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Phenotypic Diversity of Natural Killer (NK) Populations in Patients With NK-Type Lymphoproliferative Disease of Granular Lymphocytes

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Using monoclonal antibodies (MoAbs) termed GL183 and EB6, directed to a novel family of natural killer (NK) specific triggering molecules, four functional subsets of NK cells have been recently defined ($GL183^+EB6^-$; $GL183^+EB6^+$; $GL183^-EB6^+$; $GL183^-EB6^-$). In healthy individuals, all these subsets are represented in variable portion. The expression of EB6 and GL183 surface antigens has been analyzed in a series of 14 patients with lymphoproliferative disease of granular lymphocytes (LDGL) characterized by a chronic $CD3^-CD16^+$ lymphocytosis. Our data showed that in 11 of 14 cases, the proliferation was specifically sustained by one of the four possible subsets of granular lymphocytes (GLs) (seven cases: $EB6^-GL183^-$; three cases: $EB6^+GL183^-$; one case: $EB6^-GL183^+$). In the remaining three cases, a pattern was demonstrated that is consistent with that of healthy individuals (ie, the presence of all four subsets). When expressed on GL surfaces, in the majority of cases tested both EB6 and GL183 MoAbs behave as functional surface molecules as assessed in the redirected killing of P815 target cells. We also provided evidence that $EB6^+GL183^+$ proliferating cells show a definite (type 1) in vitro NK specificity as do their normal counterparts. The unique expansion of a defined subset of NK cells in most patients with LDGL suggests that the pathologic noxa leading to GL proliferation selectively acts on a specific subset of NK lymphocytes.

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THE LYMPHOPROLIFERATIVE disease of granular lymphocytes (LDGL) is a disorder characterized by a chronic lymphocytosis sustained by granular lymphocytes (GLs).[1-3] In the majority of cases, proliferating GLs belong to the T-cell lineage and express the $CD3^+CD16^+$ phenotype. However, in nearly 15% of cases, patients' GLs originate from the expansion of natural killer (NK) cells expressing the $CD3^-CD16^+$ phenotype.

Although the characteristics of T-cell subsets accounting for the proliferation of $CD3^+$ LDGL have been extensively investigated,[1-3] a similar approach has not yet been made possible for $CD3^-$ NK-type LDGL because no specific NK cell subpopulations have been recognized up to now. Two monoclonal antibodies (MoAbs), termed EB6 and GL183, have been recently reported to recognize two different antigens expressed on NK cell subsets belonging to the same 58-Kd molecular family.[4,5] Using these reagents, normal $CD3^-CD16^+CD56^+$ NK cells can be separated into four subsets: single-positive phenotypes (either $EB6^+GL183^-$ or $EB6^-GL183^+$), double-positive phenotype ($EB6^+GL183^+$), and double-negative phenotype ($EB6^-GL183^-$). Because the expression (or lack of expression) of each of these antigens is a stable property of NK cells, not modified by cell activation, proliferation, or cloning,[4,5] the analysis of the distribution of these antigens in proliferating NK cells of patients with LDGL can provide information on the events inciting the GL proliferation.

In this study, we investigated the expression of EB6 and GL183 molecules on proliferating GLs and their ability to mediate redirected cytotoxicity against P815 target cells in 14 patients with $CD3^-$LDGL. We also correlated the above-quoted surface marker expression with the specific pattern of cytotoxicity previously reported for normal NK subsets.[6] Our results indicate that in 11 of 14 cases, the pattern of EB6 and/or GL183 molecule expression (or the lack of expression) on proliferating $CD3^-$ GLs is restricted to only one subset. This indicates that a preferential expansion of discrete NK subsets occurs in the majority of patients with $CD3^-CD16^+$ LDGL.

MATERIALS AND METHODS

Patients. Fourteen patients with LDGL (seven men and seven women ranging in age from 33 to 74 years) were studied. Each patient met the criteria for LDGL, being characterized by a chronic proliferation (>6 months) of more than 2,000 GLs/mm^3.[2] Chronic diseases, in particular chronic infectious disorders, have been frequently reported to be associated with LDGL.[7] In our patients, an associated disease was documented in eight cases; in case no. 3, an ovarian carcinoma was diagnosed in 1989; seven patients had a previous history of liver infections, including type B (case nos. 1, 2, 12, and 13) or type C (case nos. 6, 8, and 14) hepatitis. Case no. 12 suffers from type A hemophilia. None of the patients was receiving any therapy at the time of this study.

