whereas the anti-CD2 mAb was the MAR206 mAb (IgG1).

† Cloned cells were assessed for cytolytic activity against P815 target cells at an E/T ratio of 5:1, with the exception of clone 3M12.5 (GL183<sup>-</sup>), which was used at an E/T ratio of 1:1.

§ Data are expressed as percent specific <sup>51</sup>Cr release. The numbers in parentheses indicate the values in the absence of GL183 mAb, whereas data out of parentheses indicate the values of the presence of GL183 mAb.

tative GL183<sup>+</sup> clones in the presence or absence of various stimuli. It is evident that GL183 mAb inhibited the lysis of P815 cells not only in the absence of added stimuli (referred to as "spontaneous" killing), but also when stimulatory mAbs or PHA were added to the cytolytic assay (referred to as "redirected" killing). Note also that, in the presence of GL183 mAb, the levels of target cell lysis in experiments of redirected killing were lower than the spontaneous lysis (independent upon the stimulus used). For example, clone F25.52 at an E/T ratio of 5:1 had a spontaneous lysis of 54%, whereas in the presence of anti-CD16 mAb, lysis reached 98%. Addition of GL183 mAb at the onset of the cytolytic assay reduced the spontaneous killing to 13% (from 54%), and the redirected killing mediated by anti-CD16 mAb to 28% (from 98%). Maximal inhibitory effect on the spontaneous killing was still observed with amounts of purified GL183 mAb of 0.1 ng/ml, whereas >50% inhibition was detectable at 0.01 ng/ml (Fig. 7). Thus, maximal inhibition could be obtained with ~100-fold less mAb, as compared with the amount required for maximal stimulation (10 ng/ml).

We further analyzed the inhibitory effect achieved upon addition of GL183 mAb at different time intervals during the cytolytic test. In this assay, clone F25.58 (surface phenotype: GL183<sup>+</sup>CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>CD2<sup>+</sup>CD8<sup>+</sup>) was tested for cytolytic activity against P815 target cells either in the presence or in the absence of various stimuli. As shown in Fig. 8, addition of GL183 mAb strongly inhibited cytotoxicity in both spontaneous and redirected cytotoxic assay when added during the first hour of the assay. Approximately 50% inhibition was still detectable within 2 h from the onset of the assay. The inhibitory activity of GL183 mAb in antibody-induced redirected killing could be due, at least in part, to competition for antibody binding to FcγR expressed on P815 target cells. However, the mAb MAR 21 (anti-CD7) of the same IgG1 isotype (which reacted with the effector cell surface) had no inhibitory effect (not shown). Moreover, GL183 mAb (IgG1) could also inhibit the cytotoxic activity induced by an anti-CD16 stimulatory mAb of the IgG2A isotype (not

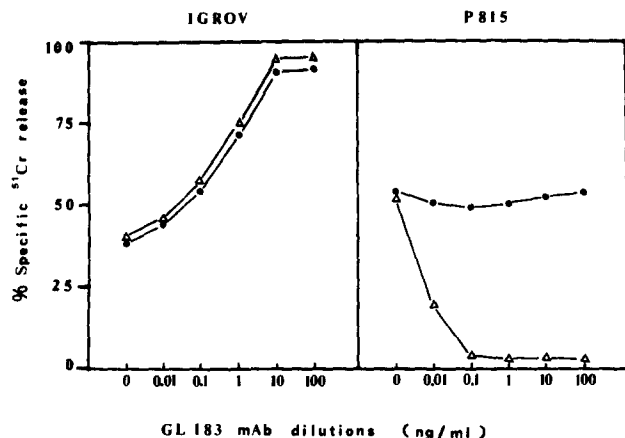


FIGURE 7. Enhancing or inhibitory effect of GL183 mAb or its F(ab)<sub>2</sub> fragments on the cytolytic activity mediated by GL183<sup>+</sup> cells against human or murine tumor cells. Clone PE5 (surface phenotype: GL183<sup>+</sup>, CD3<sup>-</sup>, CD16<sup>+</sup>, CD7<sup>+</sup>, CD2<sup>+</sup>) was tested (at an E/T ratio of 3:1) for cytolytic activity against the human IGROV and the murine P815 cell lines in the presence of graded amounts of purified GL183 mAb (Δ) or GL183 F(ab)<sub>2</sub> fragments (●).

shown). These data indicate that inhibition of mAb-induced cytolytic activity is not due to competition for the FcγR expressed by P815 cells.

