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Effect of L-Asparaginase and Hydrocortisone on Human Lymphocyte Transformation and Production of a Mononuclear Leucocyte Chemotactic Factor *in vitro*

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Summary. Human peripheral lymphocytes stimulated *in vitro* with PHA produce a soluble factor which is chemotactic for homologous monocytes. The synthesis of this factor was found to precede the blastogenic response as measured by [^{3}H] thymidine incorporation. In cultures of unseparated leucocytes the maximum of chemotactic activity was detected after 24 hr, whereas in supernatants from purified lymphocyte suspensions the maximal synthesis occurs after 72 hr. High doses of L-asparaginase from *E. coli* which have been found to prevent lymphocyte transformation completely have no influence on the production of the chemotactic factor. Therefore it seems possible that the induction of DNA synthesis by PHA and its effect on the production of a chemotactic factor depend on different biochemical mechanisms. In contrast hydrocortisone leads to a dose-dependent inhibition of both DNA synthesis and chemotactic response.

INTRODUCTION

Peripheral lymphocytes stimulated *in vitro* with mitogens produce a variety of soluble substances with biological activities (for review see Pick and Turk, 1972). Altman, Snyderman, Oppenheim and Mergenhagen (1973) have recently described a quantitative assay for a chemotactic factor produced by human peripheral leucocytes after addition of phytohaemagglutinin (PHA) or purified protein derivate (PPD). They have shown that lymphocytes produce this factor and that the synthesis of the chemotactic factor precedes DNA synthesis induced by PHA. L-Asparaginase from *E. coli* is known to cause a dose-dependent inhibition of DNA synthesis in PHA-stimulated lymphocyte cultures (Ohno and Hersh, 1970). The blastogenic response of human peripheral lymphocytes can also be inhibited by corticosteroids (Nowell, 1961; Caron, 1969).

The aim of this report was to investigate the effect of L-asparaginase and hydrocortisone on the production of a human lymphocyte chemotactic factor.

MATERIALS AND METHODS

Lymphocyte culture system

Our lymphocyte culture system has been described elsewhere in detail (Rühl, Kirchner

and Bochert, 1971). Heparinized peripheral blood from healthy adults was sedimented with 10 per cent dextran (Macrodex^{R}, Knoll AG, Germany) and the white cell rich plasma removed. A total volume of 3 ml of cell suspension containing 3×10^6 viable cells was incubated in 16 x 125 mm disposable plastic tubes (Falcon) in complete culture medium. The medium used was RPMI 1640 (Biocult) supplemented with 5 per cent heat-inactivated fresh human serum, L-glutamine (2 mM/ml), penicillin (100 u/ml) and streptomycin (100 μg/ml). Leucocyte cultures for the chemotaxis assay were set up without any serum. In some experiments purified lymphocyte suspensions were prepared by passing the white cell-rich plasma through nylon fibre columns according to the technique of Brandt, Börjeson, Nordén and Olsson (1962). The cultures were incubated at 37° in an atmosphere of 5 per cent CO_2 and 95 per cent air for various periods (generally 24 h for the chemotaxis assay and 48 h for the lymphocyte stimulation test). Test substances were dissolved in sterile saline and added in various concentrations at the beginning of the culture period. The following cultures were started: control cultures with 0·1 ml saline, PHA alone (PHA-P, Difco), PHA + L-asparaginase (L-asparaginase from *E. coli*, Behringwerke, Germany) and PHA +

