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Human natural killer cells: a convenient purification procedure and the influence of cryopreservation on cytotoxic activity

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The recognition of natural killer cells as a lymphoid subpopulation with a distinct set of surface markers has led to the development of a variety of antibody-based purification methods. In this paper we describe a rapid, three-step negative selection protocol for the purification of human natural killer (NK) cells from the mononuclear cell fraction, which is obtained by the centrifugation of peripheral blood on Ficoll-Paque. Subsequently, monocytes and B lymphocytes are removed by adherence to nylon wool and T lymphocytes by panning with anti-CD3. With this procedure, $CD3^-$, $CD16/56^+$ NK cells are purified about five-fold, from $12 \pm 3\%$ in the starting population to a final purity of $61 \pm 11\%$. A further increase to $\geqq 70\%$ is obtained, if an extra Ficoll centrifugation step is included. The recovery of NK cells (50%) is significantly higher than is usually achieved by previously described procedures. Furthermore, we show that activation of cytotoxicity, with concomitant changes in target specificity, occurs when frozen/thawed NK effector cells are kept in culture in order to regain their pre-freezing cytotoxicity levels.

Key words: Natural killer cell; Panning; LAK cell; Cryopreservation

Introduction

Natural killer (NK) cells have long been defined operationally, by their ability to mediate spontaneous cytotoxicity against various transformed and virus-infected cells, without restriction by the major histocompatibility complex (Ortaldo et al., 1977; Herberman, 1986; Trinchieri, 1989). During the last 10 years NK cells have gradually been recognized as a distinct lymphoid subpopulation, initially by their large granular morphology (Saksela et al., 1979) and, more recently, by a $CD3^-$, $CD16^+$ and/or $CD56^+$ cell surface phenotype (Lanier et al., 1986; Ritz et al., 1988; Robertson and Ritz, 1990). These studies have shown that human NK cells comprise 10–15% of the peripheral blood lymphocytes (PBL).

Two different approaches are generally used for the purification of NK cells: (i) fractionation by Percoll density gradient centrifugation (Timonen et al., 1982), (ii) antibody-mediated positive or negative selection (Cosentino and Cathcart, 1987; Pflueger et al., 1990; Naume et al.

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Abbreviations: $E:T$, effector:target; NA, non-adherent; NK, natural killer; PBMC, peripheral blood mononuclear cells; PBL, peripheral blood lymphocytes; SDS, sodium dodecyl sulphate.

1991). In most cases these techniques are applied after first removing monocytes and B cells by adherence steps (Julius et al., 1973; Shah and Dickson, 1974; Mosier, 1984).

Comparison between experiments carried out with different NK cell preparations is complicated, because NK cytotoxicity of peripheral blood lymphocytes can differ significantly from donor to donor (Pross and Maroun, 1984; Trinchieri, 1989). Therefore, a method to isolate and to preserve pooled NK cells for later experiments would be helpful, because it would permit quantitative comparison of NK cytotoxicity between different experiments. Cryopreservation of functional human NK cells has been described by several authors, but again relatively impure NK cell preparations were used (Pross and Maroun, 1984; Gratama et al., 1985; Fujiwara et al., 1986).

In this paper, we describe purification by the stepwise elimination of unwanted cells. Monocytes and B cells are removed by adherence and T cells by panning (Wysocki and Sato, 1978). After these negative selection steps the final population contains on average 61% NK cells ($CD3^-$, $CD16/56^+$). Non-mononuclear cells, which constituted a major contamination in the purified fraction, could be easily removed by centrifugation on Ficoll.

In addition, we have explored the cryopreservation of purified NK cells and investigated the implications of freezing/thawing for the regeneration of NK activity (lysis of K562 cells) and the development of LAK activity (lysis of NK-resistant Daudi cells).

