Biochemical Pharmacology, Vol. 21, pp. 3095–3106. Pergamon Press, 1972. Printed in Great Britain.

STUDIES ON LEUKOKININS—II

STUDIES ON THE FORMATION, PARTIAL AMINO ACID SEQUENCE AND CHEMICAL PROPERTIES OF LEUKOKININS M AND PMN*

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(*Received* 21 *October* 1971; *accepted* 29 *April* 1972)

Abstract—Two high molecular weight, pharmacologically active peptides have been isolated from incubates of human kininogen and enzyme fractions of macrophages and polymorphonuclear leukocytes. The incubations were carried out at pH 4·0. The peptides, while having pharmacological properties similar to those of bradykinin, do not contain bradykinin as part of their molecules. The name leukokinin is designated for these peptides. Leukokinin-M (macrophage enzyme-mediated) has 25 amino acids and a molecular weight of 2826·5. The partial amino-terminal sequence of NH_2-Arg-Ala-Ser-?-?-Lys- has been found for this peptide. Leukokinin-PMN (polymorphonuclear cell enzyme-mediated), formerly known as PMN-kinin, was found to have 21 amino acids and a molecular weight of 2416. Experiments using subcellular fractionation have indicated that the major leukokinin-forming (leukokininogenase) activity of the white cells is in the nuclear membrane-debris fraction. It is suggested that the leukokinin system may be a prime chemical mediator release mechanism of white cells at the inflammatory site.

ONE OF the outstanding features of inflammation is the eventual presence of white cells at the site of inflammation. Depending on the length of time inflammation has progressed, the population of white cells varies. Thus polymorphonuclear leukocytes are usually the first white cells to arrive, followed by mononuclear cells and lymphocytes. Menkin[1] was the first to propose that white cells may be involved in the liberation of peptides which may affect the responses known as the inflammatory response. Greenbaum and Yamafuji,[2] Greenbaum and Kim[3] and Melmon and Cline[4] reported that polymorphonuclear leukocytes may have properties that contribute to kinin formation. Greenbaum *et al.*[5] demonstrated the presence of enzymatic activity in polymorphonuclear leukocytes, macrophages, malignant cells and, more recently, in

* This investigation was supported by funds from United States Public Health Service Training Grant GM-00438, from USPHS Grant HE-12738, from the Health Research Council of the City of New York, from the National Science Foundation, from the Sandoz Foundation and from Capes, Brazil. Studies on various aspects of this investigation were submitted to the Graduate Faculties of Columbia University as part of the dissertations of J. C. and R. F. in partial fulfillment for the Ph.D. degree.

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lymphocytes,[6] which when acting on partially purified human plasma proteins at acid pH formed kinin-like material. Evidence by Greenbaum *et al.*[5] on the pharmacology and molecular weight of the kinins formed indicated that, in addition to bradykinin, a kinin was formed which differed from all known kinins. It was termed PMN-kinin, since it was first found using polymorphonuclear (PMN) cells as a source of enzyme. In the current communication, we are reporting that another related peptide of slightly larger size than PMN-kinin, but possessing biological activity similar to that of PMN-kinin, has been isolated in pure form from incubates of macrophage enzymes and partially purified human kininogen as substrate. We propose the overall general name of leukokinins for these biologically active peptides which can be formed by white cell enzymes acting at acid pH. The individual peptides so far found would be named leukokinin-PMN (PMN-kinin) which is formed by PMN cells, and leukokinin-M for the new peptide found to be formed by alveolar macrophages. The amino acid analysis of leukokinins PMN and M are given in this report as well as a partial amino acid sequence of leukokinin-M.

METHODS AND MATERIALS

Alveolar macrophages. These were obtained from anesthetized rabbits by washout of the lungs and bronchi by the method previously described.[5] This procedure yields 90–95 per cent of the cell population as macrophages, as observed by the light microscope.

