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Kinin-forming activity of human lymphocytes

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BRADYKININ and related vasoactive peptides have been implicated as chemical mediators of inflammation, pain and allergic reactions.[1-3] Recent evidence from this laboratory and others has indicated that kinin-forming enzymes in polymorphonuclear leukocytes (PMN) and macrophages may play an important role in inflammatory states.[4-7] Since lymphocytes are also involved in certain inflammatory and immune reactions, it seemed reasonable to search for such enzymes in this cell type.

Lymphocytes were isolated according to the procedures of Rabinowitz.[8] Human donor blood from the Columbia-Presbyterian Hospital Blood Bank was sedimented at room temperature with the aid of 5% dextran in 0·9% saline (1 ml/5 ml blood). The plasma and buffy coat layers (approximately 150 cm^3) were applied to a 3 × 55 cm column of alternating layers of glass beads and glass wool, which adsorbed other white cells and platelets as well as most red blood cells while allowing lymphocytes to pass through. The column yielded approximately 10^7 lymphocytes per ml of eluate and approximately one RBC for every three lymphocytes. No granulocytes were observed. The lymphocyte-RBC suspension was centrifuged at 250 *g* and the pellet resuspended in isotonic saline. The cells were washed three times with saline with intermittent centrifugation. The RBC contaminate was removed in large part (although not completely) by treatment of the cells with a hypotonic (0·45%) solution of sodium chloride and subsequent centrifugation. The lymphocyte pellet was then washed in cold 0·25 M sucrose, centrifuged and then suspended in isotonic sucrose and either subjected to repeated freezing and thawing (×6) or lysed with a glass homogenizer by hand. The lysate was centrifuged at 250 *g* to sediment nuclei and cell debris. The 250 *g* supernatant fluid was centrifuged at 15,000 *g* for 15 min at 4°, which gave a 15,000 *g* supernatant and pellet. The 15,000 *g* pellet was suspended in 5 ml of isotonic sucrose and subjected to six serial freezing and thawing procedures in order to lyse any lysosomes present.

Kinin-forming activity in the whole lysate and in its subcellular fractions was determined by incubation for 3 or 15 hr at pH 4·0 with human kininogen purified from plasma as previously described (1·0 mg kininogen released 0·5 μg bradykinin with trypsin[9]). These conditions were found previously to favor the reactions between this particular substrate and PMN and macrophage kinin-forming enzymes.[4] The kinin found was assayed on the guinea pig ileum or estrus rat uterus. The isolated tissues were usually pretreated with atropine and diphenhydramine. Carboxypeptidase B and chymotrypsin inactivation of the activity were taken as the final indication that a peptide had been formed.

Figure 1 demonstrates the formation of a kinin-like material by whole cell lysates of lymphocytes as measured by the response of the rat uterus. Only the complete reaction mixture had activity; control mixtures of kininogen incubated without enzyme and of the enzyme incubated without kininogen, as well as a lysate of red blood cells, showed no activity. As also indicated, the biological activity of this material is destroyed after incubation with carboxypeptidase B or chymotrypsin.

Each milliliter of the 15-hr reaction mixture produced kinin-like material which was equivalent to 250–300 ng bradykinin (rat uterus equivalents). In later experiments, however, it was found that the incubation period could be shortened to 3 hr, the shorter incubation period producing 60–85 per cent of the activity found after the prolonged incubation.

When the potencies of the lymphocyte-kinin on the guinea pig ileum and rat uterus were compared with that of bradykinin, the results indicated that lymphocyte-kinin had relatively greater potency on the estrus rat uterus than on the guinea pig ileum (approximately 2:1). This difference is similar to that observed with macrophage and PMN kinins,[7] suggesting that, like these latter kinins, lymphocyte-kinins differ from bradykinin.

The various fractions of the lysate were studied for their kinin-forming enzymes. Table 1 indicates the distribution of kinin-forming activity in the 250 *g* ("nuclei and debris"), 15,000 *g* pellet and the 15,000 *g* supernatant fractions. Activity was found in all three fractions and the relative amount of activity per fraction was similar to that found in PMN cells and macrophages.[7]

Table 2 lists the inhibitory effects of various agents on lymphocyte kinin-forming enzyme (whole cell lysate). Of the agents tested, only soybean trypsin inhibitor had an inhibitory effect. Like the PMN and macrophage kinin-forming enzymes, lymphocyte enzyme was unaffected by high concentrations of Trasylol, an agent known to inhibit salivary kallikrein.