Phenotypic evaluation. Peripheral blood mononuclear cells (PBMCs) were isolated through a Ficoll-Hypaque gradient (Pharmacia LKB, Uppsala, Sweden) and washed twice in phosphate-buffered saline according to the method previously reported.[8] The resulting cell population was represented by 55% to 95% of GLs, as evaluated by May-Grünwald–Giemsa–stained cytospins. PBMCs were studied for the expression of cell surface antigens with direct one-color or two-color analysis using a panel of fluorescein-conjugated (FITC) or phycoerythrin-conjugated (PE) mouse MoAbs, as previously reported.[8] The following FITC- or PE-conjugated MoAbs were used: anti-CD3 (OKT3) (Ortho Diagnostic, Raritan, NJ); anti-CD16 (Leu-11a), anti-CD56 (Leu-19), and anti-CD57 (Leu-7) (Becton Dickinson, Sunnyvale, CA); purified KD1 (IgG2) MoAb that reacts with Fc receptor for IgG (CD16).[9] EB6 (IgG1) and GL183 (IgG1) MoAbs identify two molecules expressed in subsets of NK cells; their specificity and char-

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Table 1. Surface Phenotype of PBMCs From Patients With LDGL

Case No.	CD3 (OKT3) MoAb	CD16 (KD1) MoAb	$CD3^-$, $CD16^{+*}$	EB6 MoAb	GL183 MoAb	EB6 MoAb Plus GL183 MoAb†	CD56 (Leu-19) MoAb	CD57 (Leu-7) MoAb
1	17	77	76	0	68	68	2	42
2	28	67	66	61	3	62	57	72
3	30	63	62	59	2	59	57	64
4	36	47	45	46	0	46	9	41
5	43	42	41	1	2	1	44	26
6	36	69	68	0	3	3	54	67
7	21	71	70	0	2	2	20	26
8	14	88	88	3	2	2	47	44
9	39	57	56	0	0	0	3	49
10	43	44	44	0	1	1	38	23
11	37	59	57	2	3	4	59	48
12	19	80	79	26	38	48	24	74
13	24	70	69	55	33	58	67	70
14	16	70	70	24	21	36	44	77
Controls GL‡ n = 3	2 ± 1	88 ± 5	88 ± 5	29 ± 4	22 ± 3	37 ± 4	87 ± 4	21 ± 4

* Percentage of cells expressing the $CD3^-CD16^+$ phenotype using two-color analysis.

† Sample tubes contained both EB6 and GL183 MoAbs.

‡ Controls were represented by purified NK cells.

acterization have been previously described in detail.[4,5] For two-color analysis, cells were stained with EB6 or GL183 MoAbs followed by FITC-conjugated goat anti-mouse IgG1 (Caltag Laboratories, South San Francisco, CA) and PE-OKT3 (IgG2) or purified KD1 MoAb followed by PE-conjugated goat anti-mouse IgG2 (Caltag Laboratories). Because a comparison of the distribution of EB6 and GL183 molecules by double staining and FACS analysis was impaired by the fact that both MoAbs belong to IgG1 subclass, we also investigated the percentage of cells stained with a mixture of both MoAbs. Cells were analyzed using a FACScan (Becton Dickinson) and data were processed by using the Consort 30 program, as previously reported.[8] Ten thousand cells bearing the typical lymphocyte scatters were scored.

Granular lymphocyte purification. Purified NK cells from normal donors (n = 3) and case no. 14 were obtained using a modified version of the method previously described.[10] The cell suspension was initially depleted of adherent cells by incubation for 45 minutes in plastic Petri dishes at 37°C in an atmosphere of 95% air and 5% CO_2. LGLs were further purified by removing $CD3^+$ and $CD19^+$ lymphocytes using magnetic microspheres coated with anti-mouse IgG (Dynebeads; Dynal, Norway), according to the method already reported.[11] Briefly, after incubation (45 minutes at 4°C) of the cell suspension obtained as above with CD3 (OKT3, Ortho) and CD19 (Leu-12, Becton Dickinson) MoAbs, 40×10^6 beads were mixed with 10×10^6 cells/mL for 30 minutes at 4°C under continuous slow rotation. The $CD3^+$ and $CD19^+$ cells rosetting with antibody-coated beads were then isolated and removed applying a magnetic system on the outer wall of the test tubes for 2 minutes. Following this multistep negative selection procedure, more than 95% of the cells were viable when tested by the trypan blue exclusion test and were found to show GL morphology.

