Immunology 1979 **35** 959

Why do protease inhibitors enhance leucocyte migration inhibition to the antigen PPD?

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Received 16 *January* 1978; *accepted for publication* 23 *March* 1978

Summary. Epsilon amino caproic acid (EACA) and Aprotinin significantly increase the inhibition to PPD in the leucocyte migration test (Burden, Stacey, Wood & Bell, 1978). This is not due to increased leucocyte inhibition factor (LIF) generation. The proteolytic inhibitors prevent the loss of activity of LIF due to prolonged incubation in the presence of serum. Both have a significant effect on the target cells (Polymorphonuclear leucocytes) used in the assay to exaggerate the migration inhibition to LIF.

These actions can be explained by decreased proteolytic breakdown of LIF.

INTRODUCTION

Two protease inhibitors, epsilon amino caproic acid (EACA) and Aprotinin, may provide a means of increasing the sensitivity of the leucocyte migration test (LMT) since they have been found to exaggerate consistently the inhibition of leucocyte migration by the antigen partially purified derivative of tuberculin (PPD) (Burden *et al.*, 1978). This enhancing effect was only found in tests where PPD alone produced significant inhibition. Therefore it was assumed that neither EACA nor Aprotinin provoked lymphokine release. It seemed unlikely that they were responsible for unmasking antigen receptor sites since they produced no effect in tests on patients previously known to be Heaf positive but who had subsequently become anergic.

To investigate the site of action further the LMT was divided into two parts. In the standard LMT a mixture of lymphocytes and polymorphonuclear leucocytes (PMNL) are incubated with an antigen. The effector lymphokine, leucocyte inhibition factor (LIF), is produced by the lymphocytes and is simultaneously assayed by its ability to inhibit the migration of the PMNL. Therefore, it seemed logical to divide the LMT into LIF generation and LIF assay. LIF was generated by culturing lymphocytes with antigen and subsequently obtaining LIF-containing supernatants. The LIF activity present could then be separately assayed by the inhibition of migration of PMNL. By using suitable controls it was possible to study the effect of the protease inhibitors on (a) LIF generation; (b) LIF stability in the culture medium; (c) and LIF assay.

MATERIALS AND METHODS

The culture medium used was Eagle's MEM containing antibiotics and 10% foetal calf serum at pH of 7·2. PPD (Weybridge) was used in a working concentration of 150 μg/ml; EACA (KABI) at 0·01 M,

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0019-2805/78/1200-9590$02.00

and Aprotinin (Bayer) 100 iu/ml. The tests were performed with glass microhaematrocrit capillaries (Hawksley) and plastic migration wells (Sterilin).

Cell separation

To produce relatively pure populations of either lymphocytes or polymorphonuclear leucocytes, blood was anticoagulated with preservative free heparin and sedimented with plasmagel (Laboratorie Roger Belon) for 15 min at room temperature. The buffy coat was then removed and carefully layered onto Ficoll/Hypaque. Centrifugation (20 min at 250 *g*) separated lymphocytes from PMNL and the two cell populations could then be harvested. Cells were washed three times with phosphate buffered saline and then resuspended in medium. Stained films of the cell suspensions were examined by light microscopy and if there was more than 5% contamination with the other cell type, the experiment was abandoned.

Lymphokine generation

Lymphokine containing supernatants were generated in an PPD-lymphocyte culture either for 4 or 24 h periods. Lymphocytes, at a concentration of 1×10^5/ml were incubated at 37°, either with PPD or medium alone (for control cultures). At the end of the culture period the cells were spun down and the supernatants obtained. PPD was then added to the control supernatant.

The effect of EACA or Aprotinin upon lymphokine generation. To find the effects of the protease inhibitors on lymphokine generation either EACA or Aprotinin was added to a lymphocyte suspension which was then cultured for only 4h, centrifuged, and the cell free supernatant obtained. Controls were produced by incubating the lymphocyte suspension for the same time in medium and then adding the relevant drug to the cell free supernatant obtained after centrifugation.

Lymphokine stability. The cell free supernatants, either controls or containing lymphokines, could then be used for LIF assay. Although this could be set up immediately, it was also possible to perform an intermediate incubation by keeping aliquots at 37° for either 4 or 24 h before assay. This enabled the stability of LIF in medium containing serum to be determined. The effect of the protease inhibitors on lymphokine stability was studied by adding them to the cell free supernatants before the 4 or 24 h culture period. To obtain the requisite control the drugs were added to the supernatants following either 4 or 24 h culture. LIF assay was then performed.

LIF assay

Fresh PMNL suspended in medium were placed into capillaries the ends of which were sealed with plasticine and centrifuged for 5 min at 150 *g*. The capillaries were cut just below the cell fluid interface and inserted into the wells (one capillary per well) using a minimum of six capillaries in each experimental group. The supernatant to be tested was added to the well. The wells were sealed with glass cover slips and incubated for four hours at 37°. The migration areas were drawn using a microscope with a camera lucida attachment and measured by planimetry.

The effect of either EACA or Aprotinin on LIF assay was determined by adding EACA, Aprotinin, or an equal volume of isotonic saline (for controls) to the cell free supernatants.

RESULTS

The reproducibility of this two stage LMT was investigated by performing three separate assays in six volunteer subjects known to be Heaf positive and with migration indices using the standard LMT and the same concentration of PPD of 0·71 ± SD 0·07. The detailed results are shown in Table 1. There was no significant difference between the migration index achieved in any one subject on each of the occasions they were tested. The mean migration index in the two stage LMT was not significantly different from that obtained for the same subject using the standard LMT.

