Human Natural Killer Cell Receptors for HLA-Class I Molecules. Evidence That the Kp43 (CD94) Molecule Functions as Receptor for HLA-B Alleles

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Summary

GL183 or EB6 (p58) molecules have been shown to function as receptors for different HLA-C alleles and to deliver an inhibitory signal to natural killer (NK) cells, thus preventing lysis of target cells. In this study, we analyzed a subset of NK cells characterized by a p58-negative surface phenotype. We show that p58-negative clones, although specific for class I molecules do not recognize HLA-C alleles. In addition, by the use of appropriate target cells transfected with different HLA-class I alleles we identified HLA-B7 as the protective element recognized by a fraction of p58-negative clones. In an attempt to identify the receptor molecules expressed by HLA-B7–specific clones, monoclonal antibodies (mAbs) were selected after mice immunization with such clones. Two of these mAbs, termed XA-88 and XA-185, and their $F(ab')_2$ fragments, were found to reconstitute lysis of $B7^+$ target cells by B7-specific NK clones. Both mAbs were shown to be directed against the recently clustered Kp43 molecule (CD94). Thus, mAb-mediated masking of Kp43 molecules interferes with recognition of HLA-B7 and results in target cell lysis. Moreover, in a redirected killing assay, the cross-linking of Kp43 molecules mediated by the XA185 mAb strongly inhibited the cytolytic activity of HLA-B7–specific NK clones, thus mimicking the functional effect of B7 molecules. Taken together, these data strongly suggest that Kp43 molecules function as receptors for HLA-B7 and that this receptor/ligand interaction results in inhibition of the NK-mediated cytolytic activity. Indirect immunofluorescence and FACS® analysis of a large number of random NK clones showed that Kp43 molecules (*a*) were brightly expressed on a subset of p58-negative clones, corresponding to those specific for HLA-B7; (*b*) displayed a medium/low fluorescence in the p58-negative clones that are not B7-specific as well as in most $p58^+$ NK clones; and (*c*) were brightly expressed as in the $p58^+$ clone ET34 ($GL183^-/EB6^+$, Cw4-specific). Functional analysis revealed that Kp43 functioned as an inhibitory receptor only in NK clones displaying bright fluorescence. These studies also indicate that some NK clones (e.g., the ET34) can coexpress two distinct receptors (p58 and Kp43) for different class I alleles (Cw4 and B7). Finally, we show that Kp43 molecules function as receptors only for some HLA-B alleles and that still undefined receptor(s) must exist for other HLA-B alleles including B27.

NK cells represent a lymphoid population that does not express TCRs or surface immunoglobulins and is characterized by the $CD3^-CD16^+CD56^+$ surface phenotype and by the ability to lyse some tumor or virally infected cells (1). Clonal analysis of human NK cells allowed to demonstrate a clonal heterogeneity in the ability to lyse different target cells (2–4). The molecular mechanisms involved in the susceptibility/resistance to lysis of target cells to some NK clones has recently been clarified (5–10). Thus, whereas the surface receptors and target molecules that are physiologically involved in NK cell triggering and target cell lysis are only partially understood (11–13), it has been established that the surface expression of given HLA-C alleles protects target cells from lysis mediated by two distinct subsets of NK clones (14, 15).

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/08/0545/11 $2.00
Volume 180 August 1994 545–555

Importantly, the ability to discriminate among two different groups of HLA-C alleles (represented by Cw1, Cw3, Cw7, Cw11, and by Cw2, Cw4, Cw5, and Cw6, respectively) (16, 17) is mediated by clonally distributed specific receptors that have been recently identified and analyzed in detail. These receptors belong to a new family of NK-specific p58 molecules (18, 19) that after interaction with target cells expressing the appropriate HLA-C alleles, deliver a "negative signal" resulting in inhibition of NK cell–mediated cytotoxicity and thus in target cell protection. This negative signal can be blocked by appropriate anti-p58 or anti-class I mAbs (15, 20). As a consequence, HLA-C–protected target cells became susceptible to lysis by NK clones specific for the protective HLA-C allele (15, 20). However, a fraction of NK cells, variable in size in different individuals, do not express p58 molecules (as detected by the GL183 and EB6 mAbs) (19) and thus, it is likely that they do not recognize HLA-C molecules.

In this study, we identified a subset of $GL183^-/EB6^-$ (p58-negative) clones (19) that are not specific for HLA-C molecules but rather recognize certain HLA-B alleles. Perhaps more importantly, we provide suggestive evidence that, in these clones, the surface molecule acting as putative receptor for HLA-B is represented by the Kp43 (CD94) molecule (21, 22). Finally, we show that, in individual clones, both p58 and Kp43 molecules may be involved in the delivery of a negative signal, thus providing direct evidence for the existence of NK clones with dual specificity.

Materials and Methods

Antibodies. MAbs OKT3 (IgG2a anti-CD3; Ortho Pharmaceuticals, Raritan, NJ), JT3A (IgG2a anti-CD3), HP2.6 (IgG2a anti-CD4), B9.4 (IgG2b anti-CD8), GL183 (IgG1 anti-p58), EB6 (IgG1 anti-p58), XA-141 (IgM anti-p58), KD1 (IgG2a anti-CD16), c127 (IgG1 anti-CD16), c288 (IgG1 anti-CD16), K218 (IgG1 anti-CD56), A6-220 (IgM anti-CD56), and A6-136 (IgM anti-HLA class I) (20) were used in this study.

Cloning of $CD3^-16^+56^+$ Lymphocytes. PBLs derived from normal donors were isolated on Ficoll-Hypaque gradients and cells were then incubated with a mixture of OKT3, HP2.6, and B9.4 mAbs followed by treatment with rabbit complement for 1 h at 37°C as previously described (2, 18). Viable cells were isolated and cloned under limiting dilution conditions in the presence of irradiated feeder cells 0.1% (vol/vol) PHA (Gibco Ltd., Paisley, Scotland) and exogenous IL-2 (recombinant-IL-2; Cetus Corp., Emeryville, CA) as previously described for both T and NK cell cloning (2, 23, 24).

Cytolytic Assays. 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 murine P815 cell line (either transfected with the Cw3 gene or untransfected) (14) or the C1R human cell line (8, 15) transfected or not with various HLA class I genes (kindly provided by B. Biddison, National Institutes of Health, Bethesda, MD; J. A. Lopez De Castro, Fundacion Yimenez Diaz, Madrid, Spain; and P. Cresswell, Yale University, New Haven, CT). Other target cells used in these studies were represented by a B-EBV cell line termed 81 and by a cell variant (81.OA) that lacks the expression of HLA-class I (14). We also used a homozygous cell line termed R6/12367, kindly provided by Dr. F. Christiansen (Royal Perth Hospital, Perth, Australia) (17) and a B-EBV cell line derived from donor E. C.

All these target cells were used at 5×10^3/well and the final E/T ratio is indicated in the text. Percent specific lysis was determined as previously described (2). 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 Ig. 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 (3).

Preparation of $F(ab')_2$ Fragments. This method was previously described (19). Briefly 2 ml of DEAE–Sephacel (Pharmacia LKB Biotechnology, Uppsala, Sweden)-purified mAb (1 mg/ml) were dialyzed overnight against acetate buffer (0.1 M, pH 3.8). Next, the mAb was digested with 50 μg 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 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.

