Regulation of Hematopoiesis In Vitro by Alloreactive Natural Killer Cell Clones

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Summary

Natural killer (NK) cells lyse autologous and allogeneic target cells even in the absence of major histocompatibility complex (MHC) class I antigens on the target cells. Recently, however, human allospecific NK cell clones have been generated that recognize at least five distinct specificities inherited recessively and controlled by genes linked to the MHC. Because the genetic specificity of these alloreactive NK cells in vitro appears analogous to that of in vivo NK cell–mediated murine hybrid resistance, i.e., the rejection of parental bone marrow in irradiated F_1 animals, we tested the ability of human alloreactive NK clones to recognize allogeneic hematopoietic progenitor cells. NK cells from two specificity 1 alloreactive NK clones, ES9 and ES10, significantly and often completely suppressed colony formation by purified peripheral blood hematopoietic progenitor cells from specificity 1–susceptible donors, but had no significant effect on the cells of specificity 1–resistant donors. Activated polyclonal NK cells were less efficient than the NK clones in inhibiting colony formation and had a similar effect on cells from both specificity 1–susceptible and –resistant donors. The alloreactive NK clones produced cytokines with a suppressive effect on in vitro hematopoiesis, such as interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α), when exposed to phytohemagglutinin blasts from specificity 1–susceptible, but not –resistant donors. However, the mechanism by which alloreactive NK cells inhibit colony formation is more consistent with a direct cytotoxic effect than with the production of inhibitory cytokines because antibodies (anti-IFN-γ, α-TNF-α, and -lymphotoxin) that completely blocked the inhibition by polyclonal NK cells had only a minimal effect on the inhibition by the alloreactive clones. Moreover, the alloreactive clones were directly cytolytic in a ^{51}Cr release assay against enriched preparations of peripheral blood progenitor cells from specificity 1–susceptible donors. These data indicate that the alloreactive NK cells are likely the human counterpart of the cells mediating murine hybrid resistance and that these cells might play clinically important roles in rejection or in graft-versus-leukemia reactions after allogeneic bone marrow transplantation.

NK cells lyse in vitro a variety of target cells even in the absence of MHC antigens on the target cells. NK cell–mediated killing, therefore, unlike TCR-mediated lysis, does not require MHC compatibility (1). Several lines of evidence, however, suggest that this non-MHC-restricted cytotoxicity may not be completely independent of MHC expression on target cells. In several experimental systems it was shown that the susceptibility of cells to NK cell–mediated lysis is inversely proportional to their expression of class I MHC antigens (2–6, and reviewed in reference 7). Cells from β_2-microglobulin-deficient mice do not express class I MHC antigens and are particularly sensitive to NK cell–mediated lysis; interestingly, NK cells from these animals are low in number and have a reduced lytic activity, suggesting that NK cells might be subjected to a negative selection mechanism in vivo, similarly to T cells (8). One of the hypotheses to explain this phenomenon is the "missing self hypothesis," which postulates that one function of NK cells is to recognize and eliminate cells that do not express MHC class I molecules (7). Although this hypothesis is compatible with at least some of the experimental findings, the mechanism of class I recognition by NK cells has not been elucidated, and two alternative hypotheses are often considered: (*a*) the receptor inhibition hypothesis postulating that the presence of class I on target cells delivers a negative signal to the NK cells, or (*b*) the target interference hypothesis postulating that class I antigens or their associated peptides sterically mask recognition of an NK target structure on the target cells (9). The finding that the Ly-49 antigen present on a small subset of NK cells in certain mouse strains is associated with failure

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of NK cells to lyse target cells carrying the H-2D^d antigen indicates that NK cells may be capable of a fine recognition of class I antigens (10).

In the mouse, NK cells are the primary effectors of in vivo hybrid resistance, i.e., the rejection of parental bone marrow grafts by lethally irradiated F_1 hybrids (11, 12). Unlike lysis in vitro of tumor cells, this in vivo NK-mediated reaction is highly specific, genetically restricted, and directed at cellular determinants present on hematopoietic cells only. These determinants are proposed to be the products of a class of noncodominant genes (hematopoietic histocompatibility [Hh][1] genes), of which the main one, Hh-1, maps to the MHC (H-2) between the S and D regions (13, 14). The ability of hematopoietic cells carrying relevant Hh-1 specificity to compete in vivo with hematopoietic progenitor cells and to suppress hybrid resistance (15) strongly suggests that NK cells are not only the effector cells of the resistance, but are also directly responsible for the genetic specificity of this phenomenon and can specifically recognize Hh-1.

