EFFECT OF INHIBITORS OF DNA AND PROTEIN SYNTHESIS ON PRODUCTION OF MITOGENIC FACTOR BY PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES

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The effect of inhibitors of DNA and protein synthesis on the production of mitogenic factor (MF) by human lymphocytes stimulated with phytohemagglutinin were studied. During inhibition of DNA synthesis by mitomycin MF production was not reduced. During inhibition of protein synthesis the production of MF not only was not reduced but was actually increased. It is concluded that MF pre-exists in the lymphocyte before the beginning of stimulation, not in the ready-made form, but as an inactive precursor, which is activated only in the living cell when stimulated by phytohemagglutinin, and not in need of protein synthesis at that moment.

KEY WORDS: lymphokines; mitogenic factor; inhibitors of DNA and protein synthesis.

The study of the connection between production of mediators of cellular immunity (lymphokines) [8] and various metabolic processes in the lymphocyte is of great importance both to the understanding of the molecular mechanisms triggering lymphokine production during the development of the cellular immune response and to the development of methods of influencing this process and of seeking optimal methods for obtaining lymphokines. It has recently been shown that the in vitro production of lymphokines such as migration inhibition factor [7], skin-reactive factor [10], and lymphotoxin [13] is depressed by inhibitors of protein synthesis.

The effect of inhibitors of protein and DNA synthesis on the production of one of the most important lymphokines, namely mitogenic factor (MF), was studied. Mitogenic factor, liberated in cultures of lymphocytes stimulated by phytohemagglutinin (PHA), was described by the writers previously and is a protein with molecular weight of 20,000-30,000 daltons. The MF described previously is not a product of cell destruction and it stimulates DNA synthesis in cultures of autologous and allogeneic lymphocytes for 6-7 days [2-5].

EXPERIMENTAL METHOD

Production of MF by lymphocytes was induced with PHA, using the method described previously [2], which includes inactivation of PHA residues by antisera. The lymphocytes were isolated from whole heparinized blood of healthy donors. The red cells were sedimented by dextran and the plasma with leukocytes was applied to columns with polyacrylonitrile fiber, incubated at 37°C for 40 min, and eluted with plasma of the same donor. The isolated cells were counted in a Goryaev's chamber on a Coulter-F_d counter, and in stained preparations. The washed-off lymphocytes, in a concentration of 3 million cells/ml, were incubated with PHA-P (Difco, 1 μl/ml) for 40 min, washed off twice, and cultivated in the same cell concentration in medium TS-199 (Earle-Base, IBL), enriched with L-glutamine, antibiotics, and autologous serum up to 10% (primary cultures). After 48 h of cultivation the cells were removed from the medium by centrifugation, the culture medium (CM) was filtered through millipore filters (0.3 μ, Millipore Co.), and used for determination of its mitogenic activity after neutralization of PHA residues eluted from the cells during cultivation, by anti-PHA antiserum [2]. The antiserum was added in a previously chosen concentration such that the mitogenic action was completely neutralized by PHA-P in a concentration of 1 μl/ml.

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TABLE 1. Effect of Inhibitors of Protein and DNA Synthesis on MF Production in Cultures of PHA-Stimulated Lymphocytes (M ± m)

Expt.	Characteristics of action of inhibitors on primary lymphocyte cultures (n = 3)					Mitogenic activity of CM of primary cultures for DNA synthesis in test cultures (in counts/min, n = 4)
	inhibitor	living cells (in %)	blast cells and intermediate forms (in %)	DNA synthesis (in (in counts/min)	protein synthesis (in counts/min)	
1	—	89±2,6	43±1,8	8 143±634	4 556±294	4 426±553
	Actidione	86±4,3	1,7±1,2		170±19	14 728±915
	Mitomycin C	87±0,5	44±2,4	2 070±301	4 682±558	4 292±291
	DNA synthesis in unstimulated test cultures					1 104±236
2	—	84±2,3	56±3,1	14 060±2 000	10 743±1 626	6 062±662
	Actidione	80±0,4	1,7±0,9		238±66	12 901±2 251
	Mitomycin C	83±1,8	50±1,2	76±6,9		5 749±541
	DNA synthesis in unstimulated test cultures					1 218±185
3	—	86±1,2	47±1,7	5 815±551	6 269±733	24 250±2 484
	Actidione	65±1,4	2,3±0,3	191±64	142±34	32 920±1 750
	Mitomycin C	89±2,4	41±1,4	265±58	3 266±458	20 923±1 626
	DNA synthesis in unstimulated test cultures					6 667±868
4	—	87±0,7	60±0,9		2 021±72	30 668±3 222
	Puromycin	71±1,7	3±1,7		127±13	40 570±4 659
	DNA synthesis in unstimulated test cultures					10 948±673

Legend. 1) The thymidine-^{3}H used in experiments 1 and 2 had a specific activity of 4.1 Ci/mmole and that in experiments 3 and 4 an activity of 11 Ci/mmole. 2) n = number of cultures.