Production of mAb. 5-wk-old male BALB/c mice were immunized with a cell clone termed SE192 (surface phenotype: $CD3^-CD16^+CD56^+GL183^-/EB6^-$) as previously described (18). The immunization schedule consisted of 2-wk intravenous injections of 10^7 SE192 cells. After six injections, the mice were splenectomized and immune splenocytes were fused with P3U1 myeloma cells (18). The screening of hybridoma supernatants was based on the ability to reconstitute the cytolytic function of SE192 cells against B7 protected target cells. To this end, 25×10^3 SE192 cells were cultured together with 5×10^3 target cells (for a final effector/target ratio of 5:1) in the presence of 50 μl of culture supernatants derived from the various hybridomas. The assay was performed in V-bottomed microtiter trays in a final volume of 200 μl. After 4 h, 100 μl of supernatant was removed from each well and counted in a gamma counter for the assessment of ^{51}Cr release. Percent specific target cell lysis was determined as previously described (23). According to this screening procedure, two hybridomas, termed XA88 and XA185, able to reconstitute the cytolytic activity of SE192 clone were isolated and further subcloned in limiting dilution.

Results

Identification of $GL183^-/EB6^-$ NK Clones That Recognize HLA-class I Molecules Different from HLA-C. A series of NK clones derived from donor E. C. (HLA haplotype: A1, A3; B14, B27; Cw2, C^-) (19) and characterized by the $GL183^-/EB6^-$ (p58-negative) phenotype were assessed for cytolytic activity against the 81 target cell, a previously described B-EBV–transformed cell line heterozygous for all three class I loci (14). A large fraction (90%) of the 30 representative p58-negative clones, shown in Fig. 1, were unable to kill the 81 target cells. However, the same group of clones killed efficiently the 81-OA cell variant, characterized by the loss of expression of all class I alleles (14). That indeed HLA-class I molecules represented the protective elements of the 81 cell line was confirmed by the finding that addition of the anticlass I mAb A6-136 (IgM) (20) could restore lysis of the 81 target cells (by the same clones). Since the 81 cells

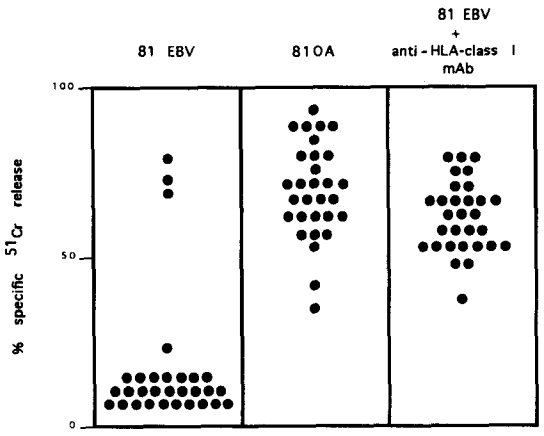

Figure 1. GL183⁻/EB6⁻ (p58-negative) NK clones recognize and are inhibited by HLA-class I molecules. *A* shows the cytolytic activity of 30 representative clones derived from donor E. C. against the 81 cell line (14) at an effector/target ratio of 10:1. In *B*, the same clones were tested against the 81OA, a class I negative variant of the 81 cell line. In *C*, the same clones were analyzed against the 81 cell line in the presence of an appropriate anticlass I mAb (A6-136 of IgM isotype) (20).

are Cw3⁺/Cw4⁺, we asked whether p58-negative clones, similar to those expressing p58 molecules (15), could recognize HLA-C molecules. To this end, three representative p58-negative clones (SE184, SE192, and SE276), which did not lyse 81 cells, were analyzed for their ability to lyse target cells expressing either Cw3 or Cw4 alleles. The first target was represented by the murine P815 cell line transfected with Cw3 (14, 15) and the second by the Cw4⁺ B-EBV cell line C1R (8, 15). These target cells had been shown (15) to be protected from lysis by GL183⁺/EB6⁺ clones (group 2, Cw3-specific) or GL183⁻/EB6⁺ clones (group 1, Cw4-specific), respectively. Since both target cells were not protected against the lysis mediated by clones SE184, SE192, and SE276, it was concluded that the elements conferring protection to 81 target cells were represented by class I molecules different from HLA-C. Five additional clones, isolated from the same donor, were analyzed for comparison: three clones (ET34, SE456, and SE273) displayed the EB6⁺/GL183⁻ phenotype and belonged to group 1 clones, specific for the Cw4 allele (3, 15). These group 1 clones displayed a strong cytolytic activity against the 81-OA variant but they did not kill the (Cw3⁺/Cw4⁺) 81 cell line. As expected (15), when analyzed against either the Cw4⁺ C1R cell line or the Cw3⁺ P815 transfectants, only the Cw4⁺ cell line was resistant to lysis (Table 1). One clone (SE362) displayed the GL183⁺/EB6⁺ phenotype and belonged to group 2 clones specific for the Cw3 allele (3, 14, 15). Thus, SE362 clone did not kill the 81 cell line (Cw3⁺/Cw4⁺) (14) nor the Cw3⁺ transfectant of the P815 cell line (15). Finally, we analyzed the SE200 clone, representative of the three p58-negative clones shown in Fig. 1, that efficiently lysed the 81 cell line. The ability to lyse 81 cells would indicate that clone SE200 does not recognize protective elements expressed by these cells. As expected, the SE200 clone lysed both C1R and the Cw3-transfected P815 cells. Taken together, these data suggest that a fraction of p58-negative clones derived from donor E. C. recognizes class I molecules different from HLA-C.

Table 1. *P58-negative NK Clones Do Not Recognize the HLA-C Alleles Expressed by the 81 Cell Line*

		Target cells‡			
Clone*	p58 Phenotype	81EBV	81OA	C1R (CW4⁺)	P815 (Cw3⁺)
SE192	GL183⁻/EB6⁻	4	73	81	72
SE276	GL183⁻/EB6⁻	2	70	76	66
SE184	GL183⁻/EB6⁻	7	84	92	76
SE200	GL183⁻/EB6⁻	62	65	68	60
SE456	GL183⁻/EB6⁺	4	81	6	73
SE273	GL183⁻/EB6⁺	4	64	2	61
ET34	GL183⁻/EB6⁺	6	91	9	84
SE362	GL183⁺/EB6⁺	5	72	ND	3

* All clones represented in this table were derived from donor E. C.
‡ Results are expressed as a percent specific ^{51}Cr-release at an E/T ratio of 10:1 (see Materials and Methods). Note that, as previously reported (14, see also in the text) the 81 cell line is Cw3⁺/Cw4⁺ whereas the 81OA cell variant is HLA-class I negative. The C1R cell line express Cw4 as the only detectable class I allele (8). The P815 used in these experiments has been transfected with the HLA-Cw3 gene.

