NATURAL KILLER-MEDIATED LYSIS OF NORMAL AND MALIGNANT TARGET CELLS, AND ITS REGULATION BY MONOCYTES

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Natural killer (NK)[1] cells have been implicated as important mediators in the prevention of experimental tumor development and metastasis (1–6). NK cells have also been suggested (7–9) as important components of the immune response against virus-infected cells. In addition, NK cells have been found to have a cytotoxic effect on certain normal target cells. Immature thymocytes (10–13), activated B lymphocytes (14), and some bone marrow cells (10, 13, 15) are known to be susceptible to lysis by NK cells or cloned killer cell lines mediating NK function. This may reflect a potential regulatory role for NK in the development and differentiation of lymphoid cells and hemopoietic stem cells. Peritoneal macrophages (10, 12, 16) and fetal fibroblasts (12) are also known to be susceptible to NK cells.

We have been studying NK-mediated lysis of lymphoma cells isolated from patients, and have found that lymphoma cells from all the patients tested were susceptible to autologous and healthy donors' short-term cultured, partially purified NK cells (17). During experiments studying the target cell selectivity of these NK cells, their broad spectrum of cytotoxicity for normal and malignant target cells has been demonstrated. In this paper, we describe the nature of these NK cells, particularly their target cell selectivity, kinetics of the development of cytotoxicity, and the monocyte-mediated regulation of the cytotoxicity. The results show that 1-d-cultured NK cells lysed all types of target cells tested, including normal T and B lymphocytes, mitogen-induced T and B blasts, monocytes, larger granular lymphocytes (LGL), various types of leukemia and lymphoma cells isolated from patients, and cultured cell lines. Freshly isolated NK cells were not or, if anything, were far less cytotoxic against the target cells, while monocytes and their supernatants added during the culture markedly inhibited the development of their cytotoxicity.

The overall results suggest that, although NK cells have ability to lyse normal

[1] *Abbreviations used in this paper:* E, sheep erythrocytes; En, neuraminidase-treated E; E:T, effector/target; F-C, Ficoll-Conray; FCS, fetal calf serum; IFN, interferon; LGL, large granular lymphocytes; mAb, monoclonal antibodies; NK, natural killer; PBMC, peripheral blood mononuclear cells.

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and malignant target cells in vivo, their lytic activity is regulated by coexisting monocytes.

Materials and Methods

Purification of Various Subsets of Cells from Peripheral Blood. The procedure for purification of various subsets of cells is depicted in Fig. 1. Peripheral blood mononuclear cells (PBMC) were obtained from healthy donors and patients with non-Hodgkin's lymphoma by Ficoll-Conray (F-C) density gradients. To obtain monocytes, mononuclear cells suspended in RPMI 1640 medium with 10% fetal calf serum (FCS) were incubated in glass dishes for 45 min at 37°C, then gently pipetted. After nonadherent cells were removed, the cells in the glass dishes were further pipetted, and loosely adherent cells were discarded. Adherent cells were left for a few hours at 4°C in phosphate-buffered saline, then vigorously pipetted. The removed cells were used as monocytes, being ≥90% α-naphthyl butyrate esterase–positive, (and completely inhibited by NaF). To avoid adherence to the plastic, harvested monocytes were left in polypropylene tubes. Glass dish–nonadherent cells were applied to columns of nylon wool and incubated for 1 h at 37°C. Nylon wool–nonadherent cells were eluted with warm medium, then added to Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient solutions as described previously (17, 18). To prepare density gradients, Percoll was mixed in various concentrations with 10% FCS-containing RPMI 1640 medium. The Percoll solutions, in volumes of 1.5 ml, were carefully layered into a 10-ml round-bottom test tube, starting with 40% Percoll (fraction 5) and graded by 2.5% concentration reductions to 30% Percoll on the top (fraction 0). After the lymphocytes were added to the Percoll solutions, they were centrifugated at 650 *g* for 20 min at room temperature. Each fraction was collected and washed twice. The lymphocytes from low-density fractions 1 and 2 were mixed and used as LGL-rich lymphocytes. The LGL-rich lymphocytes contained 52–69% (mean 60%) LGL, as determined by staining with May-Grünwald–Giemsa. High-density fraction 4 was used as T lymphocytes. This fraction was >90% positive for OKT3 monoclonal antibody (mAb), the pan-T marker. To further purify LGL, the LGL-rich lymphocytes were mixed with sheep erythrocytes (E) for 1 h at 29°C, and E rosette–negative cells were obtained by F-C sedimentation and used as purified LGL (18). Purified LGL contained 88–95% LGL. To obtain B lymphocytes, nylon fiber–retained cells were retrieved from the fiber by vigorous agitation in cold phosphate-buffered saline, then mixed with neuraminidase-treated sheep erythrocytes (En) for 2 h at 4°C. En rosette–negative cells, used as B lymphocytes, were 75–90% positive for surface Ig, and <5% positive for α-

