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Physical and functional independency of p70 and p58 natural killer (NK) cell receptors for HLA class I: Their role in the definition of different groups of alloreactive NK cell clones

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ABSTRACT **Natural killer (NK) cells express clonally distributed receptors for different groups of HLA class I alleles. The Z27 monoclonal antibody described in this study recognizes a p70 receptor specific for HLA-B alleles belonging to the Bw4 supertypic specificity. Single amino acid substitutions in the peptide-binding groove of HLA-B2705 molecules influenced the recognition by some, but not all, p70/Z27$^+$ clones. This suggests the existence of a limited polymorphism within the p70 family of receptors. The pattern of reactivity of monoclonal antibody Z27 revealed that Bw4-specific receptors may be expressed alone or in combination with different (GL183 and/or EB6) p58 molecules. Analysis of NK clones coexpressing p58 and p70 receptors allowed us to demonstrate that the two molecules represent physically and functionally independent receptors. The expression of p70 molecules either alone or in combination with EB6 molecules provided the molecular basis for understanding the cytolytic pattern of two previously defined groups of "alloreactive" NK cell clones ("group 3" and "group 5").**

Natural killer (NK) cells (1) have been shown to recognize major histocompatibility complex class I molecules on target cells (2–8). This recognition results in the generation of a negative signal, which downregulates the NK cytotoxicity and results in target cell protection (9, 10). Importantly, the analysis of human NK cell clones led to the demonstration of a clonal heterogeneity in the ability to lyse different target cells (11, 12). Thus, five different groups of NK cell clones, displaying unique patterns of allospecificities, have been defined (11) on the basis of their differential ability to lyse a panel of allogeneic target cells. In two groups of alloreactive NK cell clones ("group 1" and "group 2"), a dimorphism of HLA-C at positions 77 and 80 has been shown to control the susceptibility/resistance of target cells to lysis (13). Importantly, the ability to discriminate among these two different groups of HLA-C alleles is mediated by clonally distributed receptors that belong to a family of NK-specific p58 molecules identified by monoclonal antibodies (mAbs) GL183 (14) and EB6 (15). Group 1 clones were shown to express EB6 receptor (16) molecules, whereas group 2 clones expressed GL183 receptors (16, 17). More recently, additional putative HLA class I-specific receptors have been identified. These receptors, which are different from p58 receptors, appear to recognize two different groups of HLA-B alleles corresponding to the supertypic specificities Bw4 and Bw6 (9). Thus CD94 would recognize the Bw6 specificity (18), while a p70 molecule recognized by the DX9 mAb would recognize a fraction of NK clones specific for the Bw4 specificity (19, 20).

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In the present study by the use of an anti-p70 mAb (Z27 mAb) to the Bw4-specific NK receptor, we analyzed the receptor specificity and its functional relationship with p58 molecules.

MATERIALS AND METHODS

Production of mAb. Immunization of 5-week-old male BALB/c mice with the Bw4-specific NK cell clone SA260 (surface phenotype: CD3$^-$CD16$^+$CD56$^+$ GL183$^-$EB6$^-$) and selection of mAbs to HLA class I-specific receptors were performed as described (14, 15).

Two-Color Flow Cytofluorimetric Analysis. Analysis of peripheral blood lymphocytes (PBLs) or polyclonally activated NK cell populations for the distribution of the surface antigen recognized by the Z27 mAb, as compared to that of GL183 and EB6 antigens, was performed using two-color fluorescence cytofluorimetric analysis, as described (14, 15).

