CHARACTERISTICS OF HUMAN LARGE GRANULAR LYMPHOCYTES AND RELATIONSHIP TO NATURAL KILLER AND K CELLS

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In contrast to the several antigenic markers identified on murine natural killer (NK)[1] cells (1–3), human NK cells have so far been characterized only by surface properties and receptors (4, 5) that do not allow discrimination from many other peripheral blood mononuclear cells. Detection of human NK cells is therefore largely based on the measurement of in vitro cytolytic reactivity against certain target cells (5). Cytotoxicity assays, however, are impractical for large-scale studies of cell subpopulations, and interpretation of the results of these assays may be complicated by the spontaneous cytolytic and cytostatic activities of other cell types, such as monocytes (6), granulocytes (7), and polyclonally activated T cells (8).

Recently, evidence has been presented on the morphological association of human NK cells with large granular lymphocytes (LGL), which have a high cytoplasmic: nuclear ratio and azurophilic granules in their cytoplasm (9–12) (Fig. 1A). The central role of LGL in NK activity has been suggested by several observations: (*a*) LGL were enriched in target cell-adherent, NK cell-enriched populations (9–11), (*b*) the number of LGL binding to target K562 cells correlated with the levels of cytotoxic activity among normal donors (10), and (*c*) NK activity and LGL peaked in the same fractions obtained by discontinuous density gradient centrifugation of human peripheral blood lymphocytes (12).

Because the morphology of LGL is a potentially useful marker for human NK cells, we have initiated a series of studies to characterize LGL and to further evaluate their cytolytic activity. In the experiments reported here, we have used highly enriched populations of LGL, obtained by density gradient centrifugation (12). Evidence is presented that both spontaneous and interferon (IFN)-boosted NK and antibody-dependent cellular cytotoxic (ADCC) activities are confined to LGL-enriched fractions. Almost all LGL were found to express receptors for the Fc portion of IgG ($Fc_\gamma R$) and about 50% formed low affinity rosettes with sheep erythrocytes (E). More than 90% purity of LGL was achieved by enriching $Fc_\gamma R$-positive cells or by depleting high affinity E rosette-forming cells from the LGL-containing Percoll fractions.

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cell-mediated cytotoxicity; E, sheep erythrocytes; FBS, fetal bovine serum; $Fc_\gamma R$, receptor for the Fc part of IgG molecule; IFN, interferon; K cell, effector cell in ADCC; LGL, large granular lymphocytes; LU, lytic unit; NK, natural killer; PBL, peripheral blood lymphocytes; PBS, phosphate-buffered saline; T_G, T cell expressing $Fc_\gamma R$.

Materials and Methods

Culture Conditions. All target cells were cultured and experiments were carried out at 37°C in a humidified air atmosphere with 5% CO_2, using RPMI-1640 medium (Biofluids Inc., Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biofluids Inc.), 100 μg/ml of gentamicin, and 0.06 mg/ml glutamine.

Effector Cells. All experiments were performed with effector cells derived from normal male and female volunteers, who donated their blood for the isolation of platelets in the National Institutes of Health Plateletphoresis Laboratory. Leukocyte-enriched buffy coats were obtained from the plateletphoresis of 300 ml of blood, and the mononuclear cells were isolated by Ficoll-Isopaque (Litton Bionetics, Kensington, Md.) gradient centrifugation (13). Nonadherent cells were obtained by recovering cells nonadherent to plastic flasks, and then passing them through nylon wool columns (14). The yields of lymphocytes were usually 0.3–1.0 × 10^6/ml of blood. Nonlymphocytic contamination (mainly monocytes), as judged by morphology, varied from 0 to 2%.

Target Cells. K562, a cell line derived from a pleural effusion of a patient with chronic myelocytic leukemia in blast crisis (15), was used as the target cell for measurement of NK activity. RLβ1, a murine T cell leukemia line known to be resistant to human NK cells (16, 17), was used in ADCC assays. The target cells were sensitized for 1 h with a 1:400 dilution of rabbit anti-mouse brain hyperimmune serum (Litton Bionetics, Rockville, Md.), as previously described (17).

Percoll Fractions of Peripheral Blood Lymphocytes (PBL). PBL were separated by centrifugation on a discontinuous density gradient of Percoll, as previously described (12). Growth medium and Percoll were adjusted to 285 mosmol/kg H_2O with sterile distilled water and 10-× concentrated phosphate-buffered saline (PBS) (pH 7.4), respectively. Seven different concentrations of Percoll in medium were prepared, ranging from 40 to 57% Percoll, and each varying from the next by 2.5% concentration steps. The density of Percoll varied among batches and therefore refractive indexes were used to adjust the concentrations of Percoll to the required density. Refractive indexes for ~57 and 40% Percoll at 25°C were 1.3454 and 1.3432, respectively. After the careful layering of the gradient into 15-ml conical test tubes, 5 × 10^7 lymphocytes were placed on the top of the gradient, and the tube was spun at 550 *g* for 30 min at room temperature. Cells from the seven layers were then collected from the top with a Pasteur pipette and washed once in medium containing 2% FBS. The recovery of the cells was >80% and viability was >95%, as judged by trypan blue exclusion.

