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DIPEPTIDYLPEPTIDASE-4 AS A SURFACE MARKER OF HUMAN NATURAL KILLER CELLS

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Dipeptidylpeptidase-4 (DP-4) is a serine protease which catalyzes removal of dipeptides from the N-terminal residue of oligo- or polypeptides, if a proline, hydroxyproline, or alanine residue occupies the second position. DP-4 is found in virtually all organs and systems of the human body [6]. It is possible that DP-4 performs different functions in different situations in the body. For instance, DP-4 localized on the surface of lymphocytes is evidently connected wtih processes of lymphocyte proliferation [9]. A high level of interleukin-2 (IL-2) production by cells carrying DP-4 on their surface has been reported, when IL-2 production in populations of DP-4 cells is very low [10]. It is possible that DP-4 is a marker of IL-2-producing cells.

Natural killer (NK) cells constitute a special subpopulation of lymphocytes capable of producing lysis of cells of many autologous and allogeneic tumors, and also of nontumor, virus-infected cells without preliminary immunization and without recognition of antigens of the histocompatibility complex [7]. Besides the cytotoxic function, their ability to secrete lymphokines also has been described: for example, B-cell growth factor (BCGF), Il-2, interferon (IFN), colony stimulating factor (CSF) [8, 14].

Morphologically NK cells form a population of large granular lymphocytes (LGL) [4, 13]. They carry on their surface markers such as the Fc-receptor for immunoglobulin G ($Fc_\gamma R_1$ CD16.Leu11), HNK-1 (Leu7), and a receptor for the third component of complement (CR3, KM1, CD11) [13, 14].

The aim of the investigation was to study DP-4 activity on the surface of NK cells from human peripheral blood.

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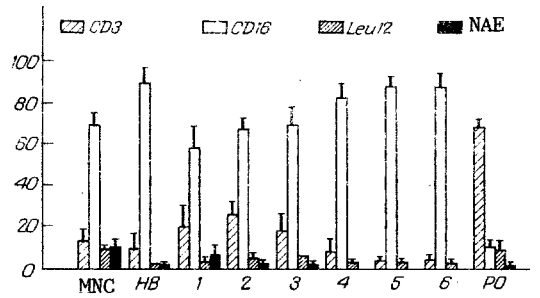

Fig. 1. Distribution of surface markers of cell population obtained during isolation of NK cells. MNC) Mononuclear cells, NW) after incubation with nylong wadding; 1-6) fractions harvested from Percoll density gradient; RF) after rosette formation. Ordinate, percentage of cells carrying corresponding marker.

EXPERIMENTAL METHOD

Mononuclear cells were isolated from buffy coat obtained from healthy blood donors [1], after which B-lymphocytes and monocytes were removed with the aid of nylon wadding [2].

To enrich the specimen with large granular lymphocytes, the cell population obtained was fractionated on a Percoll density gradient [11]. A six-step Percoll density gradient was created from solutions of Percoll in concentrations of between 40 and 52% with a step of 2.5%. The osmolaity of all the Percoll solutions in the culture medium was in the region from 285 to 290 milliosmoles/kg H_2O. The density of individual solutions was determined with the aid of density marker beads (Pharmacia, Sweden). It was higher than 1.050 g/ml for the Percoll solution with a concentration of 40%, over 1.062 g/ml for the 45% solution, and over 1.074 for the 52.5% solution. Cell fractions harvested after centrifugation from interphases were designated by the numbers from 1 to 6, starting with the fraction with lowest density. T lymphocytes were removed from fractions 2 and 3 by the method of rosette formation [12]. For this purpose, the lymphocytes were incubated with sheep's red blood cells in the ratio of 1:150 for 1 h at 29°C. Rosettes and red blood cells were then removed by centrifugation on a density gradient of lymphocyte separation medium (Flow Laboratories, England).