A Subset of p58-negative Clones Recognize HLA-B Alleles. In an attempt to better define the class I specificity of p58-negative clones, we analyzed, as target cells, the homozygous cell line R6/12367 (17) displaying the HLA A3, B7, Cw7 haplotype. Also these target cells were not lysed by the p58-negative clones SE184, SE192, and SE276. Since C1R cells transfected with A3 and B7 alleles were available, we could further investigate the susceptibility to lysis of these cell transfectants. As shown in Table 2, C1R cells transfected with A3 were

Table 2. *Some P58-negative NK Clones Recognize HLA-B7*

		Target cells‡				
Clone*	p58 Phenotype	R6/12367	C1R	C1R A3⁺	C1R B27⁺	C1R B7⁺
SE192	GL183⁻/EB6⁻	8	81	78	80	11
SE276	GL183⁻/EB6⁻	4	73	72	71	6
SE184	GL183⁻/EB6⁻	9	93	85	88	10
SE200	GL183⁻/EB6⁻	62	75	71	72	74
SE456	GL183⁻/EB6⁺	83	6	ND	6	8
SE273	GL183⁻/EB6⁺	71	4	ND	3	3

* All clones represented in the table were derived from donor E. C.
‡ Results are expressed as a percent specific ^{51}Cr-release at an E/T ratio of 10:1 (see Materials and Methods). Note that the R6/12367 cell line, as previously reported (17) is homozygous for all class I loci (A3, B7, Cw7). The C1R cell line (Cw4⁺) was used either untransfected or transfected different class I alleles.

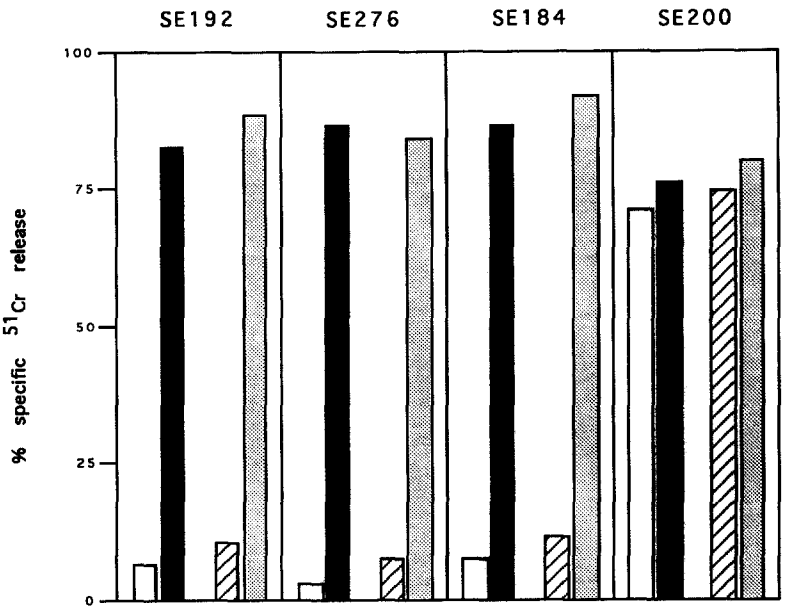

Figure 2. Anticlass I mAb reconstitute the cytolytic activity of HLA-B7–specific p58-negative clones against HLA-B7–protected target cells. Three representative clones (SE-184, SE-192, SE-276), which do not lyse the B7-protected target cells R6/12367 or C1R/B7 and the SE200 clone, were tested at an E/T ratio of 10:1 either in the presence or in the absence of the anticlass I A6-136 mAb (20). Cytolytic activity against R6/12367 target cells in the absence □ or in the presence ■ of mAb. Cytolytic activity against C1R/B7 target cells in the absence ▨ or in the presence ▩ of A6-136 mAb.

lysed, whereas C1R cells transfected with B7 (C1R/B7) were resistant to lysis. These data indicate that the protective element expressed by the R6/12367 cell line is represented by the B7 allele. Moreover, reconstitution of lysis of C1R/B7 or R6/12367 target cells was induced by the addition of the anti-HLA class I mAb A6-136 of IgM isotype (20) (Fig. 2). In addition, it is of note that C1R cells transfected with HLA-B27 (not expressed by the R6/12367 cells) were susceptible to lysis by B7-specific clones.

Selection of mAbs Able to Reconstitute the Cytolytic Activity of p58-negative NK Clones against HLA-B7–protected Target Cells. In an attempt to identify the surface receptor involved in the recognition of the B7 allele by p58-negative clones, we immunized mice with NK clones displaying this specificity. After cell fusion, the hybridoma supernatants were screened for the ability to reconstitute lysis of B7-protected target cells (R6/12367) by the NK clone SE192. Two mAbs displaying this functional property were isolated (the IgG1 XA-185 and the IgG3 XA-88). Both mAbs were found to immunoprecipitate ~70-kD molecules under nonreducing conditions and 43-kD molecules under reducing conditions. The comparative analysis of the cellular distribution and of the biochemical properties of the immunoprecipitated molecules indicated that both mAbs were directed to the previously defined Kp43 molecules recognized by the HP-3B1 mAb (21, 22). Indeed, both the XA-185 and HP-3B1 mAbs were recently clustered as CD94 (25) (5th International Conference on Human Leukocyte Differentiation Antigens, 1993, Boston, MA).

As shown in Fig. 3 *A*, mAb concentrations of ~1–5 μg/ml efficiently reconstituted the lysis of B7-protected target cells (R6/12364 cells) by clone SE192. Interestingly, these mAb concentrations were similar to those required with anti-p58 mAbs to reconstitute lysis of HLA-C-protected target cells by p58-positive NK clones (15). In addition, reconstitution of target cell lysis by p58-negative clones was induced by both anti-Kp43 mAbs and their $F(ab')_2$ fragments (Fig. 3, *A* and *B*). As shown in Fig. 3 *B*, these data were comparable to the three p58-negative clones analyzed. This figure also shows, for comparison, the reconstitution of lysis of the R6/12367 cell line ($Cw7^+$) by a group 2 $GL183^+/EB6^+$ clone mediated by anti-p58 mAbs. Although not shown, anti-Kp43 mAbs were able to reconstitute the lysis of C1R/B7 target cells by the B7-specific p58-negative clones SE184, SE192, and SE276. Remarkably, all these clones also failed to lyse C1R cells transfected with HLA-B14 or HLA-B8 (not shown) while they killed the C1R/B27 cells (Table 2). These data indicate that B14 and B8 (but not B27) alleles display a protective effect similar to B7.