The results of the current investigation showing that human lymphocytes contain kinin-forming

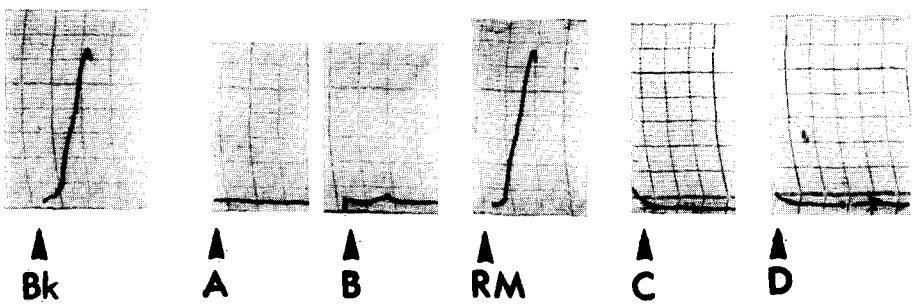

FIG. 1. Lymphocyte kinin-forming activity. The complete reaction mixture consisted of 0·2 ml of lymphocyte lysate added to 5 mg of human bradykininogen in 0·8 ml of 0·2 M acetate buffer (pH 4·0). After 3 hr, the samples were neutralized and centrifuged; 0·05-ml aliquots of the resulting supernatant fluid were added to the muscle bath. Bk = 20 ng standard bradykinin; A = 0·05 ml lysate control (no substrate added); B = 0·05 ml substrate control (no enzyme added); RM = 0·05 ml complete reaction mixture; C = 20 μg carboxypeptidase B added to 0·5 ml of the 3-hr sample and tested after 20 min; D = 20 μg chymotrypsin added to 0·5 ml of the 3-hr sample and tested after 20 min.

TABLE 1. CELLULAR LOCATION OF KININ-FORMING ACTIVITY

	Rat uterus*	Total activity (%)
250 *g* Pellet	150	46
15,000 *g* Pellet	75	23
15,000 *g* Supernatant	100	31

* Values given as nanograms of bradykinin per ml of reaction mixture per 3-hr incubation. The results are the average of three experiments.

TABLE 2. EFFECTS OF VARIOUS AGENTS ON LYMPHOCYTE KININ-FORMING ACTIVITY

Agent	Inhibition (%)
Soybean trypsin inhibitor (0·5 mg/ml)	50–70
Hydrocortisone (10^{-5} M)	0
Colchicine (10^{-4} M)	0
Trasylol (1000 units/ml)	0
Sodium salicylate (10^{-2} M)	0
EDTA (10^{-3} M)	0
Pancreatic trypsin inhibitor (1 mg/ml)	0

enzyme(s) supplement previous findings that leukocytes of the granulocytic and macrophage series, respectively, contain such enzymes[6,7] or kallikrein activators.[5] The activity of these enzymes *in vitro* however, does not lend proof either to their activity or their possible pathogenetic role *in vivo*. Nevertheless, the presence at inflammatory sites of large numbers of lymphocytes, as well as macrophage and PMN, may indicate that under appropriate conditions kinin formation and its resulting physiological effects would occur.

The activity at acid pH of the kinin-forming enzymes in lymphocytes, PMN, and macrophages is consistent with the postulate that these proteases are catheptic in nature and not kallikreins. Cathepsins D and E have been reported to be present in these cell types[10,11] and should be considered as possibly being the kininogenases under study. Previous evidence has implicated cathepsins as the kininogenases of tissues such as spleen.[12] It has been reported that intact lymphocytes do not lower kininogen levels at neutral pH.[13] The current investigation was carried out at acid pH and with broken cell preparations and by direct measurement of kinin formed. These differences may explain the apparent discrepancy of the results.

Lymphocytes are commonly seen in abundance at the sites of immune reactions, such as graft rejection and delayed hypersensitivity, as well as in such chronic inflammatory diseases as rheumatoid arthritis. In their study of tuberculin-induced delayed hypersensitivity, Pick *et al.*[14] observed that supernatants obtained from reaction mixtures of PPD with either lymphocytes or macrophages provoked an intense inflammatory reaction when injected into the skin of normal guinea pigs. The response to lymphocyte-supernatant fluid was characterized by erythema and induration, with a peak between 3 and 6 hr. At least one active substance was isolated from lymphocyte supernatant fluid. It had a molecular weight of approximately 70,000 and its activity was inhibited by pepsin. It would seem from such a description that it is possible that the inflammatory material released is the kinin-forming enzyme(s) under study in this laboratory.

