Existence of Both Inhibitory (p58) and Activatory (p50) Receptors for HLA-C Molecules in Human Natural Killer Cells

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Summary

The natural killer (NK) cell–specific p58 molecules EB6 and GL183 have been shown to represent the putative surface receptors for two distinct groups of human histocompatibility leukocyte antigen (HLA) C alleles. Interaction between p58 receptors and class I molecules expressed on target cells results in inhibition of the NK-mediated cytolytic activity and thus in target cell protection. In the present study, we show that EB6 molecules may also act as receptors mediating NK cell triggering. Activatory EB6 molecules were found to be confined only to certain donors. Moreover, in these donors, only a fraction of EB6$^+$ NK clones expressed the activatory form of EB6 molecules, while the remaining clones expressed the conventional inhibitory form. Biochemical analysis of the activatory EB6 molecules revealed a molecular mass of ~50 kD (p50), thus differing from the 58-kD inhibitory form. This difference was not due to differential glycosylation of the same protein, as revealed by deglycosylation experiments on isolated EB6 molecules. Treatment of purified p58 or p50/EB6 molecules with proteolytic enzymes, including V8-protease, chymotrypsin, and papain, showed only minor differences in the resulting peptides. Treatment with pepsin followed by two-dimensional peptide mapping demonstrated that, although the majority of peptides migrated in identical positions, differences between the two forms could be detected for at least one major peptide. Anti-EB6 monoclonal antibody (mAb)-mediated cross-linking of p50 molecules was required to trigger the cytolytic activity and the intracellular calcium ([Ca^{++}]i) increases in appropriate NK clones. Likewise, mAb-mediated cross-linking of the p58 EB6 molecules was needed to inhibit the cytolytic activity; however, in this case, no [Ca^{++}]i increases could be detected. In NK clones expressing the inhibitory p58 EB6 receptors, soluble anti-EB6 mAb prevented recognition of protective Cw4 molecules and reconstituted target cell lysis. In contrast, in clones expressing the activatory p50/EB6 receptor, EB6 masking frequently resulted in partial inhibition of the cytolytic activity against Cw4$^+$ target cells. Therefore, it appears that NK clones expressing the p50/EB6 receptors are induced to lyse Cw4$^+$ target cells upon specific interaction with Cw4 molecules. This concept was further substantiated by experiments in which target cells were represented by the HLA-negative LCL721.221 cell line transfected with the Cw4 allele. Phenotypic and functional analysis of a large number of NK clones showed that clones expressing the activatory p50/EB6 molecules consistently coexpressed inhibitory receptors for other HLA class I alleles. Taken together, our data indicate that recognition of class I molecules may result either in inhibition or in activation of NK-mediated cytolysis. However, the inhibitory pathway appears to dominate the activatory one, thus preventing lysis of class I–protected autologous normal cells.

Recent data in mouse and in humans have provided direct evidence that NK cells recognize MHC class I molecules on target cells (1–7). This recognition results in the generation of a negative signal that inhibits the NK cell-mediated cytolytic activity, thus preventing lysis of target cells (8). Clonal analysis of human NK cells has allowed the demonstration of a higher degree of complexity in HLA class I recognition (9, 10). Indeed, groups of NK clones were shown

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to specifically recognize defined groups of HLA-C alleles rather than any HLA class I molecule. These studies provided clear evidence for the existence of clonal heterogeneity among NK cells regarding their ability to recognize different HLA class I alleles. Specific recognition of class I alleles is mediated by surface receptors characterized by a clonal distribution. In humans, the first identified receptors are represented by the p58 molecules GL183 (11) and EB6 (12), which have been shown to mediate specific recognition of different groups of HLA-C alleles (13). More recently, distinct receptors specific for different groups of HLA-B alleles have been identified with the CD94 (14) and the NKB1 (15) surface molecules. All of these receptors have been shown to inhibit the cytolytic activity of NK cells upon interaction with their HLA class I ligands. In addition, a similar inhibitory effect occurred by inducing cross-linking of the receptors with specific mAbs in a redirected killing assay (13, 14). In the absence of cross-linking, (soluble) antireceptor mAbs were shown to prevent recognition of HLA class I alleles, thus resulting in lysis of class I "protected" target cells (13–15). Taken together, these data led to the concept that the expression of MHC class I molecules prevents target cell lysis by interacting with specific inhibitory receptors expressed on NK cells. In accordance with the "missing self" hypothesis (1–5), the loss (or masking) of given HLA class I alleles (e.g., in some viral infections or tumors) results in the lack of interaction with the inhibitory NK receptor and thus in target cell lysis.

In the present report, we provide evidence that the NK receptors for HLA class I may function not only as inhibitory receptors. We show that the EB6 molecule, specific for a group of HLA-C alleles, may also function as an activatory receptor. The activatory EB6 receptor displays a molecular mass of ~50 kD, thus differing from the 58-kD inhibitory receptor. The expression of activatory receptors appears always to be balanced by the coexpression of one or more inhibitory receptors.

Materials and Methods

Antibodies. mAb JT3A (IgG2a anti-CD3), HP2.6 (IgG2a anti-CD4), B9.4 (IgG2b anti-CD8), GL183 (IgG1 anti-p58), y249 (IgM anti-p58), EB6 (IgG1 anti-p58), XA-141 (IgM anti-p58), XA-185 (IgG1 anti-CD94), XA88 (IgG3 anti-CD94), Y9 (IgM anti-CD94), KD1 (IgG2a anti-CD16), K218 (IgG1 anti-CD56), A6-220 (IgM anti-CD56), 6A4 (IgG1 anti-HLA class I), and A6-136 (IgM anti-HLA class I) (14) were used in this study. F(ab')2 fragments of the relevant mAbs were derived as previously described (13).

Detection of Cytolytic Activity Mediated by NK Clones. NK clones were obtained by limiting dilution, as previously described (11, 16). The cytolytic activity of cloned NK cells was assessed in a 4-h ^{51}Cr release assay in which effector cells were tested against the following target cells: the murine P815 cell line, the human C1R cell line (13, 14), and the human HLA class I–negative LCL721.221 cell line (17), either untransfected or transfected with Cw3 or Cw4 alleles (kindly provided by Dr. Roberto Biassoni, Istituto Nazionale per la Ricerca sul Cancro and Centro di Biotecnologie Avanzate, Genoa, Italy).

These target cells were used at 5×10^3/well, and the final E/T ratio is indicated in the text. The percentage of specific lysis was determined as previously described (16). mAbs were added at the onset of the cytolytic assay before adding target cells. The mAb concentrations used in the various assays are indicated in the text.

Flow Cytofluorimetric Analysis. Cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse (GAM)[1] Ig (Southern Biotechnology Associated, Birmingham, AL). Control aliquots were stained with the fluoresceinated reagent alone. All samples were then analyzed on a flow cytometer (FACStar®, Becton Dickinson & Co., Mountain View, CA) gated to exclude nonviable cells (11).