Anti-Kp43 mAbs Reconstitute the Lysis of Autologous Target Cells by HLA-B7–specific p58-negative NK Clones. Since SE192, SE184, and SE276 clones were derived from donor E. C. ($HLA\text{-}B14^+$) (19) and an EBV cell line was available from this donor, we assessed whether the XA-185 and XA-88 mAbs could induce these clones to lyse autologous target cells. As shown in Fig. 4, autologous B-EBV cells, normally resistant to lysis by self NK clones (20), were efficiently killed in the presence of anti-Kp43 mAbs. These data indicate that Kp43 molecules are involved in the recognition of self-class I molecules and in the mechanism of protection of autologous target cells from NK cell-mediated lysis. In this context, previous data indicated that the anti-Kp43 mAb HP3B1 could induce (at least in some donors) lysis of autologous PHA blasts by polyclonal NK cell populations (22). On the basis of our present results, the above data can be interpreted as an inhibition of self-MHC recognition rather than an activation of the cytolytic activity mediated by anti-Kp43 mAbs. (Fig. 4 also shows, for comparison, two representative $GL183^-/EB6^+$ clones (SE456 and SE273) derived from the same donor and belonging to group 1 (Cw2-specific) (3, 16, 17). In this case, lysis of autologous target cells was detected in the presence of anti-p58 (EB6 or XA141 mAb) but not of anti-Kp43 mAbs (15). It is noteworthy that also these clones

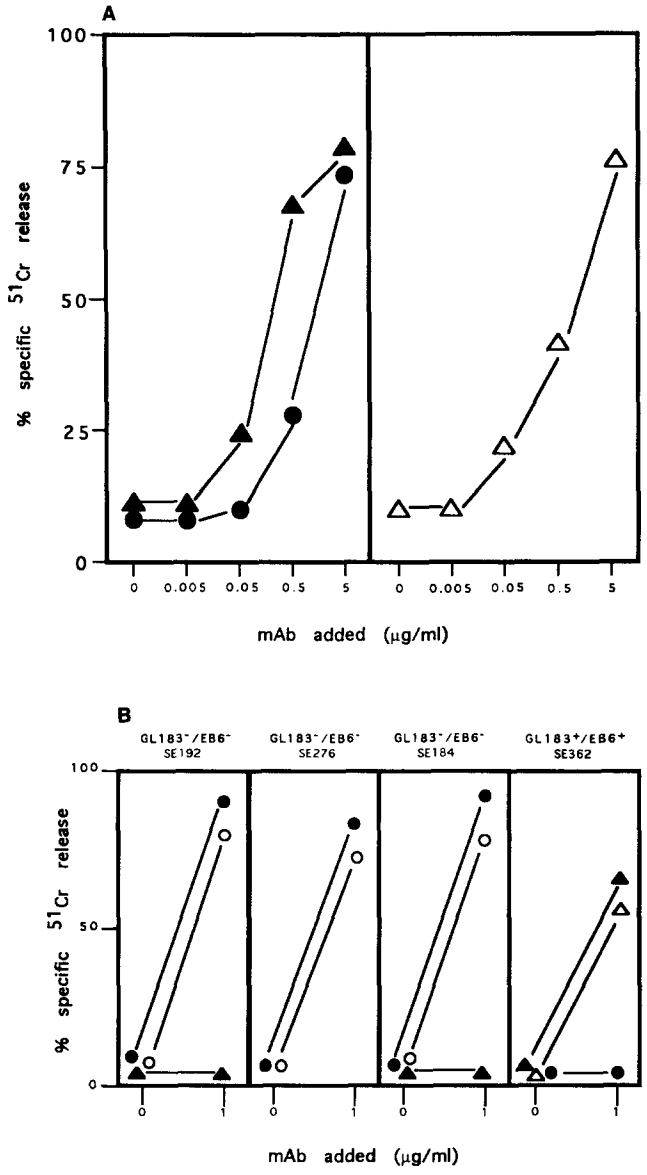

Figure 3. Anti-Kp43 mAbs reconstitute the cytolytic activity of HLA-B7-specific p58-negative clones against HLA-B7-protected target cells. In *A*, a dose-dependent effect of anti-Kp34 XA-185 (▲) or XA-88 (●) mAbs (*left*) or of the $F(ab)_2$ fragment of XA-185 (△) mAb (*right*) is shown. Effector cells are represented by the p58-negative NK clone SE-192 and target cells by the HLA-B7⁺ cell line R6/12367. In *B*, three representative, B7-specific NK clones are analyzed either in the absence or in the presence of 1 μg/ml of the anti-Kp43 XA-185 mAb (●) or of the corresponding $F(ab)_2$ fragment (O). The cytolytic activity of the p58⁺ (GL183⁺/EB6⁺) NK clone SE-362 (specific for Cw1, Cw3, and Cw7), used as control, was reconstituted by the anti-p58 GL183 mAb (▲) or the corresponding $F(ab)_2$ fragment (△) but not by the anti-Kp43 mAb XA-185 (●).

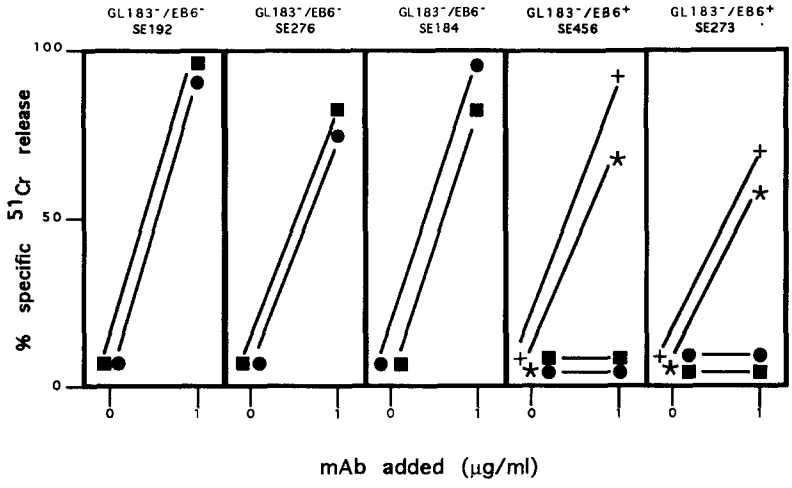

Figure 4. EBV-transformed cells are lysed by autologous NK clones in the presence of the appropriate antireceptor mAb. A B-EBV cell line derived from donor E. C. (HLA-B14⁺) was used as target cell in a cytolytic assay in which autologous NK clones displaying different specificities were tested either in the presence or in the absence of the following mAbs: the anti-Kp43 mAbs XA-185 (■) or XA-88 (●) and the anti-p58 mAbs EB6 (*asterisk*) or XA-141 (+) both directed to the same p58 molecule (EB6).

expressed surface Kp43 molecules (in medium/low amounts) (see below). Finally, clone SE200 did not kill autologous B-EBV cells and the protective effect could be reverted by the anticlass I mAb A6-136 but not by anti-Kp43 mAbs (not shown).

Anti-Kp43 mAbs Inhibit the NK-mediated Cytolysis of FcγR⁺ Target Cells. As previously shown, in redirected killing assays (i.e., using FcγR⁺ targets), addition of anti-p58 mAbs inhibited the lysis of murine unprotected (HLA-negative) P815 target cells. Thus, the mAb-mediated cross-linking of p58 molecules mimicks the effect of HLA-C molecules (15). Previous data also indicated that the anti-Kp43 mAb HP-3B1 inhibited the lysis of P815 target cells mediated by polyclonally activated NK cell populations (22). Therefore, we analyzed whether anti-Kp43 mAbs had a similar effect on the cytolytic activity of clones SE184, SE192, and SE276 against the FcγR⁺ P815 target cells. As shown in Fig. 5, lysis of P815 target cells by clones SE192 and SE184 was strongly inhibited by both XA-185 and XA-88 mAbs. In addition, both mAbs strongly inhibited the cytolytic activity induced by anti-CD16 mAbs (not shown). On the basis of these results, it appears that the functional effect of Kp43 molecules, at least in the p58-negative clones analyzed, is comparable to that of P58 molecules in P58⁺ clones (15, 18, 19). Accordingly, the inhibitory activity detected in redirected killing assays may be interpreted as the ability of anti-Kp43 mAb to mimick the interaction of Kp43 molecules with class I molecules expressed by "protected" target cells (15). The $F(ab')_2$ fragments of anti-Kp43 mAbs did not inhibit the lysis of P815 cells. This