Cytotoxic activity. The ability of patients' lymphocytes to display NK activity was assessed by the lysis of ^{51}Cr-labeled NK-sensitive K-562 targets in a 4-hour assay, as previously reported in detail.[8]

Redirected cytotoxicity against the FcγR-positive, NK-resistant P815 target cells was evaluated in the presence of EB6, GL183, and anti-CD16 KD1 MoAbs. These different reagents were used at the concentration of 1 μg/mL and resuspended in 50 μL of medium. In the experiments of redirected cytotoxicity, the above-mentioned MoAbs were added at the beginning of the test together with effector and target cells. In all instances, target cells were used at a concentration of 10×10^4/well and the results referred to the 10:1 effector: target ratio.

To investigate the specificity of target recognition, the cytotoxic activity of $EB6^+GL183^-$ proliferating cells from a highly informative patient (case no. 2) was tested against different targets of known specificity. Cells from this patient were chosen because of the strong correlation existing between EB6 expression and type 1 or type 5 NK specificity. Target cells were represented by donor 51 lymphoblastoid cell line or PHA blasts, which are susceptible to type 1-specific NK cells, and donor 82 lymphoblastoid cell line or PHA blasts, which in turn are susceptible to type 2-specific but resistant to type 1-specific NK cells.[12] PHA blasts were generated by culturing PBMCs for 4 days with 0.5% (vol/vol) PHA (GIBCO Ltd, Paisley, Scotland) in the presence of recombinant interleukin-2 (rIL-2) (100 U/mL). B-cell–enriched fractions were prepared from PBMCs by removing cells capable of forming rosettes with sheep erythrocytes and cells were then infected with the Epstein-Barr virus (EBV) to obtain stable lymphoblastoid cell lines, as previously reported.[12] B-EBV–infected cell lines maintained the same pattern of susceptibility or resistance to lysis as the PHA blasts derived from the same donors.[12] These targets were labeled with ^{51}Cr and used at 5×10^3/well at different E:T ratios.

RESULTS

Table 1 summarizes the surface antigens expressed by lymphocyte populations isolated from 14 patients with LDGL. In all patients, the majority of lymphocytes express the $CD3^-CD16^+$ surface phenotype. In addition, both percentages and absolute numbers of $CD3^+$ T lymphocytes were sharply reduced as compared with normal individual values. CD57 and CD56 antigens were expressed at variable degrees.

Analysis of the expression of EB6 and GL183 surface antigens (Table 1) indicated that in 4 of 14 patients, GLs expressed only one of these molecules (three cases were EB6 single positive and one GL183 single positive). Moreover, in seven cases, $CD16^+$ GLs were negative for both surface antigens (>90% $EB6^-GL183^-$). In the remaining three cases (nos. 12 to 14; Table 1), the pattern of expression of EB6

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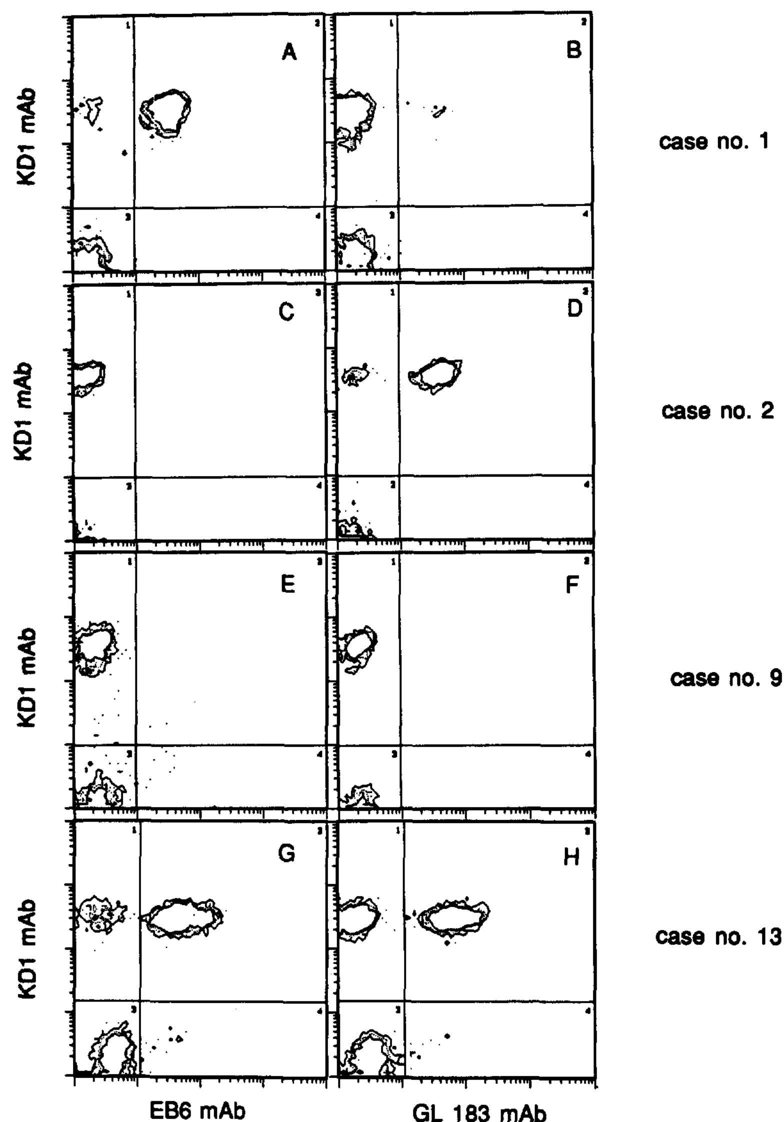

