INHIBITION OF ACTIVATION OF DNA SYNTHESIS BY IMMUNE LYMPHOCYTES IN MIXED NORMAL LYMPHOCYTE CULTURES in vitro

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Replacement of normal by immune lymphocytes as reacting cells in "one-way" mixed lymphocyte cultures (MLC) leads to inhibition of DNA synthesis. This phenomenon is observed regardless of the strain of mice, the method of immunization (in vivo or in vitro), or the method of performing the MLC test (macro- or microcultures, presence of mercaptoethanol in the medium, type of control). Unlike the cytotoxic effect of immune lymphocytes, which follows an acute course with a peak on the eighth day after immunization, inhibition of DNA synthesis in MLC is observed as early as on the second day, it continues for several weeks after immunization, and it can be produced by the addition of a small fraction (5-10%) of immune to normal lymphocytes.

KEY WORDS: mixed lymphocyte cultures; activation of DNA synthesis; immune lymphocytes; cytotoxic effect.

Activation of DNA synthesis in antigen-reactive cells (ARC) during their incubation in vitro in mixed cultures with irradiated allogeneic lymphocytes ("one-way" mixed lymphocyte culture – MLC) is a model of immunological identification which is the equivalent of the initial stage of graft rejection in vivo [17]. Preliminary immunization of mice [4, 16], rats [18], and man [3] with allogeneic lymphocytes as a rule speeds up the response to MLC and increases its intensity in the early period (1-2 days) of culture, i.e., it induces a secondary immune response. Meanwhile lymphocytes of animals immunized under certain conditions with allogeneic spleen cells [10, 13] or removed after injection of parental lymphocytes into irradiated F_1 hybrids [12, 14] inhibit activation of DNA synthesis in normal syngeneic lymphocytes at the peak of their response in MLC (fourth to fifth day of culture). It has been suggested that immunological identification is controlled by cellular and humoral interactions of ARC with special subpopulations of suppressor cells which, under different conditions, could be T or B lymphocytes and macrophages [6, 7].

The object of the present investigation was to determine the conditions for stable reproduction of inhibition of DNA synthesis by immune lymphocytes in MLC and to discover whether suppression of MLC is due to the cytotoxic effect (CE) of immune lymphocytes on target cells.

EXPERIMENTAL METHOD

Inbred C57BL/10 (abbreviated to B10), B10.D2, CC57BR, BALB/c, and A/Mv mice were obtained from the nursery of the N. F. Gamaleya Institute of Epidemiology and Microbiology. The mice were immunized by a single subcutaneous injection of ascites cells of two allogeneic sarcomas – Sa 1 (strain A) or MKh 11 (strain B10) at five points (sometimes intraperitoneally also) in a dose of 40-50 million cells per mouse. Immune lymphocytes were obtained from the regional lymph nodes 8 days after immunization [1].

Cytotoxic T lymphocytes (CTL) in MLC were generated by the method described previously [11] in plastic flasks (No. 3012, Falcon Plastics, USA) by incubating for 5 days a mixture of reacting lymph node cells with stimulating allogeneic spleen cells, irradiated in a dose of 1500 rad (cobalt source, 80 rad/min), in RPMI-1640

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TABLE 1. Stimulation of Incorporation of ^{3}H-Thymidine during Reaction of Immune Lymphocytes Obtained at Various Times after Immunization in vivo in MLC (M ± m)

Day after immunization	IS of immune lymphocytes relative to normal, %
1	98†
2	72,6†
3	40,2†
4	23,3±4,2‡
6	21,0±4,7‡
8—10	20,3±3,9‡
13—21	33,0±7,2‡
28—50	27,5±4,5‡
3—4*	10,4±4,2‡

*Secondary immunization 33-46 days after primary.
†Mean results of two experiments.
‡Mean results of three to ten experiments.

medium (Microbiological Associates, USA) containing 2 mM L-glutamine, 10 mM Hepes, 10% embryonic calf serum, $5 \cdot 10^{-5}$ M 2-mercaptoethanol, and 100 units/ml of penicillin and streptomycin.

Activation of DNA synthesis was obtained in MLC in the writers' own macro- and micromodifications [8]. Mixtures of equal volumes of reacting ($5 \cdot 10^6$ cells/ml) and stimulating lymphocytes (10^7 cells/ml) in medium RPMI-1640, containing 5% human group IV serum, 2 mM L-glutamine, $3 \cdot 10^{-5}$ M 2-mercaptoethanol, 25 mM Hepes, and antibiotics, were incubated at 37°C in an atmosphere of 5% CO_2 in a volume of 2 ml in British flasks or 0.2 ml in wells of number 3040 microplates (Falcon Plastics, USA). Three parallel tests were carried out on each mixture. As the control, either a mixture of reacting and syngeneic stimulating lymphocytes or double volumes of stimulating and reacting lymphocytes separately (monocultures) were incubated. To each sample 16 h before the end of culture 2 μCi ^{3}H-thymidine (1 Ci/mmole) in 0.05 ml of the same buffer was added. The samples were placed on ice, transferred to a Synpore 3 (Czechoslovakia) filter, and washed with physiological saline, 5% TCA, and ethanol by vacuum suction. Incorporation of ^{3}H-thymidine was determined in the Mark II (Nuclear Chicago, USA) liquid scintillation β-spectrometer.

The index of stimulation (IS) of DNA synthesis in MLC was estimated from the ratio between incorporation of ^{3}H-thymidine (in cpm) into allogeneic MLC and incorporation either into syngeneic MLC or half the sum of the incorporation into the monocultures.