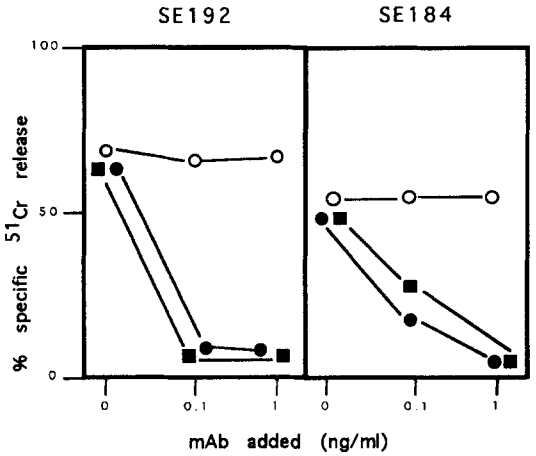

Figure 5. Anti-Kp43 mAbs inhibit the cytolytic activity mediated by HLA B7-specific p58-negative clones in a redirected killing assay. Clones SE-192 and SE-184 were tested for cytolytic activity against the unprotected (murine) FcγR$^+$ P815 target cells in the absence or in the presence of anti-Kp43 mAbs (● XA185 mAb; ○ XA-185 F(ab')$_2$; ■ XA-88 mAb).

indicates that, in order to generate the negative signal resulting in inhibition of NK-mediated lysis, cross-linking of Kp43 molecules (similar to p58 molecules) is required.

Individual NK Clones Can Use More Than One Type of Receptor for MHC Class I Molecules. As shown above (Table 1), the clone ET34, displaying the EB6$^+$/GL183$^-$ phenotype, apparently functions as a typical group 1 clone (3) when analyzed against target cells expressing the appropriate, protective HLA-C molecules (in this case the Cw4$^+$ C1R cells). On the other hand, the ET34 clone also failed to kill the homozygous R6/12367 cell line (A3, B7, Cw7) which does not express the protective HLA-C alleles recognized by classical group 1 (EB6$^+$/GL183$^-$) clones. A possible explanation of these data could be that other protective class I molecules could be expressed on the R6/12367 cell line. This is strongly suggested by the finding that lysis of the R6/12367 cells mediated by the ET34 clone was induced by the anti-HLA class I mAb A6-136 (not shown) (20). As shown in Fig. 6 *a*, the cytolytic activity of clone ET34 against the Cw4$^+$ C1R cell line occurred in the presence of anti-EB6 mAbs (EB6 or XA-141 mAbs) but not of anti-Kp43 mAbs (XA-185 or XA-88 mAbs) (Fig. 6 *A*, *left panel*). On the contrary, lysis of the HLA-B7$^+$ R6/12367 cell line by the same clone was reconstituted by anti-Kp43 mAbs but not by anti-EB6 mAbs (Fig. 6 *A*, *right panel*). B7-transfected C1R cells express both Cw4 and B7, i.e., the protective alleles independently expressed on untransfected C1R or R6/12367 target cells. Therefore, we analyzed the ability of anti-EB6 or anti-Kp43 mAbs to reconstitute the ET34-mediated lysis of these target cells. As shown in Fig. 6 *B*, the addition of either anti-EB6 or anti-Kp43 mAbs alone was not effective. However, reconstitution of lysis of C1R/B7 target cells occurred after the simultaneous addition of both mAbs. These data suggest that a simultaneous blocking of the two Cw4/EB6 and B7/Kp43 receptor/ligand interactions is required to efficiently interfere with the protective signals delivered by the C1R/B7

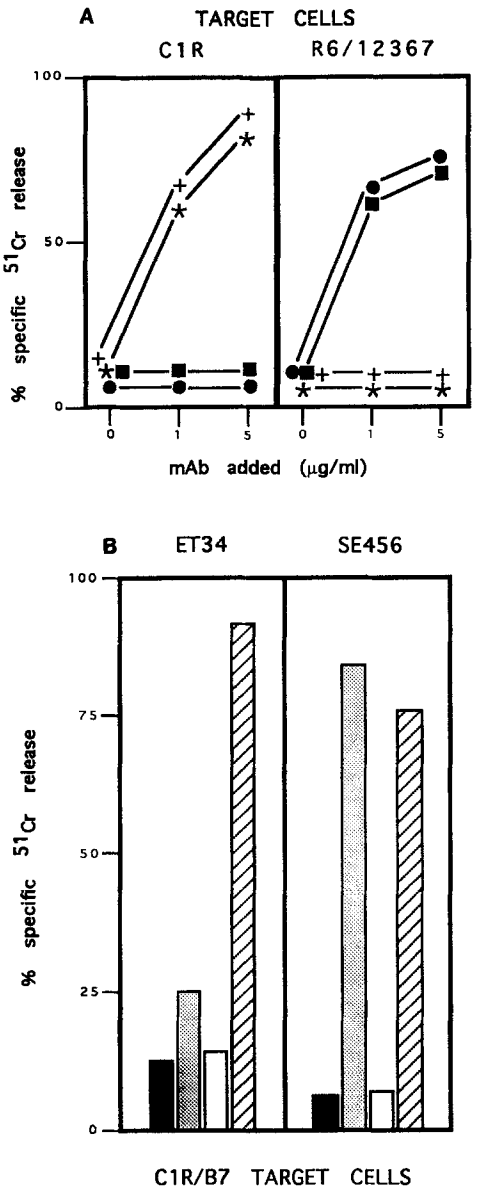

Figure 6. The ET34 NK clone express two distinct class I-specific functional receptors (p58 and Kp43). In *A*, clone ET34 (GL183$^-$/EB6$^+$/Kp43 bright) has been assessed for cytolytic activity against C1R (Cw4$^+$) or R6/12367 (A3, B7, Cw7) target cells in the presence or in the absence of anti-p58 and anti-Kp43 mAbs. Target cell lysis in the presence of the anti-p58 mAbs EB6 (*asterisk*), or XA-141 (+) anti-Kp43 mAbs XA-185 (●) or XA-88 (■). (*B*) Assessment of the cytolytic activity of clone ET34 against C1R/B7 target cells (Cw4$^+$, B7$^+$) in the presence or in the absence of mAbs. Cytolysis in the presence of both anti-Kp43 and anti-p58 mAbs (▨); of EB6 mAb (▩); of XA-185 mAb (□) or in the absence of mAb (■). *Right*, a group 1 (GL183$^-$/EB6$^+$/Kp43 dull) NK clone (SE-456) is shown for comparison.

cell line. The simultaneous blocking of both receptors was also required to allow killing of autologous B-EBV target cells by clone ET34 (not shown). Fig. 6 *B*, *right panel*, shows, for comparison, a classical GL183$^-$/EB6$^+$ clone (SE456) tested against C1R/B7 target cells. It is evident that, in this case, the addition of an anti-EB6 mAb alone is sufficient to reconstitute target cell lysis.

