

Specific Recognition of Human CD3⁻CD16⁺ Natural Killer Cells Requires the Expression of an Autosomic Recessive Gene on Target Cells

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Summary

We analyzed the recently defined ability of CD3⁻CD16⁺ cells to specifically recognize and lyse normal allogeneic target cells (PHA-induced blasts). The susceptibility to lysis by a given alloreactive natural killer (NK) clone ("1 anti-A") was expressed by PHA blasts derived from 9 of 38 random donors analyzed. In all instances, the specific lysis of "susceptible" target cells was >35% while that of "nonsusceptible" targets was <6% at an E/T cell ratio of 5:1. In addition to 1 anti-A, A anti-1 specific CD3⁻CD16⁺ clones could also be isolated from the reverse MLC combination. The relationship existing between lysis of normal allogeneic cells or tumor cells by the same CD3⁻CD16⁺ effector cell has been investigated: 1 anti-A specific CD3⁻CD16⁺ clones lysed PHA blasts of three of six cancer patients, while they lysed fresh tumor cells (ovarian carcinoma) from all six patients. The type of inheritance of the character "susceptibility to lysis" was analyzed in representative families. This analysis revealed that the character is inherited in an autosomic recessive fashion, and it is therefore different from MHC. We further investigated the type of segregation of the opposite character "resistance to lysis" (which is inherited in a dominant mode). The finding that this character segregated in all donors expressing given MHC haplotypes indicated that the gene regulating the expression of the NK-defined alloantigen is present on chromosome 6.

Natural killer cells are known as lymphocytes able to lyse tumor target cells through a non-MHC-restricted type of recognition. Since these cells lack both surface Ig and CD3/TCR molecular complex, it is generally thought that they are unable to recognize antigens (1, 2). However, recent findings appear to challenge this concept since CD3⁻CD16⁺ peripheral blood-derived NK cells were shown to be capable of specific proliferation in MLC against some allogeneic donors. Perhaps more importantly, either populations or clones of such MLC-derived CD3⁻CD16⁺ cells specifically lysed normal allogeneic target cells (PHA-induced blasts) derived from the stimulating donors (but not autologous target cells) (3). The aim of the present study was to characterize this novel NK cell-mediated specificity. We show that the character "susceptibility to lysis" is present in approximately one-fourth of random donors having different MHC haplotypes. In addition, lysis of normal allogeneic cells can be clearly distinguished from the conventional non-MHC-restricted lysis of

tumor cells. Genetic analysis shows that the character is inherited in an autosomic recessive fashion (thus differing from MHC) and is carried by chromosome 6.

Materials and Methods

Isolation of CD3⁻CD16⁺ Lymphocytes, MLC, Cell Cloning, and Evaluation of Cytolytic Activity. PBL derived from normal donors were isolated on Ficoll-Hypaque (F-H)¹ gradients and cells were then incubated with a mixture of anti-CD3 (OKT3, Ortho Pharmaceuticals, Raritan, NJ), anti-CD4 (HP26), and anti-CD8 (B9.4) mAbs followed by treatment with rabbit complement for 1 h at 37°C as previously described (3). Viable cells were isolated by F-H gradients and cultured in microwells (5 × 10⁴ cells/well) in the presence of allogeneic irradiated mononuclear cells; after 4 d, the cultures were supplemented with 100 U/ml of rIL-2. The MLC-derived cells were cloned under limiting dilution conditions in the

¹ Abbreviation used in this paper: F-H, Ficoll Hypaque.

presence of allogeneic irradiated feeder cells and 100 U/ml of rIL-2, as described (4-6). 10-20 d later the cytolytic activity was tested in a 4-h ⁵¹Cr-release assay, in which effector cells were tested against PHA blasts derived from different allogeneic (or autologous) donors. PHA blasts were obtained by culturing PBL for 4 d with 0.5% PHA (vol/vol) in the presence of rIL-2 (100 U/ml). In some experiments fresh uncultured tumor cells were used as targets. Target cells were used at 5 × 10³/well, for a final E/T cell ratio of 5:1. Percent of specific lysis was determined as previously described (3).