For the cytotoxic test a micromodification of the writers' previous method [2] was developed. Peritoneal macrophages, labeled with ^{51}Cr and washed twice, were seeded in doses of $5 \cdot 10^4$ in a volume of 0.2 ml into wells of the No. 3040 microplates 2 days before the experiment. After washing three times to remove free ^{51}Cr, lymphocytes in 0.2 ml medium No. 199, containing 5% embryonic calf serum and 10 mM Hepes were added to the wells, and to determine the maximal liberation of ^{51}Cr, a 2% solution of sodium dodecyl sulfate was added. After incubation for 16-18 h at 37°C all the culture medium from each well was transferred to tubes for counting and the radioactivity was determined in the Nuclear Chicago γ-spectrometer. The cytotoxic index was calculated by the usual equation [2].

EXPERIMENTAL RESULTS

The mean IS determined in 28 experiments at the peak of the reaction in MLC of normal lymphocytes (72-120 h) was 34.8 ± 5.9 for normal and 4.5 ± 0.8 for immune lymphocytes. In the last case the peak of the response was observed earlier (24-72 h) and its height was 9.4 ± 1.9, i.e., even at this determination it was only 27% as high as the peak for the reaction of normal lymphocytes. It will be clear from Fig. 1 (the results of three typical experiments) that IS for lymphocytes immunized in vivo increased during the first 48-72 h of culture, but then fell simultaneously with the sharp rise in IS of normal lymphocytes between 72 and 96 h of culture. Similar results were obtained in macro- and microcultures independently of the system of strains of mice (B10.D2 anti-B10; BALB/c anti-B10; B10 anti-A) and of the use of monocultures or syngeneic MLC as the

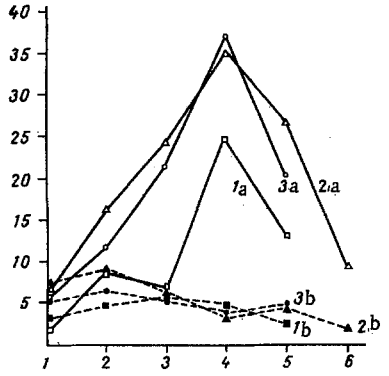

Fig. 1

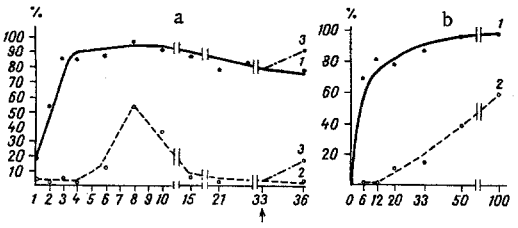

Fig. 2

Fig. 1. Dynamics of stimulation of ^{3}H-thymidine incorporation into normal and immune MLC. Abscissa, incubation time (in days) in culture before addition of ^{3}H-thymidine; ordinate, IS. 1, 2, 3) Nos. of experiment: 1) macro-, 2, 3) micromodifications of MLC. Reacting lymphocytes: a) normal, b) immune, obtained by immunization in vivo. Systems: B10.D2 anti-B10 (1 and 2), B10 anti-A (3).

Fig. 2. Dependence of CE of immune lymphocytes and of their inhibition of DNA synthesis in MLC on times of immunization (a) and proportion of immune lymphocytes in mixture with normal lymphocytes (b). Abscissa: a) days of immunization, b) proportion of immune lymphocytes in mixture with normal (in %); ordinate, decrease in IS in MLC relative to IS or normal lymphocytes (1) and CE on target cells (2). Systems: B10 anti-A (a) and B10.D2 anti-B10 (b). Arrow indicates secondary immunization; 3) secondary response.

control. The use of lymphocytes immunized in vitro, just as the addition of 2-mercaptoethanol (which stimulates DNA synthesis) to the medium, increased the difference between the reaction in MLC of normal and immune lymphocytes even more.

The decrease in IS in the "immune" MLC compared with normal was observed as early as 2 days after immunization in vivo; it reached a maximum by the fourth day, remained at an almost constant level for several weeks, and then rose again after secondary immunization (Table 1). Conversely, the dynamics of CTL generation were different: They were first found on the fifth day, reached a sharp peak by the seventh to eighth day, and then CE fell away rapidly (Fig. 2a). Inhibition of activation of DNA synthesis in MLC was thus either unconnected with CE of the immune lymphocytes on the stimulating target cells or it was produced by much smaller doses of those lymphocytes.

In fact, the addition of only 5-20% of lymphocytes from animals immunized in vivo to the normal lymphocytes led to a reduction in IS to 29 ± 4.8% of its initial level (mean results of 12 experiments). Comparison of the decrease in IS in MLC and of the decrease in CE of the immune lymphocytes depending on their relative proportion in the mixture with normal lymphocytes showed that considerably smaller doses of lymphocytes were needed to inhibit the reaction of ARC in MLC than to produce a CE on macrophages syngeneic with the cells stimulating MLC (Fig. 2b).

It has recently been shown that among lymphocytes of mice immunized with allogeneic tumors there are certain suppressor cells which inhibit CTL generation in MLC [5, 9, 15]. The phenomenon now described may also be connected with activity of these suppressors. The nature and specificity of the immune lymphocytes which inhibit DNA synthesis by normal lymphocytes and, in particular, their real differences from CTL, are currently being studied.

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