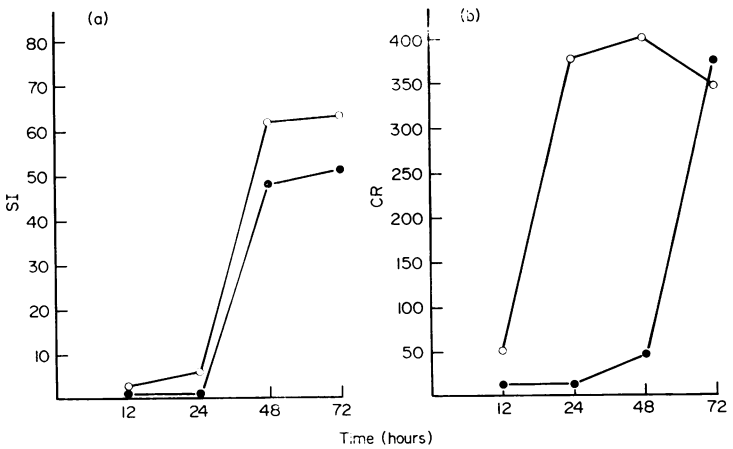

FIG. 1. Kinetics of (a) [^{3}H]thymidine incorporation and (b) production of a chemotactic factor in unseparated leucocyte cultures and in purified lymphocyte cultures. For the lymphocyte transformation test, cells were incubated with 1·0 μg/ml PHA and with 10 per cent autologous serum for 48 h; for the chemotactic assay cells were incubated with 0·3 μg/ml PHA and without any serum for 24 h. SI = stimulation index, mean of triplicate cultures. CR = chemotactic response (total number of migrated cells in twenty-five oil immersion fields), mean of triplicate samples. (○) Unseparated leucocytes. (●) Purified lymphocytes.

hydrocortisone (Actocortin^{R}, Schering AG, Germany). Two microcuries of tritiated thymidine ([^{3}H]thymidine, specific activity 2 Ci/mM) were added for the last 4 h of incubation. The cells were then treated as described previously and the radioactivity in the trichloracetic acid insoluble material was measured in a Packard scintillation counter (Rühl *et al.*, 1971). The arithmetic mean of triplicate cultures was determined and the results expressed as counts per minute per culture (cpm) or as stimulation indices (SI = cpm in treated cultures/cpm in untreated control cultures).

Chemotaxis assay

The *in vitro* chemotaxis assay was performed following the technique described by Altman *et al.* (1973). Chemotaxis chambers were purchased from Neuroprobe Incorporated,

Bethesda, USA. The two compartments of the chamber were separated by a polycarbonate filter with a 5·0 μm pore size (Nucleopore, Wallabs, Incorporated, San Rafael, California, USA). Cells tested for chemotactic responsiveness were obtained by separating human peripheral blood on a Ficoll–Hypaque gradient (Boÿum, 1968). 2×10^6 mononuclear cells/ml were filled into the upper compartment of the chamber after having been washed twice with Hanks's solution (Biocult). Experimental supernatants were obtained from leucocyte cultures after an incubation period of 24 h unless specified. The cell suspensions were centrifuged at 1500 *g* for 30 min and the supernatants stored at 4°. Control supernatants were obtained from unstimulated cultures. In some experiments either PHA, or hydrocortisone, or L-asparaginase was added to the supernatants just prior to the assay. Thirty per cent of the supernatant was mixed with 70 per cent of Hanks's solution and filled into the lower compartment of the chamber. The chambers were incubated for 90 min at 37°. Afterwards the filters were removed and stained with May–Grünwald–

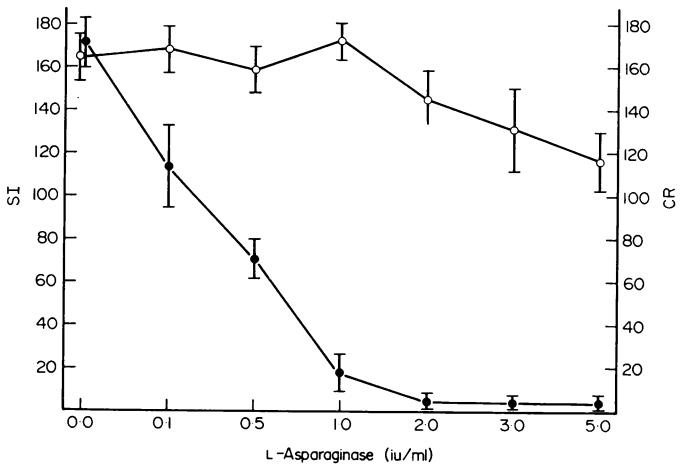

FIG. 2. Effect of L-asparaginase on lymphocyte transformation and production of a chemotactic factor. For the lymphocyte transformation test cells were incubated with 1·0 μg/ml PHA and with 10 per cent autologous serum for 48 h; for the chemotactic assay cells were incubated with 0·3 μg/ml PHA and without any serum for 24 h. L-Asparaginase was added at the beginning of the culture. SI = stimulation index, mean of triplicate cultures $\pm$ 1 s.e. CR = chemotactic response (total number of migrated cells in twenty-five oil immersion fields), mean of triplicate samples $\pm$ 1 s.e. (●) [^{3}H]Thymidine incorporation. (○) Chemotactic response.