The cells were collected by centrifugation at 500 *g*. After the supernatant was removed by aspiration, the cells were washed twice with saline, with intermittent centrifugation followed by two washings with 0·25 M sucrose and suspension in 0·34 M sucrose (5 ml sucrose per 10^8 cells; usually the amount obtained from one rabbit). The final suspension was subjected to serial freezing and thawing six times. Either the entire lysate or the more stable low speed fraction (500 *g* followed by resuspension in 5 ml of isotonic sucrose) was used. In experiments where the subcellular location of the enzyme was investigated, the 500 *g* pellet of cells obtained after the second 0·25 M sucrose wash was suspended in cold 0·34 M sucrose solution (5–6 $\times$ 10^7 cells/ml) and homogenized in a chilled 50-ml glass homogenizer (Kontes Glass Company, Vineland, N.J.). Homogenization was carried out by hand with a glass pestle for three 2-min periods. Microscopic examination indicated that about 75–90 per cent of the cells were disrupted by this procedure. After cell breakage, the homogenate was transferred to Lusteroid centrifuge tubes and centrifuged at 500 *g* for 5 min at 4°. The resulting pellet ("nuclear fraction") was washed once more with 5·0 ml of 0·34 M sucrose solution followed by centrifugation at 500 *g*. The washed pellet was then suspended in 8–10 ml of cold 0·34 M sucrose solution to constitute the "nuclear fraction", which actually contained nuclei, cell debris and some unbroken cells. The supernatant fluids obtained from the original centrifugation and the washes were combined and centrifuged (Lourdes) at 12,000 –15,000 *g* for 12 min. This resulted in a slightly opalescent supernatant fluid constituting the "extra-lysosomal fraction" and a tan pellet (lysosomal fraction). The latter was suspended in 4·0–5·0 ml of chilled 0·34 M sucrose solution and then subjected to freezing and thawing serially six times in order to destroy the integrity of the lysosomal membrane.

Human kininogen was prepared as previously described.[7] Bovine bradykininogens I and II were graciously provided by Dr. T. Suzuki, Institute for Protein Research,

Osaka University, Osaka, Japan.* Kinin-forming activity was measured by the estrus rat uterus.

Thin-layer chromatography. Thin-layer chromatography (TLC) was used to separate and identify dansyl amino acids. The dansyl (DNS) amino acid mixture to be chromatographed was taken up in 10 μl of aqueous pyridine (50%, v/v) or 20–40 μl acetone and spotted on a 20 × 20 cm glass plate coated with Silica gel H (E. Merck. Ag. Germany). The plate was developed two dimensionally by ascending chromatography using two of the following solvent systems: (1) isopropanol–methyl acetate–concentrated ammonia (90:70:40); (2) chloroform–methanol–acetic acid (75:25:5); (3) butanol–acetic acid–water (60:75:25).

The TLC was usually first developed with system 1 because many DNS-amino acids can be separated by this system. After the solvent had travelled about 15–16 cm, the plate was taken out, air-dried, turned 90° and developed by ascending chromatography in solvent system 2 or 3. The plate was then examined under ultraviolet light and comparisons were made with standard dansyl amino acids (usually run simultaneously in the second dimension).

End-group analysis of peptides by dansylation. The peptide was dried *in vacuo* and was dissolved in 15 μl of 0·2 M bicarbonate solution and mixed with 15 μl of dansyl chloride in acetone (5 mg/ml of acetone). The dansylation was allowed to proceed at 37° for 1 hr. The solution was then brought to dryness again *in vacuo* and 50 μl of 5·8 N HCl was added. The tube was sealed under vacuum and the hydrolysis allowed to proceed for 16–18 hr at 105°. After the HCl was removed *in vacuo* over NaOH, the dansyl amino acid was determined by thin-layer chromatography. This allowed the detection of 0·5 to 2 nmoles of amino acid.

Edman dansylation procedure for sequencing of peptides. This was carried out by a modification of the method of Gray.[8] Approximately 50 nmoles of the peptide were lyophilized and redissolved in 200 μl of aqueous pyridine (50%, v/v). 100 μl of a 5% phenylisothiocyanate (PTC) solution in pyridine was added and, after flushing with nitrogen, the tube was stoppered and allowed to stand for 1 hr at 45°. The PTC-peptide was dried *in vacuo* at 60° over P_2O_5 and NaOH. The PTC-peptide was dissolved in 200 μl of trifluoracetic acid and, after flushing with nitrogen, allowed to stand stoppered at 45° for 45 min. The reaction mixture was dried *in vacuo* at 60° and redissolved in 150 μl water. The by-products were removed by extraction with butyl acetate (saturated with water). 10 μl of the aqueous phase (containing 2–3 nmoles peptide) was dried *in vacuo* and subjected to dansylation and hydrolysis followed by thin-layer chromatography. The remainder of the aqueous phase was subjected to another cycle of degradation.