Biochemical Characterization of the p70 Molecules. Sepharose CnBr-coupled Z27 and GL183 mAbs were used to immunoprecipitate p70 and p58 molecules from 1% Nonidet P-40 lysates of NK clones that had been surface labeled with ^{125}I (NEN/DuPont) as described (15). Immunoprecipitates were analyzed in discontinuous SDS/PAGE gels and subjected to autoradiography using Hyperfilm-MP (Amersham). Bands were cut from the dried gel, and the eluted proteins were resuspended in 30 μl of digestion buffer [0.4 M Tris (pH 8)/1% Nonidet P-40/0.1% SDS/10 mM 2-mercaptoethanol] and subjected or not to N-Glycanase digestion [0.125 unit of recombinant N-Glycanase (Genzyme)]. Digestion was performed at 37°C for 22 hr. After digestion, samples were diluted with SDS sample buffer and analyzed by SDS/PAGE. HLA-DR was immunoprecipitated using the D1/12 mAb, and isolated α-chain was subjected to N-Glycanase digestion as a control.

Cytolytic Assays. The cytolytic activity of cloned NK cells was assessed in a 4-hr ^{51}Cr-release assay in which effector cells were tested against the C1R human cell line (16) transfected or not with various HLA class I genes including mutated HLA-B2705 molecules (kindly provided by B. Biddison, National Institutes of Health, Bethesda, MD; J. A. Lopez De Castro, Fundacion Yimenez Diaz, Madrid; and P. Cresswell, Yale University, New Haven, CT). Other target cells used in these studies were represented by phytohemagglutinin (PHA)-induced blasts derived from normal HLA-typed individuals (11).

All these target cells were used at 5×10^3 per well and the final effector-to-target cell (E/T) ratio is indicated in the text. Percent specific lysis was determined as described (11, 15).

Abbreviations: mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; PHA, phytohemagglutinin; E/T, effector-to-target cell; NK, natural killer.

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. Modulation of surface EB6 molecules was induced by overnight incubation of NK cells in the presence of XA-141 mAb as described (21).

RESULTS

Isolation of the Anti-p70 mAb. The $CD3^-CD16^+$ NK clone SA260 was used for immunization of mice. It did not express the HLA-C-specific p58 inhibitory receptors GL183 and EB6 (14–16) and lysed C1R cells ($Cw4^+$) either untransfected or transfected with the HLA-B7 allele (C1R/B7) but failed to lyse C1R cells transfected with HLA-B2705 (C1R/B27) (18) (data not shown). This cytolytic pattern suggested that clone SA260 expressed inhibitory receptors specific for the HLA-B2705 allele (belonging to the Bw4 supertypic specificity). To select mAbs specific for this receptor, the screening of the hybridoma supernatants was based on their ability to restore lysis of C1R/B27 cells by the SA260 clone. mAb Z27 was selected according to this property.

The molecules immunoprecipitated by this mAb from clone SA260 displayed an apparent molecular mass of ≈70 kDa under both reducing and nonreducing conditions and were clearly distinguishable from the p58 molecules shown for comparison (Fig. 1*A*). Both isolated p70 and p58 molecules were treated with N-Glycanase and, after this treatment, resulted in bands of ≈55 and 42 kDa, respectively (22).

Comparative Analysis of the Expression of p70 and p58 Molecules on Resting or Activated NK Cells. In these experiments, we analyzed by double fluorescence and fluorescence-activated cell sorter analysis the cellular distribution of p70 molecules in comparison to that of p58 molecules in $CD3^-CD16^+CD56^+$ bulk cultures derived from different individuals. In particular, the reactivity of the Z27 mAb was compared to that of a mixture of XA-141 (anti-EB6) and Y249 (anti-GL183) mAbs (21) (Fig. 1*B*). It is evident that some NK cells coexpressed p58 and p70 molecules while others expressed one or another surface molecule. Similar results were obtained in nine polyclonal NK cell populations, three of which are shown in Fig. 1; however, the proportions of cells coexpressing p58 and p70 molecules clearly varied in different individuals. The relative cell distribution of p70 and p58 molecules was also evaluated in fresh PBLs isolated from the same individuals. Data were comparable to those obtained in bulk cultures.