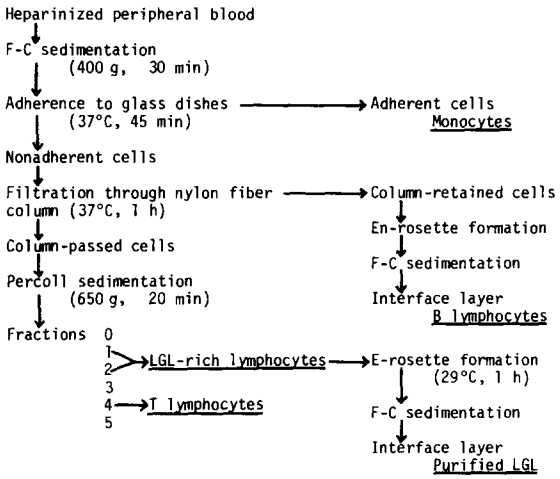

FIGURE 1. Purification procedure of various subsets of cells from the peripheral blood.

naphthyl butyrate esterase and E-rosette formation. In some experiments, T and B blasts were used as target cells. T blasts were obtained by 3-d-culture of T lymphocytes and 1/100 the number of monocytes with phytohemagglutinin-P (Gibco Laboratories, Grand Island, NY) diluted 1:1,000. B blasts were obtained by 3-d-culture of B lymphocytes with 0.1% (vol/vol) killed *Staphylococcus aureus* Cowan I bacteria. In limited experiments, B blasts were further enriched in the interface between 25% and 27.5% of Percoll solutions after centrifugation at 650 *g* for 20 min.

Characteristics of Patients and Tumor Cells. Patients 1–4 had non-Hodgkin's lymphoma. The diagnosis was based on the working formulation of the National Cancer Institute (19). Patient 1 had B cell lymphoma of large cell, immunoblastic type, patient 2 had $OKT4^+$, helper/inducer T cell lymphoma of large cell, immunoblastic type, and patients 3 and 4 had B cell lymphoma of diffuse, large cell type. The percentage of malignant cells in the lymph nodes was determined from morphology and surface markers, and constituted 80% or more in the specimens. Lymphoma cells biopsied were teased, suspended in Eagle's minimum essential medium containing 75% FCS and 10% dimethylsulfoxide, and stored in liquid nitrogen until use for target cells. Blood was obtained from the patients at least 3 wk after the cessation of therapy to minimize the effects of chemo- and radiotherapy.

Leukemic cells in the peripheral blood or bone marrow were separated from patients 5–12 with F-C gradient sedimentation. Patients with >90% leukemic cells in their peripheral blood leukocytes or bone marrow cells were selected as donors of leukemic cells. Diagnosis of acute leukemia was based on the French-American-British cooperative group designation (20).

Cell lines used for target cells were as follows: K562, an erythroleukemia; MOLT-4, a T cell acute lymphoblastic leukemia; Raji, a Burkitt's lymphoma; HL-60, an acute promyelocytic leukemia; BALL-1, a B cell acute lymphoblastic leukemia; P815, a mouse mastocytoma; and L1210, a mouse leukemia.

Assay for Cytotoxic Activity. The assay for cytotoxic activity was a 5-h ^{51}Cr-release test, as described elsewhere (17). Unless otherwise stated, effector cells were LGL-rich lymphocytes that had been incubated for 20–24 h in RPMI 1640 medium with 10% FCS at 37°C in humidified air containing 5% CO_2. Target cells were T and B lymphocytes, mitogen-induced T and B blasts, monocytes, LGL, lymphoma and leukemia cells isolated from the patients, and various cell lines. Normal unstimulated target cells were either fresh or 1-d-cultured cells. Their susceptibility to effector cells was not different. Experiments were performed in triplicate. For the labeling of target cells in the ^{51}Cr-release assay, 3 mCi of $Na_2{}^{51}CrO_4$ (sp act 20–200 mCi/mg; Japan Isotope Association, Tokyo) was diluted with 1.5 ml of RPMI 1640 medium containing 10% FCS, and 50 μCi or 300–400 μCi was incubated with 10^6 pelleted cells for 2 h. 50 μCi were used for the labeling of cultured cell lines, and 300–400 μCi for freshly isolated cells. The labeled cells were then washed three times, and used as target cells at 8×10^3 cells per microculture well. The percent specific ^{51}Cr release was calculated according to the following formula: percent release = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release), where spontaneous release was the ^{51}Cr release from 8×10^3 labeled target cells incubated alone in RPMI 1640 medium containing 10% FCS, and maximum release was that from labeled cells incubated alone in water containing 5% detergent. The standard deviation of percent specific ^{51}Cr release was <5% in most of the experiments. Maximum release of ^{51}Cr from normal unstimulated blood mononuclear cells was 1,000–4,000 cpm, that from T and B blasts was 2,000–8,000 cpm, and that from lymphoma and leukemia cells isolated from patients was 1,000–7,000 cpm. The amount of ^{51}Cr incorporated into cells depended on the specific activity of ^{51}Cr. When ^{51}Cr having specific activity of 200–400 mCi/mg Cr was used instead of 20–200 mCi/mg Cr, a much higher level of ^{51}Cr was incorporated into normal unstimulated target cells and malignant cells isolated from patients, and similar results were obtained in terms of the value of percent specific ^{51}Cr release. However, this ^{51}Cr was not used thereafter, because of its higher cost. Spontaneous release relative to maximum release was 37% (mean) in T lymphocytes, 30% in B lymphocytes, 35% in monocytes, 35% in purified