Amino acid analyses were carried out by means of the Spinco–Beckman and Technicon systems.

RESULTS

Isolation of kinins produced by macrophages

When various subfractions of rabbit alveolar macrophages were incubated with partially purified kininogen over a period of 12 hr, all fractions showed activity

* L. M. G. would like to thank Dr. Y. Yamamura, Professor of Medicine, Osaka University, for his hospitality and scientific discussions about this work during his stay in Japan. He is also indebted to Professor T. Suzuki of the Institute for Protein Research, Osaka, Japan, for making available to him both his staff and the purified bovine kininogen for the experiments described.

(Fig. 1). The low speed (nuclear debris) fraction showed the most activity. The results of this experiment indicated that the whole cell lysate might be used as a source of enzyme(s) (see Materials and Methods). Human kininogen (1·5 g) was incubated with 30 ml enzyme (equivalent to 10^9 cells) in 0·2 M acetate buffer at pH 4·0 in a final volume of 100 ml. The incubation was allowed to proceed for 16 hr at 37°. Ethanol (400 ml) at 70° was then added to the incubation mixture and the resulting material kept at 70° for 20 min. After cooling at 4°, the protein precipitant was removed by centrifugation at 7000 *g* for 15 min. The supernatant fluid was then evaporated to

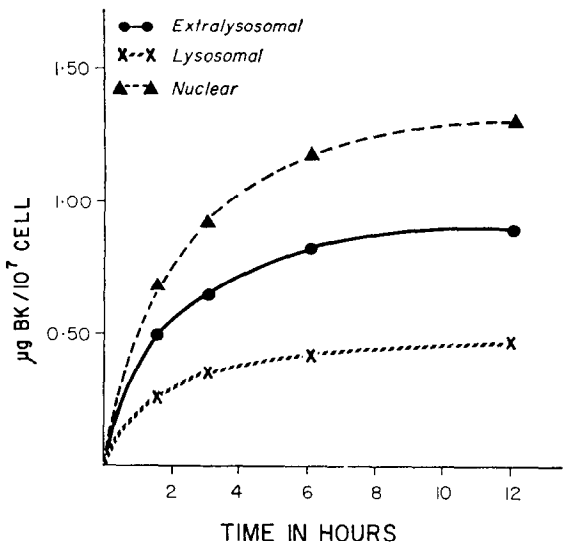

FIG. 1. Time course of the formation of biologically active material released by subcellular fractions of alveolar macrophages. Purified human kininogen (30 mg) was incubated with 0·6 ml of each subcellular fraction of alveolar macrophages in 0·2 M acetate buffer, pH 4·0, at 37° in a final volume of 5·0 ml. After 1·5, 3·0, 6·0 and 12·0 hr, a 1·0-ml sample was withdrawn, boiled for 10 min, and neutralized. An aliquot of this was assayed on the rat uterus. Ordinate: micrograms of bradykinin equivalents per 10^7 cells formed by the extra-lysosomal (●——●), the lysosomal (×-·-×) and the nuclear fraction (▲- - -▲), respectively.

dryness in a rotary evaporator (45°) in the presence of *p*-toluenesulfonic acid (to prevent adsorption to glass[9]) in a concentration to equal 10^{-3} M when the dried material was taken up in 10 ml of distilled water. The biological activity of this water-reconstituted alcohol extract was 50 μg bradykinin equivalents as tested on the estrus rat uterus. In control experiments, extracts of enzyme incubated without substrate showed no activity.

Purification of kinins on carboxymethyl-cellulose

A 2·5 × 5·0 cm "mini-column" of carboxymethylcellulose was prepared by pouring the adsorbant suspended in 0·05 M ammonium acetate buffer (at the pH of the buffer) into the column and allowing it to pack under gravity for 30 min. An additional 100 ml buffer was run through the adsorbant to wash the column. While the dimensions of this column may seem unusual, longer columns resulted in significant loss of activity

when a 2·5 cm width was used. Complete recovery of bradykinin was obtained in the above system. Ten ml of the reconstituted water extract was diluted about ten times until the conductivity was equal to that of 0·05 M ammonium actetate buffer (3·2 mmho). The sample was then applied to the column at a flow rate of 60 ml/hr; then 20 ml of 0·05 M ammonium acetate was applied to wash down the sides of the column. Fractions (4·0 ml each) were collected and elution of biologically active material (direct assay of 0·01–0·1 ml on the rat uterus) was accomplished by stepwise addition of 50 ml of 0·1 and 0·2 M ammonium acetate buffer. The results showed that the major activity was obtained in the 0·1 M fraction. The 0·1 M eluted material was lyophilized in order to reduce the volume as well as the salt concentration. The lyophilized material was then redissolved in a small volume of water (10 ml) to reduce the conductivity to that of 0·03 M ammonium acetate buffer (2·0 mmho).

