RECYCLING AND TARGET BINDING CAPACITY OF HUMAN NATURAL KILLER CELLS*

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Spontaneous or natural killer (NK)[1] cells constitute one of several naturally occurring defense mechanisms in the immune response that currently attracts the attention of a large number of investigators (1). The rationale for this interest is the idea that NK cells are operative in the protection against infections and tumors and perhaps also in the regulation of normal stem cell differentiation (1–3). Several recent reviews examined the experimental basis for these assumptions (1, 4).

Although impressive advances have been made in the understanding of the NK system since its discovery in 1975, much remains to be learned. For instance, little is presently known about the receptor and receptor binding substance(s) involved or about the regulatory mechanisms governing NK reactivity. There are some indications that glycolipids may be involved in target recognition and that interferon, prostaglandins and immune complexes may influence the cytotoxic activity (5–7). Knowledge about the regulation of the NK system is, however, far from complete and undoubtedly several more suppressor or helper factors/cells will have to be defined to uncover the whole regulatory network. Such studies must be based on adequate knowledge in quantitating all steps contributing to the overall cytotoxic reaction. NK reactivity has mostly, hitherto, been quantitated by reacting a dilution series of effector cells with a given number of target cells at effector:target ratios of, for instance, 40:1 down to 5:1. This mode of quantitation is rather inadequate considering all steps involved in a continuous cytotoxicity reaction: (*a*) recognition and binding to the target, (*b*) deposition of cytolytic enzymes on the target surface, (*c*) detachment from target cell, and (*d*) binding and initiation of killing of another target.

In this paper we have used two different ways of measuring NK cytotoxicity in order to dissect the relative importance of the various steps involved in attaining the overall result. Thus, we have used the ^{51}Cr-release assay the same way one would have used an enzyme and a substrate to determine the particular NK capacity (V_{max}) and Michaelis constant (K_m) values done previously by others. The ^{51}Cr-release assay has been combined with a newly established single cell cytotoxicity assay in agarose to allow a better quantitative analysis. With this double procedure, we believe we can estimate the following parameters: target-binding cells (using specific targets), the fraction of target-binding cells that actually goes on to killing the target, as well as the V_{max} in a given heterogenous effector population. With these three experimental

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[1] *Abbreviations used in this paper*: FCS, fetal calf serum; K_m, Michaelis constant; MIC, monolayer immune complex; MRC, maximum recycling capacity; NK, natural killer; NWP, nylon wool-column-passed; PBS, phosphate-buffered saline; TBC, target-binding cells; % TBC, the percentage of TBC; V_{max}, maximum NK capacity.

values the actual percentage of active killer cells can be estimated as well as their recycling capacity, i.e., the number of killed target cells by a single NK cell.

Our results demonstrate that certain human T cell lines are the most appropriate targets for NK measurements and that no correlation exists between the number of target-binding cells and the cytotoxic strength when these parameters are analyzed on an individual basis. Furthermore, it is clear that recycling of effector cells undoubtedly occurs and can be measured, and that the kinetics of lysis of different T cell lines vary considerably. In addition, it may be relevant to consider that the concept of "natural" killer cells is becoming more and more heterogeneous. A number of systems have been described using different target cells such as erythrocytes (8), solid tumors (9), normal cells (3, 10) and different hematopoietic malignancies (1). In the present context we define an NK cell as originally described, i.e. a cell with lymphocyte morphology, expressing a characteristic surface phenotype, that can kill certain hematopoietic tumor cell lines in a short-term cytotoxicity assay (11, 12).

Materials and Methods

Purification and Fractionation of Lymphocytes. Peripheral blood lymphocytes were obtained from normal healthy donors. Lymphocytes were collected by density centrifugation on Ficoll-Hypaque (13) and washed three times in phosphate-buffered saline (PBS). The cells were then resuspended in RPMI-1640 that contained 15% fetal calf serum (FCS). Adherent cells were removed by passage through a nylon wool column after 30 min with preincubation at 37°C in the column (NWP cells) (14). The cells obtained are referred to as effector cells. The cells used were collected on the same day as the experiment.

Enrichment of Cells with Fc Receptors. Lymphocytes were fractionated according to Fc (IgG) receptor expression using the earlier described monolayer immune-complex (MIC) technique (15). Briefly, plastic Petri dishes were coated with human IgG (0.5 mg/ml) and washed. Thereafter, a predetermined concentration of rabbit anti-IgG was added for an incubation period of 1 h. After washing of the plates, lymphocytes were added and gently spun down. Fc receptor-positive cells were firmly bound to the plastic surface and could be eluted by an additional incubation with protein A (50 μg/ml), requiring rocking for 3 h at 37°. Additional details are available elsewhere (15).

Cell Lines. Target cell lines were maintained in tissue culture in RPMI-1640 that contained 15% FCS.

Labeling of Target Cells. 50 μCi of $Na[^{51}Cr]_2O_7$ was added to ~15×10^6 target cells in 0.3 ml. The cells were then incubated at 37°C for 1 h, washed, and then incubated for an additional 20 min to lower the spontaneous release. After the last incubation, the cells were washed twice and diluted to appropriate concentration in RPMI-1640 with 15% FCS.