Fig 1. Patterns of EB6 and GL183 antigen expression on CD16+ (KD1 MoAb) lymphocytes from four different LDGL patients (case nos. 1, 2, 9, and 13). Quadrants were set up to include > 98% *of control IgG stained cells.*

and GL183 antigens was similar to that of NK cells from normal donors; that is, the percentage of cells stained with the mixture of both EB6 and GL183 MoAbs was less than the sum of the percentage of cells positive for each marker. This indicates that the coexpression of EB6 and GL183 antigens occurs in a discrete number of GLs. Figure 1 shows the pattern of expression of EB6 and GL183 surface molecules in representative patients with LDGL. $CD3^+$ lymphocytes from patients under study did not express EB6 and GL183 markers (not shown).

Because both EB6 and GL183 antigens behave as triggering surface molecules leading to activation of the NK cell functional program(s), we further investigated whether MoAbs could trigger the cytolytic activity of GLs in patients under study. As shown in Table 2, the GLs of all but one (case no. 6) of the LDGL patients analyzed, lysed the NK-sensitive K562 cells. In addition, in a redirected killing assay using the FcγR-positive P815 target cells, anti-CD16 MoAbs induced a strong cytolysis. Both EB6 and GL183 MoAbs triggered the cytolytic activity of patients' lymphocytes expressing the corresponding surface antigens (with the exception of case no. 1); thus indicating that these molecules are functional in triggering the cytotoxic machinery in the cells of our LDGL patients. These results were also confirmed by using purified $CD3^-CD16^+$ GLs from case no. 14 (Table 2).

The specificity of target recognition was investigated in a representative patient (case no. 2). As shown in Figure 2, $EB6^+GL183^-$ GLs were cytotoxic only for donor 51 lymphoblastoid cell line and PHA blasts, which are sensitive to type 1-specific NK clones.[12] By contrast, the lymphoblastoid cell line and PHA blasts obtained from donor 82 (type 2 specificity) were not lysed. These results definitely indicate that $EB6^+GL183^-$ GLs from case no. 2 expressed a type 1 specificity, as expected from previous results with alloreactive clones.[6]

DISCUSSION

In this study, we showed that in 11 of 14 cases of $CD3^-CD16^+$ LDGL, GL proliferation was specifically sustained by 1 of the 4 possible subsets of granular lymphocytes

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Table 2. Cytotoxic Activities of Fresh PBMCs and Purified GLs From Eight Patients With LDGL

Case No.	K562*	P815*				Expression of	
		Control IgG	EB6 MoAb	GL183 MoAb	KD1 MoAb	EB6 Antigen	GL183 Antigen
1	19	0	2	5	24	−	+
2	22	1	62	4	63	+	−
5	27	1	3	5	12	−	−
6	8	1	2	2	20	−	−
8	18	2	2	3	19	−	−
12	22	1	15	12	32	+	+
13	48	2	19	56	53	+	+
14	34	0	22	24	38	+	+
14 (purifed GLs)	37	2	24	27	44	+	+
Control GLs† (n = 3)	44 ± 5	2 ± 1	14 ± 4	23 ± 6	68 ± 7	+	+

* Data indicate the percentage of specific lysis at the 10:1 E:T ratio.
† Controls were represented by purified NK cells.

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as defined by the combined use of EB6 and GL183 MoAbs (one case: $EB6^-GL183^+$; three cases: $EB6^+GL183^-$; seven cases: $EB6^-GL183^-$). In the remaining three cases, the expression pattern of EB6 and GL183 antigens was consistent with that observed in healthy individuals (ie, the expression of all four NK subsets). When expressed on GL surfaces, in the majority of cases tested, both EB6 and GL183 MoAbs behave as functional surface molecules as assessed in the redirected killing of P815 target cells.

