ANALYSIS OF CHANGES IN NATURAL CYTOSTATIC AND CYTOTOXIC ACTIVITY OF MOUSE SPLEEN CELLS AFTER ADMINISTRATION OF CYCLOPHOSPHAMIDE AND α-INTERFERON

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In recent years a role of increasing importance has been ascribed to the system of natural resistance in the body's defense against tumors. This applied, in particular, to natural cytotoxic (cytolytic) activity, detectable *in vitro* as death of target cells, and to cytostatic activity, recorded as inhibition of proliferation of target cells. The cytostatic mechanism is probably no less important than the cytotoxic in defense of the body against tumors. Various cells, including macrophages, normal (natural) killer (NK) cells, and granulocytes may exhibit cytostatic activity [4, 6, 8]. Great importance is nowadays attached to the cytostatic action of cells as a mechanism capable of maintaining mutual control and adaptation of the cells of a multicellular organism [3].

The writers previously studied changes in the level of natural cytotoxic activity in the spleen and bone marrow of mice after injection of cyclophosphamide (CP) [1].

In the investigation described below the time course of natural cytotoxic and cytostatic activity of the spleen cells was compared in mice receiving CP.

EXPERIMENTAL METHOD

Experiments were carried out on male CBA mice weighing 16-20 g, obtained from the Stolbovaya, and Svetlye Gory nurseries, Academy of Medical Sciences of the USSR. CP 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, thymus, and spleen. The cells, numbering $10 \cdot 10^6$/ml, were incubated in medium RPMI-1640 with 10% fetal serum and 1% glutamine in the presence of ^{3}H-thymidine (5 μCi/ml, specific radioactivity 23 Ci/mmole) for 1 h. Radioactivity incorporated into the acid-insoluble fraction was estimated on a Packard Tricarb scintillation counter.

In two series of experiments intact spleen cells were treated beforehand with mouse leukocytic interferon (400 U/ml) for 1 h at 37°C.

The natural cytotoxic activity of the cells was determined in the test of release of ^{51}Cr from labeled target cells, which were cells of a YAC-1 mouse T lymphoma, transplanted *in vitro*.

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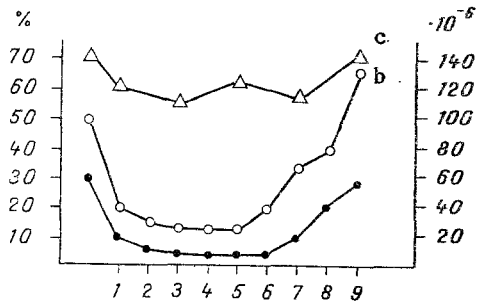

Fig. 1

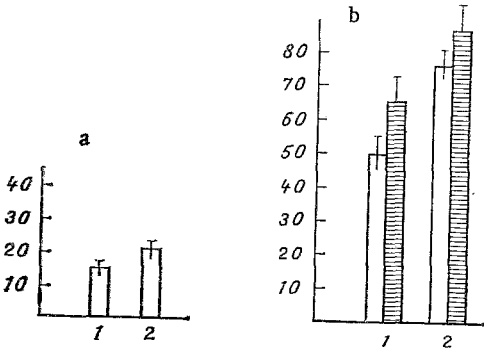

Fig. 2

Fig. 1. Time course of number of cells and level of natural cytotoxic and cytostatic activity of mouse spleen cells after treatment with CP. Abscissa, time after injection of CP (in days); ordinate: right, number of nucleated cells ($\times 10^{-6}$); left, CI (b) and CSI (c). [a, missing in Russian]

Fig. 2. Effect of pretreatment with interferon on natural cytotoxic and cytostatic activity of mouse spleen cells. Ordinate: a) CI (in %); b) CSI (in %). 1) Control; 2) treatment with interferon. Unshaded columns, inhibition of incorporation of ^{3}H-thymidine; shaded columns, ^{3}H-uridine.

