NATURAL CYTOTOXIC ACTIVITY OF MOUSE BONE MARROW AND SPLEEN CELLS DURING REGENERATION AFTER EXPOSURE TO CYCLOPHOSPHAMIDE

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Normal killer cells (NK), capable of exhibiting natural cytotoxic activity and not requiring preliminary sensitization, have been intensively studied in connection with their supposedly important role in the maintenance of the antitumor resistance of the host. Within a relatively short period of time (about 8 years) much information has been obtained about these cells, but some problems, including the histogenesis of NK, have been inadequately studied. The formation and differentiation of NK evidently take place in bone marrow [4]. Transplantation of bone marrow or fetal liver cells into lethally irradiated recipients has shown that the reactivity of the recipient's NK was determined largely by the donor's cells, arising from the stem cell of bone marrow or fetal liver. Herberman et al. [5] suggests that for differentiation of NK from precursors division is necessary, followed by the formation of nondividing precursor cells, which can differentiate or become activated rapidly into functioning NK.

The writers previously studied the cell composition of mouse bone marrow after administration of cytostatics (hydroxyurea or cyclophosphamide) which cause death of proliferating precursor cells, including proliferating stem cells [1, 2]. In the early stages after exposure to cytostatics (1st day) proliferating cells were practically absent in the bone marrow, but nonproliferating precursors (not proliferating at the time of exposure of the stem cells to the cytostatic) were preserved. By the use of this model it is possible to determine the time interval required for differentiation of cells of a particular type, provided that their life span is short.

The object of this investigation was to study the dynamics of NK activity in mouse bone marrow and spleen after injection of cyclophosphamide in a dose leading to death of all proliferating precursors, in order to establish the minimal time required for NK to differentiate from early nonproliferating precursor cells.

EXPERIMENTAL METHOD

Experiments were carried out on male CBA mice weighing 16-20 g, obtained from the "Stolbovaya" and "Svetlye Gory" Nurseries of the Academy of Medical Sciences of the USSR. Cyclophosphamide (from Serva, West Germany) was injected intraperitoneally in a dose of 250 mg/kg body weight. Daily for 9 days a cell suspension was prepared from the femoral marrow and spleen. The cells, numbering 2×10^6, were incubated in medium RPMI-1640 with 10% embryonic calf serum and 1% glutamine, in the presence of [^{3}H]thymidine (5 μCi/ml, specific activity 23 Ci/mmole) for 1 h. Radioactivity incorporated into the acid-insoluble fraction was estimated by means of a Tricarb Packard scintillation counter.

Natural cytotoxic activity of the cells was determined in the test of ^{51}Cr liberation from labeled target cells (TC), as which cells from a mouse Gac-1 lymphoma transplantable *in vitro* were used. TC numbering 5×10^6-10×10^6 were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (specific activity 1 mCi/mmole, from Amersham Corporation, England) for 60 min at 37°C. TC

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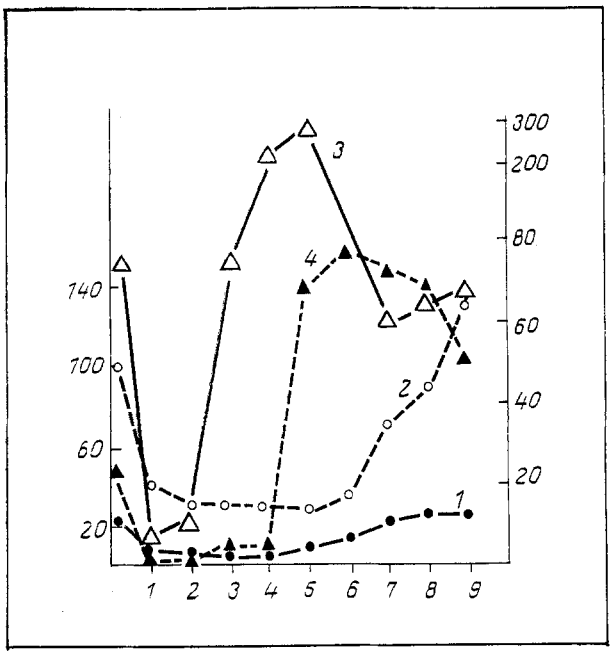