Correlation Between Expression of Inhibitory p70/Z27 Molecules and Bw4 Specificity of NK Clones. To define the reactivity of the Z27 mAb with B2705-specific clones (18), we analyzed a series of NK clones derived from six different individuals. The selection of clones was performed on the basis of their ability to lyse C1R but not C1R/B27 target cells (i.e.,

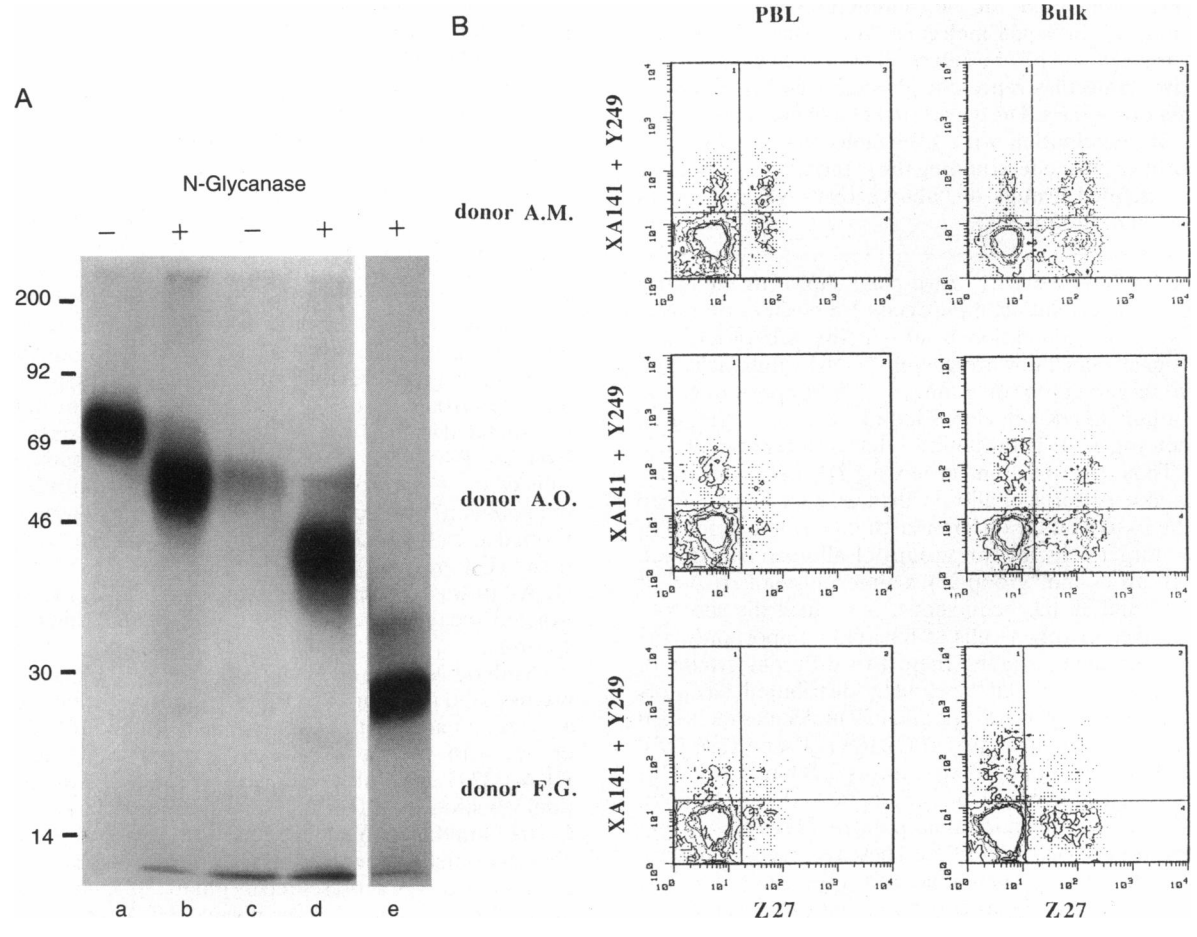

FIG. 1. Comparative analysis of p58 and p70 NK receptors. (*A*) Biochemical analysis of p70 molecules. p70 (lanes a and b) and p58 (lanes c and d) molecules isolated from surface-labeled NK clone SA260 ($p70^+/p58^-$) and clone FG32 ($p70^-/p58^+$), respectively, were subjected or not to N-Glycanase digestion. N-Glycanase-digested HLA-DR α-chain (lane e) is shown as a control. Samples were run in an SDS/11% PAGE gel under reducing conditions. (*B*) Two-color cytofluorometric analysis of the distribution of p58 and p70 molecules in PBLs and polyclonally activated NK cell populations derived from three different donors. Cells were stained with Z27 mAb followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG_1 and with a mixture of anti-GL183 (Y249 mAb; IgM) and anti-EB6 (XA-141 mAb; IgM) mAbs followed by a phycoerythrin-conjugated goat anti-mouse IgM. The contour plots were 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).

by selecting clones that were inhibited by the B27 but not by the Cw4 allele constitutively expressed in C1R cells). All the 38 clones so selected expressed Z27-reactive molecules (but not EB6 molecules, which have been shown to represent the Cw4-specific receptors) (16). Therefore, a precise correlation exists between expression of p70/Z27 molecules and specificity for B2705 molecules. To further define the pattern of reactivity of NK clones expressing the Z27 mAb-defined p70 receptor, we analyzed C1R cells transfected with one of the following HLA class I alleles: A1, A2, A3, B7, B8, B14, B27, B44, B51, B52, or B53. The cytolytic activity