Determination of Intracellular Free Calcium ([Ca++]i) Increases. Determination of $[Ca^{++}]i$ was performed as previously described (18). Briefly, CD3-CD16$^+$ clonal cell populations (2.5×10^6/ml) were loaded with the acetoxymethyl ester of Fura-2 (1 μM final concentration; Sigma Chemical Co., Cockeysville, MD) in the calcium assay buffer (17). The fluorescence of the cellular suspension was monitored with a spectrofluorimeter (LS-5; Perkin-Elmer Corp., Norwalk, CT) using a 2-ml quartz cuvette. The cell suspension was excited at 345–348 nm, and fluorescence emission was measured at 496–510 nm. All measurements were performed at 37°C using a thermostatically controlled cuvette holder and stirring apparatus. $[Ca^{++}]i$ was calculated by the method of Grynkiewicz et al. (19). Triggering of NK cells was achieved by adding into the cuvette 5 μg/ml of anti-CD16 mAb KD1 (the optimal amount of CD16 mAb was determined in each experiment starting from 20 to 0.1 μg/ml). EB6- or XA185-mediated $[Ca^{++}]i$ increases were evaluated in Fura-2–loaded CD3-CD16$^+$ NK cells upon addition of 20 μg/ml (determinated in preliminary experiments) polyclonal anti-IgG1 GAM (Southern Biotechnology Associated) after treatment of the cells with 5 μg/ml of EB6 or XA185 mAbs at 0°C for 30–45 min.

Biochemical Characterization of the EB6 Molecules. Sepharose cyanogen bromide (Pharmacia Biotech Inc., Piscataway, NJ)–coupled anti-EB6 mAb was used to immunoprecipitate EB6 molecules from 1% NP-40 lysates of NK populations or NK clones surface labeled with ^{125}I (DuPont–New England Nuclear, Wilmington, DE) as previously described (12). Immunoprecipitates were analyzed in discontinuous SDS-PAGE and subjected to autoradiography using Hyperfilm-MP (Amersham International, Little Chalfont, UK). Where indicated, bands were cut from dried gel and proteins were eluted using the centrilutor (Amicon, Beverly, MA). The eluted proteins were precipitated in 20% TCA at 4°C for 2 h, washed four times with cold (−20°C) acetone, and dried in a vacuum centrifugal concentrator (Jouan, Saint Herblain, France).

Deglycosylation of EB6 Molecules. For *N*-glycanase (Genzyme Corp., Cambridge, MA) digestion, EB6 immunoprecipitates or EB6-eluted proteins were resuspended in 30 μl of digestion buffer (0.4 M Tris, pH 8, 1% NP-40, 0.1% SDS, 10 mM 2-ME) and boiled (filtered if immunoprecipitates were used), and 0.125 U of recombinant *N*-glycanase was added to each sample. Digestion was performed at 37°C for 22 h. For neuraminidase (Sigma Chemical Co.) and *O*-glycanase (Genzyme Corp.) digestion, immunoprecipitates were resuspended in 50 μl of digestion buffer (15 mM sodium phosphate, pH 7, 10 mM calcium acetate, 1% NP-40, 0.1% SDS, 10 mM 2-ME), boiled, and filtered; 0.05 U of neuraminidase was added for 1 h at 37°C, followed by 2 mU of *O*-glycanase for 20 h at 37°C.

After digestions, samples were diluted with SDS sample buffer and analyzed by SDS-PAGE. Control (undigested) samples were subjected to the same conditions without adding of enzymes (12).

Peptide Mapping. One-dimensional peptide mapping of EB6 mol-

[1] *Abbreviations used in this paper:* $[Ca^{++}]i$, intracellular calcium; GAM, goat anti-mouse.

ecules was performed following a Cleveland modified method (20), adding 500 ng of endoproteinase Glu-C (V8-protease), papain, or chymotrypsin (Sigma Chemical Co.) to each well. Control (undigested) samples were subjected to the same conditions without adding of enzymes. Two-dimensional peptide-mapping analysis was performed as previously described (12) using Multiphor II (Pharmacia Biotech Inc.) for the first dimension.

Results

Positive versus Negative Regulation of the Cytolytic Activity Mediated by EB6 Molecules in Different NK Clones. Clones expressing the GL183$^-$/EB6$^+$ phenotype were shown to specifically recognize HLA-Cw4 and related alleles (13, 21). The interaction between EB6 (p58) receptors and HLA-Cw4 molecules results in an inhibitory signal that suppresses the cytolytic activity of these clones. Moreover, in redirected killing assays, cross-linking of EB6 molecules induced by specific mAbs (of IgG but not of IgM isotype) strongly inhibited the cytolytic activity of GL183$^-$/EB6$^+$ clones against the FcγR$^+$ P815 target cells, thus mimicking the effect of the EB6-mediated recognition of Cw4 molecules (13). Such functional behavior of GL183$^-$/EB6$^+$ clones occurs in most donors analyzed (12). However, in some donors, unexpectedly, a fraction of GL183$^-$/EB6$^+$ clones was found to kill target cells expressing Cw4 molecules. Fig. 1 shows GL183$^-$/EB6$^+$ clones isolated from two representative donors. In donor E.C. (Fig. 1 *A*), all clones failed to lyse the Cw4-protected C1R cells (13, 14), whereas, in the case of donor R.P. (Fig. 1 *B*), a large proportion of clones efficiently killed the same target cells. To analyze whether this unusual functional behavior reflected the inability of EB6 molecules to mediate inhibitory signals, GL183$^-$/EB6$^+$ clones isolated from donor R.P. or E.C. were analyzed in a redirected killing assay against P815 cells in the presence or in the absence of anti-EB6 mAb (IgG1). As shown in Fig. 2, while the cytolytic activity of all NK clones derived from donor E.C. was strongly inhibited in the presence of anti-EB6 mAb (12), an opposite effect was detected in the majority of clones derived from donor R.P. Moreover, enhancement of P815 target cell lysis occurred only in those clones that lysed (Cw4$^+$) C1R target cells, whereas inhibition of lysis consistently paralleled the inability of clones to lyse C1R cells.

Although not shown, both the inhibitory and the activatory effects were detectable at similar mAb concentrations. In addition, no opposite functional effects could be elicited in a given clone by different mAb concentrations.

We next analyzed whether the EB6-mediated triggering of cytolytic activity was consequent to mAb-mediated cross-linking of EB6 molecules. Cross-linking of p58 molecules was previously shown to be required for inducing an inhibitory effect (13). To this end, we evaluated the effect of the F(ab')$_2$ fragments of anti-EB6 mAbs under the same experimental conditions. In these experiments, the F(ab')$_2$ fragment did not modify the magnitude of the cytolytic activity, thus indicating that cross-linking of EB6 molecules is required also to induce activatory signals via EB6 molecules (not shown).

