EFFECT OF CYTOTOXIC LYMPHOCYTES OBTAINED BY in vitro IMMUNIZATION WITH SYNGENEIC LYMPHOMA CELLS ON PLURIPOTENT HEMATOPOIETIC CELLS

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UDC 612.112.94.017.4.014.46-08

KEY WORDS: cytotoxic lymphocytes; interleukin-2; syngeneic lymphoma; pluripotent hematopoietic cell.

A study of antigens common to hematopoietic precursor cells and leukemic cells is important for an understanding of the level and mechanisms of leukemogenesis and also for the discovery of antigenic markers of hematopoietic cells. An antigenic marker of acute lymphoid leukemia [7] has been discovered on early commited precursor cells of lymphopoiesis [10]. The obtaining of monoclonal antibodies against the common antigen of acute lymphatic leukemia has made their effective use possible for complement-dependent removal of lymphoblastic leukemia cells from bone marrow [9, 13]. Recognition of cell antigens by antibodies and by immune T-lymphocytes is known to take place by different molecular mechanisms and with different specificity: By means of immune T killer cells it is possible to detect cellular antigens that are not detectable serologically [1].

The aim of this investigation was to study the sensitivity of pluripotent hematopoietic cells, namely, colony-forming units in the spleen (CFU_S), to the cytolytic action of immune lymphocytes possessing specific killer activity against syngeneic T-lymphoma cells. The possibility that a common antigenic marker should exist on T-lymphocytes and CFU_S was taken into account [6]. The possibility of specific illumination of lymphoma cells from bone marrow without injury to CFU_S, with the aid of immune lymphocytes, also was studied.

EXPERIMENTAL METHOD

Female C57Bl/6 (B6) mice aged 2-4 months were obtained from the "Stolbovaya" nursery, Academy of Medical Sciences of the USSR. Syngeneic lymphoma EL-4 ($H\text{-}2^b$) was maintained in vivo. Immune killer cells were obtained on the 12th day in mixed culture (MC) of splenocytes from nonimmune B6 mice (reacting cells) and irradiated (60 Gy) syngeneic lymphoma EL-4 cells (stimulators) in the presence of interleukin-2 (IL-2) [3]. The final concentration of reacting cells was 2 million/ml. The ratio of reacting cells to stimulators was 50:1. Supernatants of cultures of human lymphocytes, stimulated by phytohemagglutinin (subsequently called IL-2), and standardized for T-growth activity, were used as IL-2 (the supernatants were supplied by the Experimental Processing of T-Growth Factor Group, directed by N. V. Varivotskaya). The supernatants were added to the cultures in a dilution of 1:10 after neutralization of trace quantities of phytohemagglutinin by rabbit antibodies [2]. The cells were cultured in RPMI-1640 nutrient medium (Gibco, USA), with the addition of 10% embryonic calf serum, 2-mercaptoethanol ($5 \cdot 10^{-7}$ M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). On the 9th day the MC was restimulated by changing the nutrient medium and by adding IL-2 and irradiated EL-4 cells. In the control cultures not containing EL-4 cells, the nutrient medium was changed and IL-2 was added. On the 12th day lymphocytes obtained from the mixed or control cultures were used to estimate their cytolytic activity, and also for treating the bone marrow or a mixture of bone marrow with EL-4 cells. To study the effect of cytotoxic lymphocytes (CTL) on CFU_S, the test lymphocytes were mixed with syngeneic bone marrow or with a mixture of bone marrow and living EL-4 cells, centrifuged (5 min, 400g), and incubated for 5 h at 37°C with periodic (every 1 h) mixing and centrifugation. The number of hematopoietic stem cells still preserved at the end of incubation was determined by the method in [14]. The cytotoxic activity (CTA) of the test lymphocytes relative to EL-4 cells, taken separately or mixed with bone marrow cells, was estimated by three methods: 1) by liberation of radioactive chromium from EL-4 target cells or control syngeneic splenocytes (activated by concanavalin A – con A) during incubation for 4 h in a 76-013-05 microplate (Linbro, England); 2) by inhibition of clonogenicity of lymphoma EL-4 cells in vitro [4]; after incubation of the

Blood Transfusion Research Institute, Minsk. (Presented by Academician of the Academy of Medical Sciences of the USSR V. I. Votyakov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 99, No. 5, pp. 604-606, May, 1985. Original article submitted April 29, 1984.

