DIFFERENCES IN KINETICS OF FORMATION OF KILLERS AND PRODUCERS OF MACROPHAGE MIGRATION INHIBITION FACTOR IN THE PRIMARY AND SECONDARY RESPONSE IN MIXED LYMPHOCYTE CULTURES

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The conditions of killer formation during the primary and secondary response were established in a one-way mixed lymphocyte culture (MLC), stimulated by irradiated or killed allogeneic lymphocytes. Migration inhibition factor (MIF) was found in the culture medium of the MLC as a pointed peak on the 2nd or 3rd day of the primary response and as a plateau after the 1st day of the secondary response, whereas killers were formed much later, reaching a maximum on the 5th and 4th days of culture respectively. The use of heated allogeneic lymphocytes instead of irradiated lymphocytes to stimulate the response in MLC had no effect on the kinetics of the MIF producers but delayed killer formation in the primary MLC.

KEY WORDS: mixed lymphocyte culture; killer cells; cells producing macrophage migration inhibition factor; primary and secondary immune response.

Effector T-cells responsible for destruction of target cells (killers) and the reaction of hypersensitivity of delayed type (producers of macrophage inhibition factor – MIF) differ in certain properties: The diameter of the cells and their ability to migrate into various lymphoid organs [16], the kinetics of formation after alloimmunization in vitro [6, 11], sensitivity to pronase and anti-θ-antibodies, properties of antigen-binding receptors [3]. The most adequate approach for the comparative study of these subclasses of T-lymphocytes is their generation in vitro in mixed lymphocyte cultures (MLC). The conditions for generation of killers in MLC has been well studied [12, 14], but MIF produced by T-cells has been found in culture medium of MLC from man [9], guinea pigs [7], rats [15], and mice [13].

The object of this investigation was to compare the formation of killers and MIF producers in the same MLC during the primary and secondary immune response.

EXPERIMENTAL METHOD

B10.D2 (H-2^d) mice were immunized by a single intraperitoneal injection of 5×10^7 spleen cells from C57BL/10 (H-2^b) (abbreviation B10), and, in some experiments, by injection of leukemia EL4 cells (2×10^7 cells, intraperitoneally) or MKh 11 sarcoma cells (10^7 cells subcutaneously at 5 points), induced and maintained by passage in C57BL/6 and B10 mice respectively. Spleen cells from normal and immune (3-8 weeks after immunization) B10.D2 mice were used as reacting lymphocytes in a one-way MLC. Spleen cells from B10 mice (B10.D2 in the control), treated by various methods – x-ray irradiation (Co, 1500 R), heating (45°C, 30 min), with glutaraldehyde (0.01%, 3 min) or with ultraviolet rays [14] – were used as stimulators of the reactions. Reacting and stimulating lymphocytes were suspended in RPMI-1629 or RPMI-1640 medium containing 10% embryonic calf serum, L-glutamine (2 mM), HEPES (12 mM, 2-mercaptoethanol (5×10^{-5} M), and antibiotics (penicillin and streptomycin, 100 units/ml of each). Next, 5×10^6 reacting lymphocytes were mixed with 1×10^6 x-irradiated stimulating cells or with 5×10^6 stimulating cells treated by other methods, and incubated in a total volume of 2 ml in wells in 25-well plates (Flow Lab., England) at 37°C in an atmosphere of 5% CO_2. At different times of incubation lymphocytes were taken from the wells, centrifuged, and counted and their cytotoxic activity was determined on macrophages from B10 mice, labeled with ^{51}Cr and cultured for 2

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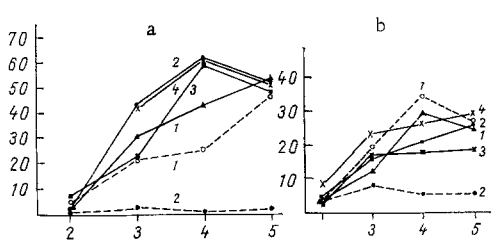

Fig. 1

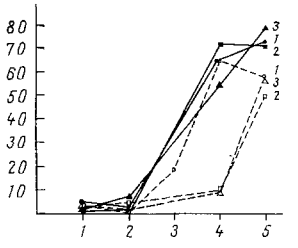

Fig. 2

Fig. 1. Formation of secondary killers during incubation of memory cells with irradiated stimulators in MLC. Here and in Fig. 2: abscissa, time of culture in MLC (in days); ordinate, cytotoxic index (A) and fraction of large lymphocytes (B). Broken lines indicate reacting lymphocytes (cells of normal B10.D2 lymph nodes), stimulated by B10 cells (1) or B10.D2 cells (2); continuous lines represent reacting lymphocytes – lymph node cells (1) or spleen cells (2, 3, 4) or B10.D2 mice 21 days after immunization with sarcoma MKh11 cells (1, 3), B10 mouse spleen cells (2), or leukemia EL4 cells (4). Stimulating cells – B10.