against C1R transfectants of a clone representative of 38 $p58^-/p70^+$ clones is shown in Fig. 2. It lysed untransfected C1R cells but failed to lyse C1R cells transfected with HLA-B alleles expressing the Bw4 supertypic specificity (B27, B44, B51, B52, and B53). In contrast, it efficiently lysed C1R cells transfected with HLA-B alleles belonging to the Bw6 supertypic specificity (B7, B8, and B14). Therefore, the surface expression of the p70/Z27 molecule appears to correlate with the recognition of HLA-B alleles characterized by the Bw4 supertypic specificity. It is of note that, in all instances, the Z27 mAb could reconstitute the cytolytic activity against Bw4-protected target cells.

Single Amino Acid Substitutions in the Peptide-Binding Groove Can Influence the p70-Mediated Recognition of the ($Bw4^+$) B2705 Allele. We further investigated whether single amino acid substitutions in the peptide-binding site of the B2705 allele could alter the recognition mediated by $Z27^+$ NK clones. Thus, two different mutants of the B2705 molecule—namely, E45T and L95I—have been analyzed (23). Notably, the E45 residue was previously shown to be involved in the stabilization of peptides bound to B2705 molecules (24–27), and both mutants were also shown to affect recognition by B2705-specific NK clones (23). The corresponding C1R transfectants were used as target cells, and clones FG22, FG50, FG55, and SA260 (all characterized by the $p58^-/p70^+$ surface phenotype) were used as effectors. As shown in Fig. 3 all clones failed to lyse C1R/B27 target cells. Of the two B2705-mutated molecules, only one (E45T) induced loss of the protective effect. In contrast, the other single amino acid substitution did not significantly affect the p70-mediated recognition. Remarkably, the E45T mutant was susceptible to lysis by only three out of four clones analyzed, thus suggesting a clonal heterogeneity in the ability of $p70/Z27^+$ clones to sense modifications of the target molecules.