Although there is no evidence for a hybrid resistance phenomenon or the existence of Hh-like genes in humans, recent reports documenting the ability of MLC-derived CD3$^-$, CD16$^+$ clones to specifically lyse PHA-induced blasts generated from the stimulating donor and a proportion of allogeneic donors, but not those generated from autologous donors (16), point to the existence of a strikingly similar system. So far, at least five distinct NK allospecificities have been identified (17, 18). Genetic analysis of these allospecificities indicated that the genes controlling the character "susceptibility to lysis" segregate independently, are inherited in an autosomal recessive mode, are linked to the MHC locus, and at least one of them maps in the MHC region between the BF complement cluster and the HLA-A locus (18–20). Thus, the genetic control of these specificities closely resembles that of Hh-1 observed in the murine system. The recent finding (21) that transfection of target cells with genomic DNA encoding the class I antigen CW3 specifically prevents recognition by allospecificity 2 NK cell clones indicates that the character "resistance to lysis" is a dominant character. This, therefore, explains the observation of pedigree analysis that the phenotype "susceptibility to lysis" is inherited as a recessive trait. Although the specificity of alloreactive NK cell clones correlates with the expression of certain NK cell surface makers, suggesting a possible role of these surface structures in target recognition and/or interaction with class I (10, 17), the exact nature of the receptors involved in class I recognition, and whether class I antigens act by masking an NK recognition site or by delivering a negative signal, remains to be investigated.

Since the hybrid resistance phenomenon has been difficult to reproduce in vitro (22), human NK clones may provide a useful tool to dissect events of potential relevance in the NK-mediated allogeneic recognition of hematopoietic cells. In the present study, using the two human alloreactive NK clones, ES9 and ES10, both recognizing specificity 1, we found that allorecognition of PHA blasts derived from donors carrying specificity 1 induces IFNγ production by the NK clones, especially in synergy with other inducers, such as IL-2 and NK cell stimulatory factor (NKSF/IL-12). Moreover, the clones inhibit in vitro colony growth of hematopoietic progenitors from specificity 1–susceptible but not specificity 1–resistant donors. Antibodies against the hematopoietic inhibitory factors IFN-γ, TNF-α, and lymphotoxin (LT) only minimally prevented the inhibition of colony formation by the alloreactive clones, whereas they completely prevented the inhibition mediated by polyclonally activated NK cells or that of specificity 1–resistant progenitors mediated by alloreactive NK clones in the presence of bystander specificity 1–susceptible PHA blasts. In ^{51}Cr release cytotoxic assays, alloreactive NK clones lysed both PHA blasts and enriched peripheral blood hematopoietic progenitor cells (PBHPC) from specificity 1–susceptible but not –negative donors. These results suggest that the NK cell allospecificity 1 is expressed on hematopoietic precursor cells and that alloreactive NK cell clones can inhibit colony growth with a direct cytotoxic mechanism.

Materials and Methods

Generation of ES9 and ES10 Alloreactive NK Cell Clones. ES9 and ES10 clones were obtained, as previously described in detail (16, 21), after cloning under limiting dilution conditions. CD3$^-$CD16$^+$ responder cells were generated in MLC culture stimulated by irradiated PBL from a normal donor. These two clones specifically lyse PHA blasts carrying the NK cell allospecificity 1. In the present study, the clones ES9 and ES10 were defrosted from a frozen stock and maintained in culture for ~1 mo in RPMI 1640 (Flow Laboratories, Irvine, Scotland) containing 10% FCS (Hyclone Labs., Logan, UT), 100 U/ml rIL-2, and 10% Lymphocult-T-LF (Biotest AG, Dreieich, Germany). The clones were confirmed by indirect immunofluorescence (flow cytometry) to be homogeneously CD16$^+$ CD56$^+$CD5$^-$CD3$^-$ at the time of assays.

Generation of Polyclonal NK Cells. PBMC from normal donors were obtained by Ficoll-Hypaque (F/H) density gradient and cocultivated with the γ-irradiated B lymphoblastoid cell line RPMI 8866 (23). After 8 d of culture, NK cell populations (>95% CD3$^-$CD5$^-$CD16$^+$CD56$^+$) were isolated by negative selection using anti-CD3, -CD5, and -CD14 antibodies and indirect immunorosetting with $CrCl_3$-treated, goat anti–mouse Ig–coated sheep erythrocytes (GαM-SRBC), as previously described (23).