^{51}Cr-release Assay. Cytotoxic assays were done with duplicate wells in V-shaped microplates (Linbro Chemical Co., Hamden, Conn.) using a total vol of 0.15 ml in each well. A dilution series of target cells with six different concentrations (diluted 1:2 or 2:3) was used with the highest target cell concentration at 4×10^5. 10^6 effector lymphocytes were added to each well. Before starting the cytotoxic assay, plates were shaken on a microshaker for 20 sec to ascertain effector and target cell contact. The cytotoxicity assay was run for 3 h at 37°C. 0.05 ml of the supernate from each well was harvested and counted in a Intertechnique gamma counter (Intertechnique, France). The percentage of killed target cells was calculated as:

$$100 \times \frac{\text{test release} - \text{spontaneous release}}{\text{80\% of total label} - \text{spontaneous release}}.$$

The number of target cells killed was determined by multiplying the percentage of killed cells in a well with the initial number of target cells in the same well.

Calculation of V_{max}. The resulting dose-response curves from the ^{51}Cr release assays resembles the Michaelis-Menten kinetics. This is expressed:

$$V = \frac{V_{max} \times T}{K_m + T},$$

where V is the number of killed target cells, T is the initial number of target cells, V_{max} is the number of target cells killed when $T \rightarrow \infty$, i.e., when the system is saturated with target cells. K_m is the number of target cells that produces one-half of V_{max}. V_{max} and K_m for different effector:target cell combinations were determined by using the Lineweaver-Burk equation:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{T} + \frac{1}{V_{max}}$$

In this equation there is a linear relationship between 1/V and 1/T. The reciprocal values of V and T were plotted and regression analysis was used (method of least squares) to obtain a straight line. V_{max} is obtained from the reciprocal of the Y intercept and K_m from the negative reciprocal of the X intercept. In Table I and Fig. 1, one experiment is given to exemplify calculation on V_{max} and K_m values as described in text above. To examine if this procedure can be simplified we correlated the obtained V_{max} values (from 19 experiments with 5 different target cells) with the corresponding values from an ordinary 5:1 ^{51}Cr test (Fig. 2). We found a correlation coefficient of 0.93. Thus it is possible to get a rough estimation of the V_{max} for 10^5 effector cells by using a 5:1 (5×10^4 lymphocytes and 10^4 target cells in triplicates) ^{51}Cr test and then using the following formula:

$$V_{max} = 1.4 \times 10^3 + 4.2 \times 10^2 \times (\text{percentage } ^{51}Cr \text{ release in 5:1}).$$

This procedure is not used here but is presented as an alternative to obtain a rough V_{max} estimation.

Conjugate Cytotoxicity Assay in Agarose. The assay was performed as described by Grimm and Bonavida (6) with minor modifications. In summary, the following procedure was followed: A 0.5% agarose solution in RPMI-1640 (with 10 mM Hepes) was prepared by heating and kept in a waterbath, at 48°C. Equal number of effector and target cells (2×10^5 cells) were mixed in a total vol of 0.2 ml of RPMI-1640 with 15% FCS in a 2.5 ml Ellerman tube. The cells were spun at 500 *g* for 2 min and incubated for 10 min at 37°C. During this incubation some of the lymphocytes bound target cells and formed conjugates. Subsequently, the pellet was resuspended by firm shaking for 10 sec on a whirlmixer. The cells were then added to 0.5 ml of the agarose solution which had been precooled to 39°C by holding the pipetted agarose for 20 sec at room temperature. The cells were then resuspended in the agarose solution with a Pasture pipette and dripped from the height of 30 cm onto plastic 60-mm Petri dishes (A/S Nunc, Copenhagen, Denmark). Thus, a thin layer of agarose that contained the cells was formed in the bottom of the dishes. After the agarose had solidified, 6 ml of RPMI-1640 with 15% FCS was added in each dish. Control dishes with the target cells alone were formed in a similar manner. Dishes were subsequently incubated at 37°C under tissue culture conditions. At appropriate time points (0, 1, 2, and 3 h) test and control plates were removed from the incubator. The medium was removed and 2 ml of trypan blue was added for 10 min. The

TABLE I

A Representative Experiment Showing Data Used for Calculations of V_{max} and K_m

Dilution number	Number of effector cells	Number of target cells (T)	Percentage killed target cells	Number of killed target cells (V)	1/T = X	1/V = Y
1	1×10^5	4×10^5	5.4	2.16×10^4	2.5×10^{-6}	4.63×10^{-5}
2	1×10^5	2×10^5	9.3	1.86 ± 10^4	5×10^{-6}	5.38×10^{-5}
3	1×10^5	1×10^5	13.2	1.32 ± 10^4	1×10^{-5}	7.58×10^{-5}
4	1×10^5	5×10^4	18.4	9.30×10^3	2×10^{-5}	1.09×10^{-4}
5	1×10^5	2.5×10^4	26.9	6.73×10^3	4×10^{-5}	1.49×10^{-4}
6	1×10^5	1.25×10^4	29.2	3.65×10^3	8×10^{-5}	2.74×10^{-4}

Regression analysis gives: $Y = a + bX$; $r = 0.997$; $a = 4.25 \times 10^{-5}$ $b = 2.87$; $V_{max} = 1/a = 2.35 \times 10^4$; $K_m = b/a = 6.75 \times 10^4$.