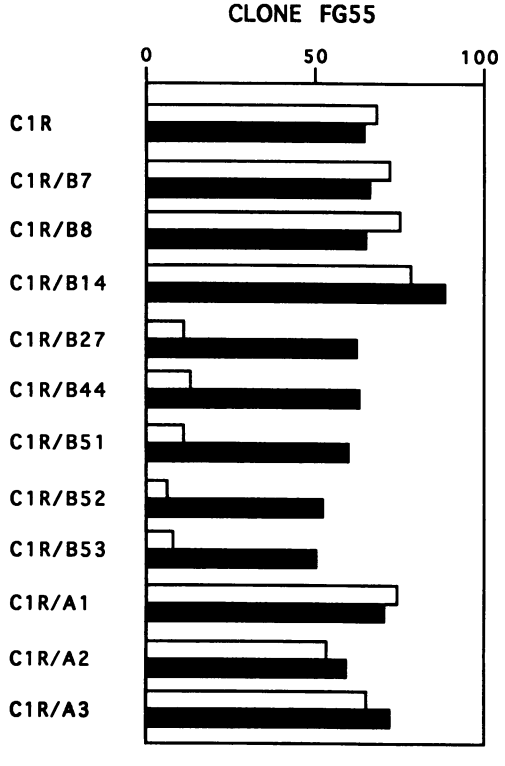

FIG. 2. Involvement of the p70 molecules identified by the Z27 mAb in the recognition of HLA-B alleles. A representative ($Z27^+/p58^-$) NK cell clone was tested for its ability to lyse C1R target cells and C1R transfected with the indicated HLA class I alleles. The cytolytic assay was performed either in the absence (□) or in the presence (■) of Z27 mAb (500 ng/ml). The E/T ratio was in all instances 4:1. Data are expressed as a percent of the specific ^{51}Cr release.

p58 and p70 Function as Independent HLA Class I-Specific Receptors in NK Clones Coexpressing the Two Molecules. We isolated $p70^+$ clones coexpressing either GL183 or EB6 (or both) receptors. Since ($Cw4^+$) C1R or C1R/B27 were used as target cells, we focused our experiments on $p70^+$ clones coexpressing (the Cw4-specific) EB6 receptors (16). These $p70^+/EB6^+$ clones were comparatively analyzed to $p70^+/EB6^-$ clones or to $p70^-/EB6^+$ clones for their cytolytic activity against C1R or C1R/B27 target cells. One clone representative of each group is shown in Fig. 4*A*. It can be seen that the $p70^+EB6^-$ clone FG50 did not lyse C1R/B27 but did lyse C1R cells. In addition, cytolytic activity against C1R/B27 target cells could be restored in the presence of the Z27 mAb. Clone FG18, which expresses the $p70^-/EB6^+$ phenotype, did not lyse either type of target cells, and cytolytic activity could be induced in the presence of anti-EB6 mAb (16).

Also clone FG123, which expresses EB6 and p70 surface molecules, failed to lyse either type of target cells; however, in this case, the anti-EB6 mAb could only restore lysis of C1R cells. Lysis of C1R/B27 could only be induced by the simultaneous addition of Z27 and anti-EB6 mAbs. These data strongly suggest that p70 and p58 molecules function as two independent inhibitory receptors specific for two distinct class I alleles. To further document this point, the EB6 molecules expressed by $p70^+/p58^+$ clones were surface modulated by overnight incubation in the presence of an appropriate anti-EB6 mAb (21). After modulation, virtually no residual EB6 molecules could be detected by either the second reagents alone or anti-EB6 mAbs followed by isotype-specific second reagents. Notably, modulation of EB6 molecules did not affect the surface expression of p70 molecules by the FG123 NK clone (Fig. 4*B*). In addition, EB6-modulated clones acquired the ability to lyse C1R cells (21) but not C1R/B27 cells. Finally, in EB6-modulated clones, addition of the Z27 mAb alone was sufficient to reconstitute the lysis of C1R/B27 cells (Fig. 4*A*).

Clones Characterized by the $p70^+/p58^-$ Phenotype Belong to the Formerly Identified "Group 3" of NK Clones. We previously described distinct groups of "alloreactive" NK clones defined on the basis of their ability to differentially lyse a panel of normal allogeneic target cells (4, 7, 11, 12). Moreover, the various groups of clones were characterized by the expression, or lack of expression, of GL183 and EB6

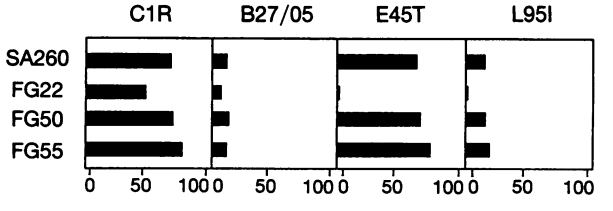

FIG. 3. Effect of single amino acidic substitutions in the peptide-binding site of the B2705 molecules on the recognition by $Z27^+$ clones. Four representative $Z27^+/p58^-$ NK clones were analyzed for cytolytic activity against C1R target cells or C1R transfected with HLA-B2705 or with one of the B2705-mutated molecules termed E45T and L95I. The E/T ratio was 4:1. Data are expressed as a percentage of the specific ^{51}Cr release.