The target cells, numbering $5 \cdot 10^6$-$10 \cdot 10^6$, were incubated with 100 μCi of $Na_2{}^{51}CrO_4$ (specific radioactivity 1 μCi/mmole, from Amersham Corporation, England) for 1 h at 37°C. Labeled target cells ($2 \cdot 10^4$) were introduced into wells of round-bottomed panels with effector cells in various proportions (total volume of the mixture 0.2 ml). The cells were incubated for 4 h at 37°C. The panels were then centrifuged at 200g for 3 min. The radioactivity of 0.1 ml of supernatant was measured by means of a "Rack-Gamma" gamma-counter. The cytotoxic index (CI) was calculated in % by the equation:

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

Cytostatic activity was assessed as the ability of the spleen cells to inhibit incorporation of ^{3}H-thymidine or ^{3}H-uridine into the acid-insoluble fraction of target cells, for which purpose mastocytoma R-815 cells immune to the cytotoxic action of NK were used. Macrophages were removed from the suspension of effector cells by incubating the cells in plastic Petri dishes at 37°C for 1 h.

Nonadherent cells were mixed with target cells in the ratio of 20:1 and cultured at 37°C. After 4 h, ^{3}H-thymidine (5 μCi/ml, specific radioactivity 5 Ci/mmole) or ^{3}H-uridine (5 μCi/ml, specific radioactivity 5 Ci/mmole) was added. After 4 h of subsequent culture the cells were sedimented on filters and the radioactivity of the acid-insoluble fraction was determined. The cytostatic index (CSI) was calculated in percent by the equation:

$$CSI = \left[1 - \frac{\text{number of counts (experiment} - \text{effector control)}}{\text{number of counts (target control)}}\right] \times 100\%.$$

Values of radioactivity of target cells and effector cells cultured separately were used as the control.

EXPERIMENTAL RESULTS

During the first 48 h after administration of CP the number of cells in the spleen fell significantly (Fig. 1). The level of natural cytotoxic activity of the cells which remained in the spleen was sharply reduced. Not until after 5 days did the number of cells in the spleen begin to increase, whereas the level of cytotoxicity of the cells rose after 6 days, and by the 9th day it had almost reached the level of cytotoxicity of the spleen cells of intact animals. Cytostatic activity of the spleen cells showed little change throughout this period after administration of CP.

Investigation of the effect of interferon, a stimulator of natural cytotoxic activity, on the cytostatic activity of the spleen cells gave the results shown in Fig. 2. Pretreatment of splenocytes from intact mice *in vitro* with leukocytic interferon caused an increase

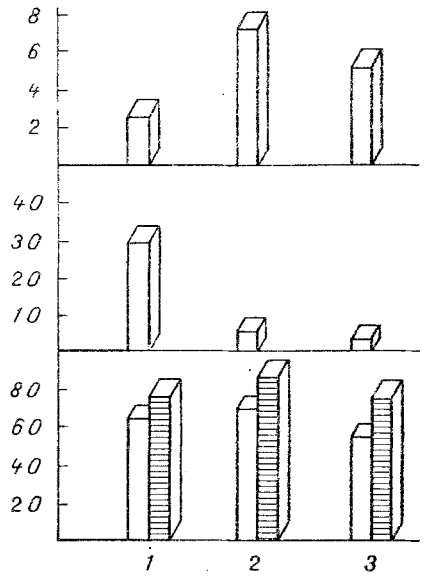

Fig. 3. Level of proliferative, cytotoxic, and cytostatic activity of mouse spleen, bone marrow, and thymus cells. Ordinate: a) incorporation of ^{3}H-thymidine (in cpm·10^{-3}); b) CI (in %); c) CSI (in %). Unshaded columns, inhibition of ^{3}H-thymidine incorporation; shaded columns, ^{3}H-uridine. 1) Spleen; 2) bone marrow; 3) thymus.

in both cytotoxic and cytostatic activity of these cells. We compared the cytotoxic and cytostatic activities of the cells of several organs, differing in the proliferative activity of their cells, in intact mice. It will be clear from Fig. 3 that the natural cytotoxic activity of spleen cells, which proliferate less actively than bone marrow and thymus cells, was considerably higher than the cytotoxic activity of the latter. Differences in the cytostatic activity of the cells of these organs were much less marked. All the cells had a quite high cytostatic action on mastocytoma R-815 cells (with respect to DNA and RNA synthesis).

