0022-1767/83/1305-2478$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 130, No. 5, May 1983
Printed in U.S.A.

STUDIES ON THE MECHANISM OF NATURAL KILLER CYTOTOXICITY

II. Coculture of Human PBL with NK-Sensitive or Resistant Cell Lines Stimulates Release of Natural Killer Cytotoxic Factors (NKCF) Selectively Cytotoxic to NK-Sensitive Target Cells

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This investigation has employed the "innocent bystander" type of experimental design to determine whether soluble cytotoxic factor(s) are released during interactions between human peripheral blood lymphocytes (PBL) and NK-sensitive target cells. PBL cocultured with NK-sensitive Molt-4 or K562 target cells in the lower well of a miniaturized Marbrook culture released natural killer cytotoxic factors (NKCF), which diffused across a 0.2-μ Nucleopore membrane and lysed Molt-4 or K562 target cells cultured in the upper chamber. Coculture of PBL with the NK-resistant Raji or WI-L2 cell lines also induced release of NKCF. These factors were selectively cytotoxic to NK-sensitive targets and lysed Molt-4 and, to a lesser extent, K562 cells. However, Raji, WI-L2, and RPMI 1788 cells were all resistant to lysis. In addition, low density fractions from Percoll density gradients that were enriched for NK effector cells also released increased levels of NKCF during coculture with Molt-4 cells. Lysis of Molt-4 and K562 targets was observed after exposure to NKCF for 48 hr and 60 to 70 hr, respectively. Cellfree supernatants containing NKCF were obtained after a short time of incubation (i.e., within 5 hr of coculture of PBL with NK target cells). The factors were nondialyzable, stable at 56°C for 3 hr, and showed partial loss of activity on storage at 4°C or −20°C for 7 days. These data suggest that NKCF may be involved in the lytic mechanism of human NK cell-mediated cytotoxicity.

Natural killing (NK)[2] by human lymphocytes against a number of tissue culture cell lines *in vitro* has been well documented. This cell-mediated lytic system differs from that of thymic (T) cell-mediated cytotoxicity (CMC) in that effector cells are present at high levels in nonimmune individuals. Thus, they may provide the host with a first line of defense against newly arising malignancies. Although the mechanism of lysis by either NK effector cells or cytotoxic T lymphocytes (CTL) is still unclear, at least two general models have been proposed. First, the effector lymphocytes may physically damage the target cell by a not well understood mechanism. Alternatively, lysis may be mediated by cytotoxins delivered to the target cell by the effector cell.

Several types of cytotoxins produced by human lymphocytes have been described. Lytic activity detectable on L-929 cells, termed lymphotoxins (LT), has been well characterized (for review, see reference 1). Human LT is a multicomponent system comprised of weakly lytic, low m.w. subunits that can be derived from more lytically active complexes. The high m.w. (>200,000) LT complexes are lytically active on additional cell lines, in particular the NK-sensitive K562 cell line (2). Furthermore, the LT complexes can associate with antigen-binding receptors that provide them with the capacity to selectively recognize target cells (3). These LT complexes are released from alloimmune human peripheral blood lymphocytes (PBL) and have been implicated as possible mediators of human T CMC (4).

Additional systems have been developed for detection of factors released from nonimmune human PBL that are cytotoxic to NK target cells. Wright and Bonavida (5) have employed miniaturized Marbrook chambers to examine the lytic capacities of materials released from human PBL upon stimulation with lectin-coated, L-929 cell monolayer. The 48-hr assay reveals that soluble cytotoxic factors produced under these conditions are lytically active on Molt-4 and, to a lesser extent K562 cells. Raji and IM-9 cells, both resistant to human NK, are not sensitive to lysis by these factors. Granger *et al.* (6) have examined the lytic capacity of materials released within 5 hr from preactivated human lymphocyte populations that are secondarily stimulated with a lectin. Lytic activity in these supernatants is detectable in an 18-hr ^{51}Cr-release assay employing Molt-4 or K562 target cells. Although the above studies show clearly that PBL can release cytotoxic factors selective for NK sensitive targets, it has not yet been determined if these factors are released after interaction between human NK cells and NK target cells. Recent results in the murine system indicate that cells with the characteristics of NK cells do release such factors during interaction with NK target cells (7). These factors have been functionally defined as natural killer cytotoxic factors (NKCF), because little is known of their biochemical nature or relationship to other more well defined cytotoxins.