Fig. 2. Formation of B10.D2 killers in MLC after different methods of treatment of B10 stimulating lymphocytes. Reacting splenic lymphocytes were normal B10 or B10 immunized with spleen cells 34 days before the experiments (continuous line). Stimulating lymphocytes irradiated in a dose of 1500 R (1), heated (2), or treated with glutaraldehyde (3).

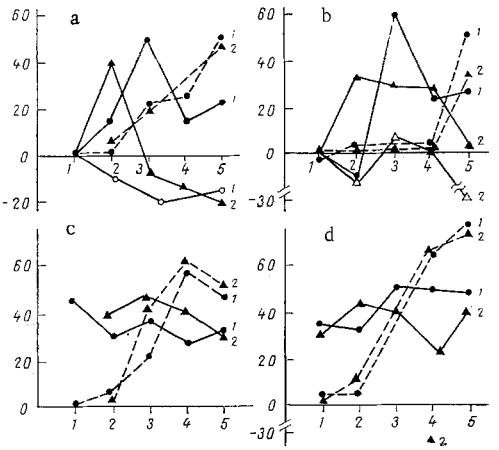

Fig. 3. MIF production and cytotoxic effects during primary (a, b) and secondary (c, d) response in MLC. Abscissa, period of culture in MLC (in days); ordinate, macrophage migration inhibition index (broken line) and cytotoxic index (continuous line). Stimulating lymphocytes were allogeneic (filled circles and triangles) or syngeneic (empty circles and triangles) – irradiated (a, c) or heated (b, d). 1, 2) Expt. Nos.

days in wells of No. 3040 microplates (Flacon Plastics, USA), at the rate of 50,000 cells per well. Lymphocytes numbering 300,000 were introduced into each well and, after incubation for 20 h, liberation of ^{51}Cr into the medium was determined [2, 4]. Meanwhile activity of MIS was determined in the medium of the same MLC as reflected in its ability to inhibit migration of macrophages from CC57Br ($H\text{-}2^b$) mice. The macrophage migration inhibition test was carried out in the microvariant [5] of the method in [10], using 1-day primary MLC as the control of migration in the medium. The area of migration of the macrophages in this medium was not less than in fresh culture medium.

EXPERIMENTAL RESULTS

In the first experiments the conditions were chosen for induction of memory cells for immunization of the mice by different methods. As Fig. 1A shows, maximal activity of primary killers was observed on the 5th day, and of secondary on the 4th day of MLC. The greatest difference in cytotoxic activity of the primary and secondary killers was observed on the 4th day of culture when spleen cells were used as reacting lymphocytes and primary intraperitoneal immunization of the mice was carried out with allogeneic spleen or with leukemia EL4. Conversely, lower memory cell activity than in the spleen was observed in the lymph nodes containing highly active killers on the 8th day after primary subcutaneous immunization of sarcoma MKh11 [1]. The cytotoxic activity of killers formed in the MLC, incidentally, did not correlate with the fraction of large lymphocytes: Their proportion was much higher in the population of primary killers than secondary (Fig. 1B).

To determine qualitative rather than quantitative differences between the primary and secondary killers, instead of irradiating the stimulating cells they were heated, treated with glutaraldehyde, or exposed to ultraviolet rays [14]. In these cases the formation of primary killers was delayed: They were absent on the 4th day and could be detected only on the 5th day in MLC (Fig. 2). Conversely, activity of secondary killers reached a maximum on the 4th day of MLC regardless of the method of treatment of the stimulating cells. The results shown in Figs. 1 and 2 were reproduced, and in subsequent experiments to study memory cells, in the absence of a primary reaction, spleen cells immune to allogeneic spleen and stimulated for 4 days with heated cells in MLC were used as reacting lymphocytes.