The question arises whether cytostatic activity of spleen cells is found only against tumor cells or whether it is also exhibited against nontumor cells. Experiments were carried out to determine the cytostatic activity of splenocytes against a number of targets of nontumor origin. Cells of mastocytoma R-815 were the most sensitive to the cytostatic action of spleen cells, whereas normal bone marrow cells were the least sensitive. Actively proliferating cells of regenerating bone marrow cells (5 days after treatment with CP) and blast cells induced by concanavalin A occupied an intermediate position. In our experiments NK had no cytostatic action on any of these cells.

Investigation of the time course of the cytotoxic and cytostatic activity of spleen cells after treatment with CP, causing death of proliferating cells, showed that effectors of natural cytotoxic activity constitute a homogeneous group as regards sensitivity to CP. Cells exerting cytostatic activity *in vitro* are evidently heterogeneous for sensitivity to CP. Despite the considerable decrease in the number of splenocytes after administration of CP, the level of cytostatic activity was not reduced, i.e., some effector cells of cytostatic activity are resistant to this cytostatic. The absence of a relative increase in cytostatic activity after treatment with CP may be evidence of the presence of a fraction of effector cells of cytostasis that are sensitive to CP.

Mouse spleen cells have the highest level of natural cytotoxic activity compared with cells of other lymphoid organs, including organs of active lymphopoiesis (bone marrow and thymus). The question arises whether this effect is connected with the ability of NK cells to limit the proliferative activity of cells. Our comparative investigation of the natural cytotoxic and cytostatic activity of spleen, bone narrow, and thymus cells showed absence of correlation between the level of proliferation of cells and their cytostatic activity. Although these organs differ considerably in their level of proliferation and cytotoxic activity, they differed only a little in the cytotoxic effect of their cells.

There are data in the literature to show that NK cells can exhibit cytotoxic activity against normal cells [5, 7]. We were unable to find a cytotoxic action of spleen cells against the target cells used. However, the cytostatic activity against normal cells was considerable, and it correlated to a certain degree with the level of proliferative activity of the target cells.

The stimulating effect of interferon and its inducers on the level of natural cytotoxic activity of mouse spleen cells is known. In the present experiments pretreatment of splenocytes with interferon caused an increase in both cytotoxic and cytostatic activity. Cells sensitive to interferon are evidently present in both populations of effectors.

Exposure to considerable stress also leads to a change in the level of cytostatic and cytotoxic activity of spleen cells, and definite correlation is observed in the time course of these two functions after stress [2].

Data on the sensitivity of the cytostatic activity of spleen cells to the effect of interferon and exposure to stress are evidence of definite similarity between the properties of effectors of cytostatic and cytotoxic activity. Meanwhile there are marked differences in the level of their activities in different lymphoid organs and in relation to different target cells.

The possibility cannot be ruled out that effectors of cytostatic activity constitute a heterogeneous population, of which cells with cytotoxic activity constitute one subpopulation. Further investigations are necessary in order to explain this problem.

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IN VIVO INDUCTION OF SPECIFIC SUPPRESSOR T CELLS IN H-2K^{bm} MUTANT MICE

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It has been shown [2] that specific supressor T cells (SSTC) in the H-2 system, appearing as a result of induction *in vivo*, are narrowly specific for a certain epitope of the H-2 molecule. SSTC can recognize determinants similar to serologic determinants [6].

In attempts to induce SSTC in mice mutant for the H-2 complex, with wild-type cells it was found that SSTC are easily formed *in vivo* by a complex mutation of the H-2D^{dm1} molecule if the corresponding mutant (M504), immunized by wild-type B10.D2 (H-2^d) cells, can also form antibodies [6]. Conversely the "point" mutation of the H-2K^{bm1} molecule does not lead to the formation either of antibodies [10], or of SSTC [6], on immunization of the mutant with wild-type C57BL/6 (H-2^b) cells, despite the effective formation of cytotoxic T lymphocytes (CTL) in the same system [10].

In the present investigation, in order to induce SSTC we used a set of mutants with clearly characterized replacement of amino acids in the H-2K^b molecule (Table 1).

EXPERIMENTAL METHOD

Mice of lines BALB/c (H-2^d), DBA/2 (H-2^d), CBA (H-2^k), AKR (H-2^k), and C56BL/6 (abbreviation B6, H-2^b) were obtained from the Stolbovaya Nursery, Academy of Medical Sciences

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