Expression of Kp43 Molecules on NK Clones Belonging to Different GL183/EB6 Phenotypic Groups. The Kp43 molecule was previously described by the use of the HP3B1 mAb, as a surface dimer of ~70 kD expressed on a fraction of TCR-γ/δ^+ lymphocytes and on virtually all IL-2–activated NK cells (21, 22). Although, in "resting" peripheral blood-derived $CD16^+$ NK cell populations, Kp43 molecule was detectable only on a cell subset (variable in size among different individuals), after culture in the presence of IL-2, it was acquired also by Kp43-negative cells. Our analysis at the clonal level substantially confirms these results, since essentially all clones were $Kp43^+$. However, remarkable variations in intensity of Kp43 surface expression were detectable in different NK clones. Thus, in donor E. C. all the $GL183^-/EB6^-$ NK clones specific for HLA-B7 were brightly stained by anti-Kp43 mAbs (two representative clones, SE276 and SE192, are shown in Fig. 7, *A* and *B*). In contrast, in $GL183^+/EB6^+$ clones, the intensity of expression of Kp43 molecules was low (Fig. 7, *G–I*). In $GL183^-/EB6^+$ clones, the expression was intermediate between p58-negative and $GL183^+/EB6^+$ clones (Fig. 7, *E* and *F*).

Interestingly, different from the other 12 $GL183^-/EB6^+$ clones analyzed in detail, the ET34 clone was brightly stained by anti-Kp43 mAbs (thus it was similar to the B7-specific, p58-negative clones). As shown above, the ET34 clone was able to recognize both Cw4 and B7 alleles, by two distinct inhibitory receptors. Another expression was represented by the p58-negative SE200 clone. Different from B7-specific clones, the SE200 clone expressed Kp43 molecules at intermediate levels. It is of note that SE200 was representative of ~10% of the p58-negative NK clones able to lyse the 81 cell line (see Fig. 1 and Table 1) as well as all cell transfectants analyzed (Table 2). Taken together these data suggest the existence of a correlation between the levels of expression of Kp43 molecules and their usage as receptors for protective class I alleles. It is also evident that, in some NK clones, Kp43 molecules may not be used as receptors involved in the generation of inhibitory signals (when expressed at medium/low levels).

Expression and Function of Kp43 Molecules in Different Donors. P58-negative clones were derived from two other donors (G. T. and L. N.) and analyzed for their HLA specificity and for the role of Kp43 molecules as receptors for MHC class I. These clones were screened in a cytolytic assay for their ability to kill C1R/B7 or C1R/B27 cell lines. Although not shown, ~45% of p58-negative clones derived from donor L. N. and 35% derived from donor G. T. were unable to lyse C1R/B7 target cells. Representative B7-specific clones from these two donors are shown in Fig. 8 (*A*, *B*, *E*, *F*, and *H*). In addition, a small fraction of clones displayed a pattern of reactivity different from B7-specific clones. Thus, clones LN35 and LN36 (from donor L. N.) (Fig. 8, *C* and *D*) and GT25 and GT40 (from donor G. T.) lysed C1R/B7, but not C1R/B27 target cells. In addition, clone GT20 (Fig. 8 *G*) did not kill either C1R/B7 or C1R/B27 target cells, but lysed untransfected C1R cells. The cytolytic activity of B7-specific clones from donors L. N. or G. T. was efficiently restored by anti-Kp43 mAbs when tested against B7-protected target cells. In Fig. 8, *A* and *B*, two representative B7-specific clones isolated from donor L. N. (LN5 and LN6) are analyzed for the cytolytic activity against C1R, C1R/B7, and C1R/B27 target cells. Both C1R and C1R/B27 cells were efficiently lysed, whereas C1R/B7 target cells were not (lysis of C1R/B7 occurred only in the presence of anti-Kp43 mAbs). A similar

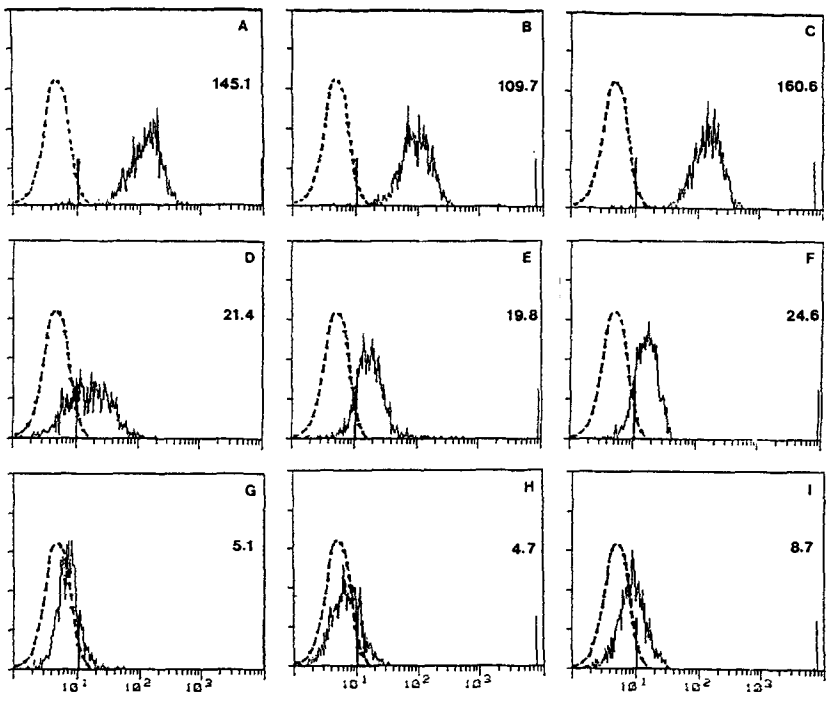

Figure 7. Expression of Kp43 molecules in different phenotypic groups of NK clones. Three distinct groups of clones were identified on the basis of the surface expression of Kp43. In *A*, *B*, and *C*, three representative B7-specific, Kp43 bright, NK clones are shown (SE-276; SE-192, and ET-34), respectively. In *D*, *E*, and *F*, clones with "medium" Kp43 fluorescence are shown. In *D*, the SE-200 clone (p58-negative) of undetermined specificity is shown. In *E* and *F* clones SE-273 and SE-456 ($GL183^-/EB6^+$) belonging to group 1 clones are shown. In panels G, H, and I, three $GL183^+/EB6^+$ clones (SE-362, SE-133, and SE-204) with low Kp43 fluorescence are shown.