Cell Preparations. PBL from 30 randomly chosen normal donors were isolated by F/H gradient and depleted of monocytes by plastic adherence (60 min at 37°C). PHA blasts were obtained by culturing PBL for 4 d with 5 μg/ml PHA (Sigma Chemical Co., St. Louis, MO) in the presence of rIL-2 (100 U/ml). Purification of PBHPC was performed as previously described (24), with some modification. Briefly, light-density cells isolated from PBL on Percoll gradient (d = 1.066) and depleted of T lymphocytes by rosetting with 2-aminoethylisothiouronium bromide (Sigma Chemical Co.)-treated SRBC were incubated (45 min at 4°C) with appropriate amounts of the following mAbs: OKT3 (anti-CD3), B36.1 (anti-CD5), B67.1 (anti-CD2), BC1 (anti-CD20), IV.3 (anti-CDw32), 3G8 (anti-CD16), B159.5 (anti-CD56), B52.1 (anti-CD14), B13.4 (antigranulocytes and antimonocytes), B59.2 (anti-CD41), and J5 (anti-CD10). After three washes with cold IMDM (Gibco Laboratories, Grand Island, NY), the cells were mixed with GαM-SRBC

[1] *Abbreviations used in this paper:* Hh, hematopoietic histocompatibility; LT, lymphotoxin; NKSF, natural killer cell stimulatory factor; PBHPC, peripheral blood hematopoietic progenitor cell.

and centrifuged for 5 min at 1,000 rpm. Nonrosetting negative cells, containing highly enriched populations of PBHPC (84–91% $CD34^+$), were recovered after F/H density gradient centrifugation. In optimal culture conditions, >30% of these cells formed hematopoietic colonies. A lower clonal efficiency (~5%) was observed in the microculture system used in this study. The different cell fractions (PBL, PHA blasts, and PBHPC) were used immediately or, in some instances, cryopreserved until used.

Cytotoxicity Assay. The cytolytic activity of the clone ES9 and ES10 or polyclonal NK cells was tested in a 4-h ^{51}Cr release assay using as target cells PHA blasts and PBHPC derived from the allogeneic donors or the K562 cell line. Percent of specific lysis was determined as previously described (25).

Proliferation Assay. Clone ES9 and ES10 NK cells were extensively washed and cultured with or without γ-irradiated (5,000 rad) PHA blasts derived from different allogeneic donors at a ratio of 5:1 for 48 h at 37°C in the absence or in the presence of rIL-2 (100 U/ml), rNKSF/IL-12 (1 ng/ml; Genetics Institute, Cambridge, MA), or rIL-2 plus rNKSF/IL-12. 6 h before harvesting, the cells were pulsed with [^{3}H]Tdr (2 μCi/well, 2 Ci/mmol; ICN Radiochemical, Irvine, CA). The cells were collected on glass fiber filters, and [^{3}H]TdR incorporation was evaluated in a beta counter.

IFN-γ and TNF-α Induction Assay. Clone ES9 and ES10 NK cells were incubated for 18 h with PHA blasts derived from the different allogeneic donors at a ratio of 5:1 in the absence or presence of rIL-2 (100 U/ml) or rNKSF/IL-12 (1 ng/nl). After incubation, triplicate 50-μl samples of cell-free supernatants were collected from each well and IFN-γ and TNF-α were measured by RIA as previously described (26, 27).

Assay for In Vitro Hematopoiesis. PBHPC, resuspended with or without allogeneic polyclonal NK cells or ES9 and ES10 clones at different NK cell/PBHPC ratios (from 5:1 to 40:1) in IMDM containing 30% FCS, 10% BSA (Sigma Chemical Co.), 5×10^{-5} 2-ME (Sigma Chemical Co.), 50 U/ml rIL-3, and 20 U/ml rGM-CSF (kindly provided by Dr. Steven Clark, Genetics Institute, Cambridge, MA) were centrifuged to allow cell-to-cell contact and then incubated for 6 h at 37°C, 5% CO_2. In selected experiments, a cocktail of neutralizing antibodies against IFN-γ, (mAb B133.3, ascites), TNF-α (mAb B154.2, ascites), and LT (polyclonal goat antiserum) was added to the culture at a final dilution of 1:500. After incubation in liquid suspension, 500-μl aliquots of 0.9% methylcellulose (Fluka Chemie AG, Buchs, Switzerland) containing 500 PBHPC were seeded in triplicates in 24-well plates (Nunc, Inc. Naperville, IL). After 14 d of culture, CFU-GM colonies were enumerated in situ using an inverted microscope.

Statistical Analysis. The data were analyzed using the STATS$^+$ (StatSoft Inc., Tulsa, OK) statistical program.

Results and Discussion

The phenotype of 30 randomly chosen normal donors was determined by testing the susceptibility of their PHA blasts to the specificity 1 NK clones ES9 and ES10 in a ^{51}Cr release cytotoxicity assay. 11 of 30 were found to be highly susceptible to lysis mediated by both clones, and therefore expressed the NK-recognized allospecificity 1, whereas two displayed an intermediate phenotype. The degree of cytolysis (50–100% specific lysis at an E/T ratio of 5:1 for the susceptible donors, <20% for the resistant donors) and the expression of the character "susceptibility to lysis" by specificity 1 NK clones in approximately one-third of the donors indicated a similar distribution of the character in the racially heterogeneous population analyzed in this study compared to that originally reported in a more homogeneous population in previous studies (19). However, we also observed two donors that were lysed with intermediate susceptibility (20–50% specific lysis). Although this rare observation may not represent a true intermediate phenotype and may simply be due to heterogeneity in the susceptibility of the PHA blast preparations to the cytotoxic mechanisms mediated by NK cells, we cannot exclude the possibility that these donors may be indicative of a previously unrecognized phenotype, possibly not present in the originally studied populations, and suggesting a new level of complexity in the genetic control of the character "susceptibility to lysis" (18, 19). For all donors, allogeneic polyclonal NK cells did not lyse the PHA blast target cells even when pure preparations of activated NK cells were used.