Data given from a typical ^{51}Cr-release assay performed as described in Materials and Methods for calculations of V_{max} and K_m. For further explanations see Materials and Methods.

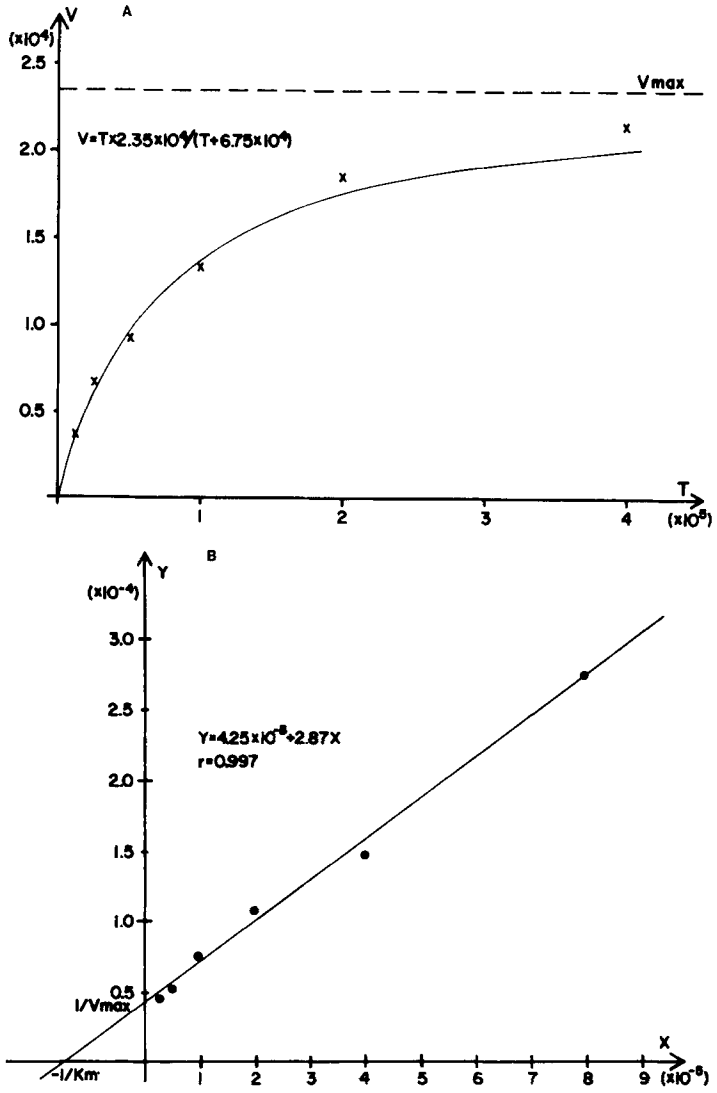

FIG. 1. (A) Data from Table I are used to exemplify the calculation of V_{max}. Initial number of target cells (T) is plotted against number of dead target cells (V) at the end of the test. The points fit well to the theoretical Michaelis-Menten saturation curve. For further explanation see text in Materials and Methods section. (B) A Lineweaver-Burk plot is used to calculate V_{max} and K_m. 1/T = X plotted against 1/V = Y. Data are taken from Table I and Fig. 1. Regression analysis is used to obtain a straight line ($r = 0.997$). V_{max} equals the reciprocal of the Y intercept and Km equals the negative reciprocal of the X intercept. For more detailed information see Materials and Methods.

dishes were washed three times for 5 min with cold PBS and finally fixed with 1% formic aldehyde. This procedure removed all the trypan blue except that in the lysed cells which were stained clearly blue. The dishes could be kept for later scoring. The percentage of conjugates was determined by counting the number of lymphocytes bound:target cells in 400 counted lymphocytes. This will be referred to as the percentage of target-binding cells (% TBC). The percentage of dead conjugates was determined by counting the number of dead target cells in 100 conjugates. The background death is determined by counting the percentage of dead cells in the control dishes at corresponding time points. Corrections are made by using the formula:

