INVOLVEMENT OF PROTEOLYTIC ACTIVITY IN EARLY EVENTS IN LYMPHOCYTE TRANSFORMATION BY PHYTOHEMAGGLUTININ

Masaki Saito*, Takiko Yoshizawa*, Takaaki Aoyagi**
and Yoshitaka Nagai*

*Department of Cell Chemistry, Institute of Medical Science, University of Tokyo, P.O. Takanawa, Tokyo, Japan and **Institute of Microbial Chemistry, Shinagawa-ku, Tokyo, Japan

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SUMMARY: The stimulation by phytohemagglutinin (PHA) of DNA synthesis in cultured blood lymphocytes of guinea pig was markedly inhibited by addition of leupeptin, a well-characterized, powerful protease inhibitor of tripeptide nature. About 30 to 40 per cent inhibition was observed at 40 μg/ml of leupeptin when leupeptin was added 30 min prior to or together with PHA. Per cent inhibition by the appropriate amount of leupeptin was proportional to the amount of PHA added in the range of 0.6 to 3.0 μg of PHA at which the per cent inhibition reached maximum. This inhibitory effect of leupeptin on PHA stimulation was abolished when the lymphocytes were preincubated with PHA for more than 10 min before addition of leupeptin or preincubated with leupeptin for more than 60 min prior to PHA addition.

In an effort to investigate the mechanism whereby lymphocytes are stimulated to transform into blastoid cells, we have examined the effect of well-characterized protease inhibitors of peptide nature, such as leupeptin, antipain etc., on the stimulation of DNA synthesis by phytohemagglutinin (PHA). Leupeptin and antipain are powerful and specific protease inhibitors first isolated from Actinomycetes by Umezawa et al. Leupeptin (acetyl- or propyl L-Leu-L-Leu-L-Arginal) inhibits the proteolysis by plasmin, trypsin, papain (1,2,3) and a lysosomal peptide hydrolase, cathepsin B (4). Antipain (OH-L-Phe-CO-L-Arg-L-Val-L-Arginal) also inhibits trypsin and papain, but differs from leupeptin in other parameters of inhibition. In a preliminary paper (5) we have reported that leupeptin markedly inhibits the stimulation of ^{3}H-thymidine incorporation by PHA but antipain dose not. The present work was performed to analyse more precisely the mechanism underlying the inhibitory effect of leupeptin on PHA stimulation of DNA synthesis in blood small

Reprint requests should be sent to Dr. Y. Nagai.

lymphocytes. It is emphasized in this report that some specific proteolytic activity is involved in the early events of lymphocyte transformation by PHA.

MATERIALS AND METHODS

Peripheral Blood Lymphocytes: Outbred adult guinea pigs weighing about 500 g were exsanguinated by cardiac puncture. Peripheral blood lymphocytes were collected aseptically from lymphocyte-rich plasma and the preparation of cell suspension was carried out as previously described (5,6).

Lymphocyte Culture: Lymphocyte culture was carried out essentially according to the same method as previously described (5,6). RPMI (Rosewell Park Memorial Institute) 1640 medium was supplemented with heat-inactivated fetal calf serum (10%, v/v) and antibiotics (streptomycin, 100 μg/ml and penicillin, 100 U/ml). All cultures contained 1 ml of cell suspension (5-9 x 10^5 small lymphocytes/ml) and were set up in triplicate. PHA (PHA-P, Difco Labs., Detroit) and/or protease inhibitor (supplied from Institute of Microbial Chemistry, Tokyo) at the given concentrations were added at various incubation periods. The culture tubes (10 x 10 mm test tube) were covered with metal lids and placed in a CO_2 incubator at 37°C gassed with a mixture containing 5% CO_2 and 95% air.