We investigated whether specificity 1–susceptible PHA blasts, in addition to being sensitive to cell-mediated cytolysis, were also able to induce the NK clones to proliferate and produce cytokines, such as IFN-γ and TNF-α. Irradiated PHA blasts from representative susceptible donors (nos. 19 and 23) and resistant individuals (nos. 22 and 24) were cultured with the ES10 clone in the absence or in the presence of rIL-2, rNKSF/IL-12, or a combination of the two cytokines. After a 48-h culture, proliferation was evaluated by [^{3}H]TdR incorporation. As shown in Table 1, the allogeneic blasts did not elicit a mitogenic signal for the ES10 clones and did not significantly affect their proliferative response to rIL-2 and rNKSF/IL-12. The inability of NK cell clones after prolonged in vitro culture to respond to allogeneic cells with proliferation, although they were still able to proliferate in response to mitogenic cytokines, contrasts with the observed in vitro expansion of alloreactive NK cells during the initial MLC and may be characteristic of the stage of maturation/differentiation of the clonally expanded cells. rNKSF/IL-12 by itself had a modest proliferative effect on the clone ES10 NK cells. As previously observed with polyclonal NK cells (28, 29), NKSF/IL-12 mediated an antagonistic effect on the IL-2-driven proliferation of the NK cells.

We investigated the ability of specificity 1–susceptible PHA blasts to induce production of IFN-γ and TNF-α by NK clones. PHA blasts from 11 representative donors were incubated 18 h with ES9 and ES10 clones (1.25×10^4/ml) at a ratio of 5:1 with or without rIL-2 or rNKSF/IL-12. The results reported in Table 2 demonstrate that PHA blasts from specificity 1–susceptible donors induced ES9 and ES10 clones to produce IFN-γ with increased production in the presence of rIL-2 and rNKSF/IL-12. PHA blasts from resistant donors or from an individual displaying an intermediate phenotype (e.g., donor no. 5) were ineffective in inducing IFN-γ production. In all five experimental conditions tested (ES9 or ES10 clones, with or without cytokines), PHA blasts from specificity 1–susceptible donors induced significantly higher levels than PHA blasts from donors lacking this specificity (student's t test, $p \leq 0.002$). When the very low concentration of NK cells (~10^4/ml) used in the experiments described in this paper is considered, the alloreactive NK cell clones appear particularly efficient in producing IFN-γ. In preliminary ex-

Table 1. *Culture with Susceptible or Resistant PHA Blasts Does Not Influence the Proliferation of Clone ES10 NK Cells*

Cytokines	PHA blasts from donor				
	None	No. 19 (73%)	No. 22 (0%)	No. 23 (72%)	No. 24 (1%)
			cpm		
None	388	424	442	426	419
rIL-2 (100 U/ml)	1,596	1,749	1,688	1,632	1,438
rNKSF/IL-12 (1 ng/ml)	668	971	595	682	585
rIL-2 + rNKSF/IL-12	638	800	229	608	901

Alloreactive (specificity 1) NK cells (clone ES10) were incubated for 48 h in medium supplemented or not with rIL-2, rNKSF/IL-12, or both cytokines and in the presence or absence of irradiated PHA blasts from four different donors, two of which (nos. 19 and 23) were susceptible to lysis by ES10 cells and two (nos. 22 and 24) that were resistant (percent ^{51}Cr release at 5:1 E/T ratio is indicated in parentheses after the donor number). Data are [^{3}H]TdR uptake measured after a 6-h pulse.

periments with a small number of donors, it was observed that not only PHA blasts, but also resting PBL from specificity 1–susceptible donors, induced the alloreactive NK clones to produce IFN-γ (not shown). This finding suggests that the molecule(s) engaged upon allospecific NK-mediated recognition may be involved in the delivery of intracellular signals leading to IFN-γ production. Low titers of TNF-α (<5 U/ml) were also found in the supernatant fluid of cultures of ES9 and ES10 cells with sensitive PHA blasts, in the presence of rIL-2 or rNKSF/IL-12 (data not shown).

