SOME PROPERTIES OF A PHYTOHEMAGGLUTININ-INDUCED LYMPHOCYTE MITOGENIC FACTOR

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The principles governing production and the effect on lymphocytes of the mitogenic factor (MF), secreted by human lymphocytes in vitro under the influence of phytohemagglutinin (PHA) and described previously by the author, were investigated. PHA was inactivated by anti-PHA antiserum and immunosorbents. Lymphocytes stimulated by PHA secrete MF in the G_1-period and at the beginning of the S-period of the cell cycle. During cultivation of the cells in a protein-free medium DNA and protein synthesis was sharply inhibited but MF production was 1.6 times higher than in media containing serum. The kinetics of the reaction of lymphocytes to MF is described: DNA synthesis (reflected in thymidine-H^3 incorporation) began on the 5th-6th day and reached a maximum on the 6th-7th day.

KEY WORDS: lymphocytes; phytohemagglutinin; mitogenic factor; kinetics of formation.

One of the most important members of the group of mediators of cellular immunity – the lymphokines [6] – is mitogenic factor (MF). MF probably participates in the interaction between different populations of lymphoid cells and in their switching to proliferation during the immune response. MF is secreted by lymphocytes in vitro under the influence of specific antigen [10] and concanavalin A [8], in the mixed reaction of lymphocytes [7]. The writer previously described an MF of human lymphocytes produced in vitro under the influence of phytohemagglutinin (PHA) [1, 2]. This factor stimulates DNA synthesis in cultures of autologous and allogeneic lymphocytes, it is not inactivated by anti-PHA antisera, it is not a product of cell breakdown [1, 2], and its molecular weight is 20,000-50,000 [2].

The principles governing liberation of MF by human lymphocytes, stimulated by PHA, and the kinetics of DNA synthesis by lymphocytes in vitro in the presence of MF were investigated.

EXPERIMENTAL METHOD

Lymphocytes were separated from heparinized blood from healthy donors. The erythocytes were sedimented with 5% dextran (molecular weight 70,000), the suspension of leukocytes in plasma was applied to columns with polyacrylonitrile fiber, and sterile lymphocytes in the eluate accounted for 90-99% of nucleated cells [3]. The secretion of MF was induced by the method described earlier [1]. Washed lymphocytes were suspended in medium No. 199 in a concentration of 3-4 million/ml, incubated at 37°C for 40 min with PHA-P (Difco) (1 μl/ml), washed twice to remove PHA, and then cultivated in the same concentration in TC-199 nutrient medium (Earle Base, IBL), with the addition of antibiotics, L-glutamine and, in some experiments, autologous heated serum (10%). To investigate the kinetics of MF production cells were removed at various times of cultivation by centrifugation and the culture fluid was filtered through millipore filters (HUFs, 0.3μ). To inactivate the trace amounts of PHA washed off with the cells, the medium was incubated for 2 h at 37°C with heated antiserum against PHA [1]. Immunosorbents for PHA, based on a product of agar and the sulfuric ester of 4-hydroxyethylsulfonyl-2-aminoanisole (HSEAA) [4, 5], to which γ-globulins (AGP), precipitated by ammonium sulfate from rabbit antiserum against PHA (AGP-sorbent), were coupled by diazotization, were used in some of the experiments. The culture fluid from lymphocytes stimu-

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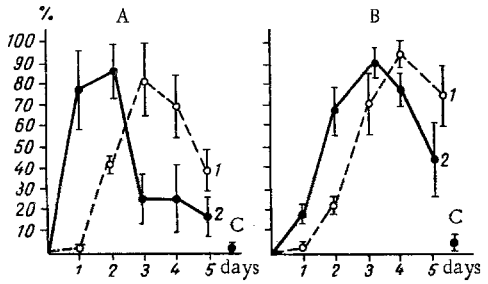

Fig. 1. Kinetics of MF complex and DNA synthesis in cultures of PHA-stimulated lymphocytes: A) PHA-lymphocytes grown in medium containing serum (mean of 3 experiments); B) in medium without serum (mean of 8 experiments); 1) DNA synthesis; 2) content of MF; C) DNA synthesis in control cultures of 10 lymphocytes. To plot the graph the maximum of DNA synthesis or of MF content in each separate experiment is taken as 100% and the corresponding values on other days were expressed in percentages of this maximum. Abscissa, period of experiment (days); ordinate, DNA synthesis and MF content (in percent of maximum).

lated by PHA (PHA-lymphocytes) was incubated for 12-15 h with the AGP-sorbent, washed frequently with TC-199, the sorbent was removed by centrifugation, and the medium was sterilized by filtration. Antiserum against PHA and the AGP-sorbent were used in preselected concentrations to ensure complete inactivation of 1 μl/ml PHA-P. The number of living cells in the cultures when antiserum against PHA and AGP-sorbent were used was the same as in the control (1-4%, test with trypan blue).

The culture fluid was tested for mitogenic activity in secondary test cultures of fresh lymphocytes after double dilution with medium TC-199. The concentration of cells in the test cultures was 0.5-1 million/ml and the final concentration of heated autologous serum was 10%. Medium No. 100 for culture fluid from unstimulated lymphocytes, cultivated in the same way as the PHA-lymphocytes, treated with antiserum against PHA or AGP-sorbent, was added to the test cultures and the control.

Thymidine labeling of the test cultures was carried out on the 6th-7th day. To each culture 2 μCi/ml thymidine-H^3 (12 Ci/mmole) was added, and after incubation for 3 h at 37°C the cells were washed twice with physiological saline at 5°C, incubated for 24 h in 5% TCA at 5°C, and precipitated on millipore filters by TCA and ethanol [3, 11]. To investigate the intensity of protein synthesis the cells were grown in MEM (minimal essential medium, IBL) in the presence of 2 μCi/ml glycine-H^3 (515 μCi/mmole) and treated by the method described above. The radioactivity of the cells on the filters was measured on a liquid scintillation counter (Nuclear Chicago) and expressed in counts/min/10^6 cells.

EXPERIMENTAL RESULTS

Cells grown in nutrient medium containing serum liberate most of their MF during the 1st-2nd day after treatment with PHA, at a time of absence or onset of DNA synthesis (Fig. 1A), i.e., in the G_1-period and at the beginning of the S-period of the cell cycle. The content of MF in the medium fell sharply until the 3rd day, when DNA synthesis by the PHA-lymphocytes reached a maximum. An identical pattern was observed if the medium containing serum was changed daily. As Fig. 2A shows, on the 2nd day summation of the MF contained previously (1st day) and that liberated later took place, whereas on the 3rd day the daily production and the total content of MF both fell. Investigation of the stability of MF obtained on the 2nd day over a period of 48 h at 37°C in culture fluid with or without serum (after removal of the PHA-lymphocyte) showed no change in activity ($P > 0.5$). The sharp decrease in the content of MF in cultures of PHA-lymphocytes on the 3rd day, which accompanied the maximum of DNA synthesis, was thus not due to spontaneous breakdown of MF, but could have been caused by production of an inhibitor or by an increase in the ability of the cells to adsorb (or inactivate) MF in the S-period of the cell cycle.

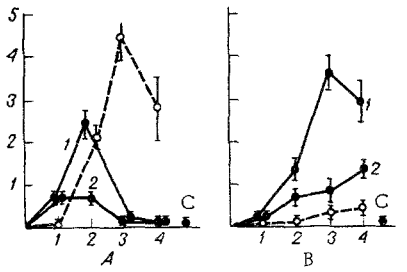

Fig. 2

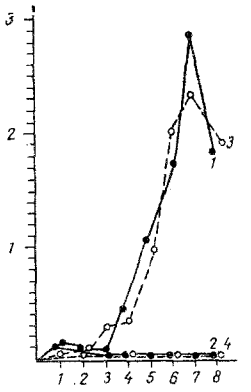

Fig. 3

Fig. 2. Kinetics of MF production and DNA synthesis in cultures of PHA-lymphocytes with daily change of nutrient medium (without disturbing the cell "layer"): A and B) the same as in Fig. 1; 1) cultures of PHA-lymphocytes without change of medium; 2) with daily change of nutrient medium; C) DNA synthesis in control cultures of test lymphocytes. Abscissa, period of experiment (days); ordinate, number of counts per minute $\times 10^{-4}$ per 10^6 cells.

Fig. 3. Kinetics of DNA synthesis of lymphocytes of test cultures in the presence of MF: 1) autologous test lymphocytes + MF; 2) autologous lymphocytes + culture medium from unstimulated lymphocytes; 3) allogeneic test lymphocytes + MF; 4) allogeneic lymphocytes + culture medium from unstimulated lymphocytes. Axes of coordinates as in Fig. 2.

The study of lymphokine production in media not containing serum facilitates the task of their immunochemical analysis. In medium without serum MF production and DNA synthesis were "delayed": MF content reached a maximum on the 3rd day and fell on the 4th-5th day in the period of maximal DNA synthesis by the PHA-lymphocyte (Figs. 1B, 2B). The maximum of the MF content in medium without serum was higher than in medium with serum ($164 \pm 11\%$; $P < 0.02$), whereas the maximum of DNA synthesis and total protein synthesis (over a period of 3 days, shown by incorporation of glycine-H^3) was lower in the medium without the serum, namely $21 \pm 7\%$ ($P = 0.02$) and $16 \pm 11\%$ ($P < 0.02$) respectively of the values in medium with serum (results of 6 experiments). This proportion was probably caused by a change in the ratio between production and inactivation of MF through the slower "departure" of the PHA-lymphocytes from the period of MF production during cultivation in medium without serum (see Fig. 2).

Investigation of the kinetics of the reaction of lymphocytes of the test cultures to MF showed that DNA synthesis began on the 5th-6th day (Fig. 3) and reached a high value, $50 \pm 13\%$ of the DNA synthesis by the same cells in response to PHA (on the 3rd day). Antiserum against PHA did not affect the course of the reaction, for when AGP-sorbent was used (i.e., with removal but not activation of the PHA residue) the reaction to MF was the same as in the presence of antiserum against PHA. This also shows that MF produced by PHA-lymphocytes was active in the total absence of the inducing agent, unlike antigen-induced MF [12].

The beginning of DNA synthesis on the 5th-6th day is a characteristic feature of reactions due to antigenic stimulation of lymphocytes, such as the "mixed" reaction, antigen-specific transformation, etc. [8]. In the present experiments autologous cells (with respect to PHA-lymphocytes, producing MF) reacted to MF with the same intensity as allogeneic cells (Fig. 3), so that the participation of histocompatibility antigens in the stimulation process can be ruled out.

Further investigations of the nature of MF and the mechanisms of its action on cells are needed. The study of these problems and the establishment of their connection with processes in vivo will give a deeper insight into the mechanisms of intercellular interaction in lymphoid tissue and will indicate ways of controlling them.

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