Finally, inhibition of cytolysis was not consequent to competitive binding of the various mAbs to the surface antigens expressed on the effector cells. Indeed, anti-CD16 or anti-CD2 mAbs (IgG2a isotype) efficiently bound to effector cells precoated with GL183 (IgG1), as assessed by anti-mouse subclass-specific second reagent (data not shown).

*Effect of F(ab)<sub>2</sub> Fragments of the GL183 mAb on the Cytolytic Activity Mediated by CD3<sup>-</sup>, GL183<sup>+</sup> NK Clones.* Since GL183 mAb was able to modulate the cytolytic function of GL183<sup>+</sup> clones, resulting in either augmentation or inhibition of the cytolytic activity, we analyzed whether both phenomena could also be induced by the F(ab)<sub>2</sub> fragment of the antibody. It has previously been shown that cloned NK cells could be activated with the F(ab)<sub>2</sub> fragments of anti-CD2 mAbs (32). On the other hand, the enhancing effects of anti-CD16 mAbs were confined to mAbs of IgG iso-

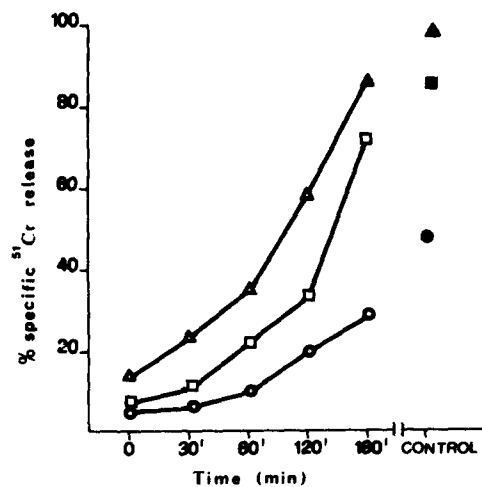


FIGURE 8. Time-course analysis of inhibition of cytolytic activities mediated by GL183 mAb. Clone F25.58 (surface phenotype: GL183<sup>+</sup>, CD3<sup>-</sup>, CD16<sup>+</sup>, CD56<sup>+</sup>, CD2<sup>+</sup>, CD8<sup>+</sup>) was tested (at an E/T ratio of 3:1) for spontaneous and redirected cytolytic activity either in the absence or in the presence of GL183 mAb. This antibody (0.2 ng/ml) was added to the cytolytic assay either at the onset or after different intervals as indicated. Controls included spontaneous killing of P815 cells mediated by clone F25.58 (●) and redirected killing in the presence of anti-CD2 mAb (■) or anti-CD16 mAb (▲). Redirected killing induced by anti-CD16 mAb was tested for inhibition with GL183 mAb (Δ), which was added at different intervals. Redirected killing induced by anti-CD2 mAb tested for inhibition with GL183 mAb (□). Spontaneous killing tested for inhibition with GL183 mAb (○).

type (18, 19), reflecting the need to bind to the Fc $\gamma$ R expressed on target cells. The F(ab')<sub>2</sub> fragments of GL183 mAb maintained the same (enhancing) effects of the whole antibody at all concentration tested (Fig. 7). On the contrary, no inhibitory effect in the cytolytic assay using P815 could be observed, thus, suggesting that, in this case, binding of mAb to surface Fc $\gamma$ R is strictly required to mediate the functional effect. The inhibitory effect of GL183 mAb could be achieved even with minimal amounts of antibody (Fig. 7). Therefore, as a wide range of antibody dilutions have been tested, it is possible to conclude that GL183 F(ab')<sub>2</sub> retains the effect of the entire antibody in enhancing, but not in inhibiting, lysis of appropriate target cells.