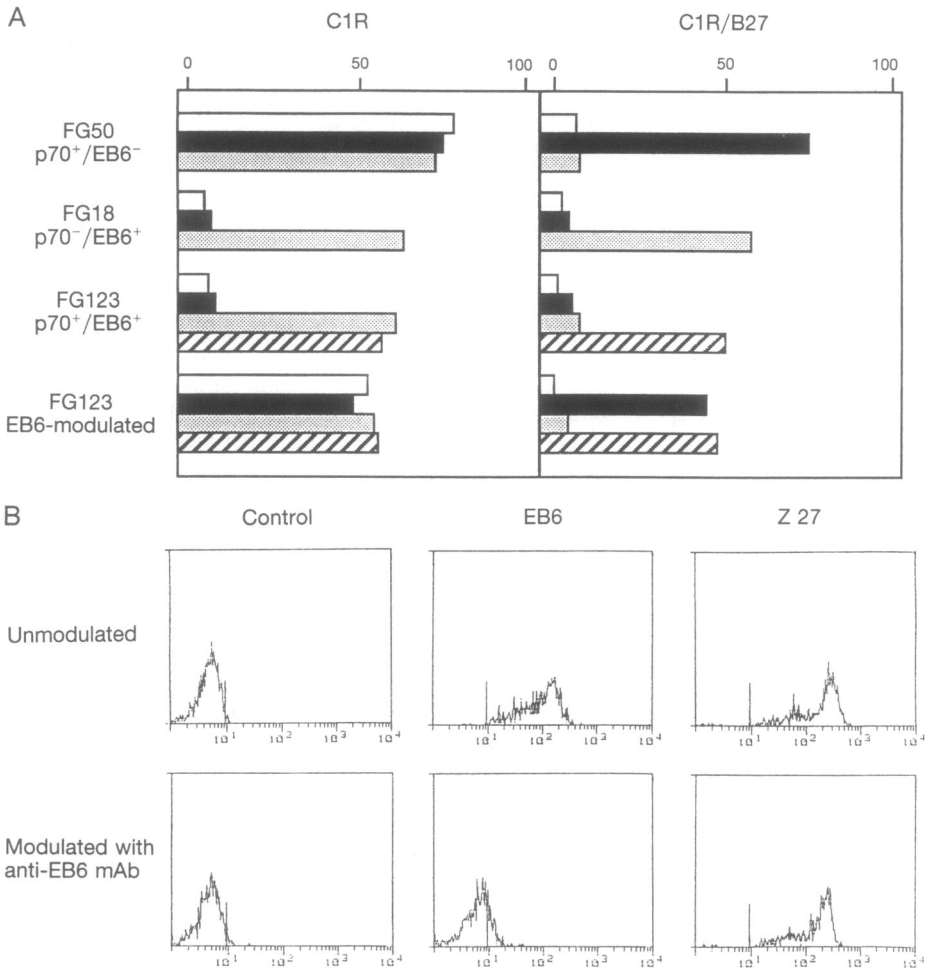

FIG. 4. EB6 and Z27 molecules independently recognize Cw4 and B2705 HLA alleles. (*A*) Three representative NK clones were assessed for cytolytic activity in a 4-hr ^{51}Cr-release assay against C1R target cells or C1R transfected with the HLA-B2705 allele. The cytolytic test was performed either in the absence of mAbs (□) or in the presence of Z27 mAb (■), EB6 mAb (▨), or a mixture of these mAbs (▧). The NK clone FG123 ($Z27^+/EB6^+$) was also analyzed for cytolytic activity after mAb-induced modulation of EB6 molecules. The E/T ratios were 4:1. Data are expressed as a percentage of the specific ^{51}Cr release. (*B*) Lack of comodulation of p70 and p58 molecules. mAb-mediated modulation of EB6 molecules was induced in clone FG123 (see *Materials and Methods*). Cells were analyzed by indirect immunofluorescence for expression of Z27 and EB6 surface molecules prior to and after EB6 modulation.