We tested whether the specificities recognized by the human alloreactive clones are expressed on hematopoietic progenitor cells, similarly to the putative Hh determinants, by analyzing whether the colony formation in semisolid medium by PBHPC from the specificity 1–susceptible donors was inhibited by clone ES9 and ES10 NK cells. Fig. 1 illustrates two experiments with one susceptible and one resistant donor at different E/T cell ratios, whereas Fig. 2 summarizes the results of all experiments. Polyclonal NK cells, as previously reported (30), only partially inhibited peripheral blood my-

Table 2. *Clone ES9 and ES10 NK Cells Produce IFN-γ in Response to PHA Blasts from Specificity 1–susceptible Donors*

PHA blasts from donor:	ES9 clone			ES10 clone			
	Percent ^{51}Cr release	Medium	rIL-2	Percent ^{51}Cr release	Medium	rIL-2	rNKSF/IL-12
		U/ml				*U/ml*	
None	NA*	3	4	NA	<1	2	3
1	53	13	26	74	19	25	39
2	77	23	39	76	31	50	70
3	95	13	23	100	25	30	44
4	12	2	3	6	5	7	6
5	33	2	4	30	3	4	5
6	74	18	24	68	20	28	27
7	7	7	5	8	<1	6	4
8	89	12	14	77	7	25	17
9	4	5	4	6	2	3	6
10	6	5	6	13	<1	7	4
11	7	2	5	6	3	9	6

Alloreactive (specificity 1) NK cell clones were tested for their cytotoxicity against PHA blasts from donor 1–11 and for the ability to produce IFN-γ when cultured with the same PHA blasts. Cytotoxicity was tested in a 4-h ^{51}Cr release assay in medium at a 5:1 E/T cell ratio. IFN-γ was measured by RIA in the cell-free supernatant fluids collected from 18-h cultures of NK clones (1.25×10^4/ml) and PHA blasts (0.25×10^4/ml) in medium or in the presence of rIL-2 (100 U/ml) or rNKSF/IL-12 (1 ng/ml). PHA blasts alone did not produce detectable IFN-γ.
* NA, not applicable.

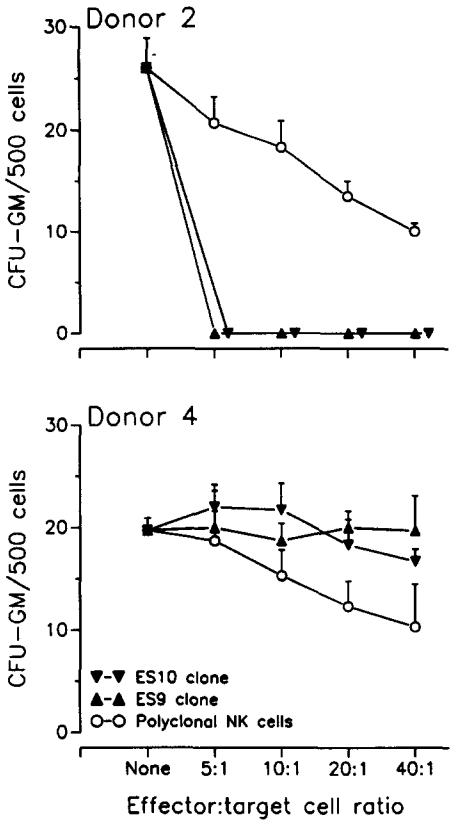

Figure 1. Inhibition of CFU-GM formation by polyclonal NK cells and alloreactive (specificity 1) NK cells clones. PBHPC (500/well in the presence of IL-3 and GM-CSF) from donor nos. 2 and 4, susceptible and resistant, respectively, to specificity 1 NK cell clones, were tested for CFU-GM formation in the presence of polyclonal NK cells (O) or ES9 (▲) and ES10 (▼) allospecific NK clones at the indicated NK cells to PBHPC ratios. Data are average + SD of triplicate determinations.

