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Separation of the pore-forming and cytotoxic activities from natural killer cell cytotoxic factor

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The influence of Ca^{2+} on the cytotoxic activity of natural killer cell cytotoxic factor (NKCF) was analyzed. When the natural killer susceptible cell line K562 was exposed to NKCF in the presence of 5 mM Ca^{2+}, two peaks of cell damage were found. The first peak was observed after 30–40 min of incubation as a result of pore formation on the surface of target cells. The second was a peak of cytolytic activity which appeared after 24 h of incubation. Upon dilution of the NKCF preparation, only the first peak was observed. Therefore, NKCF produced by large granular lymphocytes in response to K562 consists of different proteins and represents pore-forming and cytolytic activities.

Natural killer cell cytotoxic factor; Perforin; (Granular lymphocyte)

1. INTRODUCTION

NKCF was found to be released during incubation of lymphocytes with NK susceptible tumor cell lines [1]. This factor may be responsible for the cytolysis mediated by NK cells [2,3]. NKCF requires a long period of time (24–48 h) to lyse target cells [1–3]. The cytotoxic activity of NKCF is inhibited by trypsin and serum proteins and activated by interferon [4,5]. The mechanism of cytolytic action of NKCF is very poorly understood. Some data indicate that the cytolytic activity is possibly associated with proteins of 20–40 kDa [4,6].

Recently we have analysed the protein composition of NKCF by SDS-PAGE and found that NKCF is a complex mixture of different proteins with molecular masses between 20 to 70 kDa [7]. The major component with a molecular mass of 60 kDa, constituting 70–90% of the total NKCF protein, shows properties very similar to perforin isolated from cytolytic granules [8–10]: (i) their molecular masses are similar; (ii) the mobility of both proteins is highly dependent on the integrity of disulfide bonds; (iii) both proteins interact with antibodies to the C9 component of complement and perforin; (iv) antibodies to perforin inhibit the cytotoxicity of NKCF.

Therefore NKCF appeared to have a complex protein composition. The aim of the present study was to find a possible functional relationship between these proteins and the cytotoxic activities of NKCF.

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Abbreviations: NK cells, natural killer cells; NKCF, natural killer cell cytotoxic factor; LGLs, large granular lymphocytes

2. MATERIALS AND METHODS

2.1. *Tumor cells*

All cultures were routinely maintained at 37°C

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in a humidified atmosphere of 5% CO_2 in a DMEM medium, supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum. Cell line K562 (a human tumor cell line) was derived from a patient with chronic myelogenous leukemia during blast crisis.

2.2. *Lymphocytes*

Lymphocytes from the spleen of nu/nu mice were separated by centrifugation on Ficoll-Hypaque gradients. Large granular lymphocytes were purified on a Percoll gradient as described in [11].

2.3. *NKCF preparation*

Lymphocytes were adjusted to 5×10^6 cells/ml in a medium without serum and were incubated with K562 cells at 5×10^4 cells/ml at 37°C in an atmosphere of 5% CO_2 for 20 h as described in [1]. Cells were then removed by centrifugation at $300 \times g$ for 10 min and supernatants were filter sterilized through a 0.2 μm Millipore filter.

2.4. *Cytotoxicity assay*

To determine cytotoxicity we used trypan blue exclusion to count viable cells. Spontaneously staining cells constituted less than 1% of total cells. In the presence of calcium, spontaneously staining cells still constituted less than 3%. The percentage of cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{(\text{staining cells} - \text{spontaneously staining cells})}{(\text{total cells} - \text{spontaneously staining cells})} \times 100.$$

3. RESULTS

To determine the influence of a change in calcium concentration on the cytotoxic activity of NKCF, cell viability was monitored by trypan blue exclusion. In the presence of 1 mM calcium the maximum dye uptake by target cells exposed to NKCF was observed after 48 h of incubation (fig.1a). In the presence of 5 mM calcium the killing activity of NKCF is markedly accelerated, maximum activity being observed after 24 h. Possibly the most surprising finding of this study

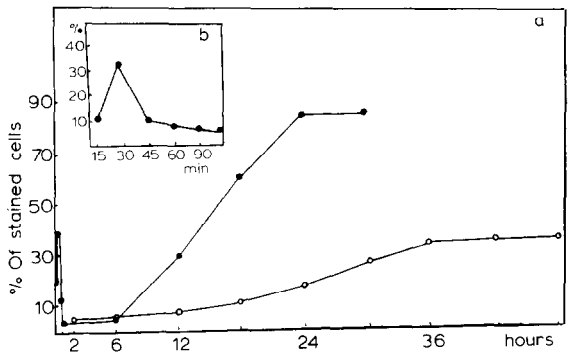

Fig.1. Ca^{2+}-dependent activity of NKCF. Cytotoxic activity of NKCF was analysed in the presence of different condition of Ca^{2+}. (a) 24 h assay in the presence of 1 mM Ca^{2+} (—○—) and 5 mM Ca^{2+} (—●—); (b) 1 h assay in the presence of 5 mM Ca^{2+}.