Preparations for fluorescence analysis were obtained as follows: 20 µl of the cell suspension (cell concentration $2 \cdot 10^6$/ml) was applied to clean slides, previously treated for not less than 1 h in a 0.03% solution of PDDA [poly-(dimethyldiallyl)-ammonium chloride] and the cells were incubated for 30 min in a humid chamber at room temperature. The medium was removed and 10 µl of the first antibodies was added. After incubation for 30 min at 4°C the liquid was drawn off and 10 µl of the second antibodies, labeled with FITC, was added. The mixture was incubated for 10 min at 4°C and the slides were washed and studied under the fluorescence microscope. To prepare specimens for flow cytofluorometry, 20 µl of the cell suspension with a cell concentration of $100 \cdot 10^6$/ml was introduced into the well of a round-bottomed 96-well planchet. Next, 20 µl of a solution of anti-CD16-antibodies was added and the sample was incubated for 30 min at 4°C, after which the cells were washed three times with PBS by centrifugation. Next, to 20 µl of the cell suspension was added 20 µl of the second antibodies (FITC-labeled mouse IgG), and the sample was incubated as described previously. Fluorescence was recorded by S. A. Rudchenko on an FACS II flow fluorometer (Becton Dickinson, USA).

To evaluate morphology, the cells were stained by Pappenheim's method. For this purpose 20 µl of cell suspension (cell concentration $2 \cdot 10^6$/ml) was applied to slides treated beforehand with PDDA. After incubation for 30 min the cells were fixed for 10 min in methanol and dried in air. The preparations were incubated for 10 min in May–Gruenwald solution, washed with distilled water, and incubated for 15 min in Giemsa solution. The preparations were dried in air and the number of LGL counted in 200 cells under the light microscope.

Activity of naphthyl acetate esterase (NAE), a marker of monocytes, was determined by the method described in [5].

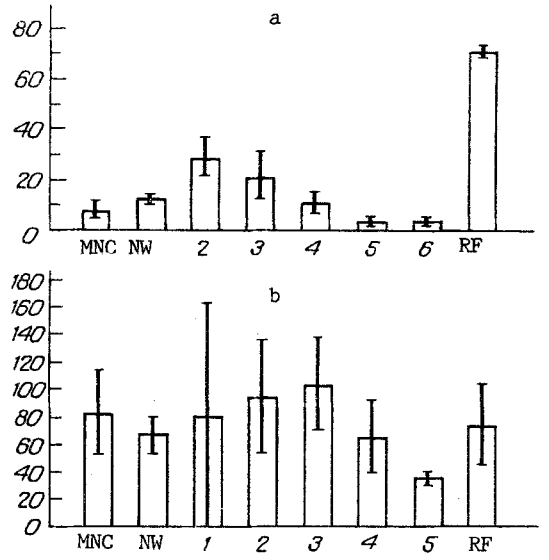

Fig. 2. Content of LGL (in percent, a) and cytotoxic activity of cells (b) in populations obtained during isolation of NK cells. Remainder of legend as to Fig. 1.

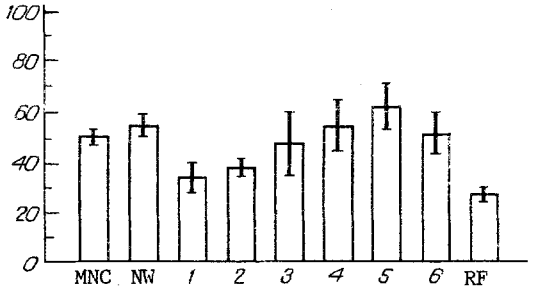

Fig. 3. Content of DP-4^+-cells (in percent) in populations obtained during isolation of NK cells. Remainder of legend as to Fig. 1.