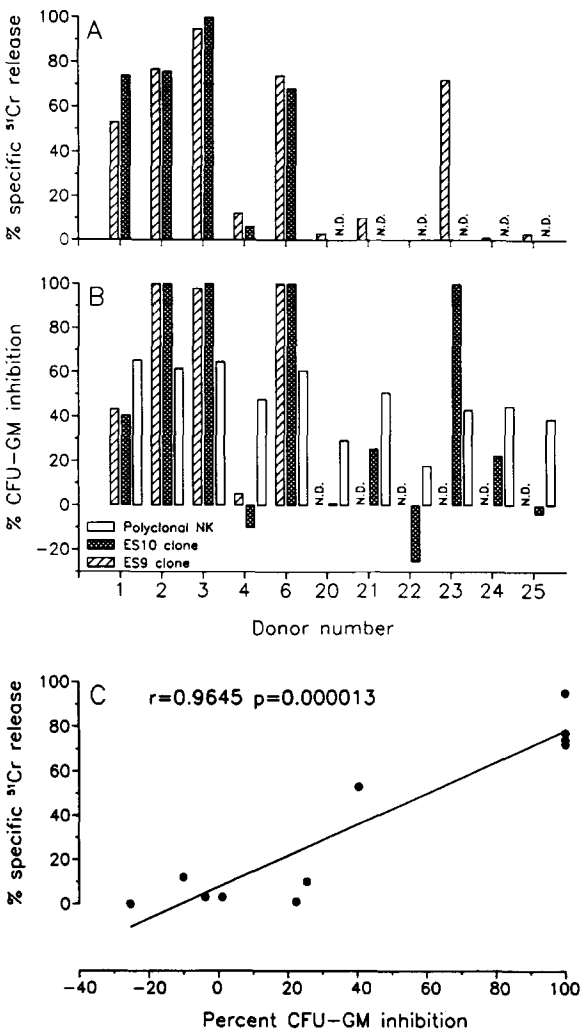

Figure 2. Sensitivity of PHA blasts and PBHPC to alloreactive NK cells. (*A*) Clones ES9 and ES10 were tested in a 4-h ^{51}Cr release assay against ^{51}Cr-labeled PHA blasts (E/T ratio 5:1) from 11 different donors. (*B*) PBHPC from the same 11 donors were tested for CFU-GM formation in the presence or absence of polyclonal NK cells (NK cells/PBHPC ratio 40:1) or clones ES9 and ES10 NK (ratio 10:1). Results are expressed as percent inhibition of CFU-GM formation in the presence of NK cells compared with PBHPC cultures without NK cells. (*C*) Correlation between cell-mediated cytotoxicity (ES9 clone) of PHA blasts and inhibition (ES10 clone) of CFU-GM formation by PBHPC from the same donors.

eloid colony formation from either susceptible or resistant donors even at an E/T ratio of 40:1. ES9 and ES10 NK clones at an E/T ratio of 10:1 significantly and often completely abrogated CFU-GM growth from donor nos. 1, 2, 3, 6, and 23 carrying the specificity 1 susceptibility phenotype, but did not significantly affect colony growth from resistant donor nos. 4, 20, 21, 22, 24, and 25 (Fig. 2 *B*). A significant correlation was observed between the sensitivity of PHA blasts to the cytotoxic effect of the alloreactive NK cells and the sensitivity of CFU-GM derived from PBHPC from the same donors to the inhibitory effect of the clones (Fig. 2 *C*). Both PHA blasts and PBHPC from donor no. 1 displayed a somewhat lower sensitivity to both ES9 and ES10 clones than cells from other specificity 1–susceptible donors. Although this reduced sensitivity might simply be due to experimental or individual variability, this donor might also represent another case of an intermediate phenotype, as discussed above. A one-way analysis of variance of these data, utilizing observed colony numbers, showed that the presence or absence of specificity 1 significantly affected inhibition of PBHPC by the ES9 clone ($F = 21.47$, $p = 0.004$), but not the inhibition by polyclonal NK cells ($F = 2.74$, NS).

The finding that PHA blasts from susceptible donors were able to induce the NK clones to produce high amounts of IFN-γ and, to a lesser extent, TNF-α, factors having a synergistic suppressor effect on in vitro hemopoiesis (31), prompted us to verify whether such cytokines were responsible for the observed colony growth inhibition. Hematopoietic progenitor assays were performed in the absence or presence of a mixture of neutralizing antibodies, anti-IFN-γ, -TNF-α, and -LT. As shown in Fig. 3, these antibodies had only a minimal effect on the ability of the ES10 clone to inhibit CFU-GM from donors carrying specificity 1, whereas they completely blocked the inhibitory activity of polyclonal NK cells on PBHPC from all four donors shown. When specificity