Flow Cytofluorometric Analysis. 10⁵ cells were stained with the appropriate mAb followed by fluoresceinated goat anti-mouse Ig. Control aliquots were stained with the fluorescent reagent alone. All samples were then analyzed on a flow cytometer (FACS STAR, Becton Dickinson, Mountain View, CA) gated to exclude nonviable cells. Results are expressed as arbitrary normalized fluorescence histograms, i.e., number of cells vs. fluorescence intensity. The mAbs used in these studies were represented by anti-Leu-4 (anti-CD3, Becton Dickinson), WT31 (directed to the TCR complex expressed by TCR- α/β -bearing cells; Sanbio, Uden, the Netherlands), BB3 (directed to the disulphide-linked form of TCR- γ/δ) (4), δ -TCS-1 (directed to the δ chain of non-disulphide-linked TCR- γ/δ) (5), MAR 206 (anti-CD2) (7), and KD1 (anti-CD16) (6).

Isolation of Fresh Tumor Target Cells. Ascitic fluid was collected by paracentesis from patients with stage III-IV ovarian carcinoma into sterile 500-ml bottles containing 5,000 U heparin. The presence of neoplastic cells was confirmed by histopathologic analysis. Cells isolated by standard F-H density gradient centrifugation and macrophages were removed by adherence to plastic flasks at 37°C, 5% CO₂, for 1 h. Tumor cells were further purified by centrifugation over discontinuous F-H density gradients as previously described (8) and frozen in several aliquots according to standard procedures.

HLA Typing. PBMC from normal donors were HLA typed using the standard National Institute of Health (Bethesda, MD) complement-mediated microcytotoxicity assay.

Results and Discussion

Specificity of Alloreactive CD3⁻CD16⁺ NK Clones. Peripheral CD3⁻CD16⁺ lymphocytes derived from individual 1 upon MLC against irradiated allogeneic cells from donor A ("1 anti-A") were shown to efficiently lyse PHA-induced blasts derived from donor A (3). To determine to which extent "1 anti-A" cloned effector cells crossreacted with randomly chosen unrelated donors, they were tested for their capability of lysing PHA blasts derived from 38 normal individuals. 9 of 38 were found to be susceptible to lysis: the degree of cytolysis was consistently high (35-85% specific lysis at an E/T ratio of 5:1) and comparable to that detected when target cells from donor A were used. On the other hand, the levels of specific lysis of "nonsusceptible" donors did not exceed 6% at the same E/T ratio.

As previously shown (3), upon stimulation in MLC against donor A, anti-A specific CD3⁻CD16⁺ clones can be derived from a number of responders. In these experiments, anti-A-specific clones were derived from representative individuals 1, 2, and 3 (resistant to lysis by 1 anti-A clones) and then tested for their pattern of reactivity against a panel of PHA blasts derived from donors B, C, D (susceptible to lysis) or donors 1, 2, 3 (resistant to lysis). It should also be noted

that not all CD3⁻CD16⁺ clones derived from a given individual reacted against A. Thus, in three representative individuals, the anti-A-specific clones were ~11, 18, and 24% of the total CD3⁻CD16⁺ clones analyzed. Table 1 shows that "anti-A" reactive clones derived from individuals 2 and 3 selectively lysed B, C, and D target cells. In addition, an identical pattern of reactivity was observed with clones that had been derived from individuals 1, 2, and 3 against donor B (Table 1). It is important to note that no clones capable of lysing autologous target cells could be detected either in donors 1, 2, and 3 (Table 1), or in donors A and B (not shown). Given the ability of NK cells of donor 1 to specifically recognize target cells of donor A, we further investigated whether NK cells from donor A could lyse target cells of donor 1. Table 2 shows the pattern of reactivity of representative NK clones generated in the reverse MLC combination (i.e., A anti-1). It is evident that also A anti-1 clones specifically lysed only