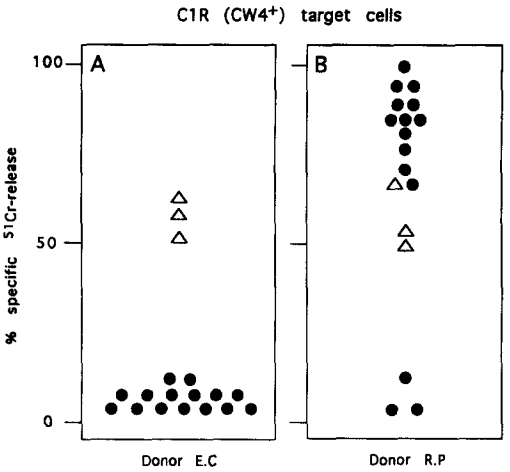

Figure 1. Cytolytic activity of GL183$^-$/EB6$^+$ clones against Cw4$^+$ target cells. In this experiment, 18 NK clones derived from donor E.C. (*A*) were compared with 18 NK clones derived from donor R.P. (*B*) for their cytolytic activity against C1R (Cw4$^+$) target cells. 15 clones from each donor were GL183$^-$/EB6$^+$ (*closed circles*), and 3 clones expressing the GL183$^-$/EB6$^-$ phenotype (*open triangles*) are shown for comparison. Data are expressed as the percentage of specific ^{51}Cr release at an E/T ratio of 5:1. Each symbol corresponds to the cytolytic activity of individual clones.

[Ca^{++}]i Increments Induced by Anti-EB6 mAbs in Clones Expressing Activatory versus Inhibitory EB6 Receptors. In view of the opposite effects on cytolysis mediated by EB6 molecules in different NK clones, we analyzed whether differences could also be detected in an early event of signal transduction, such as the [Ca^{++}]i mobilization in response to mAb-mediated cross-linking of the receptors. As shown in Fig. 3, a representative clone, characterized by an inhibitory EB6 receptor, did not display [Ca^{++}]i increments after stimulation with anti-EB6 mAb. Notably, [Ca^{++}]i increments could be induced in the same clone after stimulation with anti-CD16 mAbs. On the contrary, in clones equipped with an activatory EB6 receptor, strong [Ca^{++}]i increments in response to anti-EB6 mAbs were detected. Similar results were obtained in all NK clones analyzed (i.e., 19 clones expressing an inhibitory EB6 receptor and 8 clones equipped with an activatory receptor). These clones were derived not only from donor E.C. or R.P. but also from four additional donors.

Activatory and Inhibitory EB6 Receptors Display Different Molecular Masses. In these experiments, we analyzed the surface molecules immunoprecipitated by anti-EB6 mAb from ^{125}I-surface-labeled NK clones that expressed either inhibitory or activatory EB6 molecules. The clones analyzed were derived from donor E.C. or R.P.; from both donors we also derived polyclonal NK cell populations, which, after removal of GL183$^+$ cells, displayed a homogeneous GL183$^-$/EB6$^+$ phenotype. As shown in Fig. 4, a striking correlation existed between the type of functional response elicited via EB6 molecules (i.e., inhibition or activation) and their molecular mass as analyzed by SDS-PAGE. Thus, in clones from donor E.C. (consistently characterized by the expression of inhibitory EB6

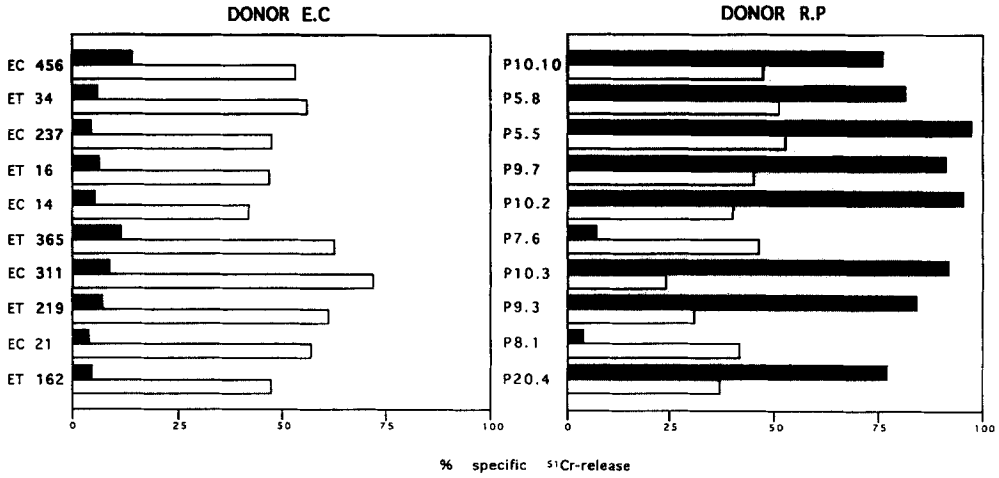

Figure 2. Anti-EB6 mAb can inhibit or activate the cytolytic activity of GL183$^-$/EB6$^+$ NK clones against P815 target cells. The cytolytic activity of 10 NK clones derived from donor E.C. and 10 NK clones derived from donor R.P. was analyzed in a redirected killing assay either in the presence (*closed bars*) or in the absence (*open bars*) of anti-EB6 mAb (IgG1). Data are expressed as the percentage of specific ^{51}Cr release at an E/T ratio of 5:1.

receptors) (12), the molecular mass of the immunoprecipitated EB6 molecules was ~58 kD (not shown), thus corresponding to the previously described p58 molecules (12). In contrast, EB6 molecules immunoprecipitated from clones isolated from donor R.P. and further selected on the basis of the expression of activatory EB6 receptors had an apparent molecular mass of ~50 kD (Fig. 4, lanes *G* and *H*). It is of note that the EB6 molecules immunoprecipitated from polyclonal GL183$^-$/EB6$^+$ NK population derived from donor R.P. displayed two distinct bands of 58 and 50 kD, respectively (Fig. 4, lanes *B* and *E*). In contrast, only the 58-kD band could be immunoprecipitated from the polyclonal GL183$^-$/EB6$^+$ NK cell population derived from donor E.C. (Fig. 4, lane *C*). These data are in agreement with the concept (substantiated by the functional analysis of >300 clones) that, in donor E.C., only the inhibitory form (58 kD) of