LGL, 19% in T blasts, 29% in B blasts, 33% in lymphoma cells, and 35% in leukemia cells. In some experiments, 500 IU/ml of interferon (IFN)-β (sp act 2×10^7 IU per milligram of protein) was added and incubated for 20–24 h with the effector cells. IFN was not directly cytotoxic to the target cells during a ^{51}Cr-release assay. Cytotoxic activity was considered positive when the value of experimental ^{51}Cr release was significantly above that of spontaneous ^{51}Cr release by the Student's *t* test.

Characterization of Effector Cells. To determine whether the lymphocytes reactive with autologous blood mononuclear cells were NK cells or not, the following methods were employed. First, we determined whether Percoll-sedimented fractions having high NK activity against K562 cells also had high cytotoxic activity against the autologous blood mononuclear cells. Effector cells were whole mononuclear cells, nylon fiber column–passed cells, and Percoll-sedimented fractions 1–4. In three similar experiments, the percentages of LGL in each of the fractions were as follows; whole mononuclear cells, 11–15%; nylon fiber–passed cells, 15–21%; Percoll fraction 1, 76–84%; fraction 2, 35–53%; fraction 3, 5–12%; and fraction 4, 2–6%. Percoll fractions 0 and 5 were not used as effector cells because these fractions contained many platelets and erythrocytes, respectively, and a small number of lymphocytes. Second, we determined whether LGL-rich effector cells depleted of NK cells by mAb Leu-11b (21), or of T cells by mAb OKT3 had cytotoxic activity against autologous blood mononuclear cells. The panning method described by Payne et al. (22) was modified as follows: 3-ml quantities of affinity-purified $F(ab')_2$ fragment goat anti–mouse IgG or IgM (Cappel Laboratories, West Chester, PA) were added to 60-mm Petri dishes (Falcon Labware, Oxnard, CA). After overnight incubation at 4°C, the antibody was harvested from the plates, and each plate was washed three times with 3 ml of cold medium. 10^7 LGL-rich lymphocytes, resuspended in1 ml of 1:10-diluted Leu-11b or OKT3 mAb were incubated on ice for 30 min, washed, resuspended in 2.5 ml of RPMI 1640 medium containing 5% FCS, and added to the antibody-coated plates. Because Leu-11b and OKT3 are IgM and IgG antibodies, respectively, Leu-11b-treated cells were added to anti–mouse IgM antibody-coated plates, and OKT3-treated cells were added to anti–mouse IgG antibody-coated plates. The plates were then incubated in a 5% CO_2 incubator at 37°C for 30 min. The nonadherent cells (Leu-11b$^-$ or OKT3$^-$ cells) were harvested by swirling the plates and removing the cells by aspiration with a Pasteur pipette. The cells were centrifuged, and the pellet was resuspended in RPMI 1640 medium containing 10% FCS. The concentration of cells was readjusted, and the cells were used as effector cells for the cytotoxicity assay. In preliminary experiments, >95% of the Leu-11b$^+$ or OKT3$^+$ cells were removed by the panning method when the cells were assayed by the indirect immunofluorescence method.

Results

Cytotoxicity of LGL-rich Lymphocytes for Autologous Normal Blood Mononuclear Cells. LGL-rich lymphocytes were cultured for 1 d and were assayed for cytotoxicity against autologous normal blood mononuclear target cells in a 5-h ^{51}Cr-release assay (Table I). Effector cells were obtained from normal individuals. Target cells were autologous T and B lymphocytes, mitogen-induced T and B blasts, monocytes, LGL, and an NK-sensitive cell line, K562. The results demonstrated that all target cells were lysed by LGL-rich effector cells. In 10 repeated experiments in which unstimulated T and B lymphocytes were simultaneously used as target cells, B cells were more susceptible in 7 experiments, and T cells in 2. B lymphocytes and monocytes had almost the same degree of susceptibility, and T lymphocytes and LGL were less susceptible in most of the experiments. To determine whether blastic cells were more susceptible than unstimulated cells, T and B blasts were compared with unstimulated T and B lymphocytes. The results suggested that the blasts were less susceptible.