is that a burst of activity is found during the first hour of incubation. In the first 15 min of incubation approx. 12% cells can be stained with trypan blue. This number increases to 30% after 30 min. Unexpectedly, after 1 h, the number of stainable cells drastically decreased to less than 10%. Only after 12 h was there a second increase in the percentage of stained cells observed, which comprised 90% of all cells after 24 h of incubation. The second peak of dye uptake is a result of cell death. However, the first peak seems to depend on temporary changes in cell membranes which make the cell penetrable to trypan blue. This conclusion is supported by the reversibility of the effect. To investigate further the nature of the first peak of activity we performed the same experiments with a

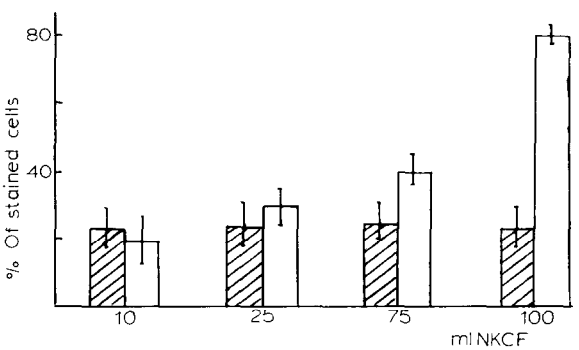

Fig.2. Dependence of cytotoxic activity of NKCF from the concentration. (▨) Cytotoxic activity after 30 min of incubation; (□) cytotoxic activity after 24 h of incubation.

diluted NKCF preparation. The amount of stained cells at 30 min was not changed even after 10-fold dilution of the NKCF preparation. In contrast, the percentage of stainable cells at 24 h drastically decreased (fig.2). The cells treated with diluted NKCF remained viable in spite of the fact that they had passed through a stage of temporary membrane damage. Thus, upon dilution the concentration of cytotoxic factors was not high enough to kill the cell, even though the concentration of factors increasing membrane permeability was still above the limiting level. In other words, the two activities of NKCF can be simply separated by dilution.

4. DISCUSSION

It is known that perforin is a protein that undergoes rapid calcium-dependent polymerisation and produces pores in the target membranes. It has been shown that perforin is capable of lysing tumor cells in the presence of calcium [12]. In the present work, we analysed the influence of calcium on the cytotoxic activity of NKCF. Our data demonstrate that NKCF-mediated killing is a two-step process. The first step seems to be reversible damage of the cell membranes which does not necessarily kill the cell. This takes place very soon after the addition of NKCF. Not more than 30% of all cells are stainable by trypan blue at early stages of incubation. However, this may depend on a rather short duration of cell membrane permeability. It cannot be excluded that in fact all cells pass through this step. Possibly the appearance of membrane lesions depends on the action of perforin which produces functional pores in the target membranes in the presence of calcium. It is known however, that nucleated cells are capable of repairing their damaged membranes. The repair mechanisms include shedding of single molecules or even fragments of membranes [13]. The pore formation on the surface of target cells may stimulate this process.

The second step is an irreversible killing of the cell which occurs much later. Almost all cells become stainable at 24 h and later die. We suggest that this step is induced by proteins different from perforin which are also present in NKCF [7]. Their concentration is much lower, giving a possible explanation for the strong effect of NKCF dilution on the second step of cell damage.

At present it is hard to say which proteins are responsible for the cytolysis. Herberman and coworkers [6] and Bonavida et al. [4] have demonstrated that the 20–40 kDa proteins are involved in cytolysis mediated by NKCF. 28–60 kDa neutral serine esterase has recently been implicated in cell killing mediated by CTL and NK cells [14,15]. Proteins with similar molecular masses can also be identified in NKCF and our preliminary data demonstrate that PMSF inhibits the cytolytic activity of NKCF. With the ability to separate the pore-forming and cytolytic activity of NKCF we can study the role of the different proteins in cell killing in more detail.

Summarising the data one may suggest that NKCF acts through short reversible opening of membrane pores by perforin, which allows other cytolytic proteins to enter the cell and induce death.

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