DP-4 activity was determined by the method described in [3]. Preparations obtained for staining by Pappenheim's method were fixed for 3 sec in a solution containing 1 part of 30% formalin and 4 parts of methanol, and cooled to −20°C. They were then washed four times for 15 sec each time in cold distilled water and dried in air. At each point, 50 ml of incubation solution of the following composition was applied to the preparation: 3.4 ml of 0.1 M phosphate buffer, pH 7.5, 2 mg of Gly-Pro-methoxy-naphthylamide, previously dissolved in 200 µl of dimethyl formamide, and 4 mg of Fast blue B, previously dissolved in 400 µl of dimethyl formamide. The specimens were incubated for 60 min at room temperature. They were then washed with distilled water, stained with methyl green, and dried in air; the number of DP-4^+-cells was counted in 200 cells under the light microscope.

To determine the cytotoxic activity [11] K-562 target cells and lymphocytes, labeled with ^{51}Cr, were incubated in ratios of 1:5 and 1:50 for 4 h at 37°C and with 5% CO_2. Total release of chromium was determined by the addition of a 1% solution of triton X-100, and spontaneous release was measured by the addition of 100 µl of complete medium to the target cells. The percentage of lysis was calculated by the formula:

$$\frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}}.$$

One lytic unit corresponded to the number of lymphocytes necessary to give 20% lysis of K-562 cells.

EXPERIMENTAL RESULTS

Among MNC, as the results of counting immunofluorescence showed, 70.2 ± 5% of cells had CD3 antigen on their surface, 13.5 ± 4.5% had CD16, and 9.5 ± 0.5% had Leu12 (Fig. 1).

The morphology of the isolated cells was studied after they had been stained by Pappenheim's method. It was shown that 8 ± 2.8% of the cells had morphological features of LGL (Fig. 2). NAE and DP-4 activity on the cell surface was assessed by the use of color tests. Cells with diffuse NAE activity on the whole cell surface (10.2 ± 3.4%) were regarded as monocytes (Horwitz, 1977). 50.5 ± 3.1% of the cells had DP-4 activity on their surface (Fig. 3). The cytotoxic activity was 82.8 ± 29.8 LU/10^7 MNC (Fig. 2).

After incubation of the MNC with nylon wadding, among the cell population there were only 1 ± 1% of monocytes and 0.7 ± 0.6% of Leu12$^+$ cells. The content of T lymphocytes was increased to 90.8 ± 6.7% and of DP-4$^+$ cells to 55.25 ± 4.4% (Fig. 1).

After centrifugation on a Percoll density gradient NK cells and monocytes accumulated in low density fractions, T lymphocytes in high-density fractions (Fig. 1). Accumulation of Leu12$^+$-cells in the low density fractions was an unexpected finding. The dimensions and density of the B- and T-lymphocytes under normal conditions were about equal. It may be that the subpopulations of larger B lymphocytes did not adhere to the nylon wadding.

After removal of the rosette-forming cells the number of CD16$^+$-cells increased to 69.8 ± 3.5% (Fig. 1), and 70.1 ± 1.15% of the cells had the LGL morphology (Fig. 2). Among cells not forming rosettes, 8.3 ± 4.9% were Luel2$^+$, 1 ± 1% were monocytes, and 10.2 ± 2.2% were CD3$^+$. Despite the increase in the number of cells with LGL morphology, the cytotoxic activity did not exceed that observed in cells of fractions 2 and 3 (74 ± 29.6 LU/10^7 effector cells, see Fig. 2). Evidently the functional activity of the cells declined in the course of the experiment. DP-4 was represented by 26.8 ± 2.5% of cells (Fig. 3). Allowing for the fact that T lymphocytes carrying DP-4 [9] accounted for only 10%, roughly 15% of NK cells may have DP-4. In the cell population obtained after sorting, with the aid of cytofluorometry, about 80% of CD16$^+$-eells and 22 ± 3.2% of DP-4$^+$-cells were found, but not more than 10% of T-lymphocytes.

These results indicate the possible existence of a small population (about 10%) of NK cells possessing DP-4. To determine the figure more accurately, it is essential to have a 100% population of NK cells. Our findings are in agreement with those published in [10], which showed that DP-4 is a marker of IL-2-producing cells.

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