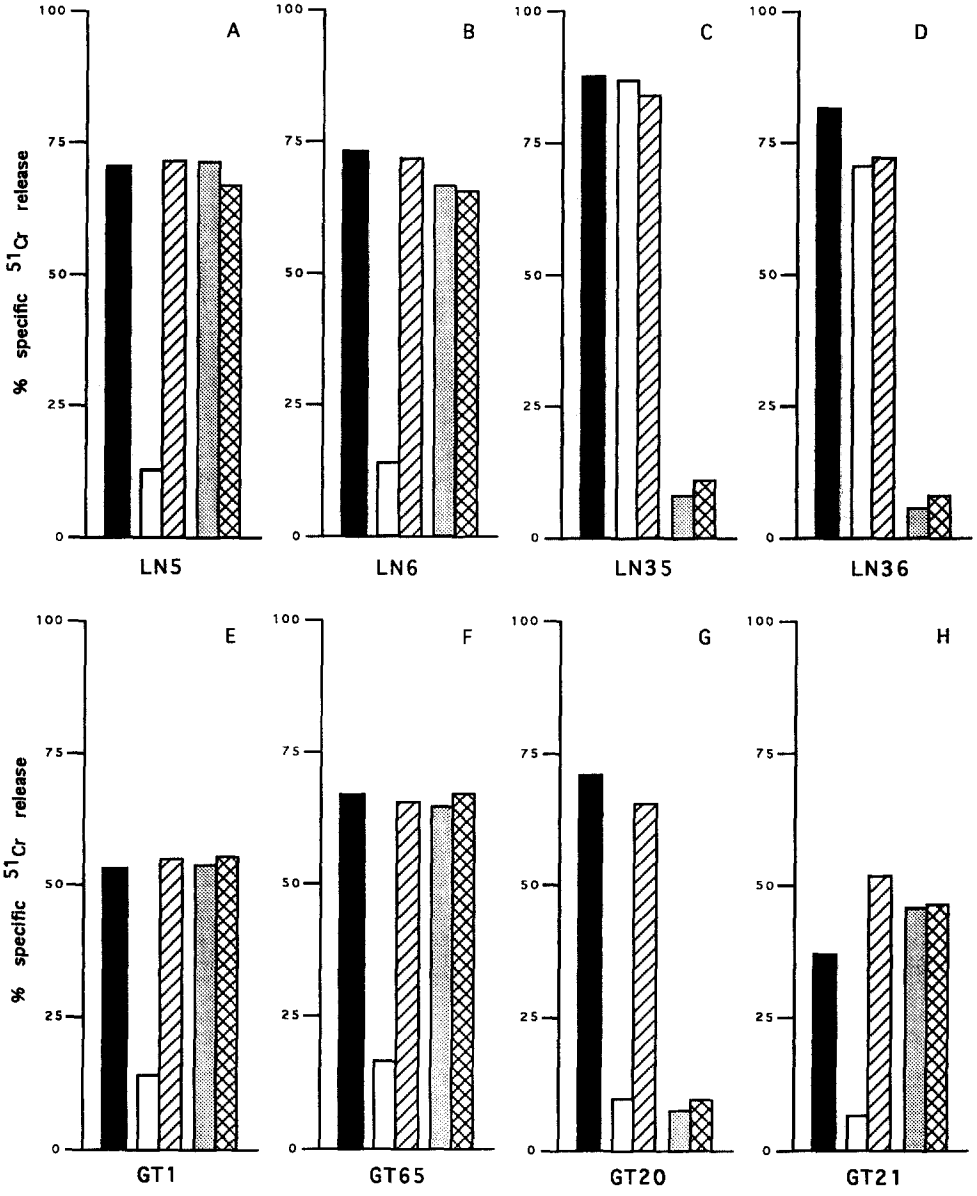

Figure 8. A fraction of p58-negative NK clones is specific for HLA-B27. Representative p58-negative clones isolated from donors G. T. and L. N. are shown. Clones were analyzed for cytolytic activity against the following target cells: (*a*) untransfected C1R cells (■); (*b*) C1R/B7 cells either in the absence (□) or in the presence (▨) of the (anti-Kp43) XA-185 mAb; (*c*) C1R/B27 cells either in the absence (▣) or in the presence (▩) of the same mAbs. In *A–D*, clones from donor L. N., LN5 and LN6 are specific for B7, whereas clones LN35 and LN36 are specific for B27. In *E–H*, clones from donor G. T., GT1, GT65, and GT21 are specific for B7, clone GT20 display an unique pattern as it fails to lyse both C1R/B7 and C1R/B27.

pattern of cytolysis, was detectable also in three representative B7-specific clones (GT1, GT65, and GT21) derived from donor G. T. (Fig. 8, *E*, *F*, and *H*). On the other hand, in the case of the two p58-negative B27-specific clones (LN35 and LN36), anti-Kp43 mAbs did not restore lysis of C1R/B27 target cells (Fig. 8, *C* and *D*). These data suggest that recognition of HLA-B27 allele is not mediated by Kp43 molecules and that a different receptor may be involved. Along this line, the NK clone GT20 (unable to kill both C1R/B7 and C1R/B27), in the presence of anti-Kp43 mAb, lysed C1R/B7 targets, but not C1R/B27 (Fig. 8 *G*). A possible explanation could be that clone GT20, similar to clone ET34, is equipped with two distinct receptors for MHC molecules: one, represented by Kp43, involved in the recognition of B7 and another, still undefined, (Kp43-like?) involved in the recognition of B27. It is noteworthy that lysis of C1R/B27 by B27-specific clones, including GT20, was reconstituted in the presence of anti-class I mAbs (not shown).

Analysis of the expression of Kp43 molecules on a series of NK clones derived from G. T. and L. N. donors revealed that B7-specific clones expressed high levels of Kp43 molecules, whereas B27-specific clones and clones of undefined specificity expressed intermediate/low levels of Kp43 surface molecules (not shown).

Discussion

In the present study we provide evidence for the existence of an additional NK receptor for HLA class I molecules. This receptor has been identified with the Kp43 molecule (21, 22) and is distinct from the formerly identified p58 molecules (18, 19). Whereas p58 molecules function as receptors for

HLA-C alleles (15), the Kp43 molecules function as receptors for (at least some) HLA-B alleles. In addition, evidence is provided that single NK clones can be equipped with more than one type of receptor for HLA class I.