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REFERENCES

1. G. P. LEWIS, *Ann. N.Y. Acad. Sci.* **104,** 236 (1963).
2. G. P. LEWIS, in *Int. Symp. Injury, Inflammation, and Immunity* (Eds. L. THOMAS, J. W. UHR and L. H. GRANT), pp. 242–253. Williams & Wilkins, Baltimore (1964).
3. H. Z. MOVAT, T. URIUHARA, N. S. TAICHMAN, H. C. ROWSELL and J. F. MUSTARD, *Immunology* **14,** 637 (1968).
4. L. M. GREENBAUM and K. S. KIM, *Br. J. Pharmac. Chemother.* **29,** 238 (1967).
5. K. L. MELMON and M. J. CLINE, *Biochem. Pharmac.* **17,** 271, (1968).
6. L. M. GREENBAUM, M. C. CARRARA and R. FREER, *Fedn Proc.* **27,** 90 (1968).
7. L. M. GREENBAUM, R. FREER, J. CHANG, G. SEMENTE and K. YAMAFUJI, *Br. J. Pharmac. Chemother.* **36,** 623 (1969).
8. Y. RABINOWITZ, *Blood* **23,** 811 (1964).
9. L. M. GREENBAUM and T. HOSODA, *Biochem. Pharmac.* **12,** 325 (1963).
10. E. PRESS, R. R. PORTER and J. CEBRA, *Biochem. J.* **74,** 501 (1960).
11. C. LAPRESLE and T. WEBB, *Biochem. J.* **84,** 455 (1962).
12. L. M. GREENBAUM and K. YAMAFUJI, *Life Sci.* **4,** 657 (1965).
13. K. L. MELMON and M. J. CLINE, *Nature, Lond.* **213,** 91 (1967).
14. E. PICK, J. KREJCI, K. CECH and J. L. TURK, *Immunology* **17,** 741 (1969).

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Pyridoxal-5′-phosphate—An inhibitor of catechol-*O*-methyltransferase *in vitro*

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NOREPINEPHRINE is enzymatically inactivated by *O*-methylation or by oxidative deamination. The former reaction is primarily extraneuronal and is catalysed by catechol-*O*-methyltransferase (COMT), a soluble magnesium-requiring enzyme which transfers a methyl group from *S*-adenosylmethionine to the catecholamine.[1] Several classes of synthetic COMT inhibitors have been characterized; substrates such as desmethylpapavarine,[2] *O*-dihydroxyphenylacetamides[3] and catechol[4] are associated with competitive inhibition of COMT. Pyrogallol[5] causes both competitive and noncompetitive[6] inhibition of the enzyme, whereas the tropolones[7] appear to inhibit COMT through chelation. Recent work has indicated that a class of compounds such as 3,5-dihydroxy-4-methoxy- and 3-hydroxy-4,5-dimethoxy-benzoic acids are associated with a mixed type of inhibition.[8]

The present communication describes inhibition of COMT *in vitro* by the naturally occurring vitamin, pyridoxal-5′-phosphate (PLP).

Female, Sprague–Dawley, 160–200 g rats were killed by a blow to the head and livers were rapidly removed and homogenized in 5 vol. of ice-cold isotonic potassium chloride solution. The homogenate was centrifuged for 30 min at 30,000 *g* in a Spinco model L centrifuge. The supernatant fraction was dialysed against 2000 vol. of 0·1 M phosphate buffer, pH 7·6, with one change over 12 hr between 0 and 4°. This enzyme preparation, with a protein concentration of approximately 25 mg/ml, was stored at −40° and was stable for weeks.

COMT was assayed by methods previously reported: 25 μl of enzyme preparation, 1 μmole magnesium chloride, 25 μl [^{14}C]-S-adenosylmethionine (specific activity of 48·5 μc/μmole at 5 μc/cm)[3] and varying amounts of norepinephrine and inhibitor were incubated for 30 min at 37° in 0·1 M phosphate buffer, pH 7·9, in a final volume of 1 ml. The reaction was terminated by addition of 0·5 ml of 0·5 M borate buffer, pH 10, and the [^{14}C]-normetanephrine formed was extracted into 10 ml of isoamyl alcohol. A 1-ml aliquot, transferred to a vial containing 1 ml ethanol and 10 ml phosphor, was counted by liquid scintillation spectroscopy in a Beckman LS250 liquid scintillation system with an efficiency for ^{14}C of approximately 75 per cent.