The present studies were undertaken to determine if human NKCF are released from freshly isolated PBL or PBL enriched for NK effector cells under culture conditions that simulate an NK (CMC) reaction. The results show that PBL cocultured with NK sensitive target cells release NKCF. Furthermore, low density PBL that are enriched for NK effector cells also release increased levels of NKCF. Although some NK resistant cells could stimulate

Received for publication November 29, 1982.
Accepted for publication January 18, 1983.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Supported in part by Grant CA12800 from the National Cancer Institute and by National Cancer Institute Training Grant CA-09120 awarded to S. Wright and Robert Kahle.
[2] CMC, cell-mediated cytotoxicity; LGL, large granular lymphocyte; LT, lymphotoxin; NK, natural killer; NKCF, natural killer cytotoxic factors; PBL, peripheral blood lymphocytes. FCS, fetal calf serum; CTL, cytotoxic T lymphocytes.

release of NKCF, only sensitive cell lines were actually lysed by the factors.

MATERIALS AND METHODS

Effector cells. Human PBL were isolated by Ficoll-Hypaque density gradient centrifugation (8). Glass-adherent cells were removed by incubating the PBL in a tissue culture bottle at 37°C for 1 hr. The resulting population was suspended at 5×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum (FCS).

Cell lines. The NK-sensitive cells used in this study were Molt-4 and K562, whereas the NK-resistant cells were Raji, WI-L2, and RPMI 1788. All cell lines were maintained in suspension culture in RPMI 1640 supplemented with 10% FCS.

Marbrook cultures. The preparation of the miniaturized Marbrook chamber has been described previously (9). The effector cell suspension (a total of 6×10^6 PBL in 1.2 ml) was cultured in the lower well. 1.2×10^5 stimulator cells were added to the effector cell suspension. Control cultures contained media without effector or stimulator cells. The bottom of the upper chamber was covered with a 0.2 μ nucleopore membrane and immersed in the effector cell suspension. 3.5×10^4 target cells were placed in the upper chamber. Cultures were set up in triplicate under sterile conditions and incubated at 37°C in 5% CO_2. At the times indicated, aliquots of the target cells were removed and their viability was assed by trypan blue exclusion. In all experiments, the viability of target cells in control cultures was greater than 85%. Percent cytotoxicity was calculated as follows:

$$\% \text{ Cytotoxicity} = \frac{\% \text{ Viability of control} - \% \text{ viability of experimental}}{\% \text{ Viability of control}} \times 100.$$

Generation and testing of cellfree supernatants. In some experiments, effector and stimulator cells were cultured for various periods of time in tissue culture flasks by using the same cell concentrations as that in the Marbrook culture. Supernatants were harvested by centrifugation followed by filtration through a 0.45 μm filter. Cytotoxic activity was assessed by placing 1.2 ml of the supernatant diluted 1:2 with fresh media into the lower well of the Marbrook chamber. Incubation and assessment of target cell viability was carried out as described previously (7).

Discontinuous percoll density gradient centrifugation. Human PBL isolated by Ficoll-Hypaque density gradient centrifugation were suspended in RPMI 1640 with 10% FCS and allowed to adhere to nylon wool columns for 1 hr at 37°C. Nonadherent cells were layered on top of the gradient that consisted of six different concentrations of Percoll ranging from 37.5% to 50% as described by Timonen *et al.* (10). After centrifugation, the different fractions (nos. 1 to 7) were collected and assayed for NK CMC in a 4-hr ^{51}Cr release assay with the use of Molt-4 target cells and for production of NKCF. In some experiments, fractions 1 and 2 yielded too few cells to be tested, and therefore such fractions were pooled as indicated. NKCF was produced by incubating 3×10^6 cells in 1 ml of RPMI 1640 along with Molt-4 stimulator cells at an effector-to-stimulator cell ratio of 50:1. After incubating in 5% CO_2 for 24 hr, cellfree supernatants were collected and assayed for cytotoxic activity in the micro supernatant assay.