Measurement of Response: After 44-48 hr cultivation, 0.5 μCi of ^{3}H-thymidine ([6-^{3}H] thymidine, 5 Ci/mmole, Daiichi Pure Chemicals Co., Tokyo) was added to each culture. 20 hours later, the culture tubes were cooled on ice and 2 ml of ice-cold Hank's solution was added to each. The cell suspension from each tube was transferred onto a glass fiber membrane (GF/C 2.5 cm, Whatman) placed in a Manifold multiplex sample collector (Millipore). Each culture tube was washed with approximately 5 ml of Hank's solution. This washing fluid was poured onto the same membrane, which was subsequently washed with a large excess of Hank's solution, physiological saline, cold 5% TCA and methanol successively. The membrane were subjected to dryness and placed into scintillation vials. The dried filter-membranes were counted for radioactivity in a toluene-based scintillation solution as previously described (5,6).

RESULTS

Fig.1 shows a steep rise in thymidine incorporation as a function of PHA dose with a peak at 9-30 μg PHA/ml-culture and with a sharp decrease above 40 μg PHA/ml-culture owing to PHA cytotoxicity. The effect of leupeptin on ^{3}H-thymidine incorporation into the acid-insoluble materials in the presence and absence of PHA was examined. Table 1 shows that 53.6 μg of leupeptin inhibited ^{3}H-thymidine incorporation by about 40 per cent when added simultaneously with PHA (30 μg), while unaffecting control cultures. When added 10 min after addition of PHA, however, leupeptin exhibited only a weak inhibitory effect, and leupeptin added more than 30 min after PHA addition showed the effect no more (Table 1 and 2). Preincubation of leupeptin with cells for more than 60 min before

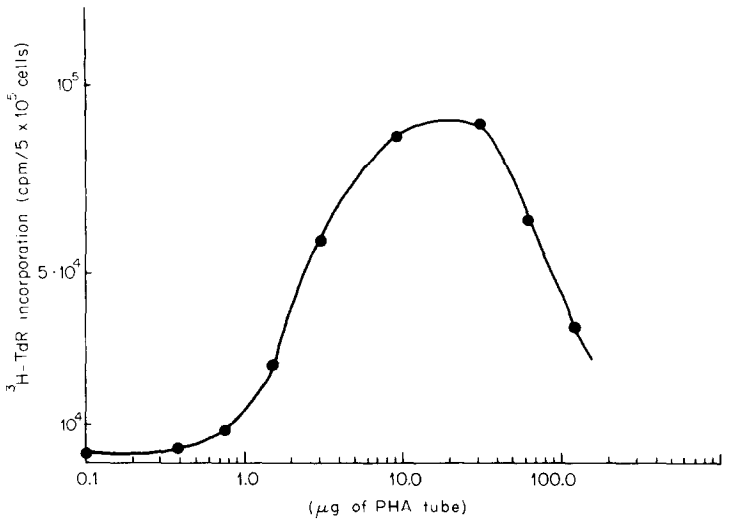

Fig.1. ^{3}H-thymidine incorporation in guinea pig blood lymphocytes cultures stimulated by phytohemagglutinin (PHA-P). Lymphocytes were cultured in vitro with various amounts of PHA-P for 48 hours at 37°C and then 0.5 μCi of ^{3}H-thymidine was added to each culture (about 5 x 10^5 small lymphocytes). Radioactivity was determined 20 hours after addition of ^{3}H-thymidine as described in the text.