molecules (15, 16). The pattern of reactivity of group 1 and group 2 clones could be explained on the basis of the expression of either EB6 or GL183 inhibitory receptors, respectively (16, 17). An additional group of clones ("group 0") coexpressed both EB6 and GL183 inhibitory receptors (17). In contrast to the groups of NK clones above, group 3 clones did not express p58 molecules (11, 12), and their ability to lyse PHA blasts from the various members of representative HLA-typed families (11) segregated independently upon the expression of HLA-C alleles. To define a possible role for the p70 receptor, we analyzed whether clones displaying the $p58^-/p70^+$ phenotype corresponded to those previously identified as group 3 clones (11, 12). To this end, the representative clone A4-7 ($p58^-/p70^+$) was assessed for cytolytic activity against all of the members of family A that had been previously utilized for the definition of the various NK-defined allospecificities. The segregation maps previously obtained with group 3 clones were identical to those obtained with clone A4-7. Remarkably, all the individuals susceptible to lysis displayed a Bw6/Bw6 haplotype, whereas individuals resistant to lysis were either Bw4/Bw4 or Bw4/Bw6 (Fig. 5). Thus, clones expressing the $p58^-/p70^+$ phenotype correspond to those formerly identified as group 3 clones.

Clones Coexpressing Inhibitory EB6 and p70 Receptors ($EB6^+p70^+$) Belong to the Formerly Identified Group 5 of NK Clones. Another group of alloreactive NK clones had been defined as group 5 (11, 12). These clones lysed only target cells from individuals susceptible to lysis by both group 1 and group 3 clones (11). Since group 1 clones expressed the $EB6^+/GL183^-/p70^-$ phenotype, whereas group 3 clones were $EB6^-/GL183^-/p70^+$ (refs. 16 and 21 and this report), we analyzed whether $EB6^+/GL183^-/p70^+$ clones could display specificity 5. As shown in Fig. 5, the representative clone A4-11, analyzed against the members of family A, displayed a cytolytic pattern that precisely correlated with that described for group 5 clones.

DISCUSSION

In the present study, by the use of the Z27 mAb, we could establish a correlation between expression of Z27-defined molecules and the specificity of NK clones for the Bw4 supertypic specificity. The Z27-defined 70-kDa molecule (p70) functions as an inhibitory NK receptor, but it is clearly distinguishable from the HLA-C-specific p58 receptors. The combined use of anti-p58 and anti-p70 mAbs has allowed the dissection of Bw4-specific NK cells into phenotypically distinct groups of clones corresponding to the previously defined group 3 and group 5 of alloreactive NK clones (11, 12).

Different NK receptors, which specifically recognize groups of HLA-C or HLA-B alleles, have been identified. These receptors are clonally distributed among NK cells and, in some instances, they can be coexpressed at the single-cell level (15–18). GL183 and EB6 (p58) molecules have been shown to represent the receptors capable of discriminating between two different groups of HLA-C alleles (16, 17), whereas CD94 molecules appear to be involved in the recognition of HLA alleles sharing the Bw6 supertypic specificity (18). In regard to the NK receptor for the Bw4 supertypic specificity, a p70 molecule recognized by the DX9 mAb has recently been proposed as a putative candidate (19, 20). A direct comparison between the Z27 and DX9 mAbs was not possible in the present study.