Micro supernatant assay. Ten thousand Molt-4 cells in 50 ml of RPMI 1640 with 10% FCS were placed in each well of sterile flat-bottomed microtiter plates. 37.5 to 150 μl of each cellfree supernatant were added to each well in triplicate. The results are reported for the 150-ml volume only because that was in the linear portion of the titration curve. One hundred fifty microliters of RPMI 1640 were added to the media control wells. After incubation for 48 hr at 37°C in 5% CO_2, aliquots of the target cells were removed and viability was determined by trypan blue exclusion. Percent cytotoxicity was calculated as follows.

$$\% \text{ Cytotoxicity} = \frac{\% \text{ Viability of control} - \% \text{ viability of test}}{\% \text{ Viability of control}} \times 100.$$

RESULTS

NK-sensitive and NK-resistant cell lines stimulate release of NKCF from nonimmune human PBL. Different allogenic cell lines were tested in the miniature Marbrook chamber for the capacity to stimulate human PBL to release NKCF. Nucleopore membranes (of pore size 0.2 μm) were used to separate the two chambers. Freshly isolated human PBL were incubated in the lower wells either alone or with Molt-4, K562, Raji, or WI-L2 cells, and Molt-4 or K562 target cells were cultured in the top chambers. The viabilities of cells in the top chambers were determined after 50 to 67 hr of culture. The results shown in Table I demonstrate that all four cell lines tested are capable of

TABLE I

Lysis of NK-sensitive targets by NKCF released from PBL cocultured with different cell lines

Expt. No.	Bottom Well Contains:	% Cytotoxicity[a]	
		Molt-4	K562
1[b]	PBL	0	n.d.[c]
	PBL + Molt-4	36	n.d.
	PBL + K562	10	n.d.
2	PBL	12 ± 1.8	n.d.
	PBL + Molt-4	26 ± 3.6	n.d.
	PBL + K562	66 ± 3.1	n.d.
3	PBL	15 ± 8.6	3 ± 1.0
	PBL + Molt-4	87 ± 1.0	28 ± 1.8
	PBL + K562	70 ± 11.4	19 ± 3.1
	PBL + Raji	32 ± 7.6	2 ± 2.5
4	PBL	0 ± 0	n.d.
	PBL + Molt-4	41 ± 1.0	n.d.
	PBL + K562	30 ± 3.8	n.d.
	PBL + Raji	38 ± 7.6	n.d.
5	PBL + Molt-4	64 ± 4.9	n.d.
	PBL + K562	38 ± 4.0	n.d.
	PBL + Raji	40 ± 6.1	n.d.
	PBL + WI-L2	42 ± 5.9	n.d.

[a] Marbrook chambers containing either Molt-4 or K562 target cells in the upper chambers were used to assess cytotoxicity after 50 hr (experiments 1, 2, and 4) or 67 hr (experiments 3 and 5) of culture.
[b] Experiment 1 was set up in duplicate instead of triplicate because of a low yield of PBL.
[c] n.d., not determined.

TABLE II

Evidence that lysis of Molt-4 is not caused by media depletion

Bottom Well Contains:	% Cytotoxicity ± SD[a]	
	0.2 μm Nucleopore	Dialysis membrane
PBL + Molt-4	39 ± 5.5	1 ± 1.2
Supernatant[b]	30 ± 8.9	3 ± 3.8

[a] Incubation time was 48 hr. The two chambers of the Marbrook culture were separated by either a 0.2 μm Nucleopore membrane or a dialysis membrane.
[b] Supernatant from 48-hr cultures of PBL + Molt-4 at 50:1 effector-to-stimulator ratio was tested as described in *Materials and Methods.*

stimulating the release of NKCF from human PBL. PBL cultured without stimulator cells occasionally release low levels of NKCF. However, experiments 1 to 4 clearly show that coculture of PBL with stimulator cells is required to generate optimal levels of NKCF. Cytotoxic activity is detectable on Molt-4 and, to a lesser extent, on K562 target cells. Since Molt-4 targets are more sensitive to the lytic activity of NKCF, this target was employed in most of the experiments reported here.