Fractionation of $Fc_\gamma R^+$ Cells by Adhesion to Monolayers of Immobilized Antigen-Antibody Complexes. PBL bearing $Fc_\gamma R$ were depleted on plastic surfaces of tissue culture flasks that were coated with immobilized antigen-antibody complexes. This procedure has been described previously (4). Two cycles of adsorptions were performed, and adherent cells were recovered by gentle scraping with a rubber policeman. The recovery of adherent cells was 10–20% of the input cells, and that of nonadherent cells was 60–70%.

Cytotoxicity Assay. Aliquots containing 2 × 10^6 target cells were labeled with 100 μCi of sodium ^{51}chromate solution (New England Nuclear, Boston, Mass.) for 1.5 h in 1 ml of medium. After washing three times, 5 × 10^3 cells in 0.1 ml of medium were pipetted to the 96-well Linbro plates (Linbro Chemical Co., Hamden, Conn.). Various concentrations of effector cells in 0.1 ml of medium were added to triplicate wells to give effector:target ratios of 20:1, 7:1, and 2:1. After incubation at 37°C for 4 h, 160 μl of supernate from each well was collected and counted for 2 min in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Fullerton, Calif.). The percentage of isotope released was calculated by the following formula:

$$\% \text{ release} = \frac{\text{cpm released from cells during incubation}}{\text{total cpm incorporated into cells}} \times 100.$$

The percentage of specific cytotoxicity (Cx %) was calculated as Cx % = a − b, where a was the percentage of release in the experimental group and b was the percentage of release in the medium control. The results are expressed as lytic units (LU)/10^7 cells, with an LU being the number of effector cells required to cause 30% lysis of target cells (17). Spontaneous release from the target cells was always <10%.

Evaluation of Cell Morphology. For the morphological analysis of the effector cell populations, 2×10^5 lymphocytes in 0.2 ml of medium were centrifuged at 900 rpm for 7 min onto microscope slides, using a Cytospin centrifuge (Shandon Southern Instruments Inc., Bewickley, Pa.). Air-dried preparations were fixed for 10 min in methanol and stained for 10 min with 10% Giemsa stain (Fischer Scientific Company, Fair Lawn, N. J.) diluted in PBS. Morphological differentials were determined by inspection of the slides by oil immersion microscopy. LGL were identified as slightly larger than small and medium-sized lymphocytes (10–15 μm). They have a relatively high cytoplasmic:nuclear ratio and weakly basophilic cytoplasm with several azurophilic granules. A kidney-shaped nucleus is frequently detected in these cells. Macrophages were distinguished from LGL on the basis of their larger size (15–20 μ), vacuolar cytoplasm, and more indented nucleus. At least 200 cells were analyzed.

Conjugate Assay. To measure the ability of the different cell populations to bind to target cells, 1×10^5 effector cells were incubated with 1×10^5 target cells in 1 ml of medium for 10 min at 37°C. The suspension was subsequently centrifuged at 130 *g* for 5 min, and the resulting pellet was suspended with a Pasteur pipette. A total of 1×10^4 cells in 0.2 ml of medium were cytocentrifuged on microscope slides and stained as described above. The proportion of lymphocytes binding to the target cells was determined by oil immersion microscopy. At least 200 cells were analyzed.

Rosettes. Lymphocyte subpopulations were mixed with E and allowed to form rosettes (E rosettes) at 4° and 29°C (18). 1 ml of E at a concentration of 3×10^8/ml was mixed with 1 ml of FBS and 1 ml of lymphocytes at 2×10^6/ml. The mixtures were centrifuged for 5 min at 100 *g* and incubated for 1 h at the temperatures indicated above, and subsequently gently resuspended. 1×10^4 lymphocytes in 0.2 ml of medium were centrifuged on microscope slides, as described above, for the morphological analysis of rosette-forming cells (19). The remainder were layered on Ficoll-Isopaque and centrifuged at 550 *g* for 30 min. Cells at the interface and in the pellet were washed in medium and assayed for cytotoxic activity as described above.

Pretreatment of Lymphocytes with IFN. The gradient fractions at a concentration of 1×10^6 cells/ml were incubated for 3 h at 37°C, either in medium alone or in medium containing 800 IU/ml of human fibroblast IFN (specific activity 2×10^7 IU/mg protein; HEM Research Inc., Rockville, Md.). After one wash, the cells were tested for cytotoxic activity and conjugate formation with K562 or antibody-coated RLδ1.

Isolation of Blood Leukocytes by Sedimentation of Whole Blood in Plasmagel. To analyze the frequency of LGL in normal leukocyte differentials, 1 ml of heparinized blood was drawn from eight healthy donors. 3 ml of Plasmagel (Roger Bellon Laboratories, Neuilly, France) was added and erythrocytes were allowed to sediment for 45 min at room temperature. Leukocyte-rich plasma was collected, washed once in PBS, and the cell concentration was adjusted to 0.6×10^6 ml. Cytocentrifuged smears were prepared from these specimens as described above.