Fig. 1. Dynamics of nucleated cells and level of DNA synthesis in bone marrow and spleen of mice receiving cyclophosphamide. Abscissa, time after injection of cyclophosphamide (in days); ordinate: left — number of nucleated cells, $\times 10^{-6}$, in femur (1) and spleen (2); right — number of counts per minute ($\times 10^{-3}$) per 2×10^6 bone marrow (3) and spleen (4) cells.

numbering 2×10^4 in 0.1 ml were mixed in the cells of a round-bottomed plate with an equal volume of effector cells (EC) in ratios of 100:1, 50:1, and 25:1. The cells were incubated for 4 h at 37°C. The plates were then centrifuged at 200g for 3 min. Radioactivity of 0.1 ml of the supernatant was measured by means of a Rack-Gamma gamma-counter. The cytotoxic index (CI) was calculated in percent by the equation:

$$CI = \frac{\text{Number of pulses (experiment} - \text{spontaneous emission)}}{\text{Number of pulses (maximal emission} - \text{spontaneous emission)}} \times 100.$$

To estimate the lytic potential of the whole organ, cytotoxicity was expressed in lytic units (Lu). The number of effector cells lyzing 20% of TC (Lu_{20}) was taken as the unit. In experiments to determine the presence of suppressor cells in the fraction of adherent cells, splenocytes were incubated in plastic Petri dishes twice, for 30 min each time, at 37°C (10^7 cells to 5 ml in a dish 10 cm in diameter). Cells not adhering to the plastic were tested for NK activity in the standard test with chromium liberation from TC.

The numerical results were subjected to statistical analysis by Student's t test.

EXPERIMENTAL RESULTS

A considerable decrease in the number of cells in the spleen was observed 24 h after administration of cyclophosphamide. The decrease in the number of cells in the bone marrow was even greater (Fig. 1). At this time an extremely low level of DNA synthesis was detected in the bone marrow, and DNA synthesis in the spleen was virtually absent. After 2 days there was some increase in the level of DNA synthesis, and this increase reached a maximum on the 5th day. The level of DNA synthesis in the spleen rose much more slowly.

In intact animals the bone marrow cells exhibited much weaker cytotoxic activity than spleen cells. The cytotoxic activity of cells from both organs was sharply reduced during the first 2 days after injection of cyclophosphamide (Fig. 2). Between the 3rd and 5th days no cytotoxic activity could be detected in the bone marrow, whereas minimal activity was

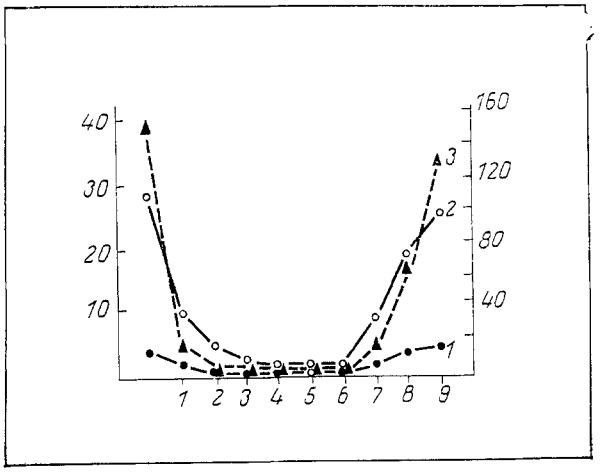

Fig. 2. Dynamics of NK activity in bone marrow and spleen of mice receiving cyclophosphamide. Abscissa, time after injection of cyclophosphamide (in days); ordinate: left — CI (in %) of bone marrow (1) and spleen (2) cells with ratio EC:TC = 50:1; right — total cytotoxic activity of NK in spleen (3), expressed in lytic units ($Lu_{2\,0}$).