Treatment of the Cells of the Primary Lymphocyte Cultures by Inhibitors of DNA and Protein Synthesis. 1. DNA synthesis was inhibited by mitomycin by the method of Bach and Voynow [6]. The lymphocytes were incubated for 20 min at 37°C with mitomycin C (Serva) in a concentration of 25 μg/ml, washed off once with medium No. 199, and then used for incubation with PHA and for investigation of MF production. 2. Protein synthesis was inhibited by actidione (Calbiochem) and puromycin (Nutr. Biochem. Corp.) in a concentration of 5 μg/ml. Higher concentrations under these experimental conditions caused only a very slight reduction in protein synthesis but substantially reduced the viability of the lymphocytes. The lymphocytes were preincubated with inhibitors for 30 min before incubation of the cells with PHA; during inhibition with PHA, during washing off the cells and cultivation the inhibitors of protein synthesis were present in the medium in the concentration specified above.

Some of the primary cultures were used to investigate DNA and protein synthesis, the viability of the cells, and their morphology in stained preparations, others were used for the study of MF production. To investigate DNA synthesis, thymidine-^{3}H (4 or 11 Ci/mmole) was added in a dose of 2 μCi/ml for 4 h at the end of cultivation. To investigate protein synthesis the cells were cultivated in Minimal Essential Medium (IBL) to which glycine-^{3}H [1] (specific activity 515 mCi/mmole), leucine-^{3}H (75 mCi/mmole), and methionine-^{3}H (49 mCi/mmole), each in a dose of 2 μCi/ml, were added at the beginning of cultivation. The "labeled" cells were treated by a modified method of Robbins [12] and the radioactivity was measured with a Packard TriCarb 2450 liquid scintillation β-spectrometer and expressed in counts/min/10^6 cells. The viability of the cells was studied by the Trypan Blue blocking method.

Removal of Inhibitors from the Culture Medium. The inhibitors were not removed from CM by dialysis for traces of inhibitors were found in it even after dialysis for 48 h. Before investigation of its mitogenic activity the CM of the primary cultures was accordingly treated by gel-filtration on chromatographic columns with Biogel P-6 (Bio-Rad), and eluted with medium TS-199. In this way the low-molecular-weight phase of CM (including inhibitors) was completely replaced by fresh medium TS-199 and the loss of proteins did not exceed 8%.

After treatment as described above the CM was sterilized by filtration through Millipore filters (0.3 μ, Millipore Co.), enriched with antibiotics, L-glutamine, and human serum (up to 15%) heated to 56°C, and then

tested for its mitogenic activity in secondary test cultures of allogeneic lymphocytes purified on columns. DNA synthesis in the test cultures was investigated on the 6th day [3] with the aid of thymidine-^{3}H. The method of labeling was similar to that described for the primary cultures.

EXPERIMENTAL RESULTS

As Table 1 shows, inhibition of DNA synthesis by mitomycin C did not affect blast transformation of the lymphocytes in the primary cultures stimulated by PHA and did not reduce MF production. DNA synthesis is thus unnecessary for MF production. Inhibition of protein synthesis led to depression of blast transformation and of DNA synthesis; under these circumstances not only was the mitogenic activity of CM not reduced, but in every case it was increased by a certain degree. If allowance is made for the protein nature of MF, these observations suggest that this was not a case of MF synthesis de novo, but of liberation of a pre-existing mitogenic protein by the stimulated lymphocytes. The present writers showed previously [2] that on incubation of lymphocytes with PHA and their subsequent rapid destruction by freezing and thawing, followed by cultivation in medium No. 199 under the same conditions as living cells, no MF was found with the CM of these cultures. In the present investigation a decrease in MF production also was observed in the case of death of cells of the primary cultures from an overdose of inhibitor. The lymphocyte evidently contains, not the ready-made MF, but its inactive precursor, which can be activated only in the living cell when stimulated by PHA and not in need of protein synthesis de novo at that moment.

The phenomenon of the increase in mitogenic activity of CM on inhibition of protein synthesis in the MF producer cells described above correlates with earlier observations of the pattern of MF production by lymphocytes in protein-free nutrient media [3]. Despite a more than fivefold decrease in protein synthesis in such media, their MF content was 1.5 times higher than in CM enriched with human serum [3]. So far as the possible mechanism of this phenomenon is concerned, it must be remembered that the CM of the stimulated lymphocytes contained not only MF, but also a combination of biologically active factors [11], including inhibitors of lymphocyte proliferation [4, 9]. In particular, the writers observed previously that CM of human lymphocytes stimulated by PHA contains an inhibitor with a molecular weight of 50,000-100,000 daltons, which sharply inhibits DNA synthesis in test cultures of lymphocytes [4]. The increase in mitogenic activity of CM may be due to a decrease in the content of these inhibitors, as a result of inhibition of their synthesis.

No information on the pre-existence of lymphokines in the unstimulated lymphocyte could be found in the literature. The MF studied by the writers is possibly unique in this respect. Meanwhile the results of the present investigation indicate that in experiments such as these there is a great risk of error as the result of the toxicity of the inhibitor or its incomplete removal from the medium. This must be taken into account both when studying the mechanisms of production of other factors of the lymphokine group and during the interpretation of existing communications.

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