 0007-4888/85/9905-0640 $09.50 © 1985 Plenum Publishing Corporation

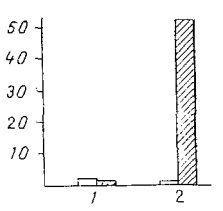

Fig. 1

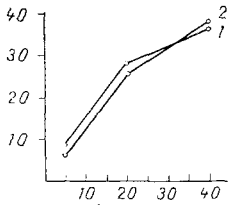

Fig. 2

Fig. 1. Characteristics of CTA of lymphocytes from control (1) and mixed (2) cultures against syngeneic lymphoma EL-4 cells (shaded columns) and against normal splenocytes, activated by con A (unshaded columns). Ordinate, CTA (in % of release of radioactive chromium from target cells.) Ratio of test lymphocytes and target cells was 20:1.

Fig. 2. CTA of lymphocytes from MC against EL-4 cells in presence of bone marrow cells. Abscissa, ratio of immune killer cells to target cells; ordinate, CTA (in % of release of ^{51}Cr from target cells). 1) CTA of lymphocytes from MC against 10^4 EL-4 cells; 2) CTA of lymphocytes from MC, mixed with equal number of bone marrow cells.

lymphoma cells with the test lymphocytes in the residue at 37°C for 5 h (ratio of CTL, bone marrow, and EL-4 cells was 120:30:1) the cells were suspended in 0.25% agarized RPMI-1640 medium, containing 20% of embryonic calf serum, and then layered above a feeder layer of 0.5% agarized medium of the same composition, containing 100,000 syngeneic splenocytes, irradiated in a dose of 15 Gy, in 1 ml. On the 10th day the number of colonies was counted. Endocolonization from bone marrow cells was not observed in the control cultures not containing EL-4 cells; 3) by the reduction in leukemogenicity of the EL-4 in Winn's tests in vivo [16]; after incubation of the test cell suspension, containing lymphoma EL-4 cells, in the residue at 37°C for 5 h (ratio of lymphocytes, bone marrow cells, and EL-4 cells was 120:30:1), the cell suspension was injected intraperitoneally into B6 mice at the rate of 10^3 original EL-4 cells per mouse, and the length of survival of the animals was determined.

EXPERIMENTAL RESULTS

Reacting cells of B6 mice, cultured in MC with irradiated syngeneic lymphoma cells in the presence of IL-2 exhibited strong cytotoxicity toward EL-4 cells but did not cause lysis of normal splenocytes activated by con A (Fig. 1). Without the addition of IL-2, immune CTL did not appear in MC (data not given) [3]. The lymphocytes from control cultures containing only IL-2 had no significant CTA in any of the five experiments (on average not more than 10% of the CTA of the immune lymphocytes, see Fig. 1). Thus during stimulation by IL-2, immune CTL distinguishing lymphoma cells were obtained from normal syngeneic activated lymphocytes, in MC of B6 mice and EL-4 cells. This proves that leukemia-associated antigens, detectable by T killer cells but not inducing a cellular immune response without the addition of exogenous IL-2, were found on T lymphoma cells.

Specific CTA of the T killer cells against ^{51}Cr-labeled target cells can be blocked by the addition of unlabeled cells possessing common antigens with the target cells [12, 15]. The possibility of neutralizing immune CTL against EL-4 cells with specific CTA in the presence of an excess of bone marrow cells was tested (Fig. 2). It was shown that immune CTL effectively "located" lymphoma cells in a mass of bone marrow cells and caused their lysis with the same activity as without addition of bone marrow. It is evident that antigens recognized by CTL were not significantly expressed on normal bone marrow cells and did not prevent recognition and lysis of malignant lymphoma cells.