Although the ability of PBL to produce NKCF may vary from donor to donor, all individuals tested can produce some NKCF. During the course of this study, PBL from 14 different individuals were tested for their ability to produce NKCF during coculture with Molt-4 stimulator cells. The mean percent cytotoxicity against Molt-4 target cells was 29% with a range of 11% to 53%. Thus, the ability to produce NKCF was found to be of general occurence in this population sample.

To ensure that lysis was not caused by media depletion, lytic activity was tested with dialysis membrane separating the two chambers (Table II). With the lower wells containing either PBL and Molt-4 cells or a supernatant from a 48-hr culture of PBL and Molt-4 cells, no lysis of Molt-4 target cells was detectable. In the same experiment, significant levels of lysis were obtained when the nucleopore membranes were used to separate the chambers. Furthermore, additional experiments utilizing cell lysates from the various stimulating cell lines indicate that the cytotoxic activity is derived from the PBL and not from the stimulator cell lines (data not shown).

The kinetics of release of NKCF was determined by incubating

PBL with Molt-4 cells and collecting supernatants after various intervals of time for up to 46 hr. The aliquots were frozen until testing on Molt-4 target cells for NKCF in a 48-hr assay. Maximum levels of lytic activity were detected in 5 hr supernatants, which were comparable to the levels obtained in 18-, 28- and 46-hr supernatants (Fig. 1).

Release of cytotoxic factors from Percoll gradient fractions enriched for NK cells. It has been demonstrated that discontinuous Percoll density gradient centrifugation can be used to enrich for low density PBL containing a high percentage of LGL that have been implicated as the human NK effector cell (10, 11). Thus, it was of interest to determine if this technique could be used to enrich for PBL which release NKCF. The results of two representative experiments are shown in Table III. Fractions from the Percoll gradient are numbered from 1 to 7 in order of increasing density according to Timonen's (10) method. The results of experiment 1 demonstrate that the low density fractions 2 to 4 contain a population of cells with significantly increased NK activity measured in a CMC assay as compared to the high density fractions 5 to 7 or to nylon wool nonadherent PBL. This is in agreement with the results of Timonen *et al.* (10). Fractions 2 to 4 also released greater levels of NKCF than did fractions 5 to 7 or nylon nonadherent PBL. The second experiment also demonstrates a high correlation between fractions enriched for NK activity in a CMC assay and production of NKCF, with fraction 3 being the most active in both assays. These data suggest that NKCF may be released from NK effector cells and that cell populations with decreased NK activity release less NKCF.

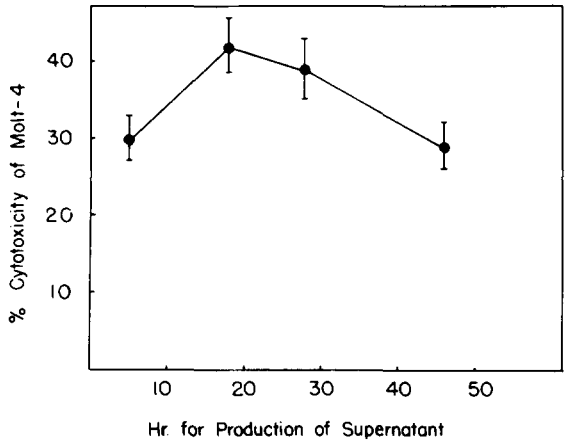

Figure 1. Kinetics of production of NKCF. Cellfree supernatants were obtained from cultures of PBL and Molt-4 after different incubation times. All samples were diluted 1:2 with fresh media and assayed simultaneously for cytotoxic activity against Molt-4 in the Marbrook chamber for 48 hr of culture.