Re-chromatography on carboxymethylcellulose

Re-chromatography was accomplished by applying the sample obtained above to a 0·9 × 100 cm column of carboxymethylcellulose and eluting the active material with a linear gradient from 0·05 to 0·4 M ammonium acetate using a 100-ml mixing chamber. The results of a typical chromatogram are shown in Fig. 2. As may be seen, four separate peaks of activity were obtained; the predominant activity was in peak IV. Ten preparations of macrophage-formed kinin were carried out and each of the four peaks obtained in each preparation was pooled with its counterparts as determined by the pattern of peaks and ionic strength needed for elution. The activity of the pooled peaks is seen in Table 1.

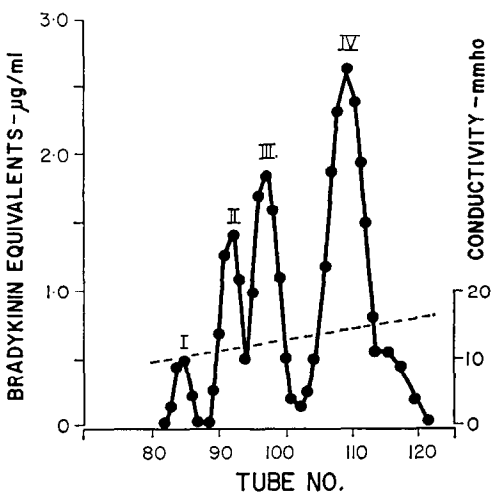

FIG. 2. Re-chromatography on carboxymethyl cellulose (CMC) of the kinins released by alveolar macrophage proteases. A 30-ml sample of the activity eluted with 0·1 M ammonium acetate containing 100 µg bradykinin equivalents (rat uterus assay) was applied to a 0·9 × 100 cm column of CMC which had previously been equilibrated with 0·05 M ammonium actetate. The flow rate was adjusted to 18 ml/hr and a gradient from 0·05 to 0·40 M ammonium acetate initiated. The volume of the mixing chamber was 100 ml. Fractions, 1·8 ml, were collected and stored at 4°. Each fraction was assayed for kinins on rat uterus. The conductivity of each fraction was also determined. The numbers I, II, III and IV indicate the separate peaks obtained.

TABLE 1. ACTIVITY OF POOLED PEPTIDES IN PEAKS I–IV

	Activity*		
Peak	Rat uterus (μg)	Guinea pig ileum (μg)	RU/GPI
I	12·4	7·9	1·6
II	44·9	10·7	4·2
III	98·5	15·6	6·3
IV	419·8	31·5	13·2

* The values represent the activity of the individually combined peaks from ten separate experiments (in each of which four peaks were obtained). The activity is measured in bradykinin equivalents. RU = rat uterus; GPI = guinea pig ileum.

Purity and end-group analysis of each peak

In order to ascertain the purity of each of the peaks, end-group analysis was carried out by marking the amino-terminal group of each peptide with dansyl chloride. The dansylated amino acid was released by acid hydrolysis of the peptide and identification was accomplished by thin-layer chromatography, using ultraviolet light to locate the dansylated product. The results of the end-group analysis of the four peaks are shown in Table 2. Peaks I and II contained several peptides. Peaks III and IV each contained only one end-group, which indicated a single peptide with arginine as its terminal amino acid. Comparison of the indices of discrimination of the two purified peaks of activity with leukokinin-PMN, bradykinin and its natural analogues is seen in Table 3. It is evident that peak IV differs in its indices of discrimination from all the other peptides. Peak IV has been termed leukokinin-M. Peak III and the previously isolated leukokinin-PMN (PMN-kinin)[5] have very similar values and consequently are considered the same.

