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Leukokinin-forming system in the ascitic fluid of a murine mastocytoma*

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NORMAL leukocytes and L1210 leukemic cells have been shown to contain enzymes which are active at acid pH in forming vasoactive peptides called leukokinins from a kininogen present in plasma.[1,2] Two leukokinins have been isolated and analyzed for amino acid content;[3] both were considerably larger than bradykinin (21–25 amino acids) and each contained only one phenylalanine. Since bradykinin contains two phenylalanines, the leukokinins do not contain bradykinin as part of their molecules. Compared to bradykinin, the leukokinins have qualitatively similar but quantitatively different pharmacological properties.[4] On a molar basis, the leukokinins are less effective in causing contraction of smooth muscle, but are more effective in lowering blood pressure and increasing vascular permeability.

In the current investigation, the ascitic murine mastocytoma P815Y[5] was examined for the leukokinin-forming system in order to determine if this system exists in such a tumor and if leukokinins play a role in tumor growth. It was found that the neoplastic mast cells contained a leukokinin-forming enzyme and that the extracellular fluid bathing these cells contained both an enzyme and substrate necessary for leukokinin formation. This communication describes the leukokinin-forming system in the extracellular fluid.

The tumor was harvested without dilution 8–10 days after transplantation.[5] Smears were made of the tumor fluid taken directly from the mice and stained with Wright's stain. Over 90 per cent of the cells were recognizable as mast cells and less than 2 per cent were granulocytes. The cells were removed from the surrounding fluid by centrifugation at 3000 *g*; the 3000 *g* supernatant fluid was centrifuged at 10,000 *g* to remove any cells possibly remaining. The 10,000 *g* supernatant fluid was termed the extracellular fluid.

To assay for kinin-forming activity, the extracellular fluid was incubated at 37°, after adjusting the pH by the direct addition of 1 N HCl. Since the fluid contained both enzyme and substrate, no further additions were necessary to start the reaction. To stop the reaction, the samples were diluted 1:5 with de Jalon's solution without glucose, heated immediately for 10 min in a boiling water bath, and centrifuged for 5 min at 3000 *g*. The supernatant fluid was neutralized and assayed on the estrus rat uterus.[1] The results were expressed as equivalents of bradykinin giving the same response.

It should be noted that storage of the extracellular fluid at −15° followed by thawing reduced the kinin-forming capacity. In addition, the formation of a gel made the material difficult to work with. Consequently, all studies of kinin formation were carried out without previous storage.

Bradykinin was obtained from Cyclo Chemical Corp.; soy bean trypsin inhibitor from the Worthington Biochemical Company; aprotinin was Trasylol from FBA Pharmaceuticals, Inc.; pepstatin was prepared by the Banyu Pharmaceutical Company and was a gift from A. Dannenberg of Johns Hopkins University.

Figure 1 shows that kinin is formed by the extracellular fluid and that the rate of kinin production by the extracellular fluid is optimal at pH 3·8. The extraordinary sharpness of the optimum suggests that this may not be the intrinsic pH dependence of the enzyme, but that several processes may be in operation. Perhaps on the acidic side the enzyme is being destroyed, and on the basic side the product may be destroyed by kininases.

The time course of the reaction in the extracellular fluid is shown in Fig. 2. The extracellular fluid is capable of producing in 22 hr an amount of kinin per milliliter which is equivalent in activity to 10 μg bradykinin, a very great total potential. The kinin released in 1 hr was 400 ng/ml, more than adequate for physiological activity such as changes in vascular permeability.

When the kinin-forming activity of the ascitic fluid was compared with that of plasma from tumor-bearing or from normal mice (Table 1), the ascitic fluid produced a much greater concentration of leukokinin than did the plasmas under the same conditions. Studies on peritoneal fluid obtained after glycogen irritation showed practically no leukokinin generating system.

Since the total blood volume and the volume of ascites obtained from each mouse were both approximately 2 ml, the total amount of kinin which could be produced by the extracellular fluid is many times greater than the amount which can be produced by either of the plasmas under the assay conditions.