Giemsa. In quantifying the chemotactic response only completely migrated monocytic cells in twenty-five random high power fields were counted and the arithmetic mean of triplicate samples was determined. The chemotactic response (CR) was determined by subtraction of the number of migrated cells in chambers containing control supernatants from the total number of cells migrated in chambers with experimental supernatants.

RESULTS

COMPARISON OF UNSEPARATED LEUCOCYTES AND PURIFIED LYMPHOCYTES

Purified lymphocyte suspensions were prepared from healthy adults by using a nylon fibre column and cultured *in vitro* in the absence of serum for various periods. Unseparated

leucocyte suspensions from the same donors were used as controls. The time course of the production of the chemotactic factor differed markedly between the two cell sources tested (Fig. 1). In supernatants from unseparated leucocyte cultures the maximum of activity was detected after 24 h of incubation without further increase after 48 or 72 h. In contrast the maximum detected in supernatants from purified lymphocyte cultures occurs after 72 h, whereas after 24 h no activity was found. Lymphocyte transformation as measured by [^{3}H]thymidine incorporation was found to be optimal in both types of cell cultures between 48 and 72 h of incubation, whereas after 24 h no activity was detected.

TABLE 1

CHEMOTACTIC ACTIVITY OF CONTROL SUPERNATANTS AND SUPERNATANTS FROM PHA-STIMULATED CULTURES AFTER ADDITION OF L-ASPARAGINASE AND HYDROCORTISONE JUST PRIOR TO THE ASSAY

Supernatant	Chemotactic activity*	
	Experiment I	Experiment II
Control	12±4	28±7
Control + L-asparaginase (0·1 iu/ml)	11±3	33±6
Control + L-asparaginase (1·0 iu/ml)	22±4	21±2
Control + L-asparaginase (10·0 iu/ml)	15±2	41±6
PHA	396±32	273±19
PHA + hydrocortisone (10·0 μg/ml)	428±13	321±18
PHA + hydrocortisone (50·0 μg/ml)	346±28	297±5
PHA + hydrocortisone (100·0 μg/ml)	372±33	257±11
PHA + hydrocortisone (200·0 μg/ml)	313±16	203±14

* Mean of triplicate samples ±s.e. expressed as cells per 25 oil immersion fields.

THE EFFECT OF L-ASPARAGINASE ON LYMPHOCYTE TRANSFORMATION AND PRODUCTION OF THE CHEMOTACTIC FACTOR

As shown in Fig. 2, a dose-dependent inhibition of DNA synthesis in stimulated cultures was observed after addition of various doses of L-asparaginase at the beginning of the culture time. The addition of as much as 0·1 iu/ml reduced [^{3}H]thymidine incorporation after 48 h markedly. Using doses between 0·5 and 5·0 iu/ml no significant transformation was observed; doses higher than 5·0 iu/ml were found to be toxic. The same figure shows the effect of L-asparaginase on the synthesis of the chemotactic factor. It was clearly demonstrated that even treatment with high doses of L-asparaginase, which inhibit the lymphoproliferative response completely, do not influence the synthesis of the chemotactic factor in PHA cultures. L-Asparaginase by itself is not chemotactic for human monocytes (Table 1).

EFFECT OF HYDROCORTISONE ON LYMPHOCYTE TRANSFORMATION AND PRODUCTION OF THE CHEMOTACTIC FACTOR

Hydrocortisone as well as L-asparaginase lead to a dose-dependent inhibition of the response to PHA. In contrast to L-asparaginase hydrocortisone also has an inhibitory effect on the synthesis of the chemotactic factor (Fig. 3). This inhibition is dose-dependent. As shown in Table 1 hydrocortisone has no inhibitory influence on the chemotactic activity of supernatants from PHA-stimulated cultures when added just prior to the assay.