Materials and methods

Cell culture

Cell culture media and additives, including heat-inactivated fetal calf serum (FCS), were purchased from Life Technologies (Gaithersburg MD, USA). The human erythromyeloid leukemia cell line K562 (ICN-Flow, Irvine, Scotland) was kept in continuous culture in RPMI 1640, supplemented with 10% FCS and 2 mM L-glutamine (culture medium). The Burkitt's lymphoma cell line Daudi (a gift of Dr. W.B.M. De Lau, Department of Immunology, University Hospital Utrecht) was cultured in culture medium supplemented with 25 mM Hepes buffer. Cells were free of mycoplasma contamination.

The isolation and purification of human NK cells

Step A – Preparation of peripheral blood lymphocytes (PBL) Human natural killer cells were isolated from peripheral blood according to a negative selection protocol (Fig. 1), based on the method described by Cosentino and Cathcart (1987). Peripheral blood mononuclear cells were obtained by centrifugation of peripheral blood (Red Cross Bloodbank Utrecht) on Ficoll-Paque for 30 min at $400 \times g$ (density 1.077; Pharmacia LKB, Uppsala, Sweden), and collecting cells from the interphase as described (Bøyum, 1968). PBMC were washed three times in medium (RPMI 1640, supplemented with 25 mM Hepes buffer, 5% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin), resuspended at 1-1.5×10^7 per ml, and incubated for 1 h at 37°C in 5% CO_2 in cell culture flasks to remove plastic-

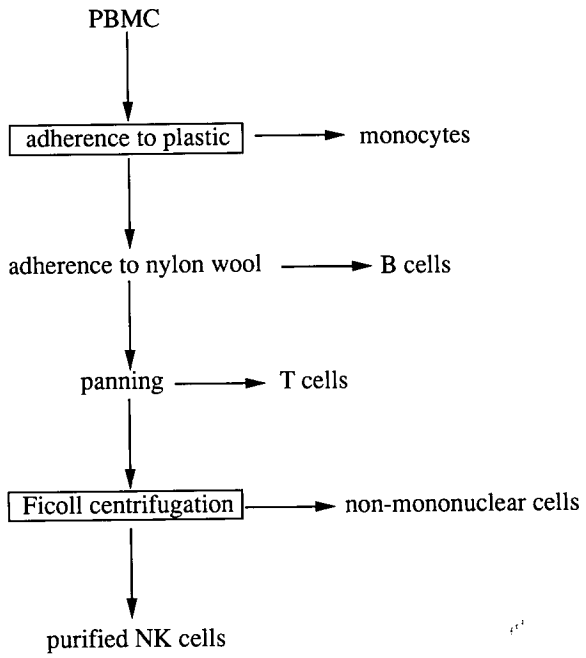

Fig. 1. Schematic representation of the negative selection protocol for NK cells. Vertical arrows depict the different steps in the procedure, horizontal arrows indicate the cell type removed at each stage. Optional steps (see text) are boxed.

adherent cells. Subsequently, peripheral blood lymphocytes (PBL), depleted of monocytes, were collected and pooled with two washes of 10 ml medium per flask.

Step B – Nylon wool filtration Nylon wool filtration of PBL was carried out essentially according to Julius et al. (1973). Scrubbed nylon wool (type 200L, Du Pont de Nemours, Wilmington, DE, USA) was washed in phosphate buffered saline (PBS: 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 8 g/l NaCl, and 0.2 g/l KCl, pH 7.2) for 2 h at 37°C, followed by extensive washing in distilled water at room temperature with five daily changes. Nylon wool columns were prepared by packing 0.6 g of dried nylon wool into 10 ml plastic syringes, and sterilized by autoclaving. Directly before use, columns were washed with medium and preincubated for 1 h at 37°C. After washing the column with fresh medium, $25–30 \times 10^7$ cells were applied in a 1–2 ml volume followed by an incubation for 45 min at 37°C. If nylon wool filtration was not preceded by plastic adherence, the procedure was slightly adapted. In that case, columns contained 0.75 g of nylon wool and incubation was performed for 75–90 min. Non-adherent (NA) cells were collected by washing the column with 25 ml of warm medium. The cells were washed once and used for panning.