Results

Distribution of LGL and of Spontaneous and IFN-boosted NK and ADCC Activities among Discontinuous Density Gradient Fractions. As previously reported (12), most of the LGL and the NK activity against K562 were found in low density fractions after discontinuous density gradient centrifugation. Since NK cells and K cells mediating ADCC have previously been shown to have similar characteristics and have been suggested to be identical cells (20, 21), it was also of interest to determine the parallel distribution of NK and K cells in these gradients. Also, because IFN has been suggested to induce effector cells from inactive precursors (5, 22, 23), it was important to determine the distribution of IFN-inducible effector cells. The characteristics of the cells in the various fractions were quite consistent among five experiments. Tables I–III summarize the results of a representative experiment. Most of the LGL and the NK and ADCC activities could be detected in fractions 2 and 3. The distribution of IFN-inducible effector cells for both NK and ADCC was the same as the distribution of LGL and the spontaneously active effector cells. In Table II, the distribution of total

TABLE I

Distribution of LGL, NK, and K Cells among Fractions Obtained by Discontinuous Density Gradient Centrifugation

Fraction*	Cells recovered × 10^{-7}	Distribution of recovered cells	LGL†	Cytotoxicity to§			
				K562	K562-IFN	RL♂1	RL♂1-IFN
		%	%	*LU*			
Input	37.5	—	18	70	248	85	331
1	1.0	3	50	31	89	8	55
2	1.6	5	83	600	3,864	700	5,463
3	4.0	13	51	316	562	366	409
4	6.8	22	24	64	165	143	186
5	7.6	25	9	<1	17	<1	5
6	5.4	17	3	<1	<1	<1	<1
7	4.6	15	<1	<1	<1	<1	<1

* Nonadherent peripheral blood mononuclear cells were fractionated into seven different fractions (1–7) by discontinuous density gradient centrifugation.

† Number of LGL per 100 cells counted in each fraction.

§ Cytotoxicity against ^{51}chromium-labeled target cells in a 4-h assay. Expressed as LU at 30% level per 10^7 cells. IFN, interferon-treated effector cells. RL♂1 refers to antibody-coated targets.

TABLE II

Distribution of NK and ADCC Activities among the Fractions Obtained by Discontinuous Density Gradient Centrifugation

Fraction*	Total LU in each fraction†				Distribution of LU among the fraction§			
	K562	K562-IFN	RL♂1	RL♂1-IFN	K562	K562-IFN	RL♂1	RL♂1-IFN
					%			
Input	262.5	930.0	318.8	1,241.3	100	100	100	100
1	3.1	8.9	0.8	5.5	1	1	1	1
2	96.0	618.2	112.0	877.1	} 85	} 91	} 81	} 84
3	126.4	224.8	146.4	163.6				
4	43.5	112.2	97.2	126.5	17	12	31	10
5	<1	12.9	<1	38.0	<1	1	<1	3
6	<1	<1	<1	<1	<1	<1	<1	<1
7	<1	<1	<1	<1	<1	<1	<1	<1
Recovery of LU, %					103	105	112	97

* See first footnote to Table I. For LGL purity, see Table I.

† LU per 10^7 cells, multiplied by the number of recovered cells. Same experiment as in Table I.

§ (Total LU in fraction/total LU in input) × 100.

LU of cytolytic activity in each fraction is shown. At least 81% of the recovered LU was detected in fractions 2 and 3, and treatment with IFN did not cause the initially weak or nonreactive high density populations to develop appreciable activity. The results indicate that both NK and ADCC activities, spontaneous and IFN-inducible, are confined to the LGL-enriched fractions.

Conjugate-forming Ability of Cells in Different Fractions Obtained by Discontinuous Density Gradient Centrifugation. As NK activity is known to be dependent on binding of effector cells to target cells (5, 9–11, 24), conjugate analyses were performed with cells

TABLE III

Distribution of LGL and Other Lymphocytes Forming Conjugates with K562 and RLδ1 among Fractions Obtained by Discontinuous Density Gradient Centrifugation

Fraction*	Total LGL	Total non-LGL	LGL-K562	LGL-K562 IFN	LGL-RLδ1	LGL-RLδ1 IFN	LY-K562	LY-K562 IFN	LY-RLδ1	LY-RLδ1 IFN
Input	18‡	80	5	5	8	8	7	6	2	3
1	50	28	—	—	—	—	—	—	—	—
2	83	15	50	44	56	58	<1	<1	<1	<1
3	51	49	39	35	28	31	7	3	<1	<1
4	24	76	10	11	17	15	4	2	<1	<1
5	9	91	3	1	5	6	6	7	11	5
6	3	97	2	2	2	2	3	2	2	7
7	<1	99	<1	<1	<1	<1	22	23	<1	<1

* See first footnote to Table I.