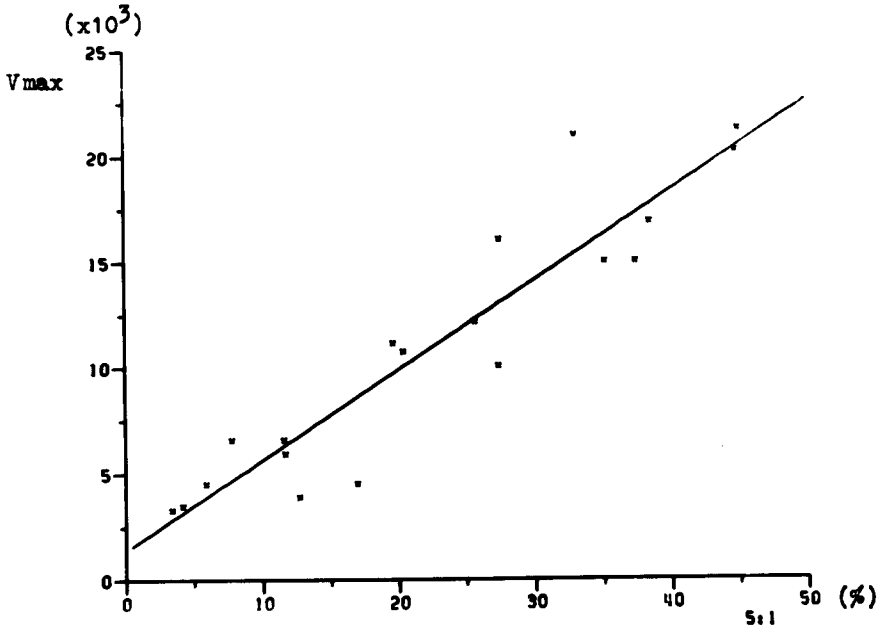

FIG. 2. Values from the standard 5:1 ^{51}Cr test (percentage of lysis) are plotted against the corresponding V_{max} values. This shows a good correlation between the two parameters ($r = 0.93$). See Materials and Methods for further discussion.

(percentage of dead target cells in conjugates) − (percentage of spontaneously dead target cells) × (percentage of dead target cells in conjugates).

Estimation of the Percentage of Active NK Cells and Maximal Recycling Capacity (MRC). The percentage of active NK cells was estimated from the agarose conjugate assay by multiplying the % TBC with the percentage of conjugates with dead target cells at the end of the assay. MRC was calculated by combining data from the ^{51}Cr-release assay and the conjugate assay. Thus, the V_{max} value was divided by the absolute number of killer cells, i.e., percentage of active NK cells multiplied with the number of effectors in the V_{max} (10^5 cells). MRC is an estimation of the average number of target cells that an active NK cell can kill in 3 h under optimal conditions.

Conjugate Assay in Microdroplets. Conjugates were formed as described above. Additionally, these were enriched by 1-*g* velocity sedimentation through fetal calf serum. The enriched conjugates were placed, in 2 μl-microdroplets, onto the inside of a plastic microplate cover using a Hamilton syringe. On the other side of the cover, a coded pattern had been drawn allowing ~150 droplets to be placed on each cover. Immediately after the drops had been placed they were overlayered with paraffin and scored for single conjugates. Only drops with a single conjugate were coded, these were consequently scored for cytotoxicity at different time points: 0, 1, 2, and 3 h. A cytotoxic event could most oftenly be seen by changes in the morphology of the target cells, if not, 0.5 μl of trypan blue could be injected into single microdroplets.

Results

The Target-binding Assay. Lymphocytes were fractionated by passage through nylon wool, which removes adherent cells, and by Fc receptor expression with the MIC technique. Roder et al. (17) have earlier shown that nylon wool passage is necessary to remove cells that bind unspecifically through their general adherent properties. The further fractionation of cells into Fc (IgG) positive or negative was of interest as

most of NK cells in the human system display receptors for Fc(IgG). Thus, one would expect that most target-binding cells (TBC) also have Fc receptors if the TBC interaction is of a specific nature.

Two different T cell lines (Molt-4 and 1301) were tested as well as the K-562 line derived from chronic myelocytic leukemia. These lines have earlier been shown to be highly susceptible to NK lysis (11). As controls, two additional lines were included; Raji, a B cell line of Burkitt's lymphoma origin, and P-815, a NK-resistant line of mouse origin.

The TBC assay was performed in two different ways, either with vigorous shaking, as described in the methods section, or with gentle resuspension. This was done to determine the strength of the cellular interaction in the TBC reaction and to detect possible low-affinity binders. From Table II it can be seen that the TBC assay, as presently performed, detects approximately the same number of TBC with all human lines, although possibly a slightly lower number against the K-562 cell line. With gentle resuspension, a somewhat higher number of cells are detected against the T lines and the K-562 line. With the B line a large increase can be seen, reflecting a low-affinity receptor present between B and T cells as earlier described (18). Subfractionation into Fc-positive and Fc-negative populations showed a substantial increase of strong binders in the Fc positive fraction, and were seemingly somewhat higher with the T cell line than with the K-562 cell line. With K-562 there is also some binding in the Fc-receptor negative cell fraction.