TABLE III

Release of NKCF and NK CMC activity of PBL fractionated by Percoll gradients

Expt. No.	Effector Cells	% Cytotoxicity Molt-4	
		NK CMC[a]	NKCF[b]
1	Nylon nonadherent PBL	52 ± 3.7	19 ± 4.7
	Fractions 2 to 4	79 ± 4.7	36 ± 5.0
	Fractions 5 to 7	41 ± 0.4	16 ± 5.9
2	Nylon nonadherent PBL	65 ± 1.5	20 ± 1.5
	Fractions 1 to 3	100 ± 10.0	30 ± 2.5
	Fraction 4	76 ± 3.0	14 ± 2.6
	Fraction 5	52 ± 2.6	11 ± 4.0
	Fraction 6	50 ± 6.9	8 ± 2.6
	Fraction 7	29 ± 1.1	8 ± 3.7

[a] CMC was assayed in a 4-hr ^{51}Cr-release assay at a 50:1 effector-to-target ratio which was found to be in the linear portion of the titration curve.

[b] Cytotoxicity mediated by 150 μl of cellfree supernatant produced by the various cell fractions was titrated for NKCF activity in the 48-hr micro supernatant assay, and the reported values are taken from the linear portion of the titration curve.

Stability and functional properties of NKCF released by PBL upon coculture with Molt-4 stimulator cells. Supernatants obtained from 48-hr cultures of PBL and Molt-4 cells were incubated at several different temperatures for various periods of time and then tested for lytic activity on Molt-4 target cells. Although the activity is stable to heating at 56°C for up to 3 hr, occasionally there was a moderate decrease in activity when stored at +4°C or −20°C for 7 days (Table IV).

The kinetics of lysis of Molt-4 and K562 target cells were also examined. When Molt-4 cells are used as the stimulating cell line in the Marbrook chamber, significant levels of lysis are detectable on Molt-4 cells within 42 hr, and the levels rise significantly after 50 hr (Fig. 2*A*). Lysis of K562 target cells is detectable only after 50 hr of incubation. When K562 target cells are used as the stimulating cell line, similar results are obtained (Fig. 2*B*). Since previous experiments (Fig. 1) indicated that significant levels of

TABLE IV

Stability of NKCF at different temperatures

Expt. No.	Exposure Time	Temperature	% Cytotoxicity Molt-4 ± SD[a]
		°C	
1	0	—	27 ± 5.7
	30 min	56	26 ± 6.1
	1 hr	56	27 ± 2.3
	2 hr	56	27 ± 7.0
	3 hr	56	30 ± 1.5
	3 days	−20	29 ± 5.2
	7 days	−20	15 ± 1.5
	7 days	4	14 ± 4.0
2	0	—	40 ± 6.0
	3 days	−20	30 ± 4.7
	7 days	−20	26 ± 5.5
	7 days	4	28 ± 7.6

[a] % Cytotoxicity was determined after 48 hr of incubation. Supernatant was produced by culturing PBL with Molt-4 for 24 hr.