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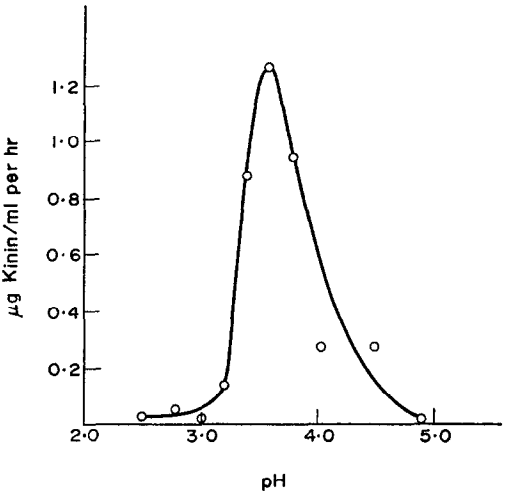

FIG. 1. pH dependence of the rate of kinin production by the extracellular fluid. The extracellular fluid was prepared from the combined tumor exudate from six mice which totaled 11·5 ml and contained $3{\cdot}9 \times 10^8$ cells. The fluid was assayed at various pH values as described in the text using a 4-hr incubation at 37°.

TABLE 1. KININ FORMATION BY EXTRACELLULAR FLUIDS AND PLASMAS

Fluid source	Kinin formed ± S.E. (ng/ml)
Extracellular fluid of P815Y tumor*	11,000 ± 3,600 (5)
Plasma from tumor-bearing mice†	720 ± 150 (5)
Plasma from normal mice	290 ± 180 (3)
Extracellular fluid 2 hr after glycogen injection‡	16 ± 10 (3)
Extracellular fluid 24 hr after glycogen injection	8 ± 3 (3)

* The samples of the extracellular fluid of the tumor were prepared as described in the text from the pooled tumor harvested from three mice.

† For each plasma sample, two or three mice were subjected to cardiac puncture using a heparinized ½ inch No. 26 gauge needle and 1·0-ml syringe. The pooled blood (0·5 to 1·5 ml) was diluted with an equal volume of saline and centrifuged at 1000 *g* to remove the cells.

‡ Two ml of 0·1% oyster glycogen in saline was injected i.p. into each normal mouse, and the animal was sacrificed after 2 or 24 hr. After 2 hr, about 0·9 ml exudate could be withdrawn from the peritoneum. The exudate contained some cells including erythrocytes which were removed by centrifugation. After 24 hr, no fluid could be withdrawn. Therefore 1 ml saline was injected into the peritoneum, and the exudate was withdrawn and centrifuged to remove the cells. The plasmas and extracellular fluids were adjusted to pH 3·8 and incubated at 37° for 20 hr. The kinin formed was assayed as described in the text, and the values reported for the plasmas have been calculated to represent the values before dilution of the whole blood. Heparin (200 units/ml) did not affect kinin formation by the tumor extracellular fluid, and neither the plasmas nor fluid contained any detectable kinin before incubation. The number of samples is given in parentheses.

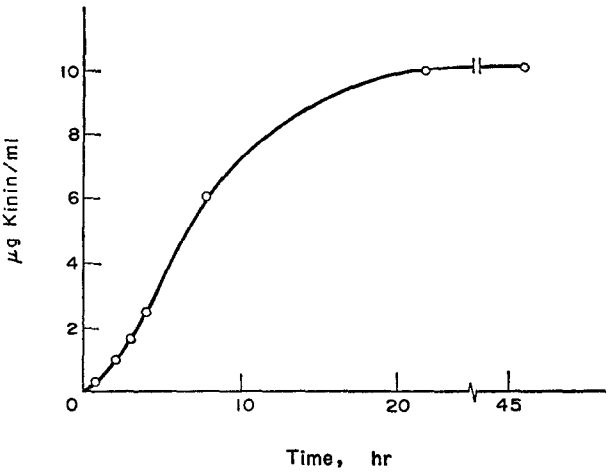

FIG. 2. Time course of kinin production by the extracellular fluid. The extracellular fluid was prepared from the combined tumor exudate from two mice which totaled 2·4 ml and contained $3·2 \times 10^8$ cells. The fluid was incubated at pH 3·8 at 37°. Aliquots were withdrawn at various times and assayed for kinin as described in the text.

The rate of kinin production by the extracellular fluid at pH 3·8 was examined in the presence of various agents. The acid protease inhibitor, pepstatin,[6] caused an 80 per cent inhibition at a concentration of 1 μM. The chelating agent, 1, 10-phenanthroline (1·0 mM), had no effect. The kallikrein inhibitors, aprotinin (500 units/ml) and soy bean trypsin inhibitor (1 mg/ml), inhibited the kinin formation by 10 and 18 per cent respectively.

The extracellular fluid was also examined for kininases. The kinin formed by the extracellular fluid was extracted in hot ethanol[1] and aliquots were incubated with fresh extracellular fluid at pH 7·5. There are kininases present in the extracellular fluid at this pH which will destroy 90 per cent of the kinin in 1 hr. The kininases were inhibited 100 per cent by 1·0 mM, 1,10-phenanthroline and 33 per cent by 1·0 mM EDTA, indicating that they are probably derived from plasma. Aprotinin (1000 units/ml), tosyl-L-phenylalanine chloromethyl ketone (1·0 mM) and $CuSO_4$ (0·1 mM) did not affect the kininases.