*GL183 mAb Induces Ca<sup>2+</sup> Mobilization in GL183<sup>+</sup> Cells.* As shown in previous studies, triggering of CD3<sup>-</sup>CD16<sup>+</sup> cell populations or clones with PHA (17), anti-CD2 mAb (17, 33), or anti-CD16 mAb (34) results in [Ca<sup>2+</sup>]<sub>i</sub> increments. Therefore, we investigated the role of free cytoplasmic Ca<sup>2+</sup> levels in the signal transduction after cell triggering with GL183 mAb. As shown in Fig. 9, when clone PE5 (surface phenotype: GL183<sup>+</sup>CD3<sup>-</sup>CD16<sup>+</sup>CD7<sup>+</sup>CD2<sup>+</sup>CD8<sup>-</sup>) was stimulated with GL183 mAb, a rapid increase of [Ca<sup>2+</sup>]<sub>i</sub> levels followed by a plateau phase could be observed. These [Ca<sup>2+</sup>]<sub>i</sub> increments were similar to those induced by PHA used as positive control. Experiments of Ca<sup>2+</sup> mobilization performed in the presence of EGTA indicated that GL183 mAb, similar to other stimuli such as PHA, anti-CD2 mAb, or anti-CD16 mAb (17, 34), induces a rapid Ca<sup>2+</sup> release from internal stores followed by an influx from the extracellular compartments (not shown).

### Discussion

In the present study, by the use of GL183 mAb, we describe a novel surface molecule expressed by a subset of human CD3<sup>-</sup>CD16<sup>+</sup> (NK) cells. More importantly, the molecules defined by GL183 mAb were shown to be involved in mechanisms of CD3<sup>-</sup> lymphocyte activation. Finally, GL183 molecules appear to exert a regulatory role in the control of cell activation induced by different stimuli, including anti-CD16 mAb, anti-CD2 mAb, and PHA.

Analysis of GL183 antigen distribution in resting PBL demonstrated that the GL183<sup>+</sup> cell population was mostly confined to the CD3<sup>-</sup>sIg<sup>-</sup> cell fraction having the morphological characteristics of LGL. In addition, double fluorescence analysis showed that expression of GL183 surface antigen characterized a subset of CD16<sup>+</sup> or CD56<sup>+</sup> cells. Since these markers are known to be expressed by essentially all NK cells (8-10), GL183 mAb appears to identify a subset of PB NK cells. It is noteworthy that GL183 antigen was also expressed on <1% CD3<sup>+</sup> cells. As shown by the clonal analysis of this very minor subset, these cells were also characterized

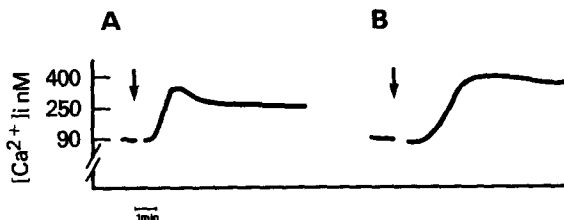


FIGURE 9. GL183 mAb induces increments of [Ca<sup>2+</sup>]<sub>i</sub> in GL183<sup>+</sup> clones. Comparative analysis of [Ca<sup>2+</sup>]<sub>i</sub> increase in the PE5 clone (surface phenotype: GL183<sup>+</sup>, CD3<sup>-</sup>, CD16<sup>+</sup>, CD7<sup>+</sup>, CD2<sup>+</sup>, CD8<sup>-</sup>) after stimulation with GL183 mAb (A) or PHA (B). The stimuli were added in presence of 1 mM extracellular Ca<sup>2+</sup>. Arrows indicate the time of the addition of the stimuli.

by the expression of CD56 and CD16 surface antigens. Thus, CD3<sup>+</sup>GL183<sup>+</sup> cells appear to belong to a previously defined minor subset of CTL characterized by the coexpression of NK cell markers (35).

