INHIBITION OF TRANSFORMATION OF PHYTOHEMAGGLUTININ-STIMULATED LYMPHOCYTES BY NEUTRAL SH-DEPENDENT SPLENIC PROTEINASE

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The effect of neutral SH-dependent splenic proteinase on the intensity of transformation of human peripheral blood lymphocytes was studied. Proteinase (1-10 μg ml) was shown to inhibit transformation of phytohemagglutinin (PHA)-stimulated lymphocytes but to have no effect on spontaneous transformation. Proteinase did not change the mitogenic properties of PHA and had no cytotoxic action. Consequently, inhibition of transformation of the stimulated lymphocytes either was the result of the direct action of proteinase on the cell or was connected with the formation of a suppressor factor.

KEY WORDS: proteinase; transformation of lymphocytes.

Much attention has recently been paid to proteinases and their inhibitors as possible regulators of cell differentiation and growth [9]. Data have been obtained in various laboratories to show that trypsin, chymotrypsin, pronase, thrombin, and other proteinases, if added to a cell culture, induce transformation and proliferation of lymphocytes [4-7, 11-13]. Neutral serine proteinases of polymorphonuclear leukocytes – elastase and cathepsin G – also have a similar action [10, 13].

Assuming that splenic proteinases can take part in the regulation of the immune response in vivo, it was decided to study the effect of neutral SH-dependent bovine splenic proteinase on transformation of human peripheral blood lymphocytes.

EXPERIMENTAL METHODS

Neutral proteinase, hydrolyzing histones, was isolated from the bovine spleen by a modified version of the method described previously [2]. Instead of on DEAE-cellulose, chromatography was carried out on CM-cellulose (from Serva) at pH 6.5 in 0.02M phosphate buffer, in the presence of 10^{-4}M EDTA and dithiothreitol (DTT), followed by gel filtration of the unadsorbed fraction on Sephadex G-75 (Superfine, from Pharmacia). The active fraction was then chromatographed on CM-cellulose at pH 5.85 in 0.02M acetate buffer. Proteinase present in the unadsorbed fraction was used in the experiments. The isolated preparations of proteinase gave three bands on electrophoresis in polyacrylamide gel. The histone-hydrolyzing activity of the enzyme was 2000 times greater than that of the extract. The experiments were carried out with three different preparations of the enzyme. Besides proteinase from bovine spleen, in two experiments a partially purified preparation of the enzyme from human spleen was used. Before the experiment the proteinase was activated by the addition of EDTA and DTT ($1 \cdot 10^{-4}$M). The final concentration of activators in the sample with the lymphocyte culture was $5 \cdot 10^{-7}$M. The same concentrations of EDTA and DTT were present in the control sample.

Lymphocytes were isolated from donors' blood by centrifugation of a leukocyte suspension in a verografin–Ficoll gradient (d = 1.078) for 40 min at 400g [3]. The cells were washed twice with medium No. 199, after which $2.5 \cdot 10^5$ cells were cultured as described previously [1]. The time of addition of the proteinase, phytohemagglutinin (PHA), and serum to the lymphocyte culture depended on the aims of the particular experi-

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TABLE 1. Effect of Splenic Proteinase on Intensity of Transformation of Human Peripheral Blood Lymphocytes

Experimental conditions	Concentration of proteinase, μg/ml	Time of addition of serum, h	Incorporation of [^{3}H]thymidine, cpm per culture*	Degree of inhibition, %
Lymphocytes + PHA	—	6	51 000	0
PHA + proteinase from bovine spleen	10	6	900	98
PHA + proteinase from human spleen	10	6	2 000	96
PHA + proteinase from bovine spleen	10	0	49 000	4
Lymphocytes (spontaneous transformation) + proteinase from bovine spleen	—	6	560	0
	10	6	540	4

*Here and in Table 2, mean of five determinations.

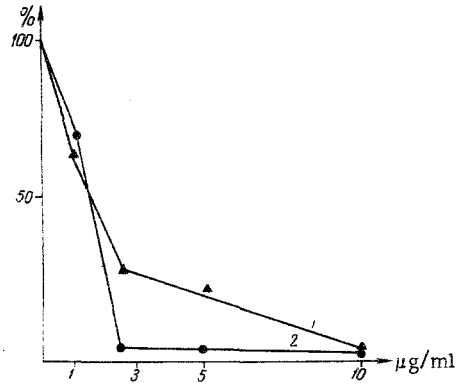

Fig. 1. Intensity of transformation of human peripheral blood lymphocytes, stimulated by PHA, as a function of proteinase concentration in culture medium. 1 and 2) Results of two experiments. Abscissa, proteinase concentration (in μg/ml); ordinate, intensity of lymphocyte transformation (in %).

ments and is given in the section Experimental Results. [^{3}H]Thymidine (10 Ci/mmole) was added to each culture in a dose of 1 μCi 3 h before the end of cultivation. The results were calculated after measuring the radioactivity of samples from five parallel cultures.

EXPERIMENTAL RESULTS

The proteinase used in the experiments is a low-molecular-weight SH-dependent enzyme, which hydrolyzes histone and casein in a neutral medium [1]. Since the activity of proteinase is inhibited by serum [1], the conditions for culture of lymphocytes ensuring manifestation of activity of the enzyme and also a high intensity of transformation of PHA-stimulated lymphocytes were selected in preliminary experiments.

Preincubation of lymphocytes with PHA in serum-free medium No. 199 for 6 h followed by addition of 5% inactivated serum was shown not to reduce the intensity of their transformation compared with the control, in which PHA and serum were added simultaneously. These conditions of culture were used in all subsequent experiments; the duration of action of proteinase was controlled by addition of serum.

TABLE 2. Dependence of Intensity of Lymphocyte Transformation on Duration of Action of Proteinase (5 μg/ml)

Duration of action of proteinase, min	Incorporation of [^{3}H]-thymidine, cpm/culture		Degree of inhibition, %
	without proteinase	with proteinase	
0	40 000	—	0
30	36 000	33 000	8
60	43 000	40 000	8
120	40 000	2 200	95
240	32 000	1 000	97
360	30 000	900	97

Proteinase from bovine spleen, acting on the lymphocyte culture for 6 h, reduced the intensity of transformation of PHA-stimulated lymphocytes but did not affect the level of spontaneous transformation (Table 1). Similar results were obtained in experiments with partially purified proteinase from human spleen (Table 1). Addition of serum to the lymphocyte culture simultaneously with the proteinase completely abolished its suppressor effect (Fig. 1). This proves that inhibition of lymphocyte transformation was connected with the proteolytic activity of the proteinase preparations.

The first step in the examination of the mechanisms whereby proteinase could modify the response of the stimulated lymphocytes was to study its cytotoxicity. Preincubation of lymphocytes for 6 h with proteinase in a concentration effectively suppressing transformation did not increase the number of dead cells compared with the control.

PHA, treated with proteinase, had the same mitogenic activity as the original preparation. Consequently, modification of PHA was not the cause of the observed inhibition of transformation.

Investigation of the effect of different concentrations of proteinase showed that in a concentration of 10 μg/ml the enzyme caused practically complete suppression of transformation in all experiments (Fig. 1). In a concentration of 1.0-2.5 μg/ml the degree of inhibition of transformation varied considerably in individual experiments, possibly on account of differences in the sensitivity of cells obtained from different donors. A study of the time of action of proteinase required for manifestation of the suppressor effect showed that the intensity of transformation fell sharply 2 h after the beginning of incubation (Table 2). In some experiments this effect appeared after 4 h.

The effect of this proteinase on lymphocytes thus differed from the action of proteinases hitherto investigated as regards both the type of the effect and the time of its appearance. Proteolytic enzymes studied previously had a stimulating action on the cells either by potentiating the effect of the mitogen [8, 10] or by inducing a mitogenic action [4-7, 11-13]. This stimulating effect was manifested usually after brief action of the proteinases (from 5 to 30 min). The proteinase studied in the present experiments, incidentally, is not an enzyme with a broad spectrum of action, for it does not hydrolyze hemoglobin, serum albumin, or several other protein synthetic substrates [1].

The results show that inhibition of transformation of PHA-stimulated lymphocytes by proteinase is not due to modification of the mitogen and is not connected with cytotoxicity. It can thus be concluded that either it is the result of the direct action of the proteinase on the cell or it is connected with formation of a suppressor factor. No data are yet available for a more detailed discussion of the mechanism of the suppressor action of this proteinase. However, the results described above show that among the cell proteinases which participate in the immune response there are enzymes which can act not only as immunostimulators [8, 10, 13, 14], but also as immunosuppressors.

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IMMUNOLOGIC REACTIVITY OF (C57BL/6 × A/Sn)F_1 MICE IN MIXED MYCOPLASMA-VIRUS INFECTION

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Mixed infection of hybrid mice, highly resistant to Rauscher virus, with this virus and Mycoplasma arthritidis was accompanied by progressive inhibition of populations of splenic rosette-forming (RFC) and plaque-forming (PFC) cells and led to induction of malignant erythroblastosis, cytologically identical with Rauscher's leukemia. During mixed infection of the hybrid mice with Acholeplasma laidlawii and Rauscher virus the immune response was almost completely suppressed on the 21st day and considerable splenomegaly was observed, but by the 62nd day of infection the RFC and PFC populations and also the weight of the spleens had regained the control level. The possible role of mycoplasmas in the induction and development of Rauscher's leukemia is discussed.

KEY WORDS: Mycoplasma arthritidis; Acholeplasma laidlawii; Rauscher leukemia virus; immune response.

Mixed infection of (C57BL/6×A/Sn)F_1 mice highly resistant to Rauscher virus with this virus and Mycoplasma arthritidis leads to induction of transplantable malignant erythroblastosis, cytologically identical with Rauscher leukemia in sensitive mice [2]. Similar infection of mice of the same line with Rauscher virus and Acholeplasma laidlawii is accompanied by short-lasting splenomegaly followed by regression and does not lead to leukemia [3], as does mixed infection with the virus and mycoplasmas. An abundance of data has been obtained to show that these agents persist in mixed infection [1-3], but there is virtually no information on immunologic reactivity.

The object of this investigation was to study the effect of mixed mycoplasma-virus infection on splenic rosette-forming (RFC) and plaque-forming (PFC) cells and on serum hemagglutinin titers in infected mice.

EXPERIMENTAL METHODS

(C57BL/6×A/Sn)F_1 mice aged 6-8 weeks were used. The mycoplasmas and virus were obtained as described previously [2, 3].

The mice were infected intraperitoneally with M. arthritidis with a titer of 10^8 colony-forming units (CFU)/ml or with A. laidlawii with a titer of 10^9 CFU/ml, in a dose of 0.5 ml, and with Rauscher virus in a titer of 10^5 ID_{50}/ml in a dose of 0.1 ml. Control mice were inoculated with broth in which the mycoplasmas were grown.

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