Pluripotent hematopoietic cells constitute only a small part of bone marrow [5], and the presence of particular antigens on them cannot therefore be detected by a direct test of neutralization of cytotoxicity. We studied the effect of immune CTL on the total number of pluripotent hematopoietic cells (CFU) detectable by Till and McCulloch's splenic colonies method [14]. The conditions of incubation of the CTL with bone marrow

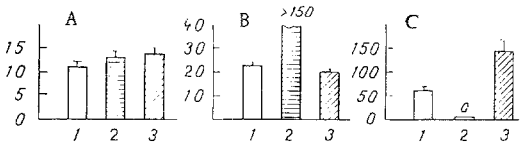

Fig. 3. Effect of incubation of test lymphocytes with mixture of bone marrow cells and EL-4 cells on number of CFU_S (A), leukemogeneity of EL-4 cells in Winn's test (B), and colony formation by EL-4 cells in agar (C). Ordinate: A) number of colonies in spleen, B) length of survival of mice (in days), C) number of colonies in agar. 1) Bone marrow cells ($3 \cdot 10^4$) + EL-4 cells (10^0), control; 2) bone marrow cells ($3 \cdot 10^4$) + EL-4 cells (10^3) + immune lymphocytes ($12 \cdot 10^4$); 3) bone marrow cells ($3 \cdot 10^4$) + EL-4 cells (10^3) + control lymphocytes ($12 \cdot 10^4$).

cells and the activity of the CTL (Fig. 2), determined as lysis of EL-4 cells, were the same as the experimental conditions described above for the investigation of the direct neutralizing activity of bone marrow cells. It was shown that on incubation of immune lymphocytes, cytotoxic against lymphoma EL-4 cells, with syngeneic bone marrow cells containing CFU_S, no decrease was observed in the number of pluripotent hematopoietic cells detectable by the method of colony formation in the spleen (the number of CFU_S in the bone marrow in the control was 9.7 ± 1.3, rising to 11.9 ± 0.7 after incubation with immune lymphocytes and 10.8 ± 1.2 after incubation with control lymphocytes). Antigens recognized by immune killer cells and specific for antigens of lymphoma EL-4 were thus absent on the CFU_S.

During interaction between immune CTL and target cells, lymphocytes are known to secrete biologically active factors, such as lymphotoxin, etc. [8], which can cause damage to surrounding cells and tissues nonspecifically. During interaction with allogeneic cells, lymphocytes have been shown to secrete factors injuring hematopoietic precursor cells [11]. The authors cited in [11] consider that these factors are an important component of the disturbance of hematopoiesis in the graft vs host reaction. In the present experiments, the possibility of selective removal of lymphoma cells from bone marrow without damage to hematopoietic stem cells was studied with the aid of immune CTL. It was found (Fig. 3) that cytolysis of syngeneic lymphoma cells by the action of immune CTL, taking place in a mixture with bond marrow cells, does not lead to a reduction in the number of CFU during incubation for 5 h. Under the conditions used, the CFU_S were thus not injured by products of interaction of immune CTL and target cells. Under these circumstances, as was shown by the survival rate of mice receiving "purified" bone marrow, and by the results of cloning EL-4 cells in semisolid agar (Fig. 3), bone marrow could be completely purified from contamination by syngeneic lymphoma cells with the aid of immune CTL.

It can be concluded from the experimental results that crossing of cell-recognizing antigens to mouse hematopoietic CFU_S or to syngeneic lymphoma EL-4 cells could not be detected with the aid of immune killer cells cytotoxic for syngeneic lymphoma EL-4 cells. Immune killer cells, mixed with bone marrow containing syngeneic lymphoma cells specifically eliminated only the lymphoma cells and did not injure the CFU_S, with the result that it was possible to purify the bone marrow from contamination by lymphoma cells and to preserve its hematopoietic potential. This suggests that if autologous antileukemic human killer cells can be obtained they will be no less effective in purifying autologous bone marrow from contamination by leukemic cells than cytotoxic monoclonal antibodies against leukemia-associated antigens.

The authors are grateful to Professor I. L. Chertkov and to Junior Scientific Assistant N. I. Drize (Central Research Institute of Hematology and Blood Transfusion) for help with determination of the number of CFU_S.

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