‡ Percentage of total or of conjugate-forming LGL and conventional lymphocytes (non-LGL) per total cells in fraction. IFN, interferon-treated effector cells.

in the different fractions obtained by discontinuous density gradient centrifugation, using K562 and antibody-coated RLδ1 as target cells. Centrifugation of conjugates on slides enabled morphological analysis of binding cells, and therefore it was possible to directly determine the binding capacities of both LGL and conventional lymphocytes in each of the fractions and to relate this to the observed cytotoxicity. A high proportion (>50%) of LGL formed conjugates with both target cells (Table III). In contrast, when RLδ1 without antibody was used as a target, conjugate-forming LGL were always <10% of the total cells in the fractions (data not shown). Pretreatment with IFN did not increase the number of LGL binding to target cells, which suggests that the boosting of reactivity was due to an increase in the rate of cytolytic reactivity and/or the induction of lytic activity in cells already capable of binding to target cells.

Despite the high percentage of LGL in the fractions with high cytolytic activity, the possibility remained that other cells in these fractions were also, or perhaps the only, effector cells. However, very few of the conventional lymphocytes in fractions 2 or 3 formed conjugates (Table III). Conjugate formation by conventional lymphocytes was observed mainly in the high density fractions, which had little or no cytolytic activity. This binding activity of small to medium lymphocytes was also detected in nonfractionated PBL, and in fact accounted for the majority (>50%) of the cells forming conjugates with K562. Binding capacity of conventional lymphocytes with nonantibody-coated RLδ1 cells was <10% of the total cells in any of the fractions (data not shown).

Expression of $Fc_\gamma R$ on LGL, and Further Enrichment of LGL by Adsorption on Monolayers of Immobilized Immune Complexes. The expression of $Fc_\gamma R$ on human NK cells reactive with K562 has been widely documented (21). However, previous studies using antibody-coated erythrocytes for binding suggested that only a portion of LGL had $Fc_\gamma R$ (8). Because adsorption of lymphocytes on monolayers of immobilized immune complexes is known to reveal even low affinity $Fc_\gamma R$ on murine NK cells (25), this method was chosen as a possibly more sensitive method for the analysis of the presence of $Fc_\gamma R$ on LGL. Two cycles of adsorption of either PBL or NK cell-enriched fractions on immune complex monolayers removed more than 90% of both cytolytic activity

and LGL (Table IV). Conversely, 95% of the adherent cells derived from the NK cell-enriched fractions were LGL. These results indicate that virtually all LGL express $Fc_\gamma R$. The nonadherent populations, depleted of most LGL, had little or no NK or ADCC activities, further strengthening the association of LGL with these effector functions.

Fractionation of $Fc_\gamma R^+$ Cells by Discontinuous Density Gradient Centrifugation. The morphological analysis of the adherent cells from the immune complex monolayer fractionation of PBL indicated that only 51% were LGL (Table IV). Because most of the non-LGL $Fc_\gamma R^+$ cells were small and medium-sized lymphocytes, attempts were made to enrich for those cells as well as for LGL by discontinuous density gradient centrifugation of $Fc_\gamma R^+$ PBL. Lymphocytes were incubated first on monolayers of immobilized immune complexes for 1 h, nonattached cells were carefully removed after several washes with medium, and the absence of nonattached cells was confirmed by inspection under an inverse microscope. The adherent $Fc_\gamma R^+$ cells were detached with a rubber policeman, washed twice in medium, and fractionated by discontinuous density gradient centrifugation. The recovered cells were then incubated for 3 h with 800 IU of IFN to produce maximal cytolytic activity. As shown in Table V, the LGL-enriched low density fractions (2 and 3) exerted the strongest cytotoxicity, whereas only a low reactivity could be detected in the high density fractions 5–7, which contained mostly small and medium-sized lymphocytes. This result indicates that $Fc_\gamma R^+$ conventional lymphocytes do not have cytolytic capacity.

E Rosette-forming Capacity of the Subpopulations of $Fc_\gamma R^+$ Lymphocytes. The majority of human NK cells have been reported to have low affinity receptors for sheep erythrocytes (18). It was therefore of interest to examine the expression of such receptors on LGL. The studies with cells separated on immune complex monolayers indicated that some $Fc_\gamma R^+$ cells had conventional lymphocyte morphology, so it was important to similarly characterize these cells. The morphology of $Fc_\gamma R^+$ PBL-forming rosettes at 4° or 29°C was therefore examined (Tables V and VI). More than 50% of LGL formed E rosettes at 4°C, but only 10–20% formed high affinity rosettes at 29°C (Table VI). In both unfractionated PBL and $Fc_\gamma R^+$ populations, the majority of non-LGL formed high affinity E rosettes. When $Fc_\gamma R^+$ non-LGL were enriched to high density fractions by discontinuous density gradient centrifugation, it could be dem-

TABLE IV

Expression of $Fc_\gamma R$ on LGL as Determined by Adherence to Immune Complex Monolayers

Fraction*	Relative lytic activity‡				LGL§ (range)
	K562	K562-IFN	RLδ1	RLδ1-IFN	
Input	100	340 (150–545)	100	204 (126–308)	10 (4–22)
Input $Fc_\gamma R^+$	496 (48–1,622)	808 (272–2,177)	153 (18–491)	396 (140–722)	51 (31–70)
Input $Fc_\gamma R^-$	0.2 (0–0.7)	0.2 (0–0.9)	0	2.7 (0.6–7)	1 (0–3)
2 and 3	100	249 (146–345)	100	318 (138–770)	78 (75–85)
2 and 3 $Fc_\gamma R^+$	96 (58–139)	179 (91–254)	94 (28–193)	161 (43–320)	95 (85–99)
2 and 3 $Fc_\gamma R^-$	0.9 (0–3)	10 (3–18)	0.8 (0–3)	4.8 (0–15)	5 (2–10)

* Nonadherent peripheral blood mononuclear cells (input) were fractionated by discontinuous density gradient centrifugation and NK cell-enriched fractions (2 and 3) were harvested. Both nonfractionated input and fraction 2 and 3 cells were subsequently fractionated into $Fc_\gamma R^+$ and $Fc_\gamma R^-$ subpopulations by adsorptions on monolayers of immobilized immune complexes.

‡ (LU/10^7 cells of each fraction and of IFN-treated cells/LU/10^7 of untreated input or fraction 2 and 3 cells) × 100. Mean (range) of five experiments is shown.

§ Number of LGL per 100 cells counted in each fraction.

TABLE V

Fractionation of $Fc_\gamma R^+$ Lymphocytes by Discontinuous Density Gradient Centrifugation

Fraction*	Cells × 10^6	LGL	Cytotoxicity ($LU/10^7$) to		E rosette-forming cells‡			
					LGL		Other lymphocytes	
			K562	RL♂1	+4°C	+29°C	+4°C	+29°C
		%				%		
1	2.2	60	2	11	50	13	94	88
2	1.8	79	116	185	53	14	92	87
3	2.1	88	183	173	57	16	96	83
4	1.8	23	15	27	56	18	95	82
5–7	2.2	3	<1	<1	62	20	90	81

* Nonadherent peripheral blood mononuclear cells were incubated on immobilized immune complex monolayers. The adherent cells were detached with a rubber policeman and fractionated by discontinuous density gradient centrifugation. The fractions were pretreated with IFN before testing for cytotoxicity.

‡ Fractions were tested for the capacity to form rosettes with sheep erythrocytes before IFN treatment. Cells were centrifuged on microscope slides and the percentage of rosette-forming cells among LGL and other lymphocytes was enumerated by oil immersion microscopy.

TABLE VI

Receptors for Sheep Erythrocytes and Fc_γ on LGL and Other Lymphocytes

Fraction*	Recovery‡	LGL	E rosette-forming cells§			
			LGL		Other lymphocytes	
			+4°C	+29°C	+4°C	+29°C
	%	%		%		
Input	100	14	57	10	95	84
Input $Fc_\gamma R^+$	16	42	53	15	81	76
Input $Fc_\gamma R^-$	70	1	NT‖	NT	97	89

* Nonadherent peripheral blood mononuclear cells (input) were fractionated to $Fc_\gamma R^+$ (input $Fc_\gamma R^+$) and $Fc_\gamma R^-$ (input $Fc_\gamma R^-$) fractions by adsorption on monolayers of immobilized immune complexes.

‡ (Number of cells recovered/number of cells in input) × 100.

§ Cells were centrifuged on microscope slides and the percentage of rosette-forming cells among LGL and other lymphocytes was enumerated by oil immersion microscopy.

‖ NT, not tested.

onstrated that these cells had little or no cytotoxic activity, and the majority of them formed high affinity E rosettes (Table V). The results indicate that most cells forming low affinity rosettes are LGL and that $Fc_\gamma R^+$ E rosette-forming cells, i.e., the cells generally referred to as the T_G population, are morphologically and functionally heterogenous.

Enrichment of LGL by the Depletion of High Affinity E Rosette-forming Cells from Low Density Fractions. Because the majority of high affinity E rosette-forming cells in low density fractions were conventional lymphocytes, it was of interest to attempt further purification of LGL by depleting high affinity rosette-forming cells from these fractions. As shown in Table VII and Fig. 1B, a considerable enrichment of LGL could be achieved by this procedure. A small number of LGL were found in the E rosette-forming pellet after separation of nonrosette-forming cells on a Ficoll-Isopaque gradient.

TABLE VII

Enrichment of LGL by Depletion of High Affinity E Rosette-forming Cells from NK Cell-enriched Populations

Fractions*	LU/10^7 cells	Recovery of cells‡	E rosette-forming§ cells		LGL
			+4°C	+29°C	
			%		%
Input	22	100	47	58	6
2 and 3	110	12	19	60	68
2 and 3^-	460	66	NT‖	NT	93
2 and 3^+	23	33	NT	NT	11
6 and 7	<1	40	45	70	1

* Nonfractionated (input) cells were fractionated by discontinuous density gradient centrifugation. NK cell-enriched fractions (2 and 3) were rosetted with sheep erythrocytes at 29°C, and rosette-forming cells (2 and 3^+) were separated from nonrosette-forming cells (2 and 3^-) by Ficoll-Isopaque centrifugation.

‡ Fraction 2 and 3^- and 2 and 3^+ recoveries are from original 2 and 3, others from input.

§ Percentage of rosette-forming cells among all lymphocytes.

‖ NT, not tested.

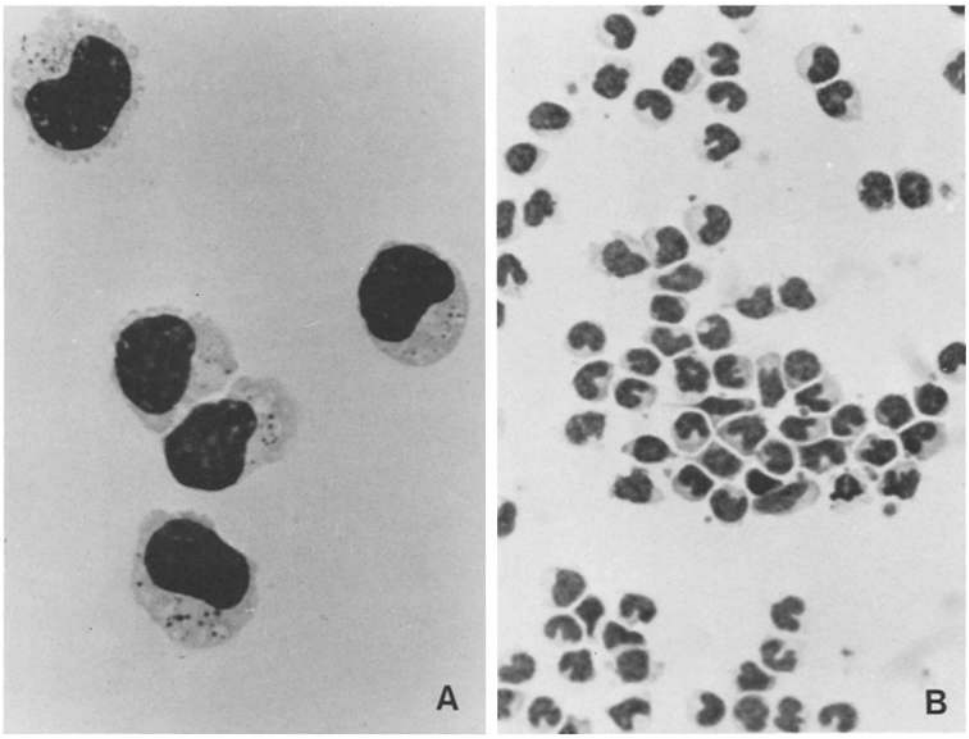

FIG. 1. Large granular lymphocytes. (A) Oil immersion microscopy (× 1,560). Note high cytoplasmic:nuclear ratio, cytoplasmic granules, and reniform nuclei in some of the cells. (B) Low-power magnification (× 585) of large granular lymphocytes isolated by depletion of high-affinity E rosette-forming cells from low density Percoll fractions.

Frequency of LGL in Peripheral Blood. To analyze the frequency of LGL in peripheral blood, cytocentrifuged cell smears were prepared from Plasmagel-purified leukocytes. This was necessary because LGL morphology is not readily detectable in conventional

hematological smears. The yield of leukocytes from the eight donors tested was 3.8–6.0 × 10^6/ml of blood (mean 4.9 × 10^6). The frequency of LGL among these cells varied from 2 to 6% (mean 3.6%). The yield of LGL by the two purification techniques used in this study was 0.3–1.0 × 10^5/ml of blood (mean 0.75 × 10^5). The average recovery of LGL can therefore be estimated to be 45%.

Discussion

Several previous studies have indicated an association between LGL and human NK cells (9–12). However, to establish the value and reliability of LGL morphology as a marker for NK cells, it is important to determine whether (*a*) all NK cells have the morphology of LGL, and conversely, whether (*b*) all LGL can function as NK cells. The focus in the present study has been on the first question, but some data have also been relevant to the second.

Our main approach has been to use various characteristics that have been demonstrated for NK cells or for LGL as the basis for separation of PBL, and to examine the proportion of LGL in the subpopulations enriched for, or depleted of, NK activity. The main separation procedure was centrifugation on a Percoll discontinuous density gradient, which was previously shown to enrich for both LGL and NK activity in the low density fractions (12). Only fractions containing a high proportion of LGL had appreciable NK activity. Furthermore, it was possible to recover most of the total lytic activity of the PBL in those fractions.

Separation of cells according to their expression of $Fc_\gamma R$ was also helpful. $Fc_\gamma R$ have been demonstrated on virtually all human NK cells reactive against K562, with a loss of cytotoxic activity upon depletion of cells adherent to monolayers of immune complexes (18). A previous study suggested that this might represent some difference from the characteristics of LGL (10). By rosetting with antibody-coated human erythrocytes, only 30–50% of LGL have detectable $Fc_\gamma R$. However, from the present results with the immune complex monolayer technique, it appears that the previous methodology was not sufficiently sensitive and that virtually all LGL express $Fc_\gamma R$. The combination of Percoll gradient centrifugation and monolyaer adsorption procedures yielded fractions containing >90% LGL and most of the NK activity of the input population.

The majority of NK cells have been shown to have low affinity receptors for sheep erythrocytes, and the remainder lack detectable E receptors (18). In contrast, most mature T cells have high affinity receptors for E, forming rosettes even at 29°C. This provided the basis for another separation procedure. Removal of high affinity E rosette-forming cells from the low-density fractions resulted in a subpopulation highly enriched for LGL and containing most of the NK activity of the input PBL.

Thus, by separation based on either $Fc_\gamma R$ or high affinity E receptors, it was possible to remove most conventional lymphocytes from low density Percoll fractions and this did not reduce the NK activity of these fractions. Conversely, none of the fractions enriched for conventional lymphocytes had appreciable NK activity. These results argue strongly for an intimate association of NK cells with LGL. However, a few non-LGL remained in the NK-enriched preparations and it remained possible that these cells were involved as effector cells. Conjugate analysis was helpful largely to rule out this possibility. Conjugate formation with target cells appears to be a requisite initial step for cytotoxicity by either cytotoxic T cells or NK cells (5, 9–11,

26). Within the NK-enriched fractions, the majority of LGL formed conjugates with K562, whereas only a small proportion of non-LGL bound to these target cells. In a single cell agarose cytoxicity assay, we have found that most conjugate-forming LGL produce lysis of the attached targets (T. Timonen, J. R. Ortaldo, and R. B. Herberman, manuscript in preparation). With all the evidence taken together, it appears conclusive that virtually all human NK activity against K562 target cells is attributable to LGL.

In regard to whether all LGL can function as NK cells, the evidence is less clear. Even after pretreatment with IFN, an appreciable portion of LGL did not form conjugates with K562. This raises two alternative possibilities: (*a*) some LGL may lack NK activity but have other functions, (*b*) NK cells are heterogenous, with subpopulations reacting with some target cells but not others. Those LGL not forming conjugates with K562 might be found to bind to other target cells. Previous studies with separation of PBL by adsorption to monolayers of NK-susceptible target cells support this possibility (24). NK reactivity against some targets could be completely depleted and yet appreciable reactivity against other targets could be detected. It would be of interest to combine such studies with conjugate analyses. Alternatively, examination of conjugate formation between LGL and a mixture of NK-susceptible target cells might provide a better estimate of the proportion of LGL able to interact with target cells.

During the course of these studies, several other important issues were addressed: (*a*) is the ability of IFN to augment NK activity also restricted to LGL or are there IFN-inducible precursors of NK cells present in other subpopulations; (*b*) do LGL also account for the most or all ADCC by K cells; and (*c*) what is the relationship between LGL and T_G cells or low affinity E rosette-forming cells?

There have been several indications of the existence of pre-NK cells that can be induced by IFN (5, 22, 23), and these precursors may have some characteristics that differ from those associated with spontaneously active NK cells (22, 23). The possibility that non-LGL might contain such pre-NK cells was raised by the observation that some conventional lymphocytes could form conjugates with K562. However, the failure of pretreatment with IFN to induce any detectable activity in non-LGL indicates that IFN-inducible precursors of NK cells are LGL. The treatment of LGL with IFN did not increase the proportion of cells forming conjugates with K562. Therefore it appears that the augmenting effects of IFN are beyond the steps of morphological differentiation into LGL and expression of receptors for recognition of NK-susceptible targets. It seems likely that IFN acts primarily to augment the reactivity of already active NK cells or to trigger the lytic machinery of conjugate-forming inactive NK cells. A further important implication of these data is that IFN can act directly on NK cells and cause augmentation of activity without the need for accessory cells. It will, however, be of importance to further analyze the effect of IFN on the conjugate-forming capacity of NK cells reactive to other cell types, particularly anchorage-dependent target cells, because recruitment of nonconjugate-forming pre-NK cells has been suggested in a fetal fibroblast system (22), and macrophages may play a role in the activation of NK cells cytotoxic against adherent tumor cell lines (27). It is quite possible that the use of target cells less NK-sensitive than K562 might give different results in terms of the effect of IFN on conjugate formation and the role of accessory cells in NK cell activation.

It has been suggested that NK and K cells are in the same subpopulation of cells and in fact may be identical (20, 21). The present procedures for obtaining highly enriched preparations of NK cells allowed further examination of this issue. We have found that K cell activity against antibody-coated mouse tumor target cells was distributed with the same pattern as NK cells. Thus, virtually all human K cells also appear to be within the population of LGL. This conclusion is consistent with the previous observations by Ault and Weiner (28). The fact that the majority of LGL formed conjugates with both K562 and with antibody-coated target cells provides further evidence in support of the hypothesis that some LGL can mediate both NK and ADCC activities.

Only about half of the $Fc_\gamma R^+$ cells that we obtained from monolayers of immune complexes had the morphology of LGL. Most of the conventional lymphocytes with $Fc_\gamma R$ were found in the high density Percoll fractions, the cells in these fraction lacked NK or ADCC activities and most formed rosettes with sheep erythrocytes. Thus, our separation procedure defined two morphologically distinct subpopulations of nonadherent mononuclear cells with receptors for $Fc_\gamma R$ and for E (and thus T_G cells). This evidence that T_G cells are morphologically heterogenous, and consist of both LGL and small to medium lymphocytes, is at variance with the observations of Grossi et al. (29), who suggested that T_G cells had LGL-like morphology. This discrepancy is probably due to technical differences in the detection and/or separation of T_G cells. It is interesting that our morphological division of the T_G population was accompanied by a functional division, with only the T_G cells with LGL morphology having NK and ADCC activities. It will be important to determine the distribution between the two cell types of other immune functions that have been associated with T_G cells, particularly suppression of immunoglobulin production by B cells (30) and of proliferative responses in mixed lymphocyte cultures (31).