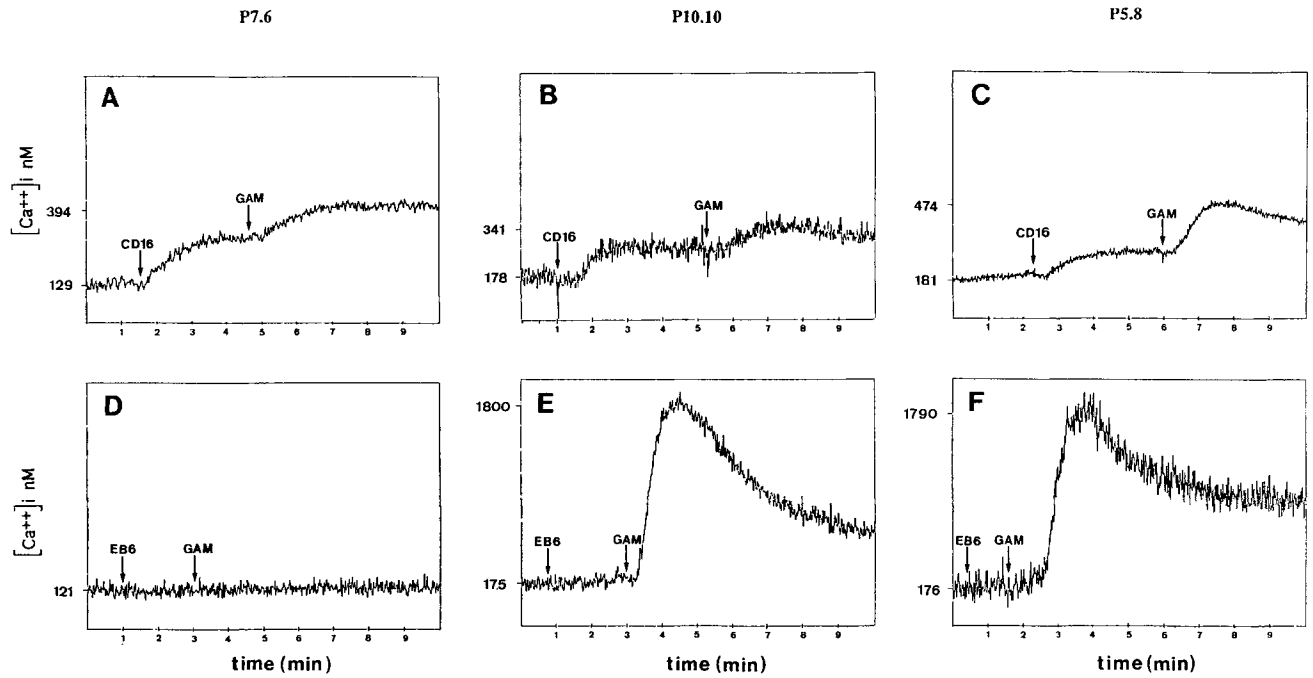

Figure 3. Comparative analysis of [Ca^{++}]i increase in NK clones expressing either inhibitory or activatory EB6 receptors. NK clones (derived from donor R.P.) characterized by either an inhibitory (clone P7.6) or activatory EB6 receptor (clones P10.10 and P5.8) were analyzed for [Ca^{++}]i mobilization after stimulation with anti-CD16 (*A–C*) or anti-EB6 (*D–F*) mAbs followed by isotype-specific GAM. While the [Ca^{++}]i increments induced by anti-CD16 mAb occurred also in the absence of GAM-mediated cross-linking, the latter was needed for the anti-EB6 mAb-mediated [Ca^{++}]i increments.

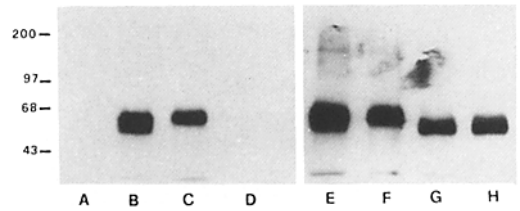

Figure 4. Two distinct molecular forms of EB6 molecules. Polyclonal NK cell populations or NK clones derived from two different donors were surface labeled with ^{125}I, lysed in 1% NP-40, immunoprecipitated with Sepharose CnBr–coupled anti-EB6 mAbs, and analyzed in 8% SDS-PAGE under reducing conditions. Lanes *B* and *E*, a polyclonal NK cell population from donor R.P.; lane *C*, a polyclonal NK cell population from donor E.C.; lane *F*, clone P7-6 from donor R.P. expressing an inhibitory EB6 receptor; lanes *G* and *H*, clones P10-3 and P5-5, both expressing an activatory EB6 receptor. In lanes *A* and *D*, the two polyclonal NK cell populations (from donor E.C. and R.P., respectively) were immunoprecipitated with Sepharose CnBr–coupled JT3a mAb (anti-CD3) as a negative control.

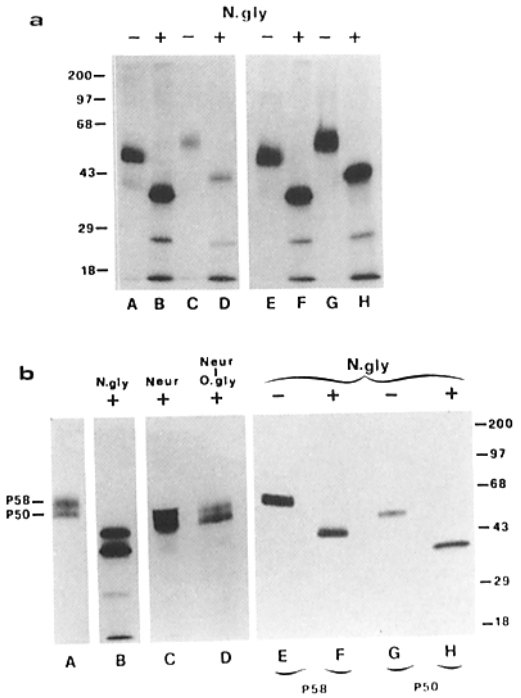

Figure 5. Deglycosylation of the EB6 molecules. (*a*) EB6 molecules immunoprecipitated from surface-labeled NK clones derived from donor R.P. (lanes *A–D*) or donor G.T. (lanes *E–H*) were subjected or not to *N*-glycanase digestion. Samples were analyzed in an 11% SDS-PAGE under reducing conditions. Lanes *A* and *B* (clone P9.3) and lanes *E* and *F* (clone G4.1) express an activatory EB6 receptor; lanes *C* and *D* (clone P8.1) and lanes *G* and *H* (clone G4.9) express an inhibitory EB6 receptor. (*b*) EB6 molecules immunoprecipitated from a surface-labeled polyclonal NK cell population derived from donor R.P. Lanes *A–D*, EB6 molecules treated with *N*-glycanase (lane *B*), neuraminidase (lane *C*), neuraminidase plus *O*-glycanase (lane *D*). In lane *A*, undigested EB6 molecules are shown. Samples were run in a 11% SDS-PAGE under reducing conditions. Lanes *E–H*, the EB6 immunoprecipitate was run in SDS-PAGE and, after 4 h of exposure, the p58 and p50 bands were separately excised from the gel, eluted, digested or not with *N*-glycanase, and analyzed in an 11% SDS-PAGE under reducing conditions. Lanes *E* and *F*, P58 EB6 molecules; lanes *G* and *H*, P50/EB6 molecules.