Step C – Panning NA cells were pelleted and resuspended at 3×10^7 per 30 μl of a solution of the mouse monoclonal anti-CD3 antibody anti-Leu-4 (undiluted: 50 μg/ml, Becton Dickinson, Mountain View, CA, USA). After 45 min on ice with regular resuspension, cells were washed three times with PBS containing 5% FCS (PBS/FCS) and finally resuspended at 10^7 per ml in PBS/FCS.

Sterile bacteriological petri dishes (Ø 10 cm, Greiner, Alphen a/d Rijn, Netherlands) were coated with 10 ml of a 5 μg/ml solution of a goat anti-mouse IgG antibody (Fc-specific, M-2650, Sigma Chemical Co., St. Louis, MO, USA) in PBS for 8 h at room temperature. After coating, the dishes were washed three times with PBS/FCS and used directly or stored in PBS/FCS at 4°C.

Panning was performed by pouring 3 ml of the anti-Leu-4 labeled cell suspension onto each dish. Dishes were incubated for 45 min at room temperature, followed by redistribution of the cells by gentle swirling and a second incubation of 45 min. Subsequently, the supernatant was removed and combined with two washes of medium from the dishes. After washing in medium, these cells were used as the purified NK effector cells. In some cases the NK cells were subjected to another centrifugation on Ficoll-Paque for 20 min at $400 \times g$ to remove contaminating non-mononuclear cells (see results section). The viability of cells was checked throughout the procedure by trypan blue dye exclusion and found to be over 95%.

Flow cytometry

Two-color flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson). Cells were stained with a Simultest reagent (Becton Dickinson), consisting of the T cell specific monoclonal antibody (MoAb) anti-Leu-4 (anti-CD3) labeled with fluorescein and the NK cell-specific MoAbs anti-Leu-11c (anti-CD16) and anti-Leu-19 (anti-CD56), both labeled with phycoerythrin. Light scatter gates were selected manually, based on the forward and right angle scattering properties of the cell population. In this way, lymphocytes, monocytes, and a remaining population consisting mainly of erythrocytes and granulocytes could be discriminated (Jackson and Warner, 1986; Loken, 1986).

Lymphocytes were subdivided on the basis of their fluorescent staining properties. Cells with Leu-4^+,Leu-$11c/19^-$ phenotype were considered to be T cells, while NK cells were defined as Leu-4^-,Leu-$11c/19^+$ (Trinchieri, 1989; Robertson and Ritz, 1990). Lymphocytes which were not stained with the Simultest reagent were considered to be B cells. Staining thresholds were determined by the analysis of cells stained with isotype-matched control antibodies.

Storage of NK cells

Cryopreservation of NK effector cells was performed essentially according to Gratama et al. (1985). Generally, cells were washed, resuspended at $1–2 \times 10^7$/ml in culture medium and cooled on ice. An equal volume of ice-cold freezing medium (50% RPMI 1640, 30% FCS and 20% DMSO) was slowly added over a 5–10 min

period. Subsequently, the preparation was dispensed into freezing vials, transferred to a polystyrene box, and placed at −80°C. Frozen vials were stored at −80°C.

Vials to be thawed were placed in a 37°C water bath. Immediately after complete thawing, samples were diluted 1/10 by dropwise addition of medium at room temperature. After two additional washes with medium, cells were either directly used as effectors or resuspended at 2×10^6 cells per ml in medium with 20% FCS and cultured at 37°C in 5% CO_2 during a 'recovery period' (see results section). In some cases 100 U/ml of recombinant human IL-2 (Sanofi, Toulouse, France) was added during the recovery period.

Chromium release assay

Effector cells were tested for natural killer cytotoxicity against K562 cells and lymphokine-activated killer activity against NK-resistant Daudi cells in a chromium release assay. Target cells (10^6) were labeled with 3.7 MBq $Na_2^{51}CrO_4$ in sodium chloride solution (Amersham Int., Amersham, UK) for 1 h at 37°C. After removal of excess ^{51}Cr by two washes with medium, cells were preincubated in excess medium for 30 min at 37°C in 5% CO_2, washed once, resuspended at 10^5/ml, and used as targets. Effector cells and 10^4 ^{51}Cr labeled target cells were mixed at various effector: target cell ratios in 200 μl medium and plated in 96-well roundbottom microtiter plates. Prior to incubation, plates were centrifuged for 5 min at $100 \times g$ to facilitate cell-to-cell contact. After incubating for 4 h at 37°C in 5% CO_2, the plates were again centrifuged and 100 μl aliquots of the supernatants were collected and counted in a gamma counter. Specific cytotoxicity was calculated with the formula