Reduced Cytotoxicity of Freshly Prepared LGL-rich Lymphocytes Against Normal

TABLE I
Autologous Cellular Lysis by LGL-rich Lymphocytes

Exp.	Target cells	Cytotoxicity at effector/target (E:T) ratios of:	
		80:1	20:1
		%	
1	T lymphocytes	24	7
	B lymphocytes	44	23
	Monocytes	41	17
	LGL	25	15
	K562 cell line	ND*	71
2	T lymphocytes	51	19
	T blasts‡	23	16
	B lymphocytes	40	19
	B blasts‡	28	7
3	B blasts‡	36	29
	B blasts, enriched§	29	5

LGL-rich lymphocytes obtained from normal individuals were cultured for 1 d and assayed for their cytotoxicity against autologous normal blood mononuclear cells and a K562 cell line.
* ND, not done.
‡ Morphologically identified blasts were 70–80% pure.
§ B blasts were enriched to 95% by Percoll density gradient centrifugation.

and Malignant Target Cells. Because LGL-rich lymphocytes were cultured for 20–24 h before use as effector cells in the cytotoxicity assay, we wished to determine whether LGL-rich effector cells were also cytotoxic without short-term culture. Table II shows that fresh effector cells did not lyse, or less efficiently lysed autologous B lymphocytes, monocytes and patients' tumor cells, the higher lytic activity being demonstrated in 20–24 h of culture. In patient 3, 1-d-cultured effector cells, but not fresh effector cells, lysed autologous lymphoma cells.

Demonstration of Cytotoxicity with Medium Supplemented with AB Serum and Autologous Serum. Because 1-d-cultured effector cells but not freshly prepared effector cells efficiently lysed the target cells, we questioned whether certain factor(s) contained in FCS would induce the cytotoxic activity in vitro. Thus, medium supplemented with 10% heat-inactivated human AB or autologous serum was used throughout the experiments. Table III shows that cytotoxic activity was also demonstrated for autologous B lymphocytes and monocytes, and allogeneic lymphoma and leukemia cells using medium containing human AB and autologous serum. Freshly prepared effector cells were not cytotoxic or only slightly cytotoxic, as was shown in the experiments using FCS-supplemented medium (data not shown).

Characterization of Cytotoxic Effector Cells. The identity of the effector cells responsible for the observed cytotoxicity was determined. First, we conducted an experiment to determine whether a highly enriched population of NK cells had cytotoxic activity for autologous blood mononuclear cells (Table IV). The fractions enriched in LGL showed a considerable increase in cytotoxicity for autologous cells and K562 cells, and the cytotoxicity was almost parallel to the

TABLE II

Reduced Cytotoxicity of Freshly Prepared LGL-rich Lymphocytes Against Normal and Malignant Target Cells

Target cells	Cytotoxicity mediated by:			
	Fresh effector cells at E:T ratios of:		1-d-cultured effector cells at E:T ratios of:	
	80:1	20:1	80:1	20:1
	%			
B lymphocytes	7*	5*	46*	14*
Monocytes	2	2	42*	20*
Lymphoma cells:				
Patient 1	5*	0	44*	24*
2	8*	ND	42*	ND
3	0	0	13*	3

LGL-rich lymphocytes from a normal individual and patient 3 were prepared, and their lytic activity was assayed, both immediately, and after 1 d of culture, for autologous B lymphocytes, monocytes, and lymphoma cells (patient 3) and for allogeneic lymphoma cells (patients 1 and 2). Effector cells for lymphoma cells of patients 1 and 2 were from the normal donor.

* Percentage of cytotoxicity in a ^{51}Cr-release assay in which the value of released ^{51}Cr was significantly ($p < 0.05$) higher than that of spontaneously released ^{51}Cr.

TABLE III

Effective Induction of Cytotoxic Effector Cells by Medium Supplemented with Human AB Serum or Autologous Serum

Exp.	Serum	Target cells	Cytotoxicity at E:T ratios of:	
			80:1	20:1
1	AB	B lymphocytes	42	14
		Monocytes	49	7
		Lymphoma cells		
		Patient 2	30	17
		Patient 3	14	6
2	Autologous	B lymphocytes	29	7
		Monocytes	15	8
		Lymphoma cells from patient 2	26	20
		Leukemic blasts from patient 7	19	5

LGL-rich lymphocytes from normal individuals were cultured for 1 d and assayed in their cytotoxicity for autologous B lymphocytes and monocytes, and allogeneic lymphoma and leukemia cells. Medium supplemented with 10% heat-inactivated human AB serum or autologous serum was used throughout the experiments, except when T lymphocytes were removed by E rosetting during the procedure of target B cell preparation, in which case FCS-supplemented medium was used.