The times of formation of MIF producers and killers in MLC differed significantly. The MIF concentration in the medium rose to a pointed peak on the 2nd or 3rd day of the primary response when all stimulators – both irradiated (Fig. 3a) or heated (Fig. 3b) were used. At these times killer activity was minimal (Fig. 3a) or (when heated stimulators were used) it was not detected until the 4th day inclusive (Fig. 3b). Conversely, on the 5th day of culture maximal killer activity was combined with a minimal MIF concentration in the medium.

During the secondary response these differences in MLC were more demonstrative still: MIF appeared from the 1st day and was held at constant level throughout the period of culture, whereas no killers appeared on the 1st or 2nd days and they reached a maximum by the 4th day of MLC by whatever method the stimulating cells were treated – by irradiation (Fig. 3c) or heating (Fig. 3d). Both these activities were minimal or could not be detected when the reacting lymphocytes were integrated with syngeneic stimulators.

The formation of MIF producers thus precedes killer formation during both primary and secondary immunization in vitro in the H-2 system. The writers obtained similar results when mice were immunized with allogeneic cells in vivo [6]. This result agrees with the dynamics of changes in the MIF concentration in the culture medium of MLC in mice [13] and rats [15], whereas in man [9] and guinea pigs [7] MIF formation in the primary MLC is slower and steadier.

It is not yet clear whether MIF producers and killers reflect successive stages of differentiation of T-cells in response to antigen or whether they reflect independent lines of such differentiation. Since some MIF producers formed in MLC, like killers, have the $Ly=1^{-}23^{+}$ phenotype [13], it can be tentatively suggested that they are one line of differentiation, and that MIF producers reflect an intermediate stage of differentiation of killers in response to antigen, i.e., they are less mature cells formed without proliferation and DNA synthesis [8, 9]. The conditions of selective detection of each of these two categories of T-cells established by the present investigation enable this problem to be studied, for example, by the preparation of antisera against each of them.

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INDUCTION OF T-SUPPRESSORS DURING IMMUNIZATION WITH ALLOGENEIC SPLEEN CELLS IN THE MOUSE H-2 SYSTEM

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The nature of suppressor cells contained in a suspension of splenic lymphocytes immunized with allogeneic spleen cells and inhibiting activation of DNA synthesis in mixed lymphocyte cultures was studied. The suppressor cells were resistant to mitomycin C and carrageenin, were not inactivated by treatment with rabbit anti-B- and anti-Ig or mouse antibodies (anti-Mls serum) against B-lymphocytes, in the presence of complement, but were eliminated by rabbit antilymphocytic globulin and also by antibodies against T-lymphocytes (rabbit ATG and anti-θ-serum). The T-suppressors studied were concentrated in the large lymphocyte fraction in a ficoll gradient. Blocking of activation of DNA synthesis by these cells has a well-marked nonspecific component.

KEY WORDS: mixed lymphocyte culture; T-suppressors; activation of DNA synthesis; carrageenin.

Immunization of mice with allogeneic spleen cells or tumor cells leads to the formation of suppressor cells of macrophagal and T-cell origin, which block activation of DNA synthesis in a one-way normal mixed lymphocyte culture (MLC) [7, 13]. The writers previously showed that suppressors can be induced by immunization with an allogeneic tumor not consisting of T-cells [6].

In the investigation described below the conditions of induction of T-suppressors during intravenous immunization of mice with allogeneic spleen cells and certain properties of T-suppressors of this type were studied.

EXPERIMENTAL METHOD

B10.D2 (H-2^d) or BALB/c (H-2^d) mice were immunized by a single intravenous injection of 90 million allogeneic spleen cells from C57BL/10 (H-2^d) (abbreviation B10) or C57BL/6 (H-2^b) mice. In the control, 90 million syngeneic spleen cells were injected. Lymphocytes were obtained from the spleen on the 4th day after immunization. The MLC test was carried out in a modification [3] of the micro method in [10], by incubating mixtures of lymph node cells with irradiated (Co, 1500 rad) allogeneic spleen cells and, in the control, syngeneic spleen cells, in No. 3040 Microplates (Falcon Plastics). The samples were applied to filters after culture for 112 h and 16 h after addition of 1 μCi ^{3}H-thymidine. Incorporation of thymidine was determined in a scintillation β-spectrometer.

To determine suppressor cell activity normal reacting lymphocytes were mixed with immune cells in the ratio of 1.5 : 1. To maintain a constant density in the culture, the corresponding number of normal lympho-

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