That expression or lack of expression of GL183 surface antigen represents a stable phenotypic characteristic of NK lymphocytes was clearly demonstrated by the clonal analysis of purified PB-derived GL183<sup>+</sup> or CD3<sup>-</sup>GL183<sup>-</sup> lymphocyte populations. Indeed, all clones (>500) derived from GL183<sup>+</sup> populations expressed GL183 surface molecules. Moreover, in no instances had clones derived from CD3<sup>-</sup>GL183<sup>-</sup> lymphocyte fractions acquired GL183 surface antigen during culture.

Since a certain degree of heterogeneity has been well documented among CD3<sup>-</sup> NK cells, particularly with respect to cytolytic function (36), we investigated whether CD3<sup>-</sup>GL183<sup>+</sup> cells differed from CD3<sup>-</sup>GL183<sup>-</sup> cells in their ability to lyse tumor target cells. Although not shown, freshly isolated peripheral blood CD3<sup>-</sup>GL183<sup>+</sup> and CD3<sup>-</sup>GL183<sup>-</sup> cells displayed similar levels of cytolytic activity against the classical NK-sensitive target K562. Analysis of the two subsets at the clonal level showed again similar cytolytic activities against K562. However, when tested against NK-resistant target cells, GL183<sup>-</sup> clones displayed a higher cytolytic activity than those expressing GL183 surface antigen. On average, GL183<sup>+</sup> cloned cells were approximately fivefold less effective than GL183<sup>-</sup> ones in lysis of NK-resistant tumor target cells. These data support the concept that expression of GL183 antigen discriminates between two distinct subsets of CD3<sup>-</sup> NK cells that, after clonal expansion, mediate different levels of cytolytic activity against NK-resistant tumor cells.

Morphological analysis of GL183<sup>+</sup>CD16<sup>+</sup> and GL183<sup>-</sup>CD16<sup>+</sup> lymphocytes after isolation from peripheral blood or after clonal expansion indicated that both cell types display LGL morphology (data not shown).

GL183 mAb was selected on the basis of its ability to modulate the cytolytic function of CD3<sup>-</sup>CD16<sup>+</sup> lymphocytes clones. The ability of GL183 mAb to enhance target cell lysis was not restricted to the ovarian carcinoma IGROV cells used in the screening of hybridoma supernatants, but was also detectable in a panel of human target cells of different histotype (Fig. 5). Again, an exception was provided by the K562 erythroleukemia target cells. In fact, no enhancement of K562 lysis by GL183<sup>+</sup> clones could be observed either in the presence of GL183 mAb (rather there was a small inhibition) or in the presence of other stimuli such as PHA or anti-CD16 mAb (not shown). The finding that GL183 mAb efficiently triggers GL183<sup>+</sup> effector cells to lyse a number of human tumor targets indicates that GL183 surface molecules are able to mediate cell activation upon binding with specific mAbs. These data are further substantiated by the analysis of TNF- $\alpha$  production and by the study of Ca<sup>2+</sup> mobilization in response to this mAb. Thus, production of TNF was induced by GL183 mAb in the presence of PMA, and levels of [Ca<sup>2+</sup>]<sub>i</sub> increase similar to those observed in the presence of PHA could be obtained by addition of soluble GL183 mAb. Also, CD2 and CD16 surface molecules have been shown to mediate CD3<sup>-</sup>CD16<sup>+</sup> cell activation characterized by Ca<sup>2+</sup> mobilization and triggering of the lytic machinery upon binding of specific mAbs (18-20). However, the distribution of CD2 and CD16 molecules is clearly different from the distribution of GL183 antigen (which is restricted to a smaller cell fraction). In addition, cocapping experiments designed to study whether GL183 determinants were associated to other surface molecules clearly indicated that GL183 antigen is not physically associated to CD16 or CD2 surface molecules.

While GL183 mAb enhanced the cytolytic activity of GL183<sup>+</sup> clones against human tumor cells, an opposite effect was observed against murine target cells. Thus, addition of GL183 mAb sharply inhibited the spontaneous lysis of P815, P3UI, and M12 murine target cells by GL183<sup>+</sup> clones. In addition, while the F(ab')<sub>2</sub> fragments of GL183 mAb could efficiently enhance the lysis of human target cells (whether FcγR<sup>+</sup>, as the U937 cell line, or FcγR<sup>-</sup>, as the IGROV ovarian carcinoma line), they had no inhibitory effect on the cytolysis of the FcγR<sup>+</sup> murine target cells (P815). These results suggest that different mechanisms may be involved in the process of induction-inhibition of cytolysis mediated by GL183 mAb. For example, the different effect induced by GL183 mAb on the lysis of human or murine target cells may reflect differences in the type and/or number of cell-cell interaction molecules involved in the process of binding of effector cells to human or murine target cells. Whatever the molecular basis of these phenomena could be, the inhibitory effect induced by GL183 mAb on the lysis of murine target cells further supports the concept that GL183 molecules are involved in mechanisms of CD3<sup>-</sup> NK cell activation. In addition, in the cytolytic assay against murine P815 cells, GL183 mAb had an effect that was clearly opposite that of other stimuli, including PHA, anti-CD16 mAb, anti-CD2 mAb (19). These data allowed us to analyze the possible functional interactions existing among different pathways of CD3<sup>-</sup> cell activation. We found that GL183 mAb strongly inhibited the target cell lysis also when added in combination with stimulatory mAbs (anti-CD2 or anti-CD16) or PHA. These data suggest that GL183 molecules may exert a regulatory control on other pathways of CD3<sup>-</sup> CD16<sup>+</sup> lymphocyte activation. Although the molecular mechanisms involved in this regulatory control remain to be defined, the downregulation induced by GL183 mAb on other pathways of CD3<sup>-</sup> cell activation is reminiscent of that detectable in CD3<sup>+</sup> cells after mAb-induced modulation of the CD3/TCR molecular complex (37).

Preliminary biochemical characterization of surface molecules immunoprecipitated by GL183 mAb demonstrated that GL183<sup>+</sup> clones may express different forms of GL183 mAb-reacting molecules. Thus, in SDS-PAGE, some clones displayed a single 58-kD chain, whereas in the majority of the clones analyzed, a broad 55–58-kD band could be observed. Two-dimensional PAGE analysis allowed us to identify, within this broad band, two distinct chains displaying slightly different molecular masses and PI. In addition, this type of analysis showed that the 58-kD chains of different clones migrate to identical positions. It is clearly necessary to comparatively analyze the 55- and 58-kD chains in order to determine to what extent they are related one to another. In this context, deglycosylation of isolated chains together with peptide mapping analysis should clarify whether, indeed, the two chains represent different molecular products. So far, no difference in the functional capability (cytolytic activity or TNF production) or in the surface phenotype (i.e., expression or lack of expression of CD2, CD8, or CD4 antigens) has been detected among clones expressing one or another form of GL183-reactive molecules. In the murine system, recent evidence has been provided for the existence of at least two distinct NK subsets based on the reactivity with a mAb termed SW5E6 (38). Thus, SW5E6<sup>+</sup> cells represent ~40–50% of murine peripheral NK cells and ~50% of IL-2-expanded NK cells. In addition, the SW5E6 mAb was shown to immunoprecipitate a 54-kD molecule from the surface of murine NK cells. These data are clearly compatible with those obtained in the human system with the GL183 mAb, with the exception that,



in the mouse, two 54-kD chains are usually bound together by disulphide bonds, whereas the 55-kD molecule recognized by GL183 mAb is found associated to a 58-kD chain in the absence of detectable disulphide bonds. It is possible that GL183 and SWE56 recognize similar types of molecules, although the subsets that are defined may not necessarily display the same functional characteristics (38). A surface molecule involved in NK cell activation has been recently identified on rat LGLs. In this case, however, neither the molecular mass nor the cellular distribution of this molecule was reminiscent of that recognized by GL183 mAb (39).

Taken together, our data suggest that GL183 surface molecules may exert a role in the activation/regulation of the cytolytic function of human NK cells. In view of the restricted subset distribution of GL183 molecules and of their involvement in the activation of this cell subset, it is possible that GL183 molecules may represent a receptor-like structure involved in the cytolytic function of these cells. One may speculate that GL183 could represent a monomorphic "receptor" molecule uniquely expressed by this subset. Alternatively, the antigenic determinant recognized by GL183 mAb may be part of a polymorphic portion of a still undefined molecule expressed by all CD3<sup>-</sup> NK cells. If this holds true, surface structures analogous to GL183 molecules should be expressed also by CD3<sup>-</sup>GL183<sup>-</sup> cells, and mAbs should be identified that are capable of recognizing these molecules. Experiments aimed to identify "functional" surface structures expressed by CD3<sup>-</sup>GL183<sup>-</sup> lymphocytes are in progress in our laboratory.

### Summary

The GL183 mAb was obtained by immunizing BALB/c mice with the E57 clone (CD7<sup>+</sup>CD2<sup>+</sup>CD3<sup>-</sup>CD16<sup>+</sup>CD56<sup>+</sup>) derived from human peripheral blood NK cells. In human peripheral blood, GL183-reactive cells ranged between 2 and 12% (mean 6.5%) in 10 different donors. Double fluorescence and FACS analysis showed that GL183<sup>+</sup> cells were consistently included in the CD56<sup>+</sup> or CD16<sup>+</sup> cell populations. Moreover, since only a fraction of CD56<sup>+</sup> or CD16<sup>+</sup> cells (~40%) coexpressed GL183 surface antigen, reactivity with GL183 mAb appears to define two subsets within the CD3<sup>-</sup> lymphocyte population expressing NK cell markers. Although, the majority of GL183<sup>+</sup> cells were CD3<sup>-</sup>, ~1% expressed CD3 surface antigens. As shown by clonal analysis, these infrequent CD3<sup>+</sup>GL183<sup>+</sup> cells coexpressed CD56 and CD16 antigens. Cloning of CD3<sup>-</sup>GL183<sup>+</sup> or CD3<sup>-</sup>GL183<sup>-</sup> cell populations under limiting dilution conditions yielded clonal progenies that maintained their original surface phenotype. Therefore, expression or lack of expression of GL183 surface antigens represents a stable phenotypic property of a subset of human CD3<sup>-</sup> NK cells. Immunoprecipitation experiments and two-dimensional PAGE analysis indicated that GL183-reactive molecules were represented in different clones either by a single 58-kD chain or, more frequently, by two chains of ~55 and ~58 kD, respectively. Analysis of GL183<sup>+</sup> or GL183<sup>-</sup> NK clones for their ability to lyse human (IGROV I) or murine (P815) tumor target cells indicated that GL183<sup>-</sup> clones were, on average, fivefold more efficient in inducing target cell lysis. GL183<sup>+</sup> and GL183<sup>-</sup> clones produced comparable levels of TNF- $\alpha$  in response to PHA plus PMA or anti-CD16 mAb plus PMA. Importantly, production of TNF- $\alpha$  was also induced by stimulation of GL183<sup>+</sup> clones with GL183 mAb plus PMA. These data indicated that GL183 antigen could mediate cell triggering. This concept was confirmed

by the analysis of  $\text{Ca}^{2+}$  mobilization, as GL183 mAb induced (in GL183<sup>+</sup> clones) increments of  $[\text{Ca}^{2+}]_i$  comparable with those induced by PHA. Moreover, GL183 mAb, or its  $\text{F}(\text{ab}')_2$  fragments, strongly enhanced the cytolytic activity of GL183<sup>+</sup> clones against a panel of human tumor target cells, including U937, Raji, IGROV I, M14, and A549. In contrast, GL183 mAb, but not the  $\text{F}(\text{ab}')_2$  fragments, sharply inhibited the cytolytic activity of the same clones against P815, M12, and P3U1 murine target cells. In this case, the effect of GL183 mAb (inhibition) was opposite that of PHA or of stimulatory anti-CD2 or anti-CD16 mAbs, which consistently enhanced the target cell lysis. When added in combination with one or another of the stimuli above, GL183 mAb sharply inhibited their enhancing effects. These data suggest that GL183 surface molecules may also exert a regulatory role on the human NK cell activation.

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