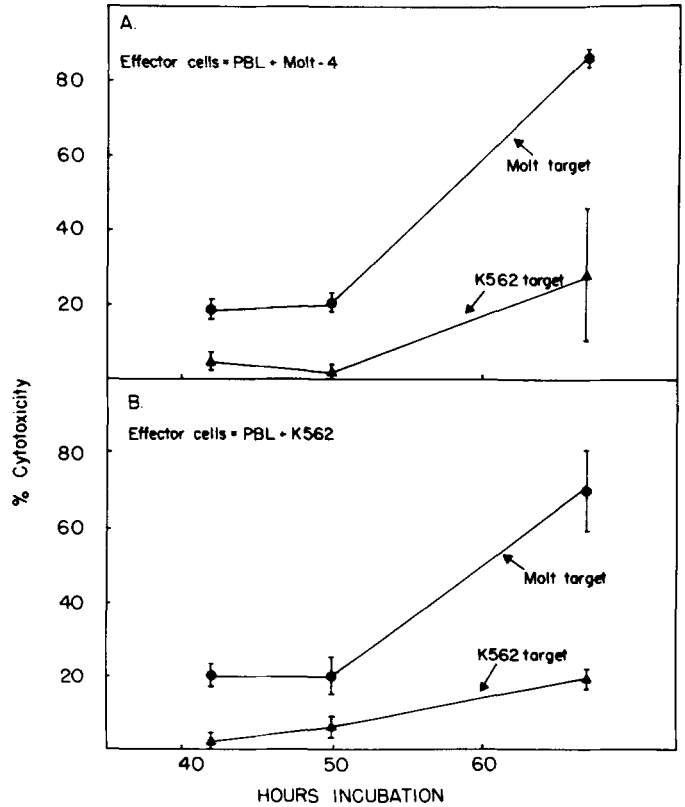

Figure 2. Kinetics of target cell lysis by NKCF. The percent cytotoxicity against Molt-4 or K562 target cells was determined after different lengths of incubation in the Marbrook culture in which the lower well contained PBL and Molt-4 (*A*) or PBL and K562 (*B*).

NKCF are released within 5 hr of culture, the extended incubation period required to obtain target cell lysis must be caused mainly by the kinetics of lysis as opposed to the kinetics of NKCF release.

The target cell spectrum of NKCF was examined by using Molt-4, K562, Raji, WI-L2, or RPMI 1788 cell lines (Fig. 3). In this representative experiment (of three total), PBL cocultured with Molt-4 stimulator cells released cytotoxic factors that are lytically active on Molt-4 and K562 cells, but not on the NK-resistant Raji, WI-L2, or RPMI 1788 cell lines. Additional experiments revealed a similar spectrum of target cell sensitivity when cellfree supernatants containing NKCF were placed in the lower wells of the Marbrook chamber instead of the PBL suspension (Table V).

DISCUSSION

The present studies demonstrate that NKCF are released from PBL during coculture with NK-sensitive and NK-resistant cells and that these factors are selectively cytotoxic to NK targets. Furthermore, the cell fractions from a Percoll density gradient that showed the highest levels of NK activity also released the highest levels of NKCF. This evidence is consistent with the possibility that NKCF are released from NK effector cells.

Previous work using alloimmune cytotoxic T lymphocytes suggested that soluble mediators were not involved in CMC reactions since "innocent bystander" cells were not lysed (13). Because of the lack of antigen specificity in the NK system, it has not been possible to test the innocent bystander effect by using the standard ^{51}Cr-release assay. However, with the use of the miniaturized Marbrook culture, we have found that innocent bystander cells separated from effector cells by a Nucleopore membrane are, in fact, lysed by NKCF released from PBL cultured under conditions which simulate an NK CMC reaction. We believe this observation warrants a reconsideration of the possible role of soluble lytic mediators in the mechanism of CMC, particularly in the NK system.

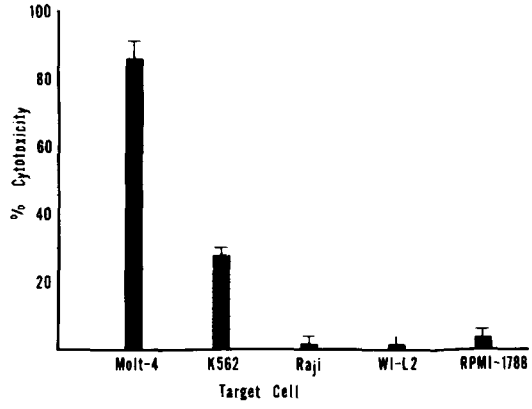

Figure 3. Target cell specificity of NKCF PBL were cocultured with Molt-4 stimulator cells in the lower well of the Marbrook chamber. After 67 hr of incubation, the viability of the various target cells in the top chamber was assessed. The % cytotoxicity obtained with the various target cells in a typical 4-hr NK CMC assay at a 50:1 effector-to-target cell ratio is as follows: Molt-4, 54 ± 5.5; K562, 28 ± 3.2; Raji, 0 ± 0; WI-L2, 3 ± 0.6; RPMI 1788, 7 ± 1.2.

TABLE V

Target cell specificity of NKCF in cellfree supernatants

Target Cell	% Cytotoxicity[a]
Molt-4	44 ± 2.9
K562	21 ± 2.4
Raji	0 ± 0.6
WI-L2	4 ± 2.1

[a] Percent cytotoxicity was determined after 66 hr of incubation of target cells in cellfree supernatants diluted 1:2 with fresh media.