The data given in Table II does thus show the following: (*a*) a subpopulation of NWP lymphocytes had high-affinity receptors for all tested human lines, the size of which is equally large against the differnt cell lines; (*b*) low-affinity receptors could also be detected against all lines but only showed a specific increase when NWP, Fc-receptor positive and -negative were compared, cells and when tested with a human B cell line; (*c*) the highest expression of Fc receptors was found among TBC binding

TABLE II

Capacity of NWP, Fc Receptor-positive, and Fc Receptor-negative Peripheral Lymphocytes to Bind to Cell Lines of Different Origin

Cell line	Origin	Susceptibility to spontaneous cytotoxicity	TBC	Bound lymphocytes in cell fraction		
				NWP	Fc positive	Fc negative
					%	
Molt-4	T cell line from ALL	High	Strong	9.3	38.1	0.3
			Weak	12.8	43.9	3.1
1301	T cell line from ALL	High	Strong	8.5	34.4	0.9
			Weak	10.1	36.2	2.9
K-562	Myeloid cell line from CML	High	Strong	7.4	21.2	2.2
			Weak	9.2	25.2	4.7
Raji	B cell line from Burkitt's lymphoma	Low	Strong	8.9	21.4	3.2
			Weak	63.4	38.2	71.8
P-815	Mastocytoma cell line from mouse	Low	Strong	0.3	2.1	0.1
			Weak	1.3	4.2	0.3

A representative experiment is given showing the percentage of TBC of three different lymphocyte subpopulations with five different cell lines. Lymphocytes were fractionated as described in the Materials and Methods section. Strong/weak binders indicate either resuspension of TBC with minimal force or with vigorous mixing.

to T cell lines. As Fc receptor expression is a property of most fresh human peripheral NK cells, it seem that the TBC assay is preferably done with T cell lines; additional results showing a larger fraction of active killers against these targets than against K-562 and susceptible B targets (data shown below) reinforces this impression.

Active NK Cells Among TBC to Different Target Cells. Even if it could be shown that a subpopulation of NWP lymphocytes did bind to NK susceptible target cells and the overwhelming majority of these had Fc receptors, this would not validate the TBC assay as a test for NK cells. It also had to be shown that the cells that did bind also went on to kill the targets. For this purpose, single conjugates, i.e., one lymphocyte binding to one target cell, were immobilized in agarose and tested for target cell death at different time points. Fig. 3 demonstrates that ~50% of the T cell conjugates contained active NK cells and that killing proceeded with different speed against different target cels. HD-Mar, a T cell line from Hodgkin's disease, was killed more rapidly than the standard target Molt-4; 1301 showed a comparatively slower rate of lysis. With the K-562 cell line, a much lower fraction of conjugating lymphocytes went on to killing, the lytic kinetics resembled that of the more-susceptible T cell lines.

To verify that the agarose technique did not functionally alter the ability of the conjugating lymphocytes to act as killer cells, an alternative conjugate-killing assay was employed. Isolated conjugates were thus studied in microdroplets as described in the Methods section. Fig. 4 demonstrates that also when using this technique, roughly 50% of conjugating cells went on to killing, also it was noted that a smaller fraction of the TBC release their targets before the lytic event and that in some instances no lysis occurred after effector cell detachment.

Estimation of Absolute Number of NK Cells and their Maximal Recycling Capacity (MRC). Table III gives a summary of different experiments using Molt-4, 1301 and K-562 as target cells. V_{max} estimations show that Molt-4 is the most susceptible target,

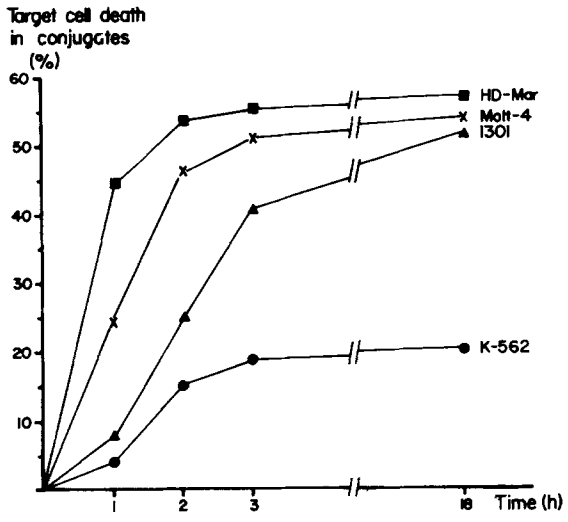

FIG. 3. Kinetics of cytotoxicity in agarose-immobilized conjugates with three different T cell lines and with the K-562 cell line. The single-cell cytotoxicity assay was performed as described in Materials and Methods.