The chemical and pharmacological nature of the kinin produced at acid pH by the extracellular fluid was found to be similar to that of the leukokinins previously described.[2,3] The kinin was destroyed by chymotrypsin and carboxypeptidase B, but not by trypsin and pepsin. The action on the uterus was not blocked by the antihistamine, diphenhydramine, or the serotonin antagonist, methysergide. The kinin was extremely potent in increasing vascular permeability in the rabbit as measured by the bluing reaction.[7] Using amounts of bradykinin and leukokinin which were equivalent in the smooth muscle assay, the leukokinin was fifty times more active in the permeability test than bradykinin.

The presence of a kinin-forming enzyme, a substrate, and kininases has been demonstrated in the extracellular fluid of the ascites tumor. The kinin-forming enzyme has a pH optimum and a response to inhibitors similar to those of the leukokininogenases described previously.[1,2] The properties of the kinins formed are similar to those of the leukokinins.[4] Experiments on kinin formation showed that the extracellular fluid was capable of producing on the average of 11 μg/ml (bradykinin equivalents) when assayed on the rat uterus. Since the luekokinins were 50 times more effective in the vascular permeability test than bradykinin, the extracellular fluid, when assayed in terms of vascular permeability, can produce an amount of kinin equivalent to 500 μg/ml of bradykinin—an impressive amount. The significance of leukokinin formation in ascitic fluid would be to promote an increase in vascular permeability, increasing the peritoneal fluid volume and the influx of nutrients into the cavity.

Compared to the ascitic fluid, the plasmas of normal and tumor-bearing mice could generate only relatively small quantities of leukokinin. This means that: (a) all the necessary components for leukokinin generation are present in the ascitic fluid but not in plasma; or (b) plasma contains an inhibitor which retards leukokinin formation, but which is not in the ascitic fluid. This biochemical difference may be an integral part of the pathology involved in ascites formation and might be used as a target for drugs such as pepstatin to control ascites formation.

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Oxidative demethylation of ^{14}C-griseofulvin by liver microsomes of rats and mice

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GRISEOFULVIN is an orally effective antifungal agent and is used widely in the treatment of fungal infections in animals and man.[1] The metabolism of griseofulvin *in vivo* has been studied in man, rat, mouse, rabbit and dog.[2–7] Limited metabolic studies of griseofulvin in tissue slices[8,9] and liver microsomes[10] have also been reported. However, the metabolism of griseofulvin by liver microsomes has not yet been elucidated. It was therefore of interest to determine the kinetic parameters for *O*-demethylations of griseofulvin in the rat and mouse liver microsomes.

Charles River male mice (20 g) and rats (180 g) were sacrificed by decapitation. Livers were homogenized and the homogenate was centrifuged at 9000 *g* for 20 min and the precipitate was discarded. The microsomes were sedimented by centrifugation at 100,000 *g* for 60 min. The enzyme activities of 4-demethylation and 6-demethylation of griseofulvin were determined by incubation of ^{14}C-griseofulvin with the liver microsomes, $MgCl_2$ ($5{\cdot}5 \times 10^{-3}$M) and NADPH-generating system (1 mg NADP, 8·8 mg glucose 6-phosphate and 2 Kornberg units glucose 6-phosphate dehydrogenase) in 0·05 M potassium phosphate buffer, pH 7·4. The reactions were stopped by acidifying the mixtures to pH 1, and griseofulvin and its metabolites were extracted with diethyl ether. The ^{14}C-labeled griseofulvin, 4-desmethylgriseofulvin and 6-desmethylgriseofulvin were separated by thin-layer chromatography using chloroform–methanol (10:1, v/v) as the solvent system. The radioactivity associated with each component was then measured by liquid scintillation counting.

Griseofulvin was converted to 4-desmethylgriseofulvin and 6-desmethylgriseofulvin by rat and mouse liver homogenates (Table 1). Both demethylating enzyme activities were localized in the microsomal fraction. When the liver microsomes were boiled, there was no evidence of conversion of griseofulvin to its demethylated derivatives. NADPH-generating system was essential for these enzymatic reactions. A linear relationship was obtained between the metabolic rate and the enzyme concentration for both demethylation reactions in the rat and mouse (Fig. 1). The amount of product formed for both reactions and in both species increased linearly during the first 8 min, and then declined (Fig. 2).