$$\% \text{ specific lysis} = \frac{\text{cpm test} - \text{cpm spontaneous}}{\text{cpm maximal} - \text{cpm spontaneous}} \times 100\%$$

where cpm spontaneous and cpm maximal were determined in the presence of medium alone and 1% SDS, respectively. Spontaneous release was always below 10% of maximal release. Results of cytotoxicity assays are presented as typical examples from a series of at least three similar experiments. Individual experiments were always performed in triplicate.

For quantitative comparison of cytotoxicity levels, the cytotoxicity values at different effector: target cell ratios were converted to lytic units according to Bryant et al. (1992).

Results

The purification of human NK cells

A schematic representation of the sequence of negative selection steps, which was used to eliminate cell types, other than NK cells, from the starting peripheral blood mononuclear cell preparation, is depicted in Fig. 1. Several variations on this basic scheme, indicated as boxed areas, were applied (see below). This procedure resulted in a purified NK cell preparation, in which the proportion of NK cells was increased about five-fold, compared to the PBMC (Table I). This increase in NK cell content was also evident from the difference between the overall recovery of 10%, compared to a recovery of about 50% for the NK cells.

Analysis of the composition of the intermediate fractions (PBL, NA) revealed that the plastic

TABLE I

ANALYSIS OF THE NK CELL PURIFICATION METHOD

Analysis of the NK cell purification procedure (Fig. 1) at different stages. Recovery percentages ([a]) are expressed relative to the number of cells present in the Ficoll interphase fraction (mononuclear cells). The recovery of NK cells ([b]) and % NK cells ([c]) are derived from FACS analyses of the fractions as described. Values are given as means ± SD ($n = 5$). PBMC: peripheral blood mononuclear cells; PBL: peripheral blood lymphocytes (monocyte-depleted); NA cells: non-adherent cells (B cell depleted); NK cells: purified natural killer cells (T cell depleted).

Purification step	Resulting purification stage	% total cell recovery [a]	% NK cell recovery [b]	% NK cells [c]
Ficoll centrifugation	PBMC	100	100	12 ± 3
Plastic adherence	PBL	79 ± 17	93 ± 13	16 ± 5
Nylon wool filtration	NA cells	60 ± 14	86 ± 11	18 ± 8
Panning	NK cells	10 ± 2	50 ± 5	61 ± 11

adherence step contributed less to the removal of monocytes than the nylon wool filtration. In general, the numbers of monocytes decreased to 50% relative to the starting fraction (PBMC) after plastic adherence and to about 10% after nylon wool filtration. Parallel experiments were performed, in which part of the mononuclear cells went through both plastic and nylon wool adherence and another part only through the latter procedure. There were no significant differences between these procedures, with regard to either the extent of contamination with monocytes and B cells or the yield of NK cells. Therefore, the plastic adherence step was omitted in later experiments.

In the purified NK cell population the contaminating cells were $11 \pm 5\%$ T cells ($CD3^+$, $CD16/56^-$), $4 \pm 2\%$ B cells (unstained cells in the lymphocyte gate), and $3 \pm 2\%$ monocytes. In addition, relatively high numbers (10–25%) of other cells were present, which, apart from a few percent dead cells, apparently originated from the granulocyte/erythrocyte layer of the Ficoll gradient. The presence of erythrocytes was indicated by a reddish coloring of the cell pellet. An attempt was made to remove these cells by subjecting the purified NK cell fraction to another Ficoll centrifugation. Most of the cells with light scatter characteristics of non-mononuclear cells were readily removed by this extra step (Fig. 2). In three separate experiments, the extra Ficoll centrifugation decreased the percentage of cells in the non-mononuclear cell gate from $13 \pm 10\%$ to $1.5 \pm 1.2\%$. The NK cell content increased from $58 \pm 5\%$ before Ficoll centrifugation to $69 \pm 5\%$ afterwards. Recovery of NK cells during this step was always about 90%.