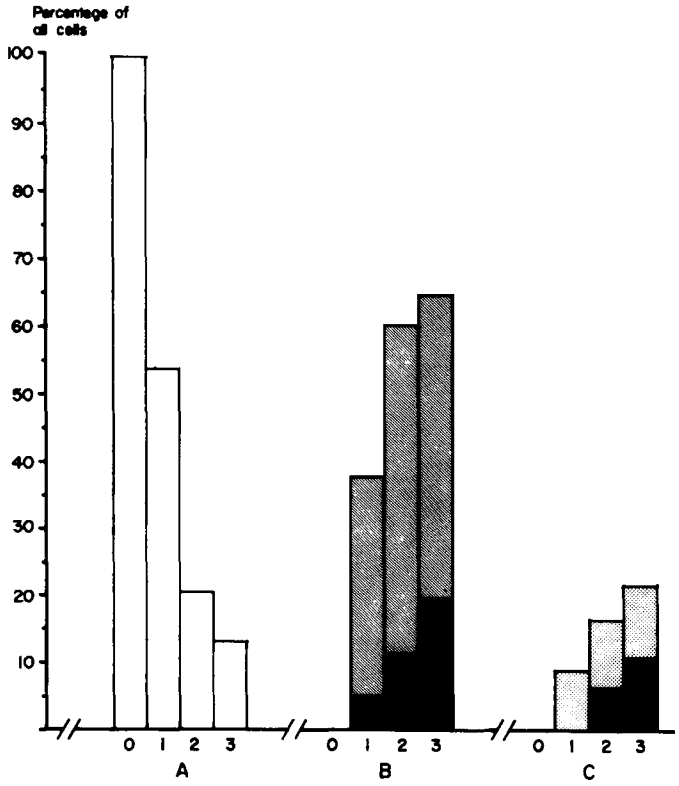

FIG. 4. Kinetics of cytotoxicity in conjugates isolated in microdroplets as described in Materials and Methods. (□) viable conjugates (with the target cell line Molt-4); (▩) dead conjugated target cells; (■) control target cell death. (▨) Target cell death among those targets that have detached from effector cells before lysis has occurred. (A) viable conjugates at 0, 1, 2, and 3 h; (B) target cell death in conjugates (control cell death given in the shaded part); and (C) target cell death among detached targets.

followed by K-562 and 1301, respectively. As also seen in Fig. 3, a much smaller fraction of K-562 binders went on to killing compared to those cells that bound to Molt-4 or 1301. This leads to a lower estimate of active NK cells with the K-562 cell line as compared with the other lines. However, as the ^{51}Cr release with K-562 was rather high, the estimated MRC was highest with this cell line. The estimated numbers of active NK cells indicate small subpopulations in the peripheral blood and a fairly high MRC of these, between 1.4 and 3.8 in the native, unstimulated state.

For comparative reasons, an alternative approach was also taken to estimate the number of absolute killer cells and their MRC. Cytochalasin B was added 15 min after the cytotoxicity test was started to prevent further target binding and lytic events (19). From preliminary experiments, it was clear that most of the lymphocyte-dependent steps already had occurred at this time point for the initial round of killing to occur; this was in agreement with findings with immune T cell killing or antibody-dependent killing. In Table IV, as explained in the legend, the estimated number of active killer cells equaled the cytotoxicity occurring after cytochalasin B inhibition and estimated recycling equaled the mean killing in media divided by mean killing with cytochalasin B inhibition. These values show a fairly good correlation with

TABLE III

V_{max}, TBC, Fraction of Dead Conjugates, and Estimation of Active NK and Their Maximal Recycling Capacity with the Molt-4, 1301, and K-562 Cell Lines

Experiment	Target cell	V_{max}	TBC	Fraction of dead conjugates	Estimated number of active NK cells	Estimated MRC
		$\times 10^3$	%	%	%	
1	Molt-4	14.9	5.5	55	3.0	4.9
2	Molt-4	10.6	6.5	48	3.1	3.4
3	Molt-4	11.1	11.1	52	5.7	1.9
4	Molt-4	5.7	6.0	44	2.6	2.2
5	Molt-4	8.0	6.8	65	4.4	1.8
6	Molt-4	6.9	5.1	47	2.4	2.9
Mean		9.5	6.8	52	3.5	2.8
1	1301	2.1	8.3	32	2.7	0.8
2	1301	2.6	7.7	38	2.9	0.9
3	1301	4.3	6.0	45	2.7	1.8
4	1301	5.8	7.5	34	2.6	2.3
Mean		3.8	7.4	37	2.7	1.4
1	K-562	3.9	10.3	20	2.1	1.9
2	K-562	1.4	7.8	11	0.9	1.6
3	K-562	4.0	7.2	17	1.2	3.3
4	K-562	10.7	6.8	23	1.6	6.8
5	K-562	4.7	7.9	19	1.5	3.1
6	K-562	12.1	8.1	24	1.9	6.2
Mean		6.1	8.0	19	1.5	3.8

The combined data from six different experiments are shown. For explanation of estimations of the different parameters see Materials and Methods.

corresponding values in Table III. The estimated recycling capacity is somewhat lower with cytochalasin B inhibition than with the agarose-V_{max} technique, a circumstance that may be related to the fact that recycling calculated by V_{max} determinations are given as maximal recycling capacity—as the V_{max} value is a theoretical value based on a fully saturated system—in contrast to the cytochalasin B inhibition experiments in which the absolute cytotoxicity values, at an effector:target ratio of 1:1, are used. The higher number of TBC and absolute NK values in Table V may be related to chance fluctuations and some leaky cytotoxicity occurring in spite of cytochalasin B inhibition. An estimation has also been done, in Table IV, of the fraction of TBC that actually go on to killing. The mean value is very similar to the corresponding value in Table III with only slight variation with most values and with only two extreme values (donor 6: 0.13 and donor 11: 0.91) most probably reflecting true biological variations.