concentration of LGL. Secondly, LGL-rich lymphocytes were treated with mAb Leu-11b or OKT3, and NK cells or T cells were depleted by the panning method. The nonadherent Leu-11b$^-$ or OKT3$^-$ cells were tested for their cytotoxicity on autologous B lymphocytes and monocytes, and K562 cells. As shown in Table V, treatment with Leu-11b markedly inhibited the cytotoxicity, whereas the removal of OKT3$^+$ cells did not inhibit the cytotoxicity, indicating

TABLE IV

Characterization of Cytotoxic Effector Cells by Percoll Discontinuous Gradient Centrifugation

Effector cells	E:T ratio*	Cytotoxicity for different target cells					
		T	B	B blasts	Monocytes	LGL	K562*
				%			
Whole mononuclear cells	80:1	−2	−2	−2	−8	−6	26
	20:1	−1	−1	−2	−5	−2	10
Nonadherent cells	80:1	−2	7	ND	−1	4	32
	20:1	2	4	ND	−3	−1	12
Percoll fraction 1	80:1	22	55	35	45	24	79
	20:1	12	26	6	10	7	50
2	80:1	7	36	15	16	8	52
	20:1	1	11	−1	−2	1	18
3	80:1	3	2	2	−3	−2	7
	20:1	2	0	−3	−5	−1	2
4	80:1	3	1	2	−5	0	2
	20:1	1	−1	1	−4	1	1

* E:T ratios were 80:1 and 20:1, except for K562 target cells, in which the E:T ratios were 20:1 and 5:1.

TABLE V

Characterization of Cytotoxic Effector Cells by Negative Selection with mAb

Treatment of effector cells	Cytotoxicity against target cells					
	B lymphocytes at E:T ratios of:		Monocytes at E:T ratios of:		K562 cells at E:T ratios of:	
	80:1	20:1	80:1	20:1	20:1	5:1
			%			
Control	23	13	31	4	49	12
Leu-11b	8	5	11	−2	4	0
OKT3	21	14	40	7	61	19

LGL-rich lymphocytes were incubated with Leu-11b or OKT3 mAb, and Leu-11b$^+$ or OKT3$^+$ cells were depleted by adhering to anti-Ig–coated plates. In control treatment, cells without mAb treatment were applied to anti-Ig–coated plates.

that mainly Leu-11b$^+$ NK cells mediated cytotoxicity for autologous blood mononuclear target cells.

Regulation of Cytotoxicity by Monocytes and Their Supernatants. Because whole mononuclear cells did not lyse autologous normal mononuclear cells, whereas fractions enriched with LGL did so efficiently (Table IV), we questioned whether the latter positive results were obtained because of the removal of suppressor cells during the preparation of LGL, and/or enrichment of the effector cell population. Because monocytes were removed during the preparation of effector cells, and because there are some reports (23–27) indicating monocyte-mediated NK suppression, we tested whether monocytes added in culture could suppress the development of lytic activity for normal and malignant target cells. As shown in Fig. 2, monocytes added to autologous LGL-rich effector cells at the beginning of culture significantly suppressed the lytic activity for autologous B lymphocytes

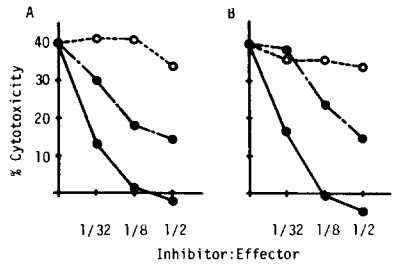

FIGURE 2. Inhibition of NK activity by monocytes. LGL-rich effector cells from normal individuals were cultured for 1 d with and without graded numbers of autologous monocytes or T lymphocytes, and assayed for their cytotoxicity against autologous B lymphocytes (*A*) and autologous monocytes (*B*), with an E:T ratio of 80:1. Monocytes (●——●) or T lymphocytes (○----○) were added at the beginning of and throughout the culture, and monocytes (●— – —●) were added only during the cytotoxicity assay.