Table 1. Reproducibility of the two stage method. Three experiments on each of 6 Heaf positive subjects

Subject	Migration index ± SD
1	0·76 ± 0·11
2	0·69 ± 0·07
3	0·54 ± 0·09
4	0·65 ± 0·13
5	0·73 ± 0·12
6	0·74 ± 0·08

Table 2. The effect of intermediate incubation of LIF containing supernatants: two experiments were performed on each of six subjects

Intermediate incubation time (h)	Mean migration index ± 1SD	Significance
0	0·66 ± 0·12	—
4	0·70 ± 0·10	NS
24	0·84 ± 0·09	P <0·01

Table 4. The effect of protease inhibitors on LIF assay; two experiments were performed on each of six subjects

Protease inhibitors	Migration index Mean ± 1SD	Statistical significance
0	0·65 ± 0·11	—
EACA	0·51 ± 0·09	P<0·01
Aprotinin	0·53 ± 0·12	P<0·01

LIF stability

Twelve experiments, two for each subject, were performed. The results are shown in Table 2; there was a significant loss of activity when lymphokine containing supernatants were subjected to a 24 h incubation before LIF assay. However, there was no significant loss of activity with a 4 h incubation.

Three experiments on each of four subjects were then performed to find the effect of the protease inhibitors on this loss in LIF activity. The results are shown in Table 3 demonstrating that both EACA and Aprotinin reduced the loss in LIF activity.

At 4 h there was no significant difference between the results achieved with supernatants in which a protease inhibitor was added prior to the further incubation compared to supernatants in which the inhibitor was added after the incubation.

These results were important for the structure of the further experiments because using a 4 h culture period clearly avoided any significant effect on the results from loss in LIF activity during the time of the test. Further experiments were needed since the migration indices with protease inhibitor present during the 24 h intermediate culture period, were significantly less than the index achieved by immediate assay with no protease inhibitor.

LIF generation

The effect of the protease inhibitors was therefore assessed by using a 4 h culture time. Twelve experiments were performed, three on each of four subjects. Significant inhibition of migration occurred, despite the limited culture time and therefore we assumed LIF was released. Neither EACA or Aprotinin had a significant effect: the mean MI for EACA was 0·84 ± 0·16, EACA control 0·85 ± 0·19; and for Aprotinin 0·80 ± 0·10, Aprotinin control 0·83 ± 0·12. Therefore neither protease inhibitor altered lymphokine generation.

LIF assay

The effect on migration inhibition of adding EACA or Aprotinin to LIF-containing supernatants was assessed in a 4 h assay. The results are shown in Table 4. There was a significant increase in inhibition in the presence of either EACA or Aprotinin ($P<0·01$).

DISCUSSION

It appeared logical to divide the conventional LMT into two parts. Our initial experiment (Table 1) showed a reasonable reproducibility and the results

Table 3. The effect of the protease inhibitors on the loss of LIF effect at 24 h intermediate incubation: three experiments were performed on each of four subjects

Protease inhibitors during intermediate incubation	Present: during assay	Mean migration index ± SD —	Significance
0	EACA	0·76 ± 0·07	—
EACA	EACA	0·52 ± 0·06	P<0·01
0	Aprotinin	0·79 ± 0·09	—
Aprotinin	Aprotinin	0·56 ± 0·07	P<0·01

were similar to those obtained in the conventional test.

The second experiment studying the effect of incubation of the protease inhibitors with preformed LIF demonstrated their main action: they prevent loss of activity during prolonged incubation of a LIF containing supernatant. The most plausible explanation of this would be that the serum in the medium contains a proteolytic substance capable of LIF breakdown, which can be inactivated by either inhibitor. This substance may be plasmin, or possibly an activated complement component: EACA is capable of inhibiting the activation of the first component of complement (Soter, Austen, & Gigli, 1975).

Of importance to the further experiments was the finding that incubation of the LIF-containing supernatant for only 4 h produced no significant loss of activity, and it was possible therefore to explore the actions of the proteolytic inhibitors upon lymphokine production, and also their effect on the target cells used in the LIF assay by employing 4 h incubation times.

We found no obvious effect upon LIF generation, which confirms our previous reasoning from the observation that the inhibitors only enhance significant migration inhibition (Burden *et al.*, 1978). There was, however, a marked effect on LIF assay. This was surprising and is difficult to explain, particularly because there was no evidence of a medium effect after only 4 h incubation. A possible explanation is that the inhibitors synergise with LIF in its action on the PMNL target cell. But it may be possible to extend the concept of decreased LIF breakdown. It has been shown that EACA in heparinized whole blood decreases fibrinolysis (Moroz & Gilmore, 1976) by about 65% at a concentration of 10 μmol/ml, although it has little effect upon cell free plasma. The PMNL and contaminants such as platelets may therefore be producing proteolytic enzymes and the inhibition of this activated proteolysis might simply account for the effect of EACA or Aprotinin at this assay stage.

The use of proteolytic inhibitors, of which EACA and Aprotinin are only examples, may increase the sensitivity and selectivity of the Leucocyte Migration Test. The results however must be interpreted with some caution because although we demonstrated no increased LIF production this was only during the short 4 h incubation period, and we are unable to determine if a longer incubation would produce different results.

If the action of EACA and Aprotinin is by decreased proteolytic breakdown of LIF then as both are competitive inhibitors this action may be diminished in the presence of high levels of proteases. Our work used PPD, a relatively pure antigen, but other antigens, especially if derived from tissues, would be likely to augment proteolysis. The concentration of EACA or Aprotinin cannot be increased to counter this because of cytotoxicity (Burden *et al.*, 1978). It may be, therefore, that a non-toxic, non-competitive inhibitor of proteolysis would be more effective.

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