the EB6 receptor is expressed. On the other hand, the presence of both 50- and 58-kD EB6 molecules in the polyclonal population derived from donor R.P. is likely to reflect the clonal heterogeneity of the functional responses (i.e., the presence of NK cells expressing either the activatory or the inhibitory form of EB6 receptor). To analyze whether the 58-kD band detected in the polyclonal population derived from donor R.P. indeed correlated with the inhibitory function, we analyzed the EB6 molecules immunoprecipitated from two clones isolated from donor R.P., which expressed an inhibitory EB6 receptor (see also Fig. 1). Fig. 4 (lane *F*) shows one of these clones. It is evident that the immunoprecipitated molecules displayed a molecular mass of ~58 kD, thus indicating that, also in donor R.P., the expression of 58-kD EB6 molecules correlates with the expression of inhibitory EB6 receptors.

Deglycosylation of p58 and p50 Molecules. We next analyzed whether the differences in molecular mass between the 50- and the 58-kD molecules could be due to a differential glycosylation. In these experiments, clones displaying either activatory or inhibitory EB6 receptors were derived from two different donors (R.P. and G.T.). Similar to donor R.P., donor G.T. was characterized by $GL183^-/EB6^+$ NK clones displaying opposite functional responses to EB6-mediated triggering. EB6 molecules were immunoprecipitated from $GL183^-/EB6^+$ clones expressing either the 50- or the 58-kD form of receptor. The two immunoprecipitates were subjected to *N*-glycanase digestion and analyzed by SDS-PAGE. As shown in Fig. 5 *a*, after deglycosylation, the 58-kD molecules displayed a 42-kD molecular mass, whereas the 50-kD molecules resulted in a 36-kD band. The low molecular mass bands that are detectable in the digested samples in some instances are caused by background proteolytic activity of the *N*-glycanase enzyme. We also analyzed the 58- and the 50-kD molecules immunoprecipitated from the polyclonal $GL183^-/EB6^+$ population derived from donor R.P. In these experiments (Fig. 5 *b*), the anti-EB6 immunoprecipitates were treated with *N*-glycanase or with neuraminidase, followed or not by *O*-glycanase. In this case as well, after treatment with *N*-glycanase, the EB6 molecules displayed a 42- and 36-kD molecular mass. The fact that the 42- and the 32-kD proteins were derived from the 58- and the 50-kD proteins, respectively, was confirmed in experiments in which the two EB6-reactive molecules were excised from the gel and separately digested with *N*-glycanase. Treatment with neuraminidase slightly decremented the mobility of the two proteins, thus indicating that both are sialated. In addition, treatment with neuraminidase followed by *O*-glycanase had no further effects on mobility, indicating that no O-linked sugars are present. All together, these experiments indicated that the different molecular masses of the two anti–EB6-reactive molecules do not reflect a differential glycosylation of the same surface protein.

Comparative Analysis of p50 and p58 Molecules after Treatment with Proteolytic Enzymes. On the basis of the above results, it was important to establish whether the two anti-EB6-reactive molecules belonged to the same molecular family or to unrelated proteins. Thus, we further compared the p58 and the p50 molecules immunoprecipitated by anti-EB6 mAbs from ^{125}I-surface-labeled polyclonal NK cells derived from donor R.P. Again, the two proteins were excised from the gel and digested separately with different enzymes, including V8-protease, chymotrypsin, and papain. As shown in Fig. 6 *a*, the pattern of digestion of the two molecules was, in most instances, similar, although some differences could be detected after papain digestion (lanes *C* and *D*). To further substantiate these findings, the isolated p58 and the p50 molecules were analyzed by two-dimensional peptide mapping after treatment with pepsin. As shown in Fig. 6 *b*, all the major peptides were shared by the two EB6 proteins, although at least one major peptide unique for each form could be detected. All together, these data indicate that the p58 and the p50 molecules recognized by the EB6 mAb are representative of strictly related surface proteins.

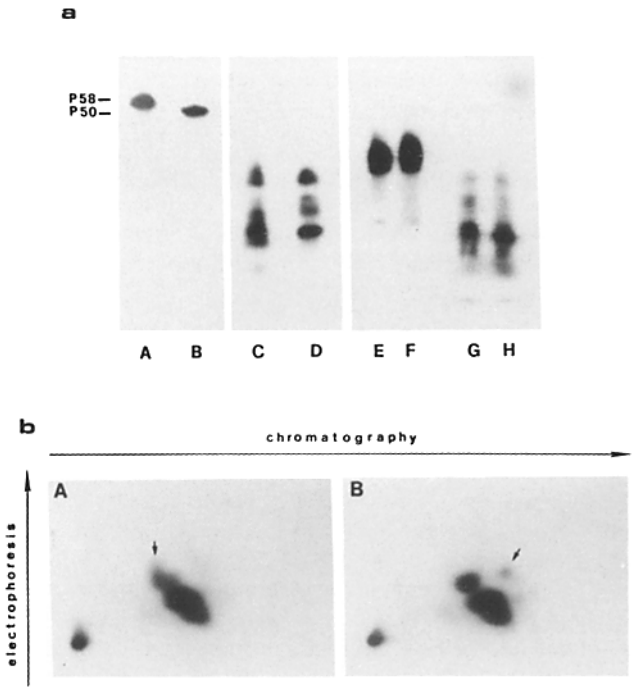

Figure 6. Proteolytic digestion of P58 and P50/EB6 molecules. (*a*) P58- and P50/EB6-reactive molecules immunoprecipitated from a polyclonal population derived from donor R.P. were separately excised from the gel. The two proteins were analyzed in a 15–20% gradient SDS-PAGE under reducing conditions, undigested (*A*, *B*) or digested with papain (*C*, *D*), V8-protease (*E*, *F*), or chymotrypsin (*G*, *H*). P58 molecules are shown in lanes *A*, *C*, *E*, and *G*, and P50 molecules are shown in lanes *B*, *D*, *F*, and *H*. (*b*) Comparative analysis of the two-dimensional peptide maps of P58 (*A*) and P50 (*B*) molecules. The two molecules were separately excised as above, eluted, and digested with pepsin. Equal amounts of samples were spotted on a silica gel plate, and peptides were separated by electrophoresis (*vertical dimension*) and chromatography (*horizontal dimension*). Arrows indicate peptides that appear to be specific for P58 and P50 molecules, respectively.

Recognition of Cw4 Molecules by the p50/EB6 Activatory Receptor. Since p50 molecules reacted with anti-EB6 mAbs and displayed a high degree of structural homology with the p58-inhibitory EB6 receptors, we investigated whether p50/EB6 molecules also specifically recognized Cw4 and related alleles. The data reported in Fig. 1, which show a strong cytolytic activity against Cw4$^+$ C1R cells, are compatible with the occurrence of an interaction between EB6 and Cw4 molecules leading to triggering of cytolytic activity. To directly assess this possibility, we analyzed two groups of NK clones expressing the inhibitory or the activatory form of EB6 molecules, respectively. Their cytolytic activity against C1R cells was evaluated either in the absence or in the presence of anti-EB6 mAb. As shown in Fig. 7, clones expressing the conventional p58 EB6 receptor (*A*) did not kill C1R cells unless anti-EB6 mAb was added (13, 21). On the contrary, clones carrying the p50/EB6 molecules (*B*) were highly cytolytic against C1R cells in the absence of anti-EB6 mAb. Upon addition of the mAb, 7 out of 12 clones were inhibited to a significant extent (i.e., >50% inhibition of cytolysis), whereas the remaining 5 were not, or were only marginally, inhibited. In both instances, clones were derived from three different donors. These data are compatible with the concept that, in a fraction of the p50/EB6$^+$ clones, the interaction between EB6 and Cw4 molecules represents a major triggering stimulus for inducing cytolytic activity. Additional still undefined activatory receptor(s) are likely to play a major role in triggering the cytolytic activity of the remaining five clones analyzed.

More conclusive evidence that the p50/EB6 receptors specifically recognize Cw4 molecules was obtained in experiments in which NK clones were analyzed for cytolytic activity against untransfected or HLA-C–transfected LCL721.221 target cells. Thus, as shown in Fig. 8, four representative p50/EB6$^+$ clones, selected on the basis of low cytolytic activity against the untransfected cell line, efficiently killed the Cw4$^+$ but not the untransfected cells or the Cw3$^+$ cell transfectants. However, two clones expressing an identical p50/EB6$^+$ phenotype (RP32 and G69) but displaying the same magnitude of cytolytic activity against both transfected and untransfected cells are shown. A likely explanation for the behavior of the latter two clones is the same as that proposed above for the five clones that were not inhibited by anti-EB6 nAbs (see Fig 7); i.e., that they may express additional triggering receptors.

Cross-talk between p58 or p50 Molecules and Other Receptors for HLA Class I Molecules. Previous studies indicated that the (inhibitory) p58/EB6 receptor can (infrequently) be coexpressed with inhibitory CD94 molecules, which function as putative receptors for some HLA-B alleles, including B7, B8, and B14 (14). However, in most clones, the p58/EB6 receptor is coexpressed with a noninhibitory form of CD94 molecule (14). On the contrary, the p50/EB6$^+$ clones derived from donor R. P. and analyzed in the present study displayed an inhibitory CD94 receptor. In addition, in this donor, clones carrying inhibitory (p58) EB6 molecules coexpressed noninhibitory CD94 molecules.

Recent studies indicated that some NK clones can be acti-

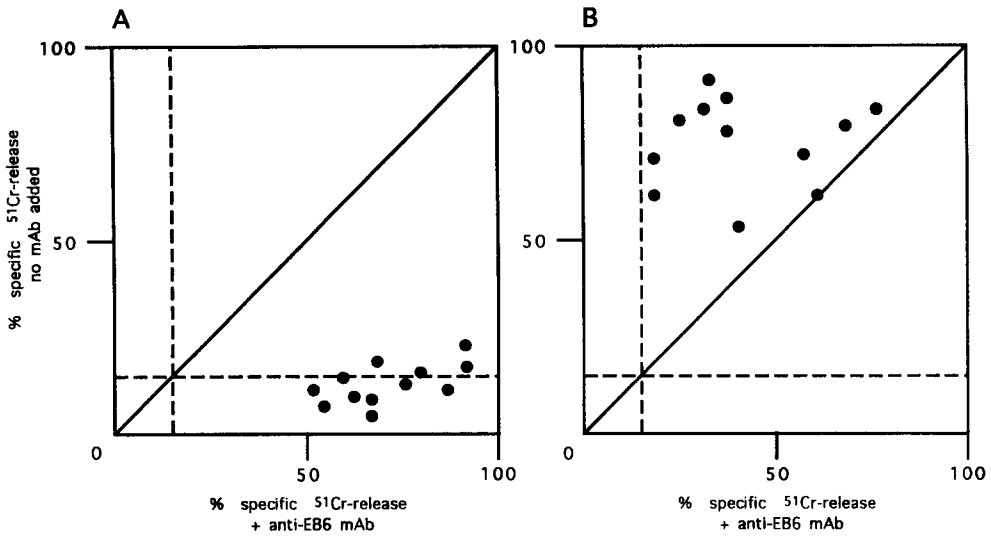

Figure 7. Effect of anti-EB6 mAb on the cytolytic activity of NK clones expressing inhibitory or activatory EB6 receptors. The cytolytic activity of 12 representative EB6+ clones expressing the inhibitory p58/EB6 receptor (*A*) was compared with that of 12 representative clones expressing the activatory p50/EB6 receptor (*B*). In both cases, clones were derived from three different donors. The cytolytic activity against C1R (Cw4+) cells was determined in a ^{51}Cr release assay either in the presence or in the absence of 1 μg/ml of anti-EB6 mAb. The dotted lines represent 3 SD above the mean spontaneous ^{51}Cr release. Each point represents the control versus the "inhibited" cytotoxicity values of a single clone. In *B*, clones resistant to the anti-EB6 mAb-mediated inhibition would thus fall close to the 45° line.

vated by anti-CD94 mAbs when analyzed in a redirected killing assay (22). Clones coexpressing EB6 and CD94 molecules were further analyzed to assess the effect of the simultaneous cell signaling via both receptors. To this end, clones were analyzed for their ability to lyse P815 target cells either in the absence or in the presence of anti-EB6 or anti-CD94 mAb, alone or used in combination. As shown in Fig. 9, target cell lysis by GL183$^-$/EB6$^+$/CD94^{low} clones (14) isolated from donor E.C. (*A*) was strongly triggered by anti-CD94 mAbs, while it was inhibited by anti-EB6 mAbs. The simultaneous addition of both mAbs resulted in a strong inhibition of target cell lysis. In control experiments, in which anti-CD94 was added together with an anti-CD56 mAb (matched for isotype, IgG1), lysis of P815 target cells was comparable to that elicited by anti-CD94 mAb alone.

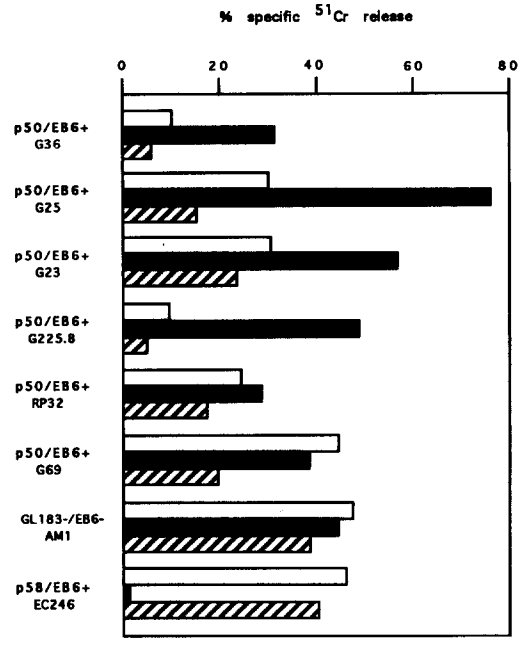

Figure 8. Specific recognition of Cw4 molecules by clones expressing the p50/EB6 receptors. In these experiments, target cells were represented by the HLA class I-negative LCL721.221 human cell line either untransfected □ or transfected with Cw4 ■ or Cw3 ▨ alleles. Six representative clones expressing the activatory p50/EB6 receptor and two control clones (AM1 displaying the GL183$^-$/EB6$^-$ phenotype and EC246 expressing the p58/EB6 inhibitory receptor) have been used as effector cells. The E/T ratio used in this experiment was 1:1.

A similar analysis was performed in GL183$^-$/EB6$^+$/CD94^{bright} clones from donor R.P. In most of these clones, anti-EB6 mAb was strongly stimulatory, whereas anti-CD94 mAb was inhibitory (Fig. 9 *B*). When the two mAbs were mixed together, a strong inhibition of target cell lysis also occurred. Thus, activation via EB6 molecules was inhibited by anti-CD94 mAb. Another clone (P7-6) derived from donor R.P. but characterized by the GL183$^-$/EB6$^+$/CD94^{low} phenotype was stimulated by anti-CD94 mAb and inhibited by anti-EB6 mAbs. The simultaneous addition of the two mAbs resulted in sharp inhibition of cytolytic activity against P815 cells in this clone as well.

In line with these results, NK clones derived from donor R.P. and characterized by activatory p50/EB6 receptors and inhibitory CD94 receptors did not lyse target cells coexpressing Cw4 and B7 alleles (14). Thus, 9 out of 10 representative GL183$^-$/EB6$^+$/CD94^{bright} clones from donor R.P. efficiently lysed C1R cells (Cw4$^+$) but not C1R cells transfected with the HLA-B7 allele (not shown). Some of these clones were unable to lyse C1R cells transfected with the HLA-B27 allele. Moreover, two out of three clones that killed C1R/B7 cells did not lyse C1R/B27 (14). In this context, we provided evidence that the putative NK receptors for HLA-

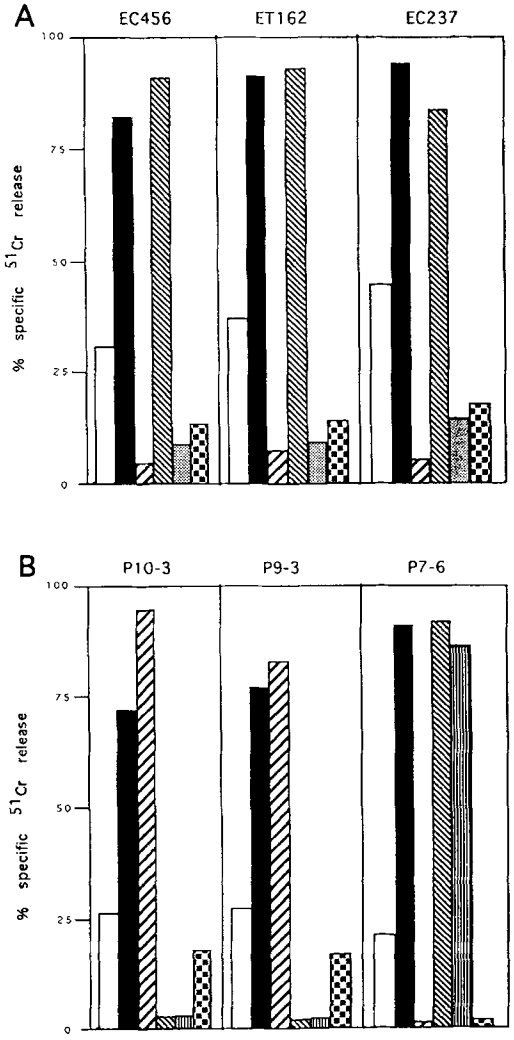

Figure 9. The cross-talk between EB6 and CD94 molecules regulates the cytolytic responses of GL183$^-$/EB6$^+$ clones. Three representative GL183$^-$/EB6$^+$ clones derived from donor E.C. (*A*) or donor R.P. (*B*) were analyzed for cytolytic activity against P815 target cells either in the absence □ or in the presence of various mAbs. (*A*) Anti-CD16 ■, anti-EB6 ▨, anti-CD94 ▧, anti-CD16 + anti-EB6 ▩, anti-CD94 + anti-EB6 ▣. (*B*) Anti-CD16 ■, anti-EB6 ▨, anti-CD94 ▧, anti-CD16 + anti-CD94 ▩, anti-EB6 + anti-CD94 ▣. Data are expressed as the percentage of specific ^{51}Cr release at an E/T ratio of 5:1.

B7 and HLA-B27, although distinct, can be coexpressed by the same NK clone (14). These data suggest that clones expressing activatory p50/EB6 receptors are negatively controlled by one or more inhibitory receptors specific for other HLA class I alleles.

Discussion

In this study, we provide evidence that EB6 molecules, known to function as inhibitory NK receptors specific for a group of HLA-C alleles (13, 21), may also function as activatory receptors mediating NK cell triggering. Biochemical analysis of anti-EB6 mAb-reactive molecules revealed that the activatory EB6 receptor displayed a molecular mass of 50 kD, thus differing from the conventional inhibitory form (58 kD). As for the induction of EB6-mediated inhibition of NK cytotoxicity (11, 12), cross-linking of p50/EB6 receptors was required to induce NK cell activation. Finally, cross-linking of the activatory but not of the inhibitory form of EB6 molecules induced [Ca^{++}]i increases.

The inhibitory form (p58) of EB6 receptor (12) has been detected in all donors analyzed so far. On the other hand, the p50-activatory receptor could be detected only in some individuals. Thus, the majority of donors (6 out of 10 donors tested) expressed exclusively the inhibitory form (e.g., donor E.C.), while others (4 donors out of 10) expressed both forms (e.g., donor R.P.). Moreover, in the latter donors, activatory and inhibitory EB6 receptors were simultaneously present and were expressed by different NK clones. Therefore, it has been possible to compare NK clones expressing either activatory or inhibitory receptors isolated from the same donor.