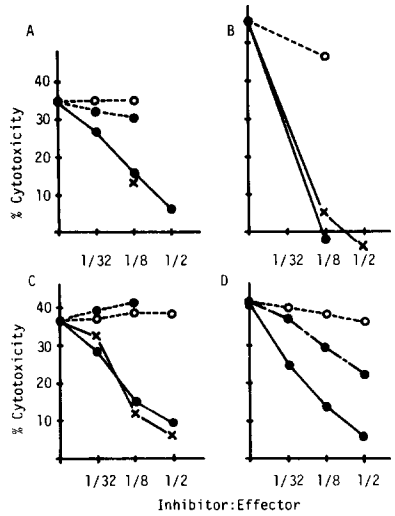

FIGURE 3. Inhibition of NK activity by monocytes. LGL-rich lymphocytes from patient 1 (*A*), patient 4 (*B*), and two normal donors (*C* and *D*) were cultured for 1 d, with and without graded numbers of monocytes or T lymphocytes, and assayed for their cytotoxicity against autologous lymphoma cells (*A* and *B*) and allogeneic lymphoma cells of patient 1 (*C*) and patient 2 (*D*). The E:T ratio was 80:1. Inhibitor cells were added as follows: autologous monocytes added throughout the period of culture (●——●) or only during the cytotoxicity assay (●— – —●); allogeneic monocytes added throughout the period of culture (×——×); and autologous T lymphocytes (○----○) and allogeneic T lymphocytes were (⊙----⊙) added throughout the period of culture. When effector cells were from the patients, allogeneic inhibitor cells added were from normal donors, and vice versa.

and monocytes, whereas monocytes added only during the cytotoxicity assay suppressed the activity less prominently. Lytic activity was not suppressed by the addition of T lymphocytes. When LGL-rich lymphocytes obtained from the patients with malignant lymphoma were cultured for 1 d with autologous patients' and allogeneic healthy donors' monocytes, monocytes from both autologous and allogeneic donors suppressed lytic activity for autologous lymphoma cells (Fig. 3). Likewise, killer activity of healthy donors' LGL-rich lymphocytes for lymphoma target cells was suppressed by the addition of autologous normal

and allogeneic patients' monocytes. The degree of suppression was almost similar between monocytes derived from the patients and those derived from healthy donors, indicating that the presence of such monocytes is not limited to patients with malignant disease.

To further clarify the mechanism of monocyte-mediated NK suppression, supernatants of 1-d-cultured monocytes and T lymphocytes were added to effector cells at the beginning of culture or only during the cytotoxicity assay. Table VI shows that supernatants of monocytes, but not those of T lymphocytes suppressed the development of cytotoxicity for autologous B lymphocytes. Cytotoxicity for allogeneic tumor cells was also suppressed. The results indicate that suppression was mediated by factor(s) released from monocytes. Supernatants added only during the cytotoxicity assay less efficiently suppressed the cytotoxicity. Thus, a more prolonged period of contact was required to induce a full degree of suppression of effector cell reactivity. When the degree of suppression was compared between monocytes and their supernatants, monocytes suppressed more prominently. This may reflect the fact that monocytes acted partly as an unlabeled competitive inhibitor for ^{51}Cr-labeled target cells, because monocytes themselves were susceptible targets of NK cells.

Lysis of Leukemic Cells by LGL-rich Lymphocytes. To examine the target spectrum of cytotoxicity of LGL-rich lymphocytes, we tested whether leukemic cells isolated from patients would be susceptible to healthy donors' LGL-rich lymphocytes. Table VII shows that all the types of leukemic cells tested were susceptible to 1-d-cultured effector cells, whereas most of them were less susceptible to freshly isolated effector cells. Moreover, monocytes added throughout the culture period completely suppressed the development of cytotoxic activity.

Susceptibility of Cell Lines to LGL-rich Lymphocytes. To further delineate the target selectivity of LGL-rich effector cells, in vitro–maintained cell lines, including those relatively resistant to lysis by NK cells, were tested for their susceptibility

TABLE VI

Suppression of Cytotoxicity by Supernatants of Cultured Monocytes

Exp.	Target cells	Duration added*	Cytotoxicity in presence of inhibitors:‡						
			None	Monocytes	Monocyte sup 2X	Monocyte sup 1X	Monocyte sup ½X	T cells	T cell sup 2X
		d				%			
1	B cells	1	41	−3	8	17	27	33	37
		CRT		14	34	37	42	36	41
2	B cells	1	39	9	19				
	Leukemic myeloblasts	1	40	8	11				

LGL-rich lymphocytes from normal donors were cultured for 1 d with or without inhibitors, and assayed for their cytotoxicity against autologous B lymphocytes and patient's leukemic myeloblasts at an E:T ratio of 80:1.

* Duration of time inhibitors were added: 1 d denotes that inhibitors were added at the beginning of culture; CRT denotes that they were added only during the ^{51}Cr-release test.

‡ Inhibitors added were as follows: autologous monocytes or T lymphocytes of ½ the number of LGL-rich effector cells; supernatants (sup) of 1-d-cultured monocytes or T lymphocytes having final concentrations of cells of two, one, and ½ times those added to LGL-rich effector cells.

TABLE VII
Lysis of Patients' Leukemic Cells by LGL-rich Lymphocytes, and Suppression of Their Lytic Activity by Monocytes

Target cells from patient*	Cytotoxicity mediated by: Fresh effector cells at E:T ratios of: 80:1	20:1	1-d-cultured cells at E:T ratios of: 80:1	20:1	80:1‡
			%		
5 (M1)	ND	ND	27§	6§	−6
6 (M1)	7§	4	31§	12§	2
7 (M2)	ND	ND	47§	30§	ND
8 (M3)	4§	4	36§	15§	3
9 (M5)	9§	6§	5§	4	−5
10 (L1)	5§	1	43§	21§	−8
11 (L1)	3	2	33§	16§	−1
12 (ATL)	4	1	8§	4	−9

LGL-rich lymphocytes from healthy individuals were assayed, immediately after preparation and again after 1 d of culture, for cytotoxicity against leukemic cells isolated from patients.