As previously shown, the protective effect of HLA-C molecules on target cells was reversed by anticlass I (20) or anti-p58 mAbs (15). This indicates that masking the receptor molecules or their ligands was sufficient to reconstitute the cytolytic activity of NK clones. However, a fraction of NK clones, did not express surface p58 receptors (recognized by GL183 or EB6 mAbs) (19). This NK subset was operationally defined as p58-negative and their specificity for MHC molecules was still undefined. Now we show that (at least in the three donors analyzed) a fraction of NK clones displaying the p58-negative phenotype recognize and is inhibited by HLA-B7 molecules. This HLA-B specificity is not limited to a single HLA-B allele as indicated by the finding that HLA-B8 and HLA-B14 also mediated target cell protection from HLA-B7–specific NK clones. On the other hand, the HLA-B27 allele did not exert a protective effect from these clones. Although an extensive genetic analysis of the protective class I alleles, paralleled by the study of a large panel of HLA-B transfectants, would be required, these data suggest that, similar to HLA-C–specific groups of NK clones (3, 15), also HLA-B–specific NK clones recognize distinct groups of HLA-B alleles. Screening of mAbs for the ability to reconstitute lysis of HLA-B7–protected target cells by p58-negative NK clones led to the isolation of two new mAbs. Importantly, both mAbs were directed to Kp43 molecules. These mAbs were analyzed on a set of representative clones displaying specificity for HLA-B7. In all instances, anti-Kp43 mAbs or their $F(ab')_2$ fragments reconstituted the cytolytic activity against HLA-B7$^+$ target cells. Similar results were obtained by using, as target cells, C1R cells transfected with HLA-B8 or B14 or an autologous HLA-B14$^+$ B-EBV cell line. On the other hand, anti-Kp43 mAbs did not reconstitute the cytolytic activity of NK clones displaying other specificities. For example, the lysis mediated by HLA-C–specific NK clones (p58$^+$) against HLA-C–protected target cells was modified (i.e., reconstituted) by anti-p58 mAbs but not by anti-Kp43 mAbs. This indicates that, although these molecules are expressed also by p58$^+$ clones, they are not involved in recognition of HLA-C molecules. Interestingly, unlike most p58$^+$/Kp43$^+$ clones analyzed, a single clone (ET34) has been identified, and analyzed in detail, in which both p58 and Kp43 molecules functioned as inhibitory receptors. This clone, similar to the previously identified group 1 clones (3), expressed the GL183$^-$/EB6$^+$ phenotype, did not kill target cells expressing Cw4, and its cytolytic activity against C1R target cells (Cw4$^+$) was reconstituted by anti-EB6 mAbs (15). However, this clone also failed to kill target cells lacking Cw4 or related protective alleles. For example, the ET34 clone did not kill homozygous cell lines displaying the A3, B7, Cw7, MHC haplotype. In this case, anti-p58 mAbs did not reconstitute the cytolytic activity. However, reconstitution of cytolytic activity was obtained by anti-Kp43 mAbs. Thus, the cytolytic activity (i.e., the specificity) of ET34 clone appears to be controlled by two distinct types of receptors for MHC class I molecules (i.e., the EB6 molecules specific for the Cw4 allele and the Kp43 molecules specific for the HLA-B7 allele). As a consequence, when analyzed on target cells expressing both protective alleles (such as the C1R/B7 cell line), the combined use of both antireceptor mAbs as required in order to reconstitute the cytolytic activity of the ET34 clone.

Our studies indicate that a different subset of p58-negative NK clones recognize a different HLA-B allele, namely the HLA-B27. In this case, however, Kp43 molecules were not involved in the recognition of the B27 allele as indicated by the failure of anti-Kp43 mAbs to reconstitute the cytolytic activity of these clones. Along this line, anti-Kp43 mAbs could reconstitute the cytolytic activity of another p58-negative clone (GT20) specific for both B7 and B27 alleles, only when tested against C1R/B7 but not C1R/B27 target cells. These data are compatible with the existence, on clone GT20, of another receptor specific for the B27 allele. In p58$^+$ clones, a precise correlation has been established between the HLA-C alleles recognized and their p58 phenotype (3, 5, 13–15, 19). In the case of clones specific for HLA-B molecules, a similar correlation between the HLA-B alleles recognized and the Kp43 surface phenotype could not be established. Indeed, essentially all cloned NK cells expressed Kp43 molecules. Nevertheless, differences could be detected in the relative fluorescence intensity of different NK clones. Remarkably, clones recognizing HLA-B7 were consistently brightly stained by anti-Kp43 mAbs. On the contrary, the HLA-B27-specific p58-negative clones as well as most clones coexpressing p58 and Kp43 receptors were characterized by a medium/low Kp43 fluorescence. A possible explanation of these observations could be that the number of interactions between HLA-B7 and Kp43 receptor molecules may be critical for the induction of the negative signal necessary to induce the protective effect. If this hypothesis is correct, NK clones expressing a relatively low number of Kp43 molecules may not be able to reach the critical number of interactions with the protective MHC molecules required to deliver an effective negative signal. Thus, negative signaling via Kp43 may not be required in clones that already express other types of functional receptors as it occurs in p58$^+$ NK clones that are already inhibited by HLA-C molecules (a remarkable exception is represented by the ET34 clone). As suggested by studies of Storkus et al. (25), also HLA-A–specific NK cells appear to exist. Thus, it is possible that a fraction of p58-negative NK clones may express a still undefined negative receptor specific for HLA-A alleles.

Our data suggest that NK clones which express a given receptor type (e.g., p58 molecules) in general, express low amounts of Kp43 molecules. On the other hand, Kp43 is brightly expressed in a significant fraction of p58-negative NK cells. In addition, as revealed by the functional behavior of the ET34 and GT20 clones, also NK cells exist which express two distinct inhibitory receptors represented by Kp43 and p58 molecules (ET34 clone), or by Kp43 and a still undefined receptor for HLA-B27 (GT20 clone). The relative frequency of these clones remains to be established. It is evident that, a NK cell equipped with two different negative

receptors will lyse target cells expressing two relevant class I alleles (e.g., autologous cells) only when both class I alleles are simultaneously masked (or downregulated) (20). Another possible explanation should be considered to explain the functional differences between NK cells expressing high v. medium/low amounts of Kp43 surface molecules. It is noteworthy that, in Kp43 "bright" clones, we could consistently detect: (*a*) The specificity for certain HLA-B alleles; (*b*) the reconstitution of the cytolytic activity against protected target cell by nti-Kp43 mAbs and (*c*) the inhibition of lysis (of $Fc\gamma R^+$ target cells) in redirected killing assays. On the contrary, in Kp43 medium/low clones, Kp43 molecules did not appear to participate in the mechanisms of protection consequent to recognition of HLA-B alleles. A possible interpretation of these data would be that the Kp43 antigen may include homologous, though structurally distinct, molecules that could confer different specificities for HLA molecules. Molecular cloning of the Kp43 molecule-encoding gene(s) will allow exploration of this possible structural heterogeneity. Along this line, recent data would suggest that, in some instances, polyclonal NK cell populations may contain cells that are triggered, rather than inhibited, by anti-Kp43 mAbs (Lopez-Botet, M., unpublished observations). Isolation and functional analysis of NK clones displaying this property should help to better understand these data. If, also in this case, anti-Kp43 mAbs mimick the functional effect of the Kp43/class I interaction, it is evident that this interaction would lead to a "positive" signal and to triggering rather than inhibition of cytolytic activity. In addition, it will be of interest to define whether these NK clones, different from those inhibited by the interaction with HLA-B7, express medium-low levels of Kp43 surface molecules.

In conclusion, distinct NK cells are capable of recognizing different class I molecules. This occurs via defined receptor structures. While p58 receptors mediate recognition of HLA-C molecules, Kp43 receptors are involved in recognition of (at least one) HLA-B alleles. Since we recently showed that all clonogenic NK cells recognize class I molecules (20), it is predictable that NK clones which do not belong to the HLA-C or HLA-B allele-specificity defined so far, may be specific for different groups of HLA-A or B alleles. These cells may be equipped with a still undefined third type of receptor or with receptors belonging to either the p58 or the Kp43 molecular family, but not reactive with the available mAbs. The finding that single NK cells may also express two different types of receptors, displaying different class I specificities, suggest that the NK cell repertoire for MHC may be influenced not only by the expression of different receptors, but also by the coexpression of a different second receptor.

This work was supported by grants awarded by the Associazione Italiana per la Ricerca sul Cancro (AIRC), Istituto Superiore di Sanita (ISS), Consiglio Nazionale delle Ricerche (CNR), and Progetto Finalizzato Application Cliniche della Ricerca Oncologia (ACRO). Simona Sivori is a recipient of a fellowship awarded by AIRC.

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Received for publication 16 February 1994 and in revised form 21 April 1994.

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