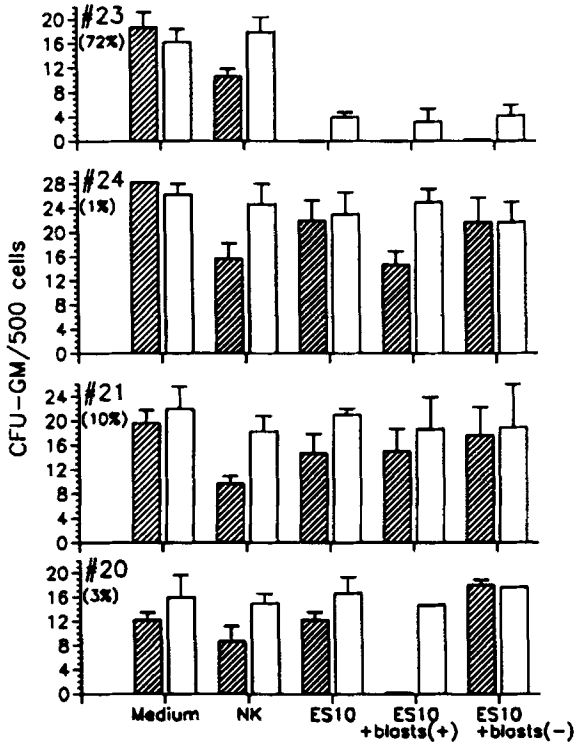

Figure 3. Role of cytokines in the inhibition of CFU-GM formation by polyclonal NK cells and alloreactive NK cell clones. Colony formation by PBHPC (500/well in the presence of IL-3 and GM-CSF) from donor no. 23, susceptible to specificity 1 NK cell clones, and from resistant donor nos. 24, 21, and 20 was measured in medium (*striped bars*) or in the presence of a mixture of anti-TNFα, -LT, and -IFN-γ antibodies (*open bars*). No NK cells (*Medium*), polyclonal NK cells (*NK*), or cells of the ES10 NK clone (*ES10*) (5×10^3/well) were added to the indicated cultures. In the indicated cultures containing PBHPC and ES10 cells, PHA blasts (1,000 cells/well) from NK allospecificity 1-positive [*blasts* (+)] or negative [*blasts* (−)] donors were also added. Results are expressed as average + SD from triplicate experiments. The number in parentheses under the donor number indicates the percent ^{51}Cr release obtained when a cytotoxic assay was performed using clone ES9 effector cells and PHA blasts from the indicated donors as target cells (E/T ratio 5:1). These results are representative of six donors tested.

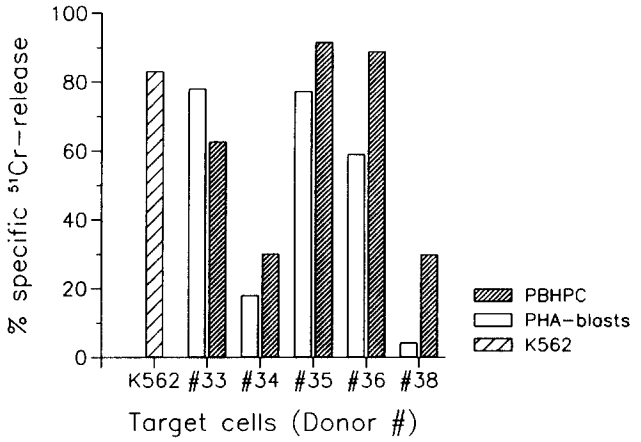

Figure 4. Sensitivity of PHA blasts and PBHPC to the cytotoxic activity mediated by ES10 alloreactive NK cell clones. Cells from the ES10 NK clone were used as effector cells (E/T ratio 4:1) against ^{51}Cr-labeled K562 cells or PHA blasts and PBHPC from donor nos. 33, 35, and 36, susceptible to specificity 1 NK clones and donor nos. 34, and 38, specificity 1 resistant, in a 4-h ^{51}Cr release assay.

1-susceptible and -resistant PHA blasts [Fig. 3, *blasts* (+) and *blasts* (−), respectively] were added to the culture of alloreactive NK clones with PBHPC, a partial to complete inhibition of specificity 1-resistant PBHPC was observed when PHA blasts from susceptible, but not resistant donors, were added (Fig. 3 illustrates the results obtained with PBHPC from three representative specificity 1-resistant donor: nos. 20, 21, and 24). Unlike the inhibitory effect of ES10 cells on specificity 1-susceptible PBHPC from donor nos. 23, the inhibition of specificity 1-resistant PBHPC (donor no. 20, 21, and 24) in the presence of bystander specificity 1-susceptible PHA blasts (*blasts* [+]) was completely prevented by the antibodies against IFN-γ, TNF-α, and LT.

These results suggest that the inhibition of specificity 1-susceptible PBHPC by alloreactive NK cell clones could be mostly due to a direct cytotoxic effect of the NK cells on the hematopoietic progenitor cells. To directly test this hypothesis, we analyzed the ability of clone ES10 to lyse ^{51}Cr-labeled PHA blasts and PBHPC from five different donors, three positive and two negative for specificity 1. As shown in Fig. 4, ES10 at an E/T ratio of 4:1 lysed >60% of both PHA blasts and PBHPC from the three susceptible donors, whereas PHA blasts and PBHPC from the two resistant donors were lysed at a much lower level. In particular, >90% of PBHPC from susceptible donor nos. 35 and 36 were lysed by ES10 cells. Because >80% of PBHPC are $CD34^+$ and >30% of them form hematopoietic colonies in vitro, these data are consistent with the hypothesis that the alloreactive NK cells can lyse hematopoietic precursor cells from specificity 1-susceptible donors. In the same experimental conditions, activated polyclonal NK cells were unable to lyse either PHA blasts or PBHPC.