* Target cells were leukemic cells obtained from patients. Diagnosis of acute leukemia was based on the FAB designation (20): M1, myeloblastic leukemia without maturation; M2, myeloblastic leukemia with maturation; M3, hypergranular promyelocytic leukemia; M5, monocytic leukemia; and L1, lymphoblastic leukemia characterized by small, homogeneous blasts. ATL is adult T cell leukemia.

‡ Monocytes of ½ the number of effector cells were added at the beginning of culture.

§ Percentage of cytotoxicity in a ^{51}Cr-release assay in which the value of released ^{51}Cr was significantly ($p < 0.05$) higher than that of spontaneously released ^{51}Cr.

to LGL-rich lymphocytes. Table VIII shows that 1-d-cultured effector cells lysed all the cell lines tested. P815 and L1210, which were resistant to lysis by freshly isolated LGL-rich lymphocytes, were also lysed by 1-d-cultured LGL-rich lymphocytes. L1210, which was resistant to lysis by IFN-activated blood mononuclear cells, was also susceptible to the 1-d-cultured LGL-rich effector cells. The addition of monocytes inhibited the development or enhancement of cytotoxicity of LGL-rich effector cells for all the cell lines tested.

Discussion

In this study, we demonstrate the presence of NK cells with a broad spectrum of cytotoxicity for normal and malignant target cells, and describe the monocyte-mediated regulation of their cytotoxicity. There has been no earlier report concerning the NK-mediated lysis of autologous normal blood mononuclear cells. There may be several reasons why such findings have not been reported previously: (*a*), NK-enriched fractions were used as effector cells in our experiments. When whole mononuclear cells, instead of LGL-rich lymphocytes, were used as effector cells, cytotoxicity was not elicited (Table IV). (*b*), T blasts were mostly used as target cells by others; when blasts and unstimulated cells were compared, blasts were less susceptible targets. (*c*), Cytotoxicity was efficiently

TABLE VIII
Lysis of Cultured Cell Lines by PBMC and LGL-rich Lymphocytes

Target cell lines	E:T ratio	Cytotoxicity mediated by:					
		Fresh PBMC	1-d PBMC	1-d PBMC plus IFN-β*	Fresh LGL	1-d LGL	1-d LGL plus monocytes‡
				%			
K562	20:1	40	51	82	61	82	60
	5:1	14	17	48	28	45	ND
MOLT-4	20:1	24	31	72	53	71	47
	5:1	12	17	39	32	48	ND
Raji	80:1	3	4	49	15	61	12
	20:1	1	4	34	6	23	ND
HL-60	80:1	1	−1	67	36	78	18
	20:1	2	−1	37	15	52	ND
BALL-1	80:1	−1	0	62	34	86	12
	20:1	1	2	53	12	50	ND
P815	80:1	1	2	54	1	22	1
	20:1	0	2	36	0	8	ND
L1210	80:1	−1	−1	4	2	33	2
	20:1	−1	0	3	1	8	ND

Freshly isolated and 1-d-cultured PBMC and LGL-rich lymphocytes were assayed for their cytotoxicity against various cultured cell lines.
* 500 IU/ml IFN-β was added to PBMC throughout the culture period.
‡ Monocytes of ½ the number of LGL-rich lymphocytes were added throughout the culture.

demonstrated only after a short period of culture; freshly prepared NK cells did not possess, or only slightly possessed such cytotoxicity. (*d*), Because of poor incorporation of ^{51}Cr into unstimulated normal target cells as compared to that of transformed cells, unstimulated cells have not usually been used as target cells by other workers. This problem of poor ^{51}Cr incorporation was partially resolved in our experiments by labeling pellets of target cells with highly active ^{51}Cr of 300–400 μCi.

With regard to the nature of effector cells, fractions enriched in LGL had higher cytotoxicity (Table IV), and depletion of Leu-11b$^+$ cells markedly reduced cytotoxicity (Table V), indicating that mainly NK cells mediated cytotoxicity for autologous normal blood mononuclear cells. Similar results were obtained in autologous and allogeneic systems of lymphoma cell lysis (17). Furthermore, competitive inhibition studies using unlabeled target cells showed that cytotoxicity was efficiently inhibited by the addition of unlabeled K562 cells, and less efficiently by L1210 cells, suggesting that killer cells reactive with autologous normal cells were included within the population of killer cells that were more reactive with K562 than L1210 cells (data not shown).