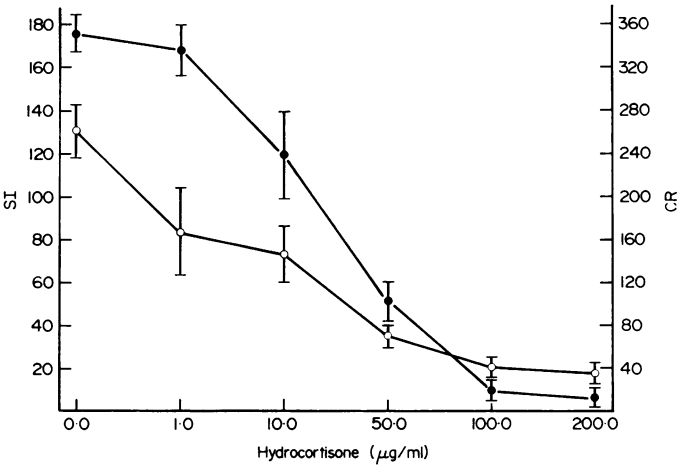

FIG. 3. Effect of hydrocortisone on lymphocyte transformation and production of a chemotactic factor. For the lymphocyte transformation test cells were incubated with 1·0 μg/ml PHA and with 10 per cent autologous serum for 48 h; for the chemotactic assay cells were incubated with 0·3 μg/ml PHA and without any serum for 24 h. Hydrocortisone was added at the beginning of the culture. SI = stimulation index, mean of triplicate cultures ± 1 s.e. CR = chemotactic response (total number of migrated cells in twenty-five oil immersion fields), mean of triplicate samples ± 1 s.e. (●) [^{3}H]Thymidine incorporation. (○) Chemotactic response.

DISCUSSION

There is increasing evidence that human peripheral lymphocytes are the source of a whole range of biologically active substances called lymphokines. It has been shown that some of these soluble lymphocyte products in man and animals have chemotactic activity. Recently Altman *et al.* (1973) reported a quantitative method to investigate a human chemotactic factor produced by stimulated lymphocytes *in vitro*. This assay seems to be a sensitive *in vitro* correlate of delayed type hypersensitivity.

Our experiments presented in this report have shown that human peripheral leucocytes stimulated with PHA *in vitro* synthesize a chemotactic factor affecting human macrophages. We were able to confirm the data given by Altman *et al.* (1973) that lymphocytes are the source of cells producing this factor. Experiments in which purified lymphocyte populations were used have shown that macrophages or polymorphonuclear cells are not necessary for the synthesis of the factor; the presence of these cells however, seems to change the kinetics of the production of the chemotactic factor in a characteristic manner.

The production of the chemotactic factor has been found to precede detectable [^{3}H]thymidine incorporation in PHA-stimulated cultures (Altman *et al.*, 1973). A comparable temporal dissociation was found in the production of the migration inhibition factor (Rocklin and Ratcliffe, 1972). Kirchner, Altman, Blaese and Oppenheim (1973) found that after irradiation with 900 rads the blastogenic response of chicken lymphocytes was completely inhibited whereas the synthesis of the chemotactic factor in stimulated cultures was not influenced.

After treatment with L-asparaginase we observed a dose-dependent inhibition of the PHA response, an effect which is well established now (Astaldi, Burgio, Krč, Genova and Astaldi, 1969; Ohno and Hersh, 1970). It has been shown that the inhibition of lympho-

cyte blastogenesis by L-asparaginase was not due to cytotoxity but rather to specific depletion of exogenous and endogenous L-asparagine (Ohno and Hersh, 1970). In contrast to the effect of L-asparaginase on DNA synthesis we found no inhibition of the production of the chemotactic factor in PHA-stimulated cultures. As shown in Fig. 2 even high doses of the enzyme have no inhibitory effect on the synthesis of the factor. In order to exclude the possibility that our preparation of L-asparaginase contains contaminants with chemotactic activity the enzyme was added to supernatants from unstimulated cultures in various concentrations. The lack of activity found in these experiments excludes this possibility. In summary our results indicate that the metabolic processes which lead to the production of the chemotactic factor were not influenced by L-asparaginase, in contrast to those inducing DNA synthesis. Comparable results were recently reported by Schlag (1971) who found that L-asparaginase has no inhibitory effect on MIF-production in guinea-pigs.

The inhibitory effect of corticosteroids on the blastogenic response is well established (Nowell, 1961; Caron, 1967; Caron, 1969), though the mode of action is still unknown. Elrod and Schrek (1966) have shown that hydrocortisone was not toxic on human peripheral lymphocytes over the range of 10–100 μg/ml and it can be assumed that corticosteroids will prevent the lymphoproliferative action of PHA on lymphocytes *in vitro* without being toxic. We found a dose-dependent inhibition of PHA-induced DNA synthesis and in contrast to the effect of L-asparaginase also an inhibitory effect on the production of the chemotactic factor. These findings are not explicable at the present time.

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