Relationship between V_{max} K_m, % TBC and the Percentage of Dead Conjugates. In Table V, the values for correlaion between V_{max}, K_m, % TBC, and the percentage of dead conjugates are given for Molt-4 and K-562, respectively. Neither with Molt-4 nor K-562 could we find any correlation between V_{max} and % TBC. Thus, counting the

TABLE IV

Estimation of NK Cell Frequency and Recycling Capacity by Inhibition of Later-occurring Cytotoxicity with Cytochalasin B

Experiment	Donor	Natural cytotoxicity ratio 1:1 in			TBC	Cyt B (+15 min) / TBC
		Media	Cyt B* (−15 min)	Cyt B (+15 min)		
		% lysis				
1	1	12.0	0.5	6.8	11.0	0.62
	2	5.6	1.6	4.4	10.3	0.43
2	3	13.7	−0.5	3.4	11.7	0.29
	4	8.8	0.5	3.8	8.2	0.46
3	5	12.3	−0.7	5.8	12.4	0.47
	6	9.3	−2.8	1.3	10.1	0.13
4	7	12.9	0.8	3.3	8.0	0.41
	8	16.4	0.4	5.2	10.7	0.49
5	9	16.7	0.3	8.1	12.7	0.64
	10	15.9	0.9	8.3	13.3	0.62
6	11	17.8	−0.9	7.0	7.7	0.91
	12	8.3	0.3	6.5	15.8	0.41
7	13	21.7	−0.3	12.9	21.0	0.61
	14	25.2	−1.9	9.7	19.3	0.50
	Mean values	14.0	−0.1	6.2	12.3	0.50

Estimated mean number of killer cells: 6.2%. Estimated mean recycling index: $\frac{14.0}{6.2} = 2.3$.

The combined data from seven separate experiments with Molt-4 as target cell are shown. Cytochalasin B added 15 min before the target cells (−15) totally blocked cytotoxicity. By adding cytochalasin B after 15 minutes of effector:target contact (+15), only one round of killing is allowed to proceed, thus corresponding to immobilizing effector cells in agarose. From the data, the number of active killer cells and their apparent recycling capacity can be estimated as shown in the Table.

* Cyt B, cytochalasin B.

TABLE V

Correlation Matrix for Molt-4 and K-562

	Correlation matrix for Molt-4				Correlation matrix for K-562			
	V_{max}	K_m	% TBC	Percentage of dead conjugates	V_{max}	K_m	% TBC	Percentage of dead conjugates
V_{max}	1.0				1.00			
K_m	−0.26	1.00			0.45	1.00		
% TBC	0.19	−0.23	1.00		−0.32	−0.20	1.00	
Percentage of dead conjugates	0.28	0.11	0.04	1.00	0.87	0.53	0.02	1.00

Correlation matrix for Molt-4 and K-562. For each cell type V_{max}, K_m, % TBC, and the percentage of dead conjugates are correlated in all possible combinations. Correlation coefficients are based on the data in Table III (individual K_m values not presented in Table III). Note the lack of correlation between V_{max} and % TBC.

percentage of binding lymphocytes does not give a proper estimation of the overall activity. The K_m did not seem to be related to any of the other three variables either with Molt-4 nor K-562. In the enzyme model, K_m reflects the affinity between the

TABLE VI

TBC Are Not Recruited during the 3-h Test Period

Experiment	Target cell	TBC before fractionation	TBC after fractionation		TBC after 3-h incubation	
			Upper fraction	Lower fraction	Upper fraction	Lower fraction
1	Molt-4	4.0	1.0	7.0	1.9	6.0
	K-562	4.2	1.8	8.6	1.5	7.3
2	Molt-4	4.6	1.4	6.4	0.8	4.8
	K-562	3.3	1.0	5.3	0.4	5.5

Two separate experiments are shown in which TBC were fractionated by 1-*g* velocity sedimentation on undiluted FCS and thereafter tested for target binding capacity at time 0 and 3 h. It can be seen that no recruitment of binders occur from the TBC-negative fraction and that the positive fraction stays approximately the same during the 3-h test period.

enzyme and the substrate. In our experiments, K_m was a little lower for K-562 (average 1.3×10^4) than for the T cell line (average: 3.2×10^4). This might reflect a difference in affinity between effector and target cells but can also be due to the fact that fewer of the lymphocytes that bind K-562 kill their target cells than those that bind T cell lines. Finally, the correlation between the percentage of dead conjugates and V_{max} was high for K-562, but low for Molt-4. This will be further discussed elsewhere.[2]

Discussion

This work demonstrates that ~10% of NWP peripheral lymphocytes have the ability to bind to NK-susceptible cell lines. Of these, some 50% will normally go on to killing T cell targets and a smaller fraction to kill the myeloid cell line K-562, commonly used as a NK target. Most of conjugating cells also expressed receptors for IgG, a marker for human NK cells. Consequently, the TBC assay in agarose can be used with human lymphocytes to identify NK cells and to quantitate their number. In fact, the TBC assay is probably, at present, the only assay which positively identifies NK cells on the single-cell level, the definition being an effector cell attached to a dead target cell after 3 h of incubation in agarose.