With regard to the nature of target cells, some T and B lymphocytes, T and

B blasts, monocytes, and LGL were lysed by NK cells. Thus it may be important to elucidate exactly which T cells or other cells are susceptible to NK cells. Because not all of the LGL are NK cells (18), LGL other than NK cells may be susceptible to NK cells, or this might be one of the negative regulatory mechanisms of NK activation. In addition to normal target cells, NK cells showed lytic activity for malignant cells isolated from patients. Although we did not determine whether patients' NK cells would be able to lyse autologous leukemic blasts, NK cells lysed autologous lymphoma cells in all the patients tested (17), and NK cells from normal individuals lysed lymphoma (17) and leukemia cells (Table VII). Included among the susceptible malignant cells were T and B lymphoma cells, leukemic myeloblasts, promyelocytes, monoblasts, and lymphoblasts. Leukemic monoblasts from patient 9 were less sensitive to lysis than normal monocytes. Of various cell lines tested, murine L1210 leukemic cells, which were resistant to lysis by IFN-activated blood mononuclear cells (presumably IFN-activated NK cells), were also lysed by short-term cultured LGL-rich lymphocytes (Table VIII). Thus LGL-rich lymphocytes lysed NK-resistant cell lines, indicating their broad spectrum of target cell selectivity.

Kinetic studies revealed that lytic activity was readily demonstrated after 1 d of culture, and freshly prepared effector cells did not lyse, or less efficiently lysed target cells (Table II). Furthermore, a similar level of cytotoxicity for autologous B lymphocytes was persistent after 3 d of culture, and the cytotoxicity was present even after 1 wk of culture, although the level of cytotoxicity was lower (data not shown). Because FCS has been reported (28–30) to augment NK activity, medium supplemented with AB serum or autologous serum was used, and similar results were obtained (Table III). Thus, it is unlikely that certain factor(s) included in FCS induced the cytotoxic activity in vitro. The possibility that cytophilic Ig, which might inhibit the lytic activity of NK cells in vivo, might be released from the surface of NK cells after a short term of culture, thus enhancing the cytotoxicity in vitro, cannot be ruled out (31). However, the overall results could be explained if monocytes coexisting with LGL in the peripheral blood are inhibiting NK activity in vivo, and the removal of monocytes unmasks the presence of killer cells in a short-term culture. Indeed, there have been many reports (23–27) describing monocyte-mediated NK suppression. In our experiments, monocytes added at the beginning of culture markedly inhibited the development of cytotoxicity (Fig. 2). Using medium supplemented with human AB serum or autologous serum, monocytes added at the beginning of culture also inhibited the development of cytotoxicity (data not shown). Furthermore, supernatants of cultured monocytes inhibited the development of cytotoxicity (Table VI). Thus, monocyte-mediated inhibition was shown to be due to factor(s) released from monocytes. The nature of the inhibitory factor(s) remains to be elucidated. In preliminary experiments, indomethacin or catalase added to monocytes did not abrogate the monocyte-mediated inhibition, suggesting that neither prostaglandins (23, 25) nor hydrogen peroxide (24) plays an important role. Because monocytes were susceptible targets for NK cells, the possibility that target structures present on the surface of monocytes and recognized by NK effector cells were shed into the medium during culture, subsequently blocking the receptor sites of effector cells, cannot be ruled out.

Whether NK cells having the ability to lyse autologous normal cells and malignant cells are actually present in vivo is an important issue. Although indirectly suggested, the NK lytic process seems to be involved in the mechanism of NK suppression of antibody-producing cells in vivo (32), and animal experiments indicate NK-mediated lysis of tumor cells in vivo (4, 33, 34). Therefore, if monocytes play a central role in NK regulation, changes in these regulatory mechanisms may lead to the development, exacerbation, or regression of such diseases as malignant neoplasms and autoimmune diseases.

Summary

After depletion of monocytes, natural killer (NK) cells were partially purified from peripheral blood by Percoll density gradient sedimentation. The NK cells were then cultured for 1 d and assayed for their cytotoxicity against various types of normal and malignant target cells. All types of target cells tested were found to be susceptible to NK cells. The susceptible targets were autologous T and B lymphocytes, mitogen-induced T and B blasts, monocytes, large granular lymphocytes, autologous or allogeneic lymphoma and leukemia cells isolated from patients, and cultured cell lines, including those resistant to interferon-activated lymphocytes. Such a broad spectrum of cytotoxicity was demonstrated in 1 d of culture, and freshly prepared NK cells were not cytotoxic, or, if anything, were less cytotoxic. Monocytes and their supernatants, added throughout the course of culture, markedly inhibited the development of their cytotoxicity. These results may suggest that, although NK cells having ability to lyse autologous normal and malignant target cells are present in vivo, their lytic activity is regulated by coexisting monocytes.

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