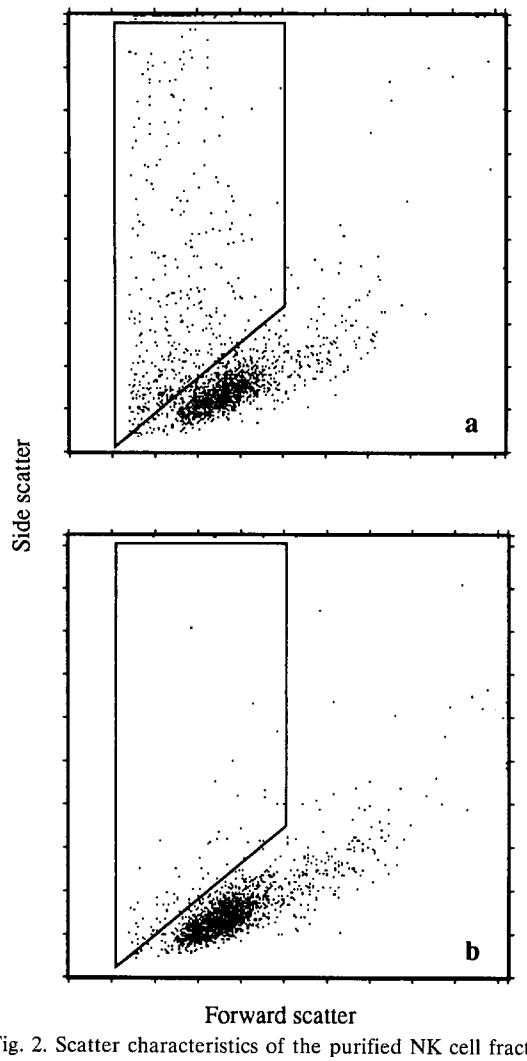

Fig. 2. Scatter characteristics of the purified NK cell fraction before/after Ficoll centrifugation. FACS analysis was performed on a purified NK cell preparation before (*a*) and after (*b*) Ficoll centrifugation. Granulocytes and erythrocytes are in the gate.

Cytotoxicity

The relative increase in the number of NK cells during the purification procedure was accompanied by a strongly enhanced cytotoxicity against the NK-sensitive cell line K562 (Fig. 3). The cytotoxicity of the purified NK cells was $53 \pm 11\%$ for six different preparations from different donors, at an effector : target ($E:T$) ratio of 10 : 1. This level of cytotoxicity is comparable to other reports for highly purified NK cell fractions (Cosentino and Cathcart, 1987; Naume et al., 1991). Furthermore, the cytotoxicity of the purified NK fraction at an $E:T$ ratio of 10 : 1 is comparable to that of PBMC at an $E:T$ ratio of 60 : 1 (Fig. 3). This is in agreement with the six times higher NK cell content of the former fraction. Apparently, the isolation procedure does not affect the cytolytic activity of the individual effector cells.

Storage of NK cells

The isolation of NK cells involves a relatively laborious protocol and more purified NK cells can be produced from a buffy coat than are generally used in one series of experiments. Therefore, we have investigated the feasibility of several storage procedures, with regard to the recovery of viable and active NK cells.

Short term ($\approx$ 24 h) storage of NK cells at 4°C resulted in a slight reduction of the cytotoxicity against K562 to 78 $\pm$ 22% ($n = 3$) of the original level. Additional experiments showed that the pattern of NK cytotoxicity after storage at 4°C was similar to that of fresh NK cells, albeit at a somewhat lower level. Therefore, storage at 4°C was applied when effector cells were to be used within 24 h of isolation.

When NK cells were kept at 37°C, a significant increase in their cytotoxic capacity to 176 $\pm$ 13% ($n = 3$) of the control level was found. This phenomenon has also been observed in experiments in which cryopreserved lymphocytes were thawed and recultured to regain their pre-freezing cytotoxicity levels (Pross and Maroun, 1984; Fujiwara et al., 1986). We investigated whether the increase in lytic activity against K562 cells, which occurred at 37 °C, was accompanied by other signs of activation, such as the onset of lytic activity against the NK-resistant cell line Daudi. These experiments were performed with different effector cell fractions, ranging from mononuclear cells to purified NK cells.

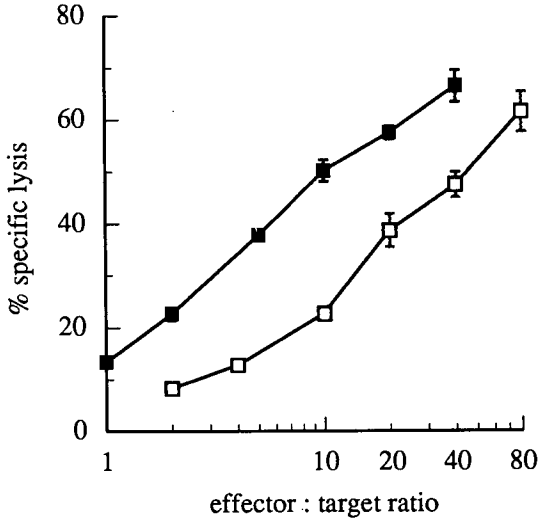

Fig. 3. Natural killer cytotoxicity before and after purification of NK cells. The cytotoxicity of mononuclear cells (□, 10% NK cells) and of purified NK cells (■, 63% NK cells) was tested in a 4 h chromium release assay. Bars indicate the standard deviation between triplicate determinations.

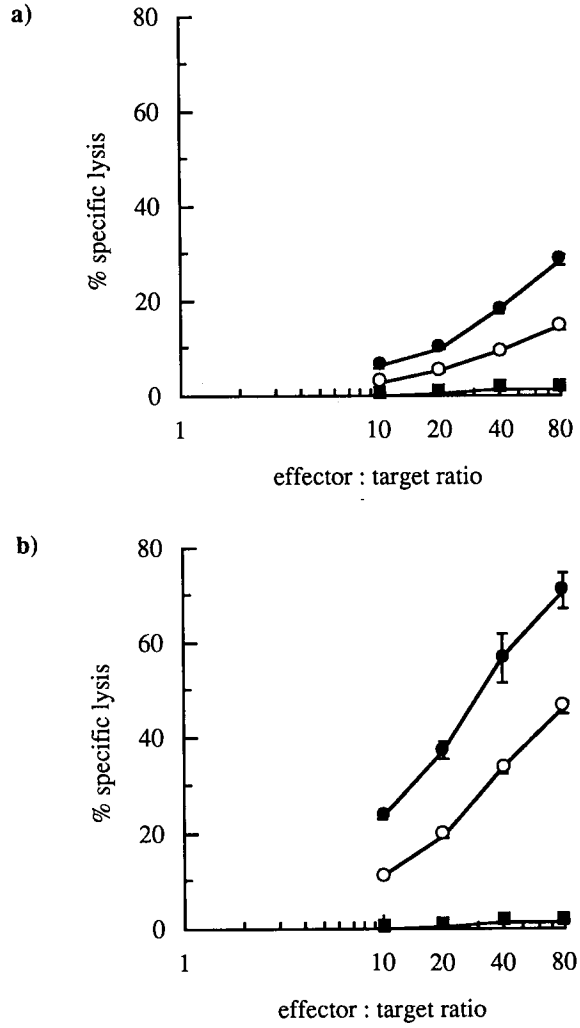

Fig. 4. Cytotoxicity of frozen/thawed effector cells (NA cells) against Daudi target cells. *a*: effector cells were thawed, cultured (see text) for 0 h (■), 24 h (○), or 48 h (●), and subsequently tested for cytotoxicity against Daudi target cells. *b*: effector cells were thawed, cultured (see text) for 0 h (■), for 24 h in the presence of 100 U/ml rIL-2 (○), or preincubated in medium for 24 h followed by the same IL-2 stimulation (●), and subsequently tested for cytotoxicity against Daudi target cells. Bars indicate the standard deviation between triplicate determinations.