Recent evidence has demonstrated that T_G cells (32) and NK cells (33) express the OKM1 antigen, which has been associated with the myelomonocytic lineage. A nonlymphocytic nature of T_G and NK cells has therefore been suggested (32, 33). However, several lines of evidence indicate that LGL (and thus NK cells and part of T_G cells) are related to T cells. In addition to the E rosette-forming capacity described here, LGL are known to be negative for surface immunoglobulin and positive for sodium-fluoride resistant alpha-naphthyl-acetate-esterase, a characteristic of T cells (9). We have recently examined the histochemistry of LGL further. Like T cells, they are negative for lysozyme, peroxidase, and alpha-naphtyl-butyrate-esterase, and positive for tartrate-sensitive acid phosphatase (T. Timonen, E. Jaffe, J. R. Ortaldo, and R. B. Herberman, unpublished data). Obviously, the lineage of LGL cannot be established on the basis of the available data. In vitro cultures of the highly purified subpopulations of the LGL might give further insight into this controversial issue.

The emerging evidence that human NK and K cells are LGL presents a series of important implications and opportunities. First, identification of LGL in Giemsa-stained cytocentrifuge preparations is a technically simple procedure, requiring relatively few cells. Enumeration of these cells in various disease states and during the course of various treatments may add a new dimension to the differential counting of leukocytes and the evaluation of the NK cell population. Second, enumeration of LGL forming conjugates with target cells provides a direct method for determining the number of cells capable of recognition and potential lysis. It should be noted that

evaluation of morphology as well as binding to targets is critical, since conventional lymphocytes that formed conjugates did not appear to function as effector cells or as IFN-inducible precursors. Thus, a total count of conjugate-forming PBL could be misleading in regard to the size of the effector cell population. Third, the Percoll discontinuous density gradient centrifugation procedure and the other cell separation methods used here provide the possibility of further dissection of human lymphocyte subpopulations. The combination of a Percoll gradient and adsorption on immune complex monolayers results in highly purified preparations of LGL. With the same technique, it is similarly possible to obtain highly purified populations of $Fc_\gamma R^+$ or $Fc_\gamma R^-$ cells, virtually devoid of LGL. E rosetting of LGL-containing fractions at 29°C provides an alternative method for obtaining highly purified populations of LGL, without the need for interaction of cell surface receptors on LGL with their ligands. This may avoid the potential activation or inhibition of the fractions of LGL during the isolation procedure. These separated cell subpopulations should be particularly valuable for understanding the nature of T_G cells and their relationship to other mononuclear cells. Fourth, the isolated LGL should also be useful for detailed studies of the mechanisms of NK and ADCC, and the nature of the regulatory processes affecting these activities. Furthermore, these separated populations should facilitate biochemical studies on the nature of the recognition structures of the effector cells.

Summary

Recent evidence has demonstrated an association between a subpopulation of peripheral blood mononuclear cells, morphologically identified as large granular lymphocytes (LGL), and natural killer (NK) activity. We have now evaluated more directly the role of LGL in both NK activity and antibody-dependent cellular cytotoxicity (ADCC), by using highly enriched populations of LGL, obtained by centrifugation of peripheral blood mononuclear cells on Percoll discontinuous density gradients. Both spontaneous and interferon-augmented NK and ADCC activities were exclusively associated with the LGL-enriched, low density fractions. The majority of LGL formed conjugates with NK-susceptible and antibody-coated target cells. Approximately 20% of small conventional lymphocytes also formed conjugates with the target cells for NK, but this was not associated with cytotoxic activity. Virtually all LGL were found to have receptors for the Fc portion of IgG ($Fc_\gamma R$). The frequency of LGL among blood leukocytes was 2–6%. LGL could be enriched to an average purity of 95% by combining discontinuous density gradient centrifugation with subsequent adsorptions of the low density fractions on monolayers of immobilized immune complexes. About 50% of LGL were found to be $Fc_\gamma R$-bearing T cells (T_G), forming low affinity rosettes with sheep erythrocytes at 4°C. Only 10–20% of LGL formed high affinity rosettes with sheep erythrocytes at 29°C. LGL could be enriched to a purity of >90% by depleting high affinity rosette-forming cells from low density Percoll fractions. LGL were only a subpopulation of T_G cells, because some lymphocytes with conventional morphology also adhered to the immobilized immune complex monolayers and formed high affinity rosettes with sheep erythrocytes. Separation of these cells from LGL by discontinuous density gradient centrifugation indicated that they are not cytotoxic, suggesting a morphological and functional subdivision of T_G cells. The verification in this study that virtually all human NK and K cells have a characteristic morphology adds a useful parameter to the monitoring of human

lymphocytes, and the ability to purify these cells by simple physical procedures should be invaluable in their further characterization.

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