Amino acid analyses

Amino acid analysis of leukokinin-M. Approximately 6×10^{-9} moles of peak IV (estimated by the visual intensity of fluorescence of the dansylated end-group as compared to the intensity of control DNS-amino acids) was lyophilized; 300 μl of

TABLE 2. N-TERMINAL AMINO ACIDS

Peak	DNS-amino acid
I	Alanine, serine, arginine, glutamine*
II	Glutamine, serine, alanine
III	Arginine
IV	Arginine

* Where more than one DNS-amino acid was found, they are listed in order of the intensity of fluorescence.

TABLE 3. INDICES OF DISCRIMINATION OF PEAKS III AND IV AS COMPARED TO KININS*

	RU/GPI	RD/GPI	RBP/GPI
Bradykinin	1·0	1·0	1·0
Peak III	6·3	3·3	35·0
Peak IV	13·2	2·3	23·0
Kallidin	2·1	1·8	6·0
Met-Lys-bradykinin	3·4	2·2	15·0
Leukokinin-PMN†	5·9	2·9	33·0

* The data represent the average of three to six determinations. RU = rat uterus; RD = rat duodenum; RBP = rabbit blood pressure; GPI = guinea pig ileum.
† Data from Greenbaum *et al.*[5]

5·7 N HCl was added, the tube was sealed under vacuum, and the hydrolysis was allowed to proceed for 24 hr at 105°. The solution was brought to dryness in a rotary evaporator and HCl removed by addition of water and rotary evaporation several more times. The final residue was dissolved in 0·1 ml water and subjected to automated amino acid analysis. The results are seen in Table 4. Since most amino acids were present in an amount near to 0·01 μmole, this value was set equal to 1·0 in order to calculate the amino acid residues present in the sample of peptide.

TABLE 4. AMINO ACID ANALYSIS OF LEUKOKININ-M*

Amino acid	μM	No. of residues†	Total wt.
Alanine	0·0226	2	178·2
Arginine	0·0311	3	522·6
Aspartic acid	0·0109	1	133·1
Cysteine 1/2		0	
Glutamic acid	0·0159	2	294·1
Glycine	0·0208	2	150·2
Histidine	0·0096	1	155·2
Isoleucine	0·0043	0	
Leucine	0·0112	1	131·2
Lysine	0·0289	3	438·6
Methionine		0	
Phenylalanine	0·0078	1	165·2
Proline	0·0292	3	345·3
Serine	0·0150	2	210·2
Threonine	0·0127	1	119·1
Tyrosine	0·0089	1	181·2
Valine	0·0210	2	234·2
	Total	25	3258·5
	Water of bond formation		−432·0
	Mol. wt		2826·5

* Twenty-eight μg bradykinin equivalents (rat uterus) of peak IV was subjected to amino acid analysis (see text).
† Using 0·01 μM leucine as one residue.

Based on this analysis, the peptide of peak IV is composed of 25 amino acids; the mol. wt is 2826·5. It may be noted that no methionine residue and only one phenylalanine residue was found. This latter finding was surprising, since it indicated that bradykinin *per se* is not part of the molecule (bradykinin has two phenylalanine residues).

Amino acid analysis of leukokinin-PMN. In a previous publication,[5] a kinin which differed from bradykinin was isolated from incubates of rabbit peritoneal polymorphonuclear leukocytes and kininogen by procedures similar to that described above for macrophages. The kinin was termed PMN-kinin (present nomenclature, leukokinin-PMN). Amino acid analysis of this peptide is seen in Table 5. While this peptide

TABLE 5. AMINO ACID ANALYSIS OF LEUKOKININ-PMN*

Amino acid	μM	No. of residues	Total wt
Alanine	0·0112	1	89·1
Arginine	0·0183	2	348·4
Aspartic acid	0·0083	1	133·1
Cystine 1/2		0	
Glutamic acid	0·0111	1	147·1
Glycine	0·0161	2	150·2
Histidine	0·0117	1	155·2
Isoleucine	0·0031	0	
Leucine	0·0083	1	131·2
Lysine	0·0195	2	292·4
Methionine	0·0033	0	
Phenylalanine	0·0116	1	165·2
Proline	0·0263	3	345·3
Serine	0·0156	2	210·2
Threonine	0·0073	1	119·1
Tyrosine	0·0181	2	362·4
Valine	0·0080	1	117·1
	Total	21	2776·0
	Water of bond formation		−360·0
	Mol. wt		2416·0

* Sample was 1·0 ml of the peak activity equivalent to 50·0 μg bradykinin equivalents (rat uterus) obtained by procedures discussed in ref. 5 from a 0·9 × 100 cm column of carