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CELLULAR REGULATION OF SECRETION OF MITOGENIC FACTOR BY HUMAN LYMPHOCYTES IN VITRO

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The principles governing production of mitogenic factor in human lymphocyte cultures stimulated by phytohemagglutinin (PHA) were studied. The mitogenic activity of the culture media were tested in the presence of antibodies against PHA. Irradiation of the lymphocytes sharply increased their ability to produce mitogenic factors. Removal of phagocytic cells by means of iron carbonyl also led to marked stimulation of production of the factor. Irradiations and removal of phagocytic cells were shown to stimulate production of mitogenic factor by different mechanisms.

KEY WORDS: lymphokines; mitogenic factor.

Mitogenic factor (MF) is produced by human and animal lymphocytes in response to nonspecific or antigenic stimulation and is detected by its ability to induce proliferation of lymphocytes in culture. It is difficult at present to decide what type of intercellular interaction in immunogenesis in vivo is mediated through the liberation of MF. Meanwhile a secondary nonspecific mitogenic signal is necessary for the response of B-cells to be induced by antigen [6]. It has recently been shown that mitogenic lymphokines in vitro induce formation of killer cells in response to soluble transplantation antigens and, in the absence of antigens, may induce polyclonal stimulation of precursor cells of T-killers [8, 9]. Antibodies against MF suppress the proliferative response in mixed lymphocyte cultures [7]. Obtaining highly active MF is an essential condition for the study of its properties and role in the development of immune reactions. It must be emphasized that in vitro MF is produced inconstantly, often with low activity [10].

The writers showed previously that secretion of MF is sharply increased when protein synthesis is inhibited in stimulated lymphocytes [3]. However, the heterogeneity of MF [4, 7] arouses misgivings that the factor obtained under these conditions may have an incomplete spectrum of biological activity.

The present writers have suggested that variability of MF production during stimulation of lymphocytes is attributable to the existence of certain mechanisms controlling its production, and the aim of the present investigation was to discover these mechanisms.

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TABLE 1. Effect of Irradiation and Removal of Phagocytic Cells on MF Production (M ± m, n = 4)

Expt. No.	State of cells in primary culture stimulated by PHA	Cells concentration, 10^6/ml	Mitogenic activity of supernatants of primary cultures (stimulation index)		
			dilution of supernatant in test culture		
			1:2	1:4	1:8
1	Untreated	3	8,88±0,60	10,27±1,25	7,14±2,92
	Irradiated	3	22,41±6,62	26,34±3,33	20,11±4,0
	Purified from PC	3	24,81±1,77	27,02±2,87	18,48±1,99
2	Untreated	4	6,72±1,62	3,13±0,6	1,51±0,26
	Untreated	2	4,91±0,36	2,73±0,87	2,25±0,54
	Untreated	1	0,80±0,11	1,31±0,23	0,99±0,11
	Irradiated	4	9,62±0,62	10,11±0,88	7,06±0,45
	Irradiated	2	8,87±1,06	4,98±0,85	3,80±0,46
	Irradiated	1	4,30±0,50	2,78±0,29	2,10±0,28
	Irradiated + untreated	4	8,74±1,01	3,66±0,80	2,49±0,46
	The same	2	10,37±0,65	6,51±0,69	4,28±0,80
	The same	1	1,10±0,24	1,04±0,25	0,95±0,10
3	Untreated	3	3,64±0,66	2,38±0,47	2,65±0,25
	Purified from PC	3	6,84±0,75	3,28±0,82	3,04±0,77
	Irradiated	3	11,82±0,73	9,26±0,63	6,74±0,64
	Irradiated	1,5	10,09±0,71	6,58±1,46	3,70±0,73
	Irradiated + untreated	3	11,67±0,70	4,96±0,37	5,33±0,97
	Purified from PC and then irradiated	3	27,62±1,72	17,54±2,12	10,36±0,65
4	Untreated	3	2,89±0,57	2,43±0,13	
	Irradiated	3	16,21±0,85	18,11±0,49	
	Irradiated	1,5	7,21±0,68	7,72±0,40	
	Irradiated + untreated	3	24,38±6,10	27,66±4,06	
	Irradiated + adherent to plastic	3	17,34±0,64	23,81±1,23	
	Purified from PC	3	4,96±0,30	4,42±0,68	
	Purified from PC + adherent irradiated	3	1,62±0,26	1,66±0,17	
	Purified from PC and then irradiated	3	15,49±0,57	20,57±1,87	
5	Untreated	3	23,01±4,51	10,75±0,70	
	Purified from PC	3	36,25±2,78	38,15±2,54	
	Purified from PC + untreated	3	16,54±1,32	10,79±1,21	
6	Untreated	3	11,32±2,30		
	Purified from adherent cells	3	19,51±1,97		

EXPERIMENTAL METHOD

Cultures of human peripheral blood lymphocytes were used. MF production was induced with the aid of phytohemagglutinin (PHA), and the mitogenic activity of supernatants of lymphocyte cultures stimulated by PHA was investigated in test cultures of allogeneic lymphocytes in the presence of antibodies against PHA [1, 2]. Lymphocytes isolated from blood by centrifugation in a Ficoll–Verografin solution (1.077 g/ml) and washed off in a concentration of 3×10^6 cells/ml were incubated for 40 min at 37°C with 30 μg/ml PHA-P (Difco) in medium No. 199 without serum, with the addition of 10mM HEPES buffer (Microbiological Assoc.), in a volume of 5 ml, directly in plastic Petri dishes (Flow Lab). The cells were sedimented in the same dishes at the end of incubation by centrifugation at 1000 rpm (in special centrifuge attachments), washed once to remove PHA, and cultured at 37°C in medium 199 without serum, made up in Earle's solution (Gibco), enriched with L-glutamine and antibiotics [1, 2], and in an atmosphere of 5% CO_2 in air for 72 h [2]. These cultures were designated primary. The medium of the primary cultures was filtered through millipore filters (0.3 μ, Millipore Co.) and used for determination of MF activity in test cultures. Lymphocytes of the primary cultures were irradiated in a dose of 1600 R on the RUM-11 apparatus (190 kV, 10 mA, 0.5 mm Cu, 46 R/min) on ice in serum-free medium with HEPES buffer, directly in plastic dishes, in which incubation was then carried out with PHA and culture (thus preventing loss of adherent cells during irradiation).

To remove phagocytic cells (PC) the suspension of isolated monocytes was incubated in medium 199 with HEPES buffer and 10% unheated autologous serum with a suspension of iron carbonyl particles measuring 2-5 μ [500 mg to 20 ml medium, cell concentration $(5\text{-}6) \times 10^6$/ml] for 40 min at 37°C on a water bath, with careful and constant shaking. The particles of iron and the cells phagotosing it were removed by means of a powerful permanent magnet. Adherent cells were obtained by incubation of 5 ml of the monocyte suspension in medium 199 with 10% autologous serum (3×10^6 cells/ml) for 1 h at 37°C in plastic dishes; nonadherent cells were washed out by pipeting twice. In some experiments primary cultures were prepared from a mixture of equal numbers of cells treated by different methods, which were then incubated with PHA and cultured as described above.

MF activity was investigated in test cultures of allogeneic lymphocytes after dilution of the supernatants of the primary cultures with medium 199 with 10% heat-inactivated autologous serum and addition of antibodies against PHA in an amount ensuring complete inactivation of mitogenic activity of PHA-P in a concentration of 15 μg/ml [1, 2]. Antibodies were isolated from rabbit antisera against PHA by immunosorption on PHA bound with CBr-Sepharose. On the 5th-6th day [2] DNA synthesis in the test cultures was investigated by studying incorporation of ^{3}H-thymidine, and the radioactivity of cells sedimented on the millipore filters [5] was measured by means of a Tri-Carb 2450 spectrometer (Packard). One determination of MF activity included four parallel tests. Mitogenic activity was expressed by a stimulation index, the quotient obtained by dividing the index of DNA synthesis in test cultures in the presence of the test supernatant by the index of DNA synthesis in control test cultures containing PHA-P (15 μg/ml) and antibodies against PHA. This last index was indistinguishable in all cases from that in the control test cultures without PHA or was a little below it.

EXPERIMENTAL RESULTS

As Table 1 shows, irradiation of the lymphocytes sharply increased MF production. The effect was observed with different concentrations of lymphocytes in the primary cultures (experiment 2) and different dilutions of the test supernatants. Protein synthesis in the primary cultures of irradiated cells (based on ^{3}H-glycine [3]) was 89 ± 6.7% compared with the unirradiated cells ($P > 0.05$). To verify that the effect of radiation was not connected with the existence of radiosensitive suppressor cells, inhibiting MF production, an equal number of unirradiated cells was added to the primary cultures of irradiated lymphocytes (experiments 2, 3, and 4). In no case did they suppress the increase in MF production induced by irradiation (compared with primary cultures with a concentration of irradiated cells corresponding to their concentration in the "mixed" cultures. PC were not necessary for the stimulating effect of irradiation to be manifested (experiments 3 and 4). Adherent cells did not suppress the effect of irradiation (experiment 4).

Purification of the monocytes to remove PC by means of iron carbonyl significantly increased MF production (experiments 1, 3, 4, and 5). Removal of cells adherent to the plastic also stimulated MF production (experiment 6). Stimulation of MF production induced by removal of PC ceased on the addition of the original monocytes, not purified from PC (experiment 5) and also of adherent irradiated cells (experiment 4).

The results described above indicate that irradiation and removal of PC stimulate MF production in serum-free cultures of human lymphocytes treated with PHA. The effect of irradiation was not connected with the existence of radiosensitive cells suppressing MF production and was not suppressed by unirradiated cells. Irradiation evidently directly stimulated secretion of MF from stimulated lymphocytes. The effect of irradiation cannot be explained by an increase in cell mortality in the primary cultures (in the irradiated cultures 25 ± 4% of the initial number of cells survived, compared with 36 ± 5% in the unirradiated cultures ($P > 0.05$)), for destruction of lymphocytes treated with PHA prevented the appearance of active MF in the culture [1]. The action of irradiation was not mediated through its effect on PC, for the effect of removal of PC was completely suppressed by monocytes unpurified from PC, and also by irradiated adherent cells, whereas the effect of irradiation was not sensitive to the inhibitory action of PC contained in the adherent fraction of cells and unpurified monocytes. This last observation also showed that the inhibitory effect of PC was not mediated by soluble products suppressing incorporation of H^3-thymidine in the test cultures. The mechanisms of stimulation of MF production during irradiation of lymphocytes and removal of PC, and also in response to inhibition of protein synthesis [3] require further study. Whatever the case, the results of the present investigation can be used to obtain highly active MF, required to study the role of mitogenic lymphokines in immunogenesis.

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