Different experiments would suggest the existence of a heterogeneity among Bw4-specific NK receptors. Thus, recent

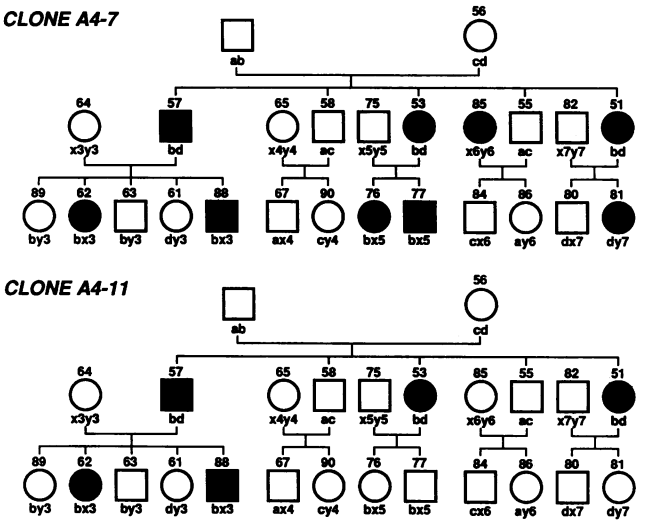

FIG. 5. Mode of inheritance in family A of the character susceptibility to lysis by NK clones expressing the $Z27^+/EB6^-$ or the $Z27^+/EB6^+$ surface phenotype. PHA-induced blasts derived from each member of this family were analyzed for susceptibility to lysis by the representative clones A4-7 ($Z27^+/EB6^-$) and A4-11 ($Z27^+/EB6^+$). The statistical analysis employed for considering target cells susceptible or resistant to lysis has been described in detail (11). Filled symbols represent susceptible donors and open symbols represent resistant (i.e., protected) donors. Circles indicate female donors and squares indicate male donors. Small letters identify serologically defined major histocompatibility complex haplotypes. Family A has been described in previous reports (7, 11, 12) and was utilized for the first definition of the various groups of "allospecific" NK clones (11, 12). Notably, the mode of inheritance of the donor's susceptibility/resistance to lysis corresponded to that of group 3 and group 5, respectively, for clones A4-7 and A4-11.

data by Malnati *et al.* (23) indicate that heterogeneity exists among B2705 (Bw4)-specific NK clones in their ability to recognize HLA-B2705 molecules mutated at single amino acids in the peptide-binding site. These experiments also showed heterogeneity in the ability to recognize a given self peptide loaded onto HLA-B2705 molecules expressed in peptide transporter-deficient target cells. In view of these data and of our present findings, it is possible that the B2705-specific NK receptors may be characterized by a limited polymorphism. It is noteworthy that the Z27 mAb-reactive molecules immunoprecipitated from all B2705-specific NK clones analyzed displayed an apparently homogeneous type of p70 molecules. This was also true for clones displaying a different capability of recognizing mutated HLA-B2705 molecules. The availability of the Z27 mAb has allowed the simultaneous analysis of the expression of p70 and p58 receptors in resting as well as in activated NK cell populations and in NK cell clones. It is noteworthy that p70/Z27 molecules were expressed in all individuals analyzed, including not only $Bw4^+$ (either homozygous or heterozygous) donors but also in Bw6/Bw6 donors. This is in line with the concept that class I allele-specific receptors can be expressed regardless of their ability to recognize self class I alleles (15, 17). However, these inhibitory receptors for non-self alleles must necessarily be coexpressed in individual NK cells with inhibitory receptors for self alleles to prevent cytolytic activity against autologous cells.

Our present experiments indicate that inhibitory Bw4-specific p70 molecules may be coexpressed with different inhibitory p58 receptors (either EB6 or GL183) and that the two receptors function independently.

We originally described groups of NK clones displaying unique patterns of cytolytic activity against different allogeneic donors (11, 12, 16) and clarified the molecular basis of the susceptibility versus resistance to lysis mediated by group 1 (16, 17, 21), group 2 (16, 17), and group 0 clones (17). Here we show that NK clones expressing the p70 receptor (but not GL183 and EB6 molecules) are responsible for the formerly identified "specificity 3," whereas those that coexpress p70 and EB6 inhibitory receptors correspond to the previously described group of clones displaying the "specificity 5."

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