Since differences in molecular mass could reflect differences in glycosylation of the same surface protein, purified p50 and p58 molecules were treated with neuraminidase, O-glycanase, and *N*-glycanase. After this enzymatic treatment, the protein backbones of the two molecules displayed a different molecular mass. Although both p58 and p50 molecules were recognized by the same anti-EB6 mAb, it was possible that they belonged to unrelated molecular species. To clarify this point, purified p50 and p58 EB6 molecules were treated with various proteolytic enzymes and analyzed in one- and two-dimensional peptide mapping. These experiments indicated that p50 and p58 molecules belong to the same molecular family, since most of the major peptides migrated in identical positions and only one peptide was different.

Our present results may offer a possible interpretation of data reported in our original paper describing the GL183 molecules (11). In this paper we showed that, from some NK clones isolated from donor G.T., two molecules could be immunoprecipitated by the GL183 mAb. Thus, while most clones expressed a 58-kD band only, the 58-kD molecule was coprecipitated in some clones with a second molecule displaying a slightly lower molecular mass. Since, in further studies of GL183$^+$/EB6$^+$ clones, we showed that GL183 and EB6 molecules could be coprecipitated by using either anti-EB6 or anti-GL183 mAb (21), it is possible that the lower band in the anti-GL183 immunoprecipitates corresponded to the presently defined p50/EB6 molecule. Indeed, recent analysis of GL183$^+$/EB6$^+$ clones derived from the same donor (G.T.) revealed the existence of clones that coexpressed an inhibitory GL183 and an activatory EB6 molecule (Moretta, A., unpublished data).

The question of how to explain the molecular and functional differences between the two forms of EB6 receptors arises. Analysis of the Ca^{++} mobilization in response to mAb-induced EB6 cross-linking clearly indicated that only the activatory EB6 receptors transduced signals resulting in [Ca^{++}]i increments. No [Ca^{++}]i increments could be detected in 20 clones expressing the inhibitory EB6 receptor, after cross-linking of the EB6 molecules.

These data suggest that p50 and p58 molecules may be coupled to different pathways of signal transduction. This may reflect differences in the cytoplasmic tails of p50 and p58 molecules, possibly related to divergent amino acid sequences within this portion of the molecules. Precise information on this matter will be soon available, since molecular cloning of different cDNAs that encode for surface proteins reacting with anti-p58 mAbs (in cell transfectants) has recently been performed (23a).

It is unclear why some individuals express only the inhibitory form of EB6 molecules while others express both the activatory and the inhibitory forms. One could speculate that, in some donors, the NK receptor repertoire may be shaped in the course of certain pathological conditions. Another possible explanation is that the MHC haplotype itself, thought to be responsible for the shaping of the NK cell repertoire, may operate not only by selecting NK receptors specific for self HLA class I alleles, but also by influencing the functional type (activatory or inhibitory) of receptor selected. Interestingly, in most instances, clones expressing a p58-inhibitory receptor coexpressed activatory CD94 or NKB1-homologous z27/p70 receptors (23b) and vice versa. Therefore, a balancing effect appears to exist between activatory and inhibitory receptors at the clonal level.

The finding that EB6$^+$ (p50) clones displayed a strong cytolytic activity against C1R (Cw4$^+$) cells suggested that the activatory EB6 receptors may play a relevant role in NK cell triggering as a consequence of the recognition of Cw4 molecules. In previous studies, a correlation between expression of given HLA-C alleles on target cells and expression of the corresponding receptor on NK clones had been suggested by the analysis of the segregation of the antigen recognized in representative families (9). However, this approach was not informative for NK clones expressing the activatory p50/EB6 molecules, because of the coexpression of other inhibitory or stimulatory receptors. Therefore, p50/EB6$^+$ clones were analyzed against a panel of target cells from HLA-homozygous donors in an attempt to lower the number of potentially recognized HLA class I alleles. A fraction of these clones lysed target cells expressing Cw4 or related alleles but not those expressing Cw3 and related alleles. However, another fraction of p50/EB6$^+$ clones could lyse only some of the HLA-homozygous target cells expressing Cw4 or related alleles. Again, a likely explanation for this heterogeneity could be the coexpression, in these clones, of inhibitory receptors for either HLA-B or HLA-A alleles expressed on target cells. We next showed that anti-EB6 mAbs strongly inhibited killing of C1R cells by a fraction of EB6$^+$ (p50) clones (Fig. 7). These data suggest that the interaction between the EB6 NK receptors and Cw4 molecules expressed on target cells plays an important role in triggering the cytolytic activity of these clones. Another fraction of clones was not inhibited or was only marginally inhibited by anti-EB6 mAbs. This implies that different activatory receptor(s) (nonspecific for MHC class I molecules) are likely to be responsible for triggering the cytolytic function of these clones. This interpretation was further supported by experiments in which p50/EB6$^+$ NK clones were analyzed against the HLA class I–negative LCL721.221 cell line, either untransfected or transfected with Cw4 or Cw3 alleles. In these experiments, increments of cytolytic activity against Cw4 transfectants could be detected only in a fraction of the clones analyzed.

A possible involvement of MHC molecules in NK cell triggering has been previously suggested by experiments on alloreactive NK cells isolated from certain strains of rats (24). Indeed, it has been shown that the genetic trait "susceptibility to lysis" cosegregated with certain MHC class I haplotypes in a dominant way. These data are compatible with the existence of activatory receptors of MHC class I in rat NK cells.

Taken together, our present results suggest that the cytolytic function of NK cells may be regulated by the balance between positive and negative signals delivered by the interaction between NK receptors and their specific MHC ligands. Thus, MHC molecules would not only be responsible for the protection of potential target cells but would also be involved in NK cell triggering. Based on our data, it appears that the positive signals delivered by MHC molecules to NK cells can be revealed only in the absence of effective inhibitory interactions. This event would occur in pathological conditions characterized by down-regulation or masking of the protective MHC allele(s), such as in tumor transformation or viral infections. Whether the MHC-induced triggering of the NK-mediated cytotoxicity can be regarded as a major pathway of NK cell activation remains to be determined. In this context, it is important to note that HLA-negative cells are highly susceptible to the NK cell–mediated lysis. Obviously, in this case, no NK cell triggering can be induced by activatory receptor–MHC interactions, and the existence of activatory receptors specific for ligands that are different from MHC molecules must be postulated. Our present data confirm the relevance of the "missing self" hypothesis as the major event in rendering autologous target cells susceptible to NK cell–mediated cytolysis (1–5). However, they show that the simultaneous expression on autologous cells of self class I alleles that interact with activatory NK receptors may be responsible for the induction or at least for the potentiation of the cytolytic activity of NK cells.

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