Previous work employed lectin to stimulate the release of cytotoxic factors (5, 6). However, the role of such factors in NK CMC required the demonstration that NK target cells stimulate the release of NKCF after interaction with the effector cell. This study has demonstrated that incubating with either NK-sensitive or NK-resistant lines can induce release of NKCF from PBL. Thus, the NK-resistant Raji and WI-L2 cell lines could stimulate release of NKCF although they were resistant to their lytic activity. The question of whether binding is required between the NK cell and stimulator cell in order to induce release of NKCF has not yet been evaluated. It is not known whether Raji or WI-L2 cells will bind to NK cells, however it has been reported that large granular lymphocyte (LGL)-enriched fractions of human PBL contain cells that can form conjugates with Raji cells (14).

These findings suggest that there may be at least two different criteria that determine whether a target cell induces release of or whether it is lysed by NKCF in an NK CMC reaction. Thus, it is possible that some cell lines are NK-resistant because they lack a binding site for NKCF. Evidence suggests that this is true for some NK-resistant murine tumor cells (7). Other absorption studies, however, indicated that some NK-resistant cells could bind cytotoxic factors released from lectin-treated PBL (6, 13). This suggests that NKCF binding alone is not sufficient to lead to target cell lysis. Other possible mechanisms by which target cells are NK resistant could be that they possess a repair mechanism or else are inherently resistant to the lytic activity of NKCF. Another interesting possibility is that factors bound to the target cell must be activated by enzymes present on the target cell membrane. This phenomenon has been observed in the LT system where it was demonstrated that L929 cells pretreated with protease inhibitors become partially resistant to the lytic activity of LT (15).

The finding that NKCF are released quite rapidly from PBL (i.e., within 5 hr) is consistent with their possible involvement in NK CMC. This also implies that preformed pools of NKCF may be present in freshly isolated PBL.

In contrast to the rapid rate of release of NKCF, lysis of the target cell required a prolonged incubation period (48 to 70 hr). If NKCF are the lytic mediators of NK CMC reactions, then they must be more lytically active when effector and target cells are in direct contact. It is possible that NKCF in the Marbrook culture become too dilute to mediate rapid target cell lysis. However, in a CMC reaction, the concentration of NKCF in the local microenvironment of the effector-target cell conjugate could be high enough to cause target cell lysis in 4 hr. It is also possible that NK cells produce short-lived factors that facilitate target cell lysis by NKCF in a short-term assay. Alternatively, it could be that in CMC reactions, NKCF are delivered directly from the effector cell to the target membrane, and molecules escaping into the soluble phase are relatively unstable. This would be similar to Ware and Granger's (4) model for alloimmune T CMC in which high molecular weight LT complexes possessing high lytic activity rapidly break down in solution into smaller molecules with decreased lytic activity.

Little is known about the biochemical characteristics of NKCF. It is possible that NKCF activity found in whole supernatants is the result of the interaction of a number of different molecules ultimately leading to cell lysis. One obvious question is whether they are members of the LT family of molecules. The observation that the m.w. of NKCF is greater than 12,000 because they do not pass through a dialysis membrane is consistent with this possibility. However, attempts to fractionate NK lytic activity derived from lectin-treated PBL by gel filtration techniques known to separate the LT classes yielded inconclusive results (M.

Weitzen, unpublished observations). Further biochemical characterization of NKCF is necessary to clarify the issue.

This investigation has demonstrated that freshly isolated human PBL can be stimulated with NK-sensitive or resistant cell lines to release NKCF which selectively lyse NK-sensitive target cells. Evidence from the density gradient fractionation experiments implicates the NK effector cell as the source of NKCF. Although conclusive proof is lacking, the available evidence is consistent with the hypothesis that NKCF may be involved in the mechanism of NK CMC. Accordingly, the studies indicate that susceptibility of a target cell to NK CMC is determined ultimately by its sensitivity to lysis by NKCF rather than its ability to induce release of the factors.

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