When the conjugate-killing assay is combined with a ^{51}Cr-release assay it is possible to calculate the number of killed target cells by a single effector cell, i.e., the recycling capacity. Given target cell saturation, corresponding to our V_{max} calculations, a maximal recycling capacity can be estimated. This estimation is, however, based on the assumption that the number and mode of action of the effector cells stays constant during the 3-h test procedure. This may be a grossly acceptable assumption as the testing time is short and as we have shown that no recruitment of binders, from nonbinders, occurs and that consequently the size of the TBC fractions stays mostly the same when depleted and enriched populations are compared at the beginning and at the end of the test (Table VI). However, the estimated recycling values do represent estimates of whole populations of cells and thus are mean values for cells that are probably heterogeneous in themselves with regard to their mode of action.

[2] Ullberg, M., and M. Jondal. Effect of interferon on human spontaneous killer activity against tumor cell lines of different origin. An analysis of absolute number, recycling capacity and interferon enhancement of effector cell recruitment and effector cell recycling. Manuscript submitted for publication.

A question of interest concerns the remaining TBC which do not go on to killing the target cells. Are they nonactive NK cells or do they belong to a non-NK population of cells? As in some instances a very high % TBC did kill (Table V). These normally non active cells may have the capacity to kill if they are given the right stimulus. We later tested the effect of the NK booster interferon on the presently studied parameters, binding, speed of killing, fraction of active killers among binders, and recycling capacity.[2] These investigations do not indicate a recruitment of killer cells active against T cell targets, but, rather an increased speed of killing, detectable against suboptimally susceptible targets with which the lymphocyte-independent lytic phase is not the rate-limiting factor, and an increased recycling capacity of the effector cells resulting in an increased overall V_{max}. Thus, interferon did not stimulate the remaining nonactive 50% of binders to kill T targets. The additional positive boosting signals for NK activity remain to be defined to conclusively answer the question of the nature of the normally nonactive TBC.

The methodology described here has been applied to a small clinical material, patients with acute multiple sclerosis in different stages of their disease, i.e., active, stable, or chronic.[3] From earlier data it was known that some of these patients are deficient with regard to NK activity (20, 21). Our preliminary results indicate that patients in the acute phase are deficient both with regard to number of TBC as well as to the speed of killing and to the recycling capacity. In contrast, stable patients have a normal number of binders which seem to kill with a normal speed but which are deficient in their recycling capacity.[3] Taken as an example for a clinical investigation, these results would indicate the need for additional work with regard to factors known to affect NK activity such as autoantibodies, immune complexes, or prostaglandins and their possible roles in the pathophysiology of this particular disease.

The specific TBC assay, as well as the ability to dissect discrete steps in the cytolytic process will also be valuable in further work on the basics of the human NK system. In our earlier work, the TBC assay has already been used in an effort to study the receptor-binding substance present on the target cells (22). When positively characterized cell lines with NK activity are established, as already preliminarily described by some groups (23, 24), a similar approach may be taken to study the antigen-binding receptor present on the effector cells. Using agarose immobilization and thereby momentarily freezing the effector-target interaction should facilitate such studies.

Summary

By combining a newly established single-cell cytotoxicity assay in agarose (16) with estimations of the maximum natural killer (NK) potential (V_{max}) by ^{51}Cr release the percentage of target-binding cells (TBC), the fraction of active killers among TBC, the kinetics of single-cell cytotoxicity, and the recycling of effector cells was studied. Using nylon wool-passed peripheral lymphocytes, ~10% of the cells will bind to NK-susceptible target cell lines. Most of these have receptors for IgG. Some 50% will go on to kill T cell targets and some 20% to kill the standard target cell K-562. As the individual NK cell is shown to have the capacity to recycle, i.e., to kill more than one

[3] Merill, J. M. Jondal, J. Seeley, M. Ullberg, and A. Siden. Deficient NK activity in patients with multiple sclerosis: an analysis of absolute number, recycling capacity and interferon enhancement in different disease stages. Manuscript submitted for publication.

target cell in the 3-h test period, and as recycling seems to vary between individuals, there is no consistent correlation between the number of TBC and ^{51}Cr-release values.

It seems as if the single-cell cytotoxicity assay, as presently performed in agarose, is a valuable complement to V_{max} determinations by ^{51}Cr-release to study the different steps involved in the cytolytic process: recognition, enzyme activation, and effector cell recycling. The discrimination between these steps will probably be necessary to define mechanisms influencing NK cells in different disease states as well as in learning more about the normal function and regulation of the human NK system.

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