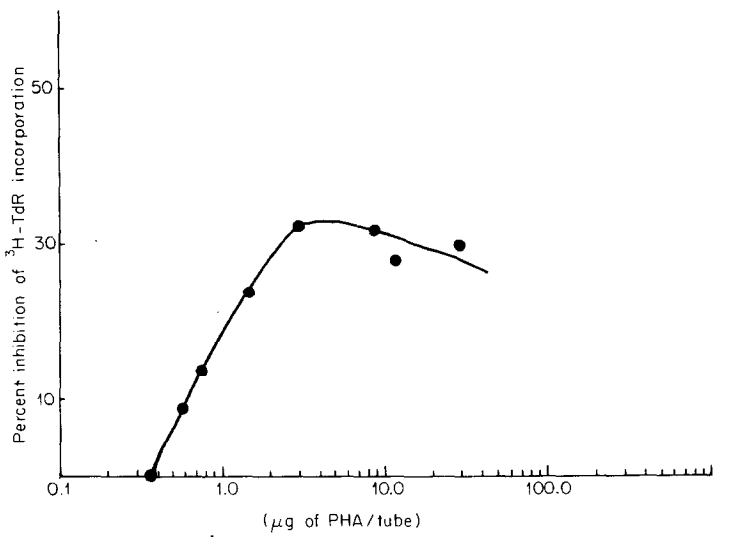

Fig.2. Inhibitory effect of leupeptin on PHA stimulation of ^{3}H-thymidine incorporation at various doses of PHA-P. Leupeptin (80 μg) was added to triplicate 1-ml cultures (about 6 x 10^5 small lymphocytes) at the time of PHA addition. ^{3}H-thymidine incorporation in the presence of PHA alone is expressed as 100 % at each PHA dose. Per cent inhibition represents:

$$100 - \frac{\text{^{3}H-TdR Uptake in the presence of PHA and Leupeptin}}{\text{^{3}H-TdR Uptake in the presence of PHA alone}} \times 100$$

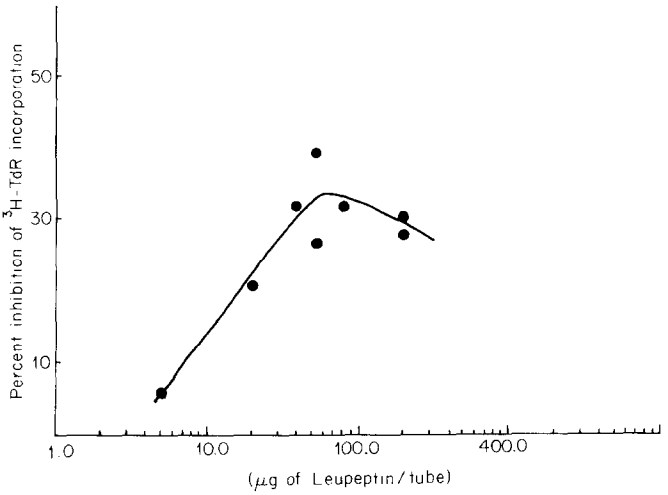

Fig.3. Inhibitory effect of leupeptin on PHA stimulation of ^{3}H-thymidine incorporation at various doses of leupeptin. Various doses of leupeptin (5 to 200 μg/culture) were added to each culture (about 6 x 10^5 small lymphocytes) at the same time of PHA addition (3 μg/culture). Per cent inhibition is expressed as shown in Fig.2.

PHA addition abolished almost comletely the inhibitory effect of leupeptin on PHA stimulation as shown in Table 1 and 2. However, its preincubation with culture medium (10% FCS-RPMI 1640) alone for a long time (more than several hours) did not exert any influence on its inhibitory effect.

The inhibitory effect of leupeptin on PHA stimulation was further investigated at various doses of PHA. The result is shown in Fig.2. Per cent inhibition of ^{3}H-thymidine incorporation by 80 μg of leupeptin increased linearly up to about 30 per cent inhibition in the range of 0.6 to 3 μg of PHA, while above a PHA dose of 3 μg it decreased gradually.

Fig.3 shows changes of the per cent inhibition of ^{3}H-thymidine incorporation at various doses of leupeptin. At about 40 to 80 μg/ml-culture, leupeptin exerted the maximal inhibitory effect on PHA stimulation of DNA synthesis, whereas above the latter dose its effect slightly decreased. More than 400 μg/ml-culture of leupeptin seemed to exhibit somewhat direct cellular damage.

DISCUSSION

From the current and the previous studies (5), it is concluded that some specific proteolytic activity is predominantly involved

Table 1

Effect of Leupeptin on ^{3}H-Thymidine Incorporation in Guinea Pig Peripheral Blood Lymphocytes Cultured *in vitro* for 68 hours

Incubated with	^{3}H-TdR Incorporation (counts/min/culture ± S.E.M.)	DNA Synthesis (%)#
No PHA	1,309 ± 75	
No PHA + Leupeptin(53.6 µg)	1,347 ± 161	
PHA (30 µg)	49,543 ± 2,284	100
Leupeptin + PHA (o min)*	30,065 ± 621	60.6
Leupeptin + PHA (120 min)**	50,112 ± 729	101.1
PHA + Leupeptin (120 min)***	50,536 ± 3,422	102.0

Leupeptin(53.6 ug) was added to triplicate 1-ml cultures (9 x 10^5 small lymphocytes) at the same time of PHA addition(*), 120 min before PHA(**), or 120 min after PHA(***). 0.5 µCi of ^{3}H-thymidine (5 Ci/mmole) was added 48 hours after PHA addition, and radioactivity was determined 20 hours after addition of ^{3}H-TdR as described in the text.
^{3}H-TdR incorporation in the presence of PHA alone is expressed as 100 %

Table 2

Preincubation Time Dependency of Inhibitory Effect of Leupeptin on PHA-Stimulation of ^{3}H-Thymidine Incorporation in Blood Lymphocytes

	Preincubation (minutes)	DNA Synthesis (%)*
	0	69.4- 72.0
Preincubated with leupeptin	30	73.8- 74.3
for various times prior to	60	100.0-103.0
PHA addition	120	93.9-100.7
	300	90.5- 97.3
Preincubated with PHA	2	78.0- 78.9
for various times prior to	10	94.6- 98.8
leupeptin addition	30	97.5-101.0
	120	102.0-102.5

*Percentage represents $\frac{\text{^{3}H-TdR Incorporation in the presence of leupeptin and PHA}}{\text{^{3}H-TdR Incorporation in the presence of PHA alone}} \times 100$

^{3}H-TdR incorporation in the presence of PHA alone is expressed as 100 %.

in the initiation process of PHA stimulation of small lymphocytes. Further, the mechanism of the inhibitory action of leupeptin was more precisely investigated in the present paper.

The inhibitory action of leupeptin on the stimulation of DNA synthesis was completely abolished by its preincubation with lymphocytes (Table 1 and 2). This phenomenon is suggested to be due to the inactivation of leupeptin during some metabolic processes in lymphocytes since it is not exhibited by the preincubation of leupeptin with the culture medium (10% FCS-RPMI 1640) alone. The action of leupeptin was not demonstrated when leupeptin was added more than 10 min after addition of the mitogen (Table 2). This implies that leupeptin affects the early metabolic changes triggered by PHA, and also supports the above conclusion.

It is of particular interest that inhibitory effects of leupeptin at various doses of PHA showed a linear increase up to about 30% inhibition in the range of 0.6 to 3 μg of PHA and reached maximum at this point with a gradual decrease above the latter dose. This observation may reflect the relative proportion of T (thymus-derived) and B (bone marrow-derived) cells in the peripheral blood of guinea pig on the ground of several reports that the PHA responsive cells in T/B mixtures were predominantly T cells (7,8), and that the B cells become able to respond to PHA by humoral factors produced by T cells (9). The action of leupeptin may possibly be related to an inhibition of the production of such humoral factor. On the other hand, the linear increase in the inhibitory effect of leupeptin may also suggest that PHA primarily gives rise to an increase in permeability of the surface membrane of lymphocytes, and that leupeptin does not exert its inhibitory activity until it enters into the cell interior. This implies that the stronger the PHA activity becomes, the more leupeptin enters into cytoplasm and the more marked inhibitory effect concomitantly appears. Recently, Averdunk (10) has reported that the stimulation of human lymphocytes by PHA is accompanied by an increase in the rate of influx of methyl α-D-glucopyranoside, potassium ions, and γ-aminobutyric acid and that the change in the influx rate of potassium ions is measurable 30 sec after the addition of PHA.

The effect of leupeptin and other protease inhibitors of peptide nature, such as chymostatin,pepstatin and antipain on the induction of blast cell transformation of lymphocytes by immunologically specific antigens as well as by PHA is now under investigation.

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