The data presented in this paper demonstrate that the specificity recognized by alloreactive NK cell clones is expressed not only on lectin-activated T lymphocytes or EBV-transformed B cells, as originally described (16, 21), but also on resting PBHPC and probably other hematopoietic cells. The specificity of alloreactive NK cells is likely determined by a pattern of class I MHC antigen expression on target cells that determines resistance or susceptibility to lysis by different NK cell clones (21). The ability of PBHPC to be recognized specifically by alloreactive NK cells suggests that, similarly to mature cells, they efficiently express the class I MHC antigens that prevent lysis by the alloreactive NK cell clones with an inappropriate specificity. These data contrast with the hypothesis that the reactivity of NK cells with hematopoietic progenitor cells reflects a proposed function of NK cells in the surveillance against primitive cells (32) by possibly recognizing low or absent class I MHC expression (7). However, the ability of autologous or allogeneic polyclonal NK cells to affect proliferation of progenitor cells, a phenomenon mostly due to secretion of inhibitory cytokines (30, 33), may differ from the ability of alloreactive NK cell

clones to inhibit colony formation with a mechanism that suggests a direct cytotoxic effect, and the physiologic relevance of each of these phenomena remains to be determined.

The ability of PHA blasts from specificity 1–susceptible donors to specifically stimulate the production of IFN-γ and other lymphokines from specificity 1–alloreactive NK cell clones demonstrates that resistant target cells are not only unable to be lysed or to trigger the cytotoxic machinery in NK cells, but also fail to induce the signal transduction mechanism leading to lymphokine production. If the hypothesis that resistance of target cells to lysis is due to a negative signal delivered to the NK cells by class I MHC antigens on target cells is correct, then these results indicate that this negative signal affects not only cytotoxicity, but also the signaling pathways leading to both cytotoxicity and lymphokine production. Antibodies against TNF-α, IFN-γ, and LT, the cytokines most commonly involved in the inhibition of hematopoiesis by lymphocytes (26, 33), only minimally ($\sim$20%) prevented the inhibition of CFU-GM by alloreactive NK cells. These results and the finding that the alloreactive NK clones efficiently lyse highly enriched preparations of PBHPC are consistent with the hypothesis that these NK cells mostly act via a direct cytotoxic effect on the progenitor cells. However, because the PBHPC preparations used in this study are not completely pure, the possibility of an indirect effect of the alloreactive NK cells can not be excluded. By contrast, alloreactive NK cell clones can at least partially inhibit PBHPC from resistant donors when bystander PHA blasts from susceptible donors are present and this inhibition is completely reversed by anticytokine antibodies. Therefore, in this latter system, the mechanism of inhibition of PBHPC by alloreactive NK cell clones appears to be analogous to that mediated by autologous and allogeneic polyclonal NK cells at much higher E/T ratios. The mechanism of induction of cytokine production in polyclonal NK cell preparations is not well characterized and the question of whether the progenitor cells themselves or other cell types present in enriched progenitor cell preparations are responsible for their induction is still an open question (30, 33).

In the mouse, in vitro models for hybrid resistance have been difficult to establish. NK cell preparations have been shown to inhibit hematopoietic colony formation in vitro in the strain combinations incompatible for Hh-1, but at levels only marginally higher than those observed in syngeneic combinations (22). Fresh or activated NK cells from athymic rats lyse and inhibit colony formation by allogeneic hematopoietic progenitor cells, but stimulated syngeneic hematopoiesis (34). Although crosscompetition experiments have shown that allodeterminants coded for by single MHC haplotypes are responsible for the specificity of alloreactive rat NK cells (35), the experimental systems using polyclonal NK cell cultures offer a limited ability to investigate the fine specificity of NK cell recognition. The development of human alloreactive NK clones has offered new opportunities for the understanding of the mechanism of target cell recognition by NK cells, and important information has already been provided by this experimental system. Our present results show that human NK cell alloreactivity is probably the human counterpart of the mouse and rat hybrid resistance phenomenon. Because of the high efficiency of alloreactive NK cells to mediate cytotoxicity and cytokine release, it is possible that they play a role in the rejection of clinical allogeneic bone marrow transplants, that, in at least a few cases, has been shown to be mediated by host NK cells (36, 37). More importantly, however, alloreactive NK cells of donor origin might be involved in the graft-vs.-leukemia reaction in transplanted patients. It has been reported that a proportion of transplanted patients with chronic myelogenous leukemia develop IL-2-activatable NK cells that lyse autologous and allogeneic leukemia cells with an intriguing pattern of selectivity that is reminiscent of that seen for alloreactive NK cell clones (38). It is of interest that the relapse rate is much lower in the patients developing this cytotoxic activity than in those not developing it (92.8% relapse free at 3 yr vs. 28.3%) (38). A better knowledge of the mechanism and specificity of alloreactive NK cell clones may therefore not only provide us with a better understanding of the regulation of the natural resistance mechanism, but may also provide information of clinical relevance that could lead to therapeutic attempts to modulate the activity of these cytotoxic cells or their use in adoptive transfer.

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