Effector cells were frozen, thawed with or without subsequent reculture, and tested for cytotoxicity against Daudi target cells. Because a programmable freezer was not available, preliminary experiments were carried out to determine an optimal 'manual' cryopreservation method. From these experiments (data not shown) we concluded that, in our hands, optimal recovery and cytotoxicity of frozen/thawed effector cells were obtained, using gradual freezing in a polystyrene box (Gratama et al., 1985), in medium containing a moderate serum concentration (20%).

The NK cytotoxicity of both crude and purified effector cells was clearly below the pre-freezing level directly after thawing and returned to this level after an overnight recovery culture (data not shown). Additional experiments, which were carried out with (partially purified) NA cells, showed that the recovery of lytic activity against K562 cells was accompanied by a 'spontaneous' development of cytotoxicity against Daudi cells, proportional to the duration of the culture period (Fig. 4*a*). Freshly thawed cells displayed virtually no activity (Fig. 4*a*). Purified NK cells spontaneously developed even higher cytotoxicity levels against Daudi target cells, proportional to the higher NK cell content (data not shown). In Fig. 4*b* it is shown that cells, which have been preincubated overnight, clearly respond more strongly to IL-2 stimulation than cells which are treated with IL-2 directly after thawing. This is an additional indication that culturing of effector cells leads to unwanted activation.

The recovery of effector cells after a freezing/thawing cycle was rather poor. Directly after thawing, $62 \pm 9\%$ ($n = 5$) of the cells were recovered, but this figure dropped to values around 50%, when the cells were cultured, regardless of whether IL-2 was present or not. Among the recovered cells the viability was high; trypan blue dye exclusion was usually better than 90%.

Discussion

In the last decade, the interaction between natural killer cells and their target cells has become a matter of considerable interest (Trinchieri, 1989). Many of these studies have been performed with rather impure effector cell preparations, impairing reliable observations of NK activity (cf., Rubin et al., 1982). A variety of antibody-based purification methods for NK cells has been developed. At the moment, Percoll gradient centrifugation is the only alternative procedure which does not use antibodies. Although it is probably the fastest procedure, a practical disadvantage of the Percoll technique is that its efficiency is very dependent on the composition and osmolarity of the different Percoll layers (Timonen et al., 1982). Furthermore, the NK cells are always divided over several fractions, thereby offering a choice between low recovery and low purity (Cosentino and Cathcart, 1987).

Positive selection

Positive selection of NK cells has been performed using magnetic beads (Pflueger et al., 1990; Naume et al., 1991) as carriers of antibodies. However, the application of this type of procedure is not feasible directly after Ficoll centrifugation, because of low recoveries and interactions of adherent cells with the antibody carriers (Pflueger et al., 1990; Naume et al., 1991). Even in an optimized immunomagnetic isolation protocol, the recovery is only 10–15% (Naume et al., 1991). The application of a specialized system with a very strong magnet (Pflueger et al., 1990) could be an (expensive) solution to this problem. Positive selection of NK cells by cell sorting is efficient only on a relatively small scale. At a sorting speed around 3000 cells/s, which offers a reasonable recovery, sorting of a buffy coat (at least 5×10^8 mononuclear cells) takes about 48 h. A general disadvantage of positive selection methods is that effector cells are obtained attached to an antibody (and a carrier). This influences the expression of cell surface molecules (Naume et al., 1991) and could affect the viability or the activity of the cells.

Negative selection

The non-adherent cell population (Fig. 1) consists almost exclusively of T cells and NK cells. Therefore, negative selection for NK cells implies antibody-mediated removal of T cells. T cells can be removed by means of complement-mediated lysis (Ledbetter et al., 1980), or binding of T cells

with antibodies to solid phases. Another way to separate T cells from NK cells is rosetting with sheep red blood cells, but the efficiency of this procedure appears to be lower than that of the antibody-based techniques (Cosentino and Cathcart, 1987).

The negative selection method described in this paper represents a good compromise between the final purity and the yield of NK cells. In our hands, the major contamination of the final effector cell population consisted of non-mononuclear cells, stemming from the granulocyte/erythrocyte layer after the Ficoll centrifugation step. Surprisingly, contamination with these cells was not mentioned by other authors (Cosentino and Cathcart, 1987; Garcia-Peñarrubia et al., 1989), although even under optimal conditions 3–4 percent non-mononuclear cells are present in the Ficoll interphase (Bøyum, 1984). Relative to an NK cell content of 10–12% this is a significant contamination. Our results demonstrate that contaminating non-mononuclear cells can be efficiently removed by centrifugation of the purified NK fraction on Ficoll (Fig. 2).

Significant non-specific losses of NK cells did not occur during the adherence steps (Table I). In this respect, we found that saturation of the nylon wool columns is crucial. The application of much less than $25–30 \times 10^7$ cells per column resulted in lower recoveries, without significant improvements in the removal of unwanted cells. The relatively low recovery during the panning step could be caused by interactions between Fc receptors on the surface of NK cells and the coating antibodies.The use of $F(ab')_2$ fragments of a goat anti-mouse antibody could be an improvement.

During the panning experiments we observed that elimination of T cells proceeded much better at room temperature than at 4°C. This was found for two different coating (goat anti-mouse) antibodies, the Fc-specific one mentioned in the experimental section and a goat anti-mouse IgG/IgM antibody purchased from Tago (nr. 4153, Tago, Burlingame, CA, USA).

Our method is convenient and does not require expensive reagents or equipment, with the exception of the almost inevitable antibodies. On the other hand, concessions are made with respect to purity. However, we have shown that the purity is sufficient for cytotoxicity studies and the additional Ficoll centrifugation step can provide even higher purities, which are desirable for single cell assays. In published procedures purities are generally higher but they are reached at the cost of recovery (Cosentino and Cathcart, 1987; Naume et al., 1991) or very high amounts of antibody (Garcia-Peñarrubia et al., 1989). With regard to the data of Naume et al. (1991), it should be noted that the very high (up to 90%) purity these authors report, can be ascribed to the unusually high NK cell content in the starting mononuclear cell preparation, which is 31% compared to a normal value of 10–15% (Trinchieri, 1989; Robertson and Ritz, 1990; this paper). In our experience, the final purity of NK cells is strictly correlated with the NK cell content of the mononuclear cell fraction.

Cryopreservation

It is generally observed that upon thawing, the cytotoxic capacity of cryopreserved NK cells is significantly impaired, but that incubation in medium during a recovery period of at least 5 h seems to abolish this decline in activity (Pross and Maroun, 1984; Gratama et al., 1985; Fujiwara et al., 1986). Contrary to the observations of Lamers et al. (1991), we found evidence for the onset of LAK activity during 'recovery culture' of frozen/thawed lymphocytes (Fig. 4). To exclude any induction of T cell-mediated cytotoxicity against Daudi cells by the anti-CD3 antibody (Geller et al., 1991), these experiments were carried out with cells which had not seen anti-Leu-4. As expected, the recovery of effector cells using the 'polystyrene box procedure' was lower than the values reported with programmed freezing (Fujiwara et al., 1986; Schmidt-Wolf et al., 1989).

Although a detailed investigation of the apparent activation of frozen/thawed effector cells was beyond the scope of this paper, an obvious explanation could be the release of endogenous lymphokines during the recovery culture period. We conclude that our results confirm earlier reports that culturing of frozen/thawed NK effector cells is necessary in order to recover their pre-freezing cytolytic capacity. However, the results depicted in Fig. 4 indicate that this 'recovery

culture' is accompanied by activation of effector cells and changes in their target cell repertoire.

Therefore, in our view, cryopreservation is not the optimal strategy to ensure reproducibility in studies concerning target cell recognition and specificity of NK cells. Instead, we advocate the application of fresh NK cells, which can be obtained relatively easily with the isolation procedure described in this paper.

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