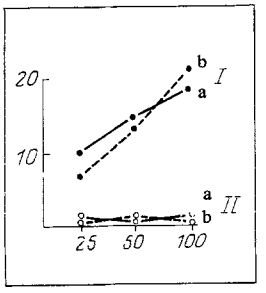

Fig. 3. Cytotoxic activity of NK before and after removal of adherent cells. Ordinate, CI (in %) of cells of normal spleen (I) and 3 days after injection of cyclophosphamide (II) with EC:TC ratio of 25:1, 50:1, and 100:1 respectively (abscissa). a) Unfractionated cells, b) fraction of nonadherent cells.

observed in the spleen at these times. When calculated in lytic units per spleen, it was reduced by 150 times. Experiments with preliminary removal of adherent cells showed that the decrease in NK activity was not connected with an increase in suppressor activity of the adherent cells (Fig. 3). NK activity reappeared in the bone marrow and increased in the spleen only on the 7th day after injection of cyclophosphamide. A sharp rise in its level was observed in the spleen on the 8th-9th day.

Consequently, 2 days after injection of the cytostatic, the bone marrow cells did not exhibit NK activity, and after 3 days only minimal cytotoxic activity could be detected in the spleen. These findings suggest that the duration of functioning of the cells as NK does not exceed 3 days after differentiation from proliferating precursors. However, bone marrow and spleen are open cell systems, and for that reason migration of NK into other organs cannot be ruled out. Riccardi et al. [6] observed a decreased in cytotoxicity after injection of cyclophosphamide, not only in the spleen but in the lungs also. Their data on the dynamics of NK activity in the mouse spleen after injection of cyclophosphamide in a

dose close to that now used, differ from those obtained in the present investigation in higher levels of activity on the 2nd and 6th days, but they also observed the maximal decrease in cytotoxicity after 4 days.

NK are sensitive to cyclophosphamide but relatively resistant to ionizing radiation. Postradiation recovery of NK activity has been shown to be slower than general cell regeneration [3]. This can be explained by the influence of regulatory cells. Macrophage-like cells perform the suppressor function for NK. In the present experiments suppressor activity of adherent cells from mouse spleen 4 days after injection of cyclophosphamide (the time of minimal NK activity) could not be found. In the bone marrow at this time activation of cell proliferation was taking place, as shown by the observed rise in the level of DNA synthesis (Fig. 1). The fraction of cells synthesizing DNA on the 5th day after treatment with the cytostatic exceeds 40% [2]. Proliferating precursors of different hematopoietic series are formed. After 7 days newly formed specialized B lymphocytes appear. For NK to differentiate from an early nonproliferating precursor, the same period is evidently required. However, the number of B lymphocytes after 8 days in the bone marrow does not exceed 10% and in the spleen 20% of their number in the intact animal, whereas the NK level at this time often reaches the values found in intact animals. The earlier recovery of function by NK than by other lymphocyte populations is interesting from the point of view of the possible participation of NK in regulation of hematopoietic cell differentiation or in control of the size of the pool of certain normal cells [3].

NK activity is thus absent from the 2nd through the 6th day in the bone marrow of mice receiving cyclophosphamide, but in the spleen its minimal level is observed between the 3rd and 6th days. Starting with the 7th day there is a rapid increase in NK activity in both organs.

LITERATURE CITED

1. L. V. Van'ko, V. V. Malaitsev, N. S. Khamitova, et al., Byull. Éksp. Biol. Med., No. 10, 460 (1978).
2. L. V. Van'ko and N. S. Suleimanova, Immunologiya, No. 4, 6 (1980).
3. G. Cudkowicz and P. S. Hochman, Immunol., Rev., 44, 13 (1979).
4. O. Haller and H. Wigzell, J. Immunol., 118, 1503 (1977).
5. R. B. Herberman and H. T. Holden, J. Natl. Cancer Inst., 62, 441 (1979).
6. C. Riccardi, T. Barlozzari, A. Santoni, et al., J. Immunol., 126, 1284 (1981).