The concept that normal NK cells do not recognize specific antigens has been recently challenged by data demonstrating that peripheral blood NK cells were able to specifically proliferate in mixed lymphocyte culture (MLC) against some allogeneic targets.[6,13,14] In fact, different NK-defined specificities (at least five) have been demonstrated,[15] indicating the existence of an NK repertoire. More interestingly, a relationship between specific target recognition and the expression of EB6 and/or GL183 molecules by effector cells has been reported.[5,6,15] As a matter of fact, in a representative patient (case no. 2), we provided definitive evidence that proliferating $EB6^+GL183^-$ GLs express the specific pattern of cytotoxicity observed for their normal counterparts.[6] Taken together, these data are consistent with a direct involvement of EB6 and GL183 molecules in the recognition of allogeneic cells. It will be important to obtain insights into the pathogenetic mechanisms leading to the in vivo GL proliferation. It might be speculated that the etiologic event (virus?) accounting for GL proliferation induces the alteration and/or expression of masked antigens that in turn can be specifically recognized by a given NK cell subset that could undergo selective expansion. The association between chronic viral infections and LDGL[7,16] frequently observed in these patients is consistent with this possibility. For the time being, however, no correlation has been demonstrated between the prevalence of a specific NK subset of proliferating GLs and the clinical characteristics of patients.

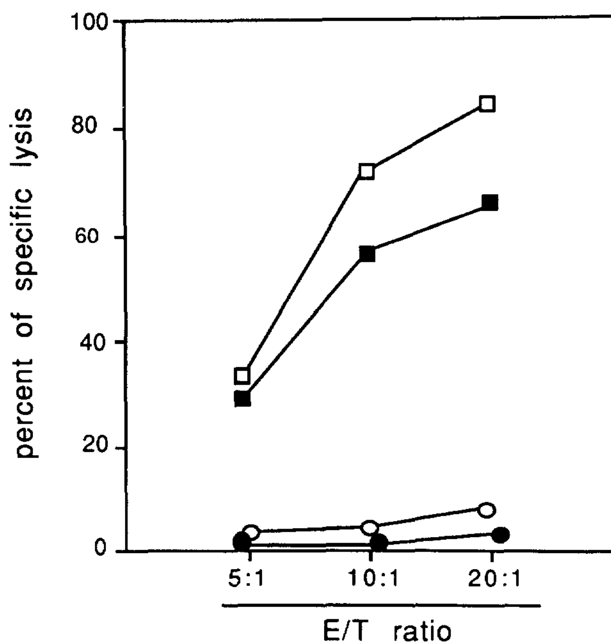

Fig 2. Cytotoxic activity mediated from case no. 2 $EB6^+GL183^-$ GLs against targets susceptible to type 1 NK specificity (donor 51 B lymphoblastoid cell line [□] and PHA blasts [■]) and targets susceptible to type 2 NK specificity but type 1 resistant (donor 82 B lymphoblastoid cell line [●] and PHA blasts [○]) at different E:T ratio.

A question still unsolved deals with the clonal, and thus putatively neoplastic, nature of $CD3^-$ LDGL. Although the analysis of T-cell receptor gene (TCR) rearrangements may be used to assess the clonality in $CD3^+$ LDGL,[2,3,17,18] this type of evaluation cannot be extended to $CD3^-$ LDGL because GLs do not rearrange TCR genes.[19,20] Using a different approach, such as chromosomal analysis, the clonal nature of $CD3^-$ GL proliferation has been demonstrated in a minority of cases studied.[21] Obviously, no definitive conclusions can be reached in terms of clonality on the basis of data presented herein. However, our results support the concept that the pathogenetic noxa accounting for the in vivo expansion of $CD3^-$ GLs preferentially acts on a specific subset of NK cells. This finding suggests that only a discrete NK subset is likely to be involved in $CD3^-$ LDGL proliferation; thus offering a new piece of information on the pathophysiology

of in vivo expanded NK cells. The issue of whether this inciting event stimulates a clonal process per se or if it initially triggers a polyclonal proliferation of a discrete NK subset, eventually resolving or progressing toward a clonal disease, deserves further studies. The demonstration of the acute transformation in a chronic $CD3^-$ LDGL in association with the appearance of additional chromosome abnormalities[22] might support such a multistep process hypothesis.

In conclusion, translating the data obtained in vitro from the analysis of clones into in vivo pathologic conditions, the NK cell compartment appears as a complex system with intriguing relationships. In this regard, because an in vitro correlation has been reported between the subset assignment of NK clones and the ability to mediate specific antigen recognition,[5,6] patients with LDGL represent an invaluable source of cells with defined specificities and can offer the possibility of better defining the functional properties of in vivo expanded NK subsets.

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