Table 1. Specificity of MLC-Derived CD3⁻CD16⁺ Cytolytic Clones

Clones*	⁵¹ Cr-labeled target cells [†] from donor:						
	1	2	3	A	B	C	D
Anti-A							
CA 8.25 (1) [‡]	0 [§]	2	0	67	45	48	66
CA 2.100 (1)	1	0	0	64	36	38	56
CA 6.50 (1)	3	1	1	75	46	58	54
TA 7.50 (2)	2	1	2	39	43	49	45
TA 10.50 (2)	0	3	2	52	40	53	54
TA 2.50 (2)	2	0	0	53	60	47	48
L 41 (3)	0	1	1	54	35	66	63
L 92 (3)	3	2	2	52	70	52	49
Anti-B							
E 168 (1) [‡]	0	0	1	51	62	59	55
G 10 (1)	2	0	0	48	55	56	52
H 12 (1)	2	1	1	61	65	57	49
TG4.25 (2)	0	0	1	65	72	58	61
TG8.50 (2)	2	3	0	49	57	52	47
TG2.50 (2)	2	2	1	45	51	48	52
L 93 (3)	0	0	0	58	75	46	47

* Clones were derived from CD3⁻CD16⁺ populations cultured in MLC against donor A or B, respectively.

[†] Target cells were represented by PHA-blasts derived from donors 1, 2, 3, A, B, C, D.

[§] Results are expressed as percent of specific ⁵¹Cr release at an E/T ratio of 5:1.

[‡] (1) Clone derived from individual 1; (2) clone derived from individual 2; (3) clone derived from individual 3.

Table 2. Specificity of CD3⁻CD16⁺ Cytolytic Clones Derived from the Reverse MLC Combination (A Anti-1)

A anti-1 clones	⁵¹ Cr-labeled target cells* from donor:						
	1	2	3	A	B	C	D
AGT 11†	345	25	40	4	4	5	2
AGD 5	44	18	35	4	3	4	0
AGD 17	52	ND	39	0	1	8	4
AGD 27	37	ND	33	0	1	6	7

† Clones were derived from CD3⁻CD16⁺ populations from donor A and cultured in MLC in the presence of irradiated feeder cells from individual 1.

* Target cells were represented by PHA-blasts derived from donor 1, 2, 3, A, B, C, D.

‡ Results are expressed as percent of specific ⁵¹Cr release at an E/T of 5:1.

some allogeneic cells; however, their pattern of reactivity was clearly different from that of 1 anti-A clones.

Lack of CD3-TCR Expression in Alloreactive CD3⁻16⁺ Clones. Phenotypic analysis of several representative CD3⁻16⁺ alloreactive clones showed a lack of reactivity with a panel of anti-CD3 mAbs or mAbs specific for different types of

TCR (Fig. 1). In addition, Northern blot analysis did not show detection of productive mRNA for TCR- α , β , and γ chains or for CD3 γ and δ chains. Conversely, CD3 ϵ mRNA was consistently present (not shown). These data are in agreement with our previous reports on cloned NK cells (3, 9).

NK Cell-mediated Recognition of Normal Allogeneic Cells Can Be Distinguished from Lysis of Tumor Cells. We next investigated the relationship existing between the NK cell-mediated lysis of normal allogeneic cells and the conventional non-MHC-restricted lysis of tumor target cells. To address this question, we analyzed alloreactive CD3⁻CD16⁺ clones for their cytolytic activity against either PHA blasts or tumor cells (ascitic ovarian carcinoma cells) isolated from a number of patients. No preferential lysis was observed against tumor cells isolated from patients whose PHA blasts were lysed by the alloreactive 1 anti-A clones. Table 3 shows that CD3⁻CD16⁺ clones lysed PHA blasts of 3 of 6 patients only, while they lysed tumor cells from all patients. In addition, tumor cells were efficiently lysed also by those CD3⁻CD16⁺ clones (used as control) that did not display any evident reactivity against normal PHA blasts (derived from donor A). These results indicate that the specific recognition of normal allogeneic cells and the MHC-unrestricted cytotoxicity of tumor cells represent two independent functional capabilities of NK cells, possibly related to different mechanisms of target cell recognition.

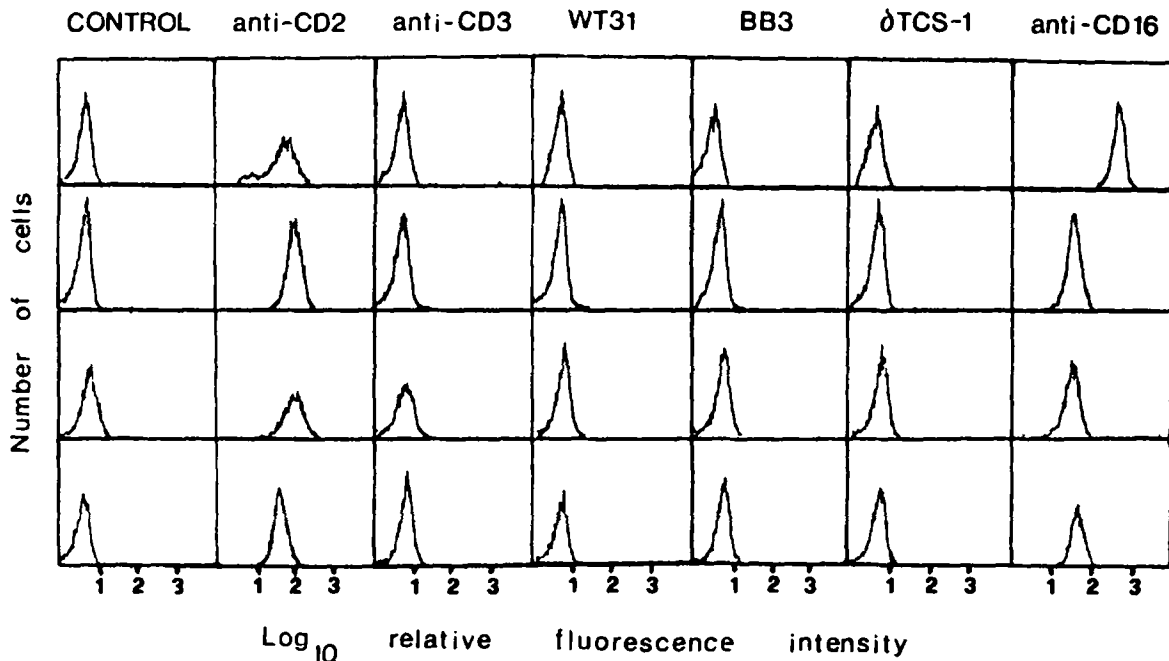


Figure 1. Lack of T cell markers in alloreactive CD3⁻CD16⁺ effector cells. Surface antigens expressed by a representative MLC-activated CD3⁻CD16⁺ polyclonal cell population (lane I) or by three clones displaying specific cytolytic activity against allogeneic PHA-blasts. (Lane II) clone G10; (lane III) clone H12; (lane IV) clone E168. The mAbs used in these studies were represented by anti-Leu4 (anti-CD3, Becton Dickinson and Co.), WT31 (directed to TCR- α/β ; Sambio, Uden, The Netherlands), BB3 (directed to the disulphide-linked form of TCR- γ/δ) (4), δ -TCS1 (directed to the δ chain of non-disulphide-linked TCR- γ/δ) (5), MAR 206 (anti-CD2) (7), KD1 (anti-CD16) (6).

Table 3. Cytolytic Activity of CD3⁻CD16⁺ Clones against Normal PHA Blasts or Tumor Cells Derived from Individual Cancer Patients

Clone (CD3 ⁻ CD16 ⁺)	⁵¹ Cr-labeled target cells											
	Donor FR		Donor IA		Donor AD		Donor PR		Donor SC		Donor RA	
	PHA blasts	Tumor cells	PHA blasts	Tumor cells	PHA blasts	Tumor cells	PHA blasts	Tumor cells	PHA blasts	Tumor cells	PHA blasts	Tumor cells
CA8.25	58	39	43	30	46	27	0	50	0	40	0	40
CA2.100	56	26	53	20	52	20	0	30	0	50	0	26
C 42	2	18	0	16	0	16	0	73	4	40	0	65
C 26	0	40	0	25	0	23	0	62	10	75	0	55
C 30	0	46	0	30	0	35	0	80	0	80	0	60

All the clones analyzed were derived from individual 1. Clones CA8.25 and CA2.100 had been preselected on the basis of their ability to lyse donor A ("1 anti-A" specificity), whereas the remaining three did not lyse donor A. Target cells were represented by peripheral blood-derived PHA-activated lymphocytes, whereas tumor cells were isolated from the ascitic fluid of six patients affected by ovarian carcinoma, as previously described by Ferrini et al. (8). Note that anti-A-specific clones lysed PHA blasts from donor FR, IA and AD, but not PR, SC and RA, whereas the remaining clones, used as control, did not lyse PHA blasts derived from any patient. In contrast all clones lysed tumor cells from all patients, independently on their anti-A specificity. Results are expressed as percent specific ⁵¹Cr-release at an E/T ratio of 10:1.

Mode of Inheritance of the Character "Susceptibility to Lysis."
To investigate whether the alloantigen recognized by CD3⁻CD16⁺ cells could be represented by molecules encoded by class I or class II MHC genes, we determined whether the character "susceptibility to lysis" was codominantly inherited (as MHC markers are) (10). To this end, target cells were derived from parents of donors A and B. In these experiments, the reactivity of alloreactive 1-anti-A or 1-anti-B CD3⁻CD16⁺ clones was compared with that of conventional CD3⁺TCR- α/β ⁺ alloreactive (anti-A or anti-B) cytolytic clones. As expected, CD3⁺ clones lysed at least one of the parent's cells. In contrast, anti-A- or anti-B-specific CD3⁻CD16⁺ clones failed to lyse target cells derived from any of these parents (Table 4). These results suggest that, different from HLA, the character examined is recessively inherited.

To gain more precise information on the mechanism of inheritance of this genetic trait, we studied a large family of 38 members spanning three generations (Fig. 2). The PHA blasts derived from these individuals were analyzed for their susceptibility to lysis by "1-anti-A" CD3⁻CD16⁺ clones. In this family, the female founder, whose blast cells were susceptible to lysis, had eight offspring; of these, four (50%) were susceptible to lysis. Recessivity of the trait "susceptibility to lysis" is shown by its segregation in the second and third generation. In fact, crosses between two resistant partners (see for example 54x78, 65x58, or 85x55) gave rise to susceptible individuals among the progeny. Furthermore, the progeny of a susceptible father (73) and of a resistant mother (52) or, vice versa, of a resistant father (75) and of a sensitive mother (53), are all resistant to lysis by alloreactive CD3⁻CD16⁺ cells. Pedigree analysis also shows that the progeny of partners who were both susceptible to lysis (for example 59x83 or 57x64) are also composed of susceptible individuals (5/5) for each mating. Although lymphocytes from the

Table 4. Cytolytic Activity of Alloreactive CD3⁻CD16⁺ or CD3⁺TCR- α/β ⁺ Clones

CD3 ⁻ CD16 ⁺ clones [‡]	⁵¹ Cr-labeled target cells*					
	A	MthA	FthA	B	MthB	FthB
CA 8.25 (1) [§]	67	0	6	45	0	1
CA 2.100 (1)	64	0	3	36	0	2
CA 6.50 (1)	75	5	0	46	1	1
E 168 (1)	51	2	0	62	0	0
G 10 (1)	48	1	2	55	1	0
H 12 (1)	61	3	2	65	0	0
TA 7.50 (2)	39	1	0	43	2	1
TA 10.50 (2)	52	3	1	40	0	2
TA 2.50 (2)	53	0	0	60	2	5
L 41 (3)	54	3	0	35	2	4
L 92 (3)	52	0	0	70	0	0
L 93 (3)	58	2	1	75	0	3
CD3 ⁺ TCR- α/β ⁺ clones						
EA2 (1)	33	34	0	ND	ND	ND
EG21 (1)	ND	ND	ND	61	2	45

* Target cells were represented by PHA blasts derived from donors A and B and from their mothers and fathers.

[§] (1) Clone derived from individual 1; (2) clone derived from individual 2; (3) clone derived from individual 3.

^{||} Results are expressed as percent specific ⁵¹Cr release at an E/T ratio of 5:1.

[‡] CD3⁺ clones EA2 and EG21 were derived from individual 1 upon stimulation in MLC against donor A or B, respectively.

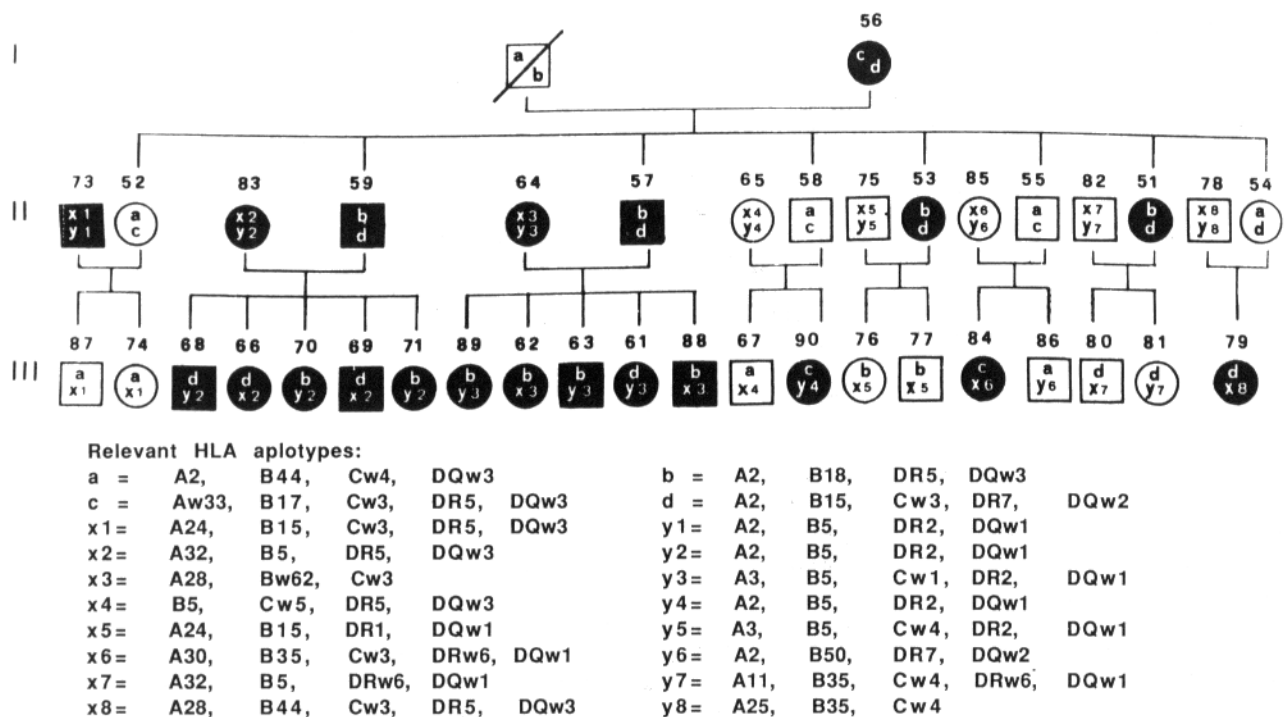


Figure 2. Mode of inheritance of the character "susceptibility to lysis" by CD3-CD16⁺ cells in a representative family. Black symbols refer to (PHA blasts of) individuals susceptible to lysis by "1 anti-A" alloreactive CD3-CD16⁺ cells, whereas resistance to lysis is represented by open symbols. Circles are used for female and squares for male donors. Letters within the symbols indicate serologically defined MHC haplotypes including markers for the A, B, C, DR, and DQ loci. The effector cells used were represented by MLC-derived CD3-CD16⁺ polyclonal population or clones from individual 1 raised against donor A.

male founder were no longer available for analysis, on the basis of 50% segregation, it can be inferred that he was heterozygous for the locus determining the phenotype examined (and must thus have been resistant to lysis). In contrast, his mate (no. 56) is homozygous for the locus causing susceptibility. From these observations it is possible to conclude that the trait "susceptibility to lysis" by alloreactive CD3-CD16⁺ cells is an autosomally inherited recessive character.

The fact that "susceptibility to lysis" is a recessive character suggests that the locus governing susceptibility or resistance to lysis does not encode an alloantigen itself, but rather a factor that negatively regulates the expression of the alloantigen. According to this hypothesis, the activity of a dominant locus determining the resistance to lysis in a given individual would inhibit the expression of the putative alloantigen (see the heterozygous individuals nos. 52 and 58 and the homozygous nos. 67, 82, or 86).

Further analysis of the pedigree (Fig. 2) indicates that the presence of MHC "a" haplotype (a = A2, B44, Cw4, DQw3) is always associated with the character "resistance to lysis." "Resistance to lysis" is also observed in all the individuals carrying the haplotypes x4, x5, y6, and y8 (Fig. 2). These data suggest that the character "resistance to lysis" segregates in all donors expressing given MHC haplotypes. As a consequence, the genes that regulate the susceptibility to lysis would be present on chromosome 6.

In conclusion, our present data indicate that CD3-CD16⁺

NK lymphocytes are capable of specific recognition and that the alloantigen recognized is clearly distinguishable from HLA antigens as indicated by its mode of inheritance. Susceptibility to NK cells has been reported to correlate with low expression of MHC class I antigen on B-EBV-transformed target cells (11, 12); however, in our experiments no differences in the amounts of HLA class I antigen could be detected on PHA-induced blast cells derived from either susceptible or resistant individuals. Although in this study we analyzed the alloantigen defined by 1 anti-A effector cells, our data indicate that at least a second NK-defined specificity exists. Thus, in addition to 1 anti-A clones, also alloreactive specific clones could be generated in the reverse MLC combination (A anti-1).

In the mouse, NK cells have been shown to reject H-2-compatible/Hh1-incompatible bone marrow grafts (the so-called hybrid resistance phenomenon) (13). Genes controlling the expression of Hh antigens were shown to be transmitted in an autosomal recessive mode and were mapped on mouse chromosome 17, telomeric to H2-S, and centromeric to H-2D (14). In view of the close similarity between these two phenomena, it is possible that the ability of human CD3-CD16⁺ cells to recognize alloantigens may represent the human counterpart of murine hybrid resistance (15, 16).

While cloned NK cells specifically lysed appropriate normal allogeneic cells (PHA blasts), the lysis of tumor target cells was clearly unrelated to this phenomenon. It would be important to define whether this may represent a major func-

tion of human NK cells, perhaps physiologically more relevant than the non-MHC-restricted cytolytic activity. The ability of NK cells to recognize alloantigen may not be physiologically important per se. However, it may reflect the ability

of these cells to specifically recognize exogenous antigens (e.g., microbial structures) either in soluble form or in association with cell surface molecules.

We thank C. Di Donato for technical help and C. Miriello for secretarial assistance.

This work was partially supported by CNR and AIRC. O. Viale is a recipient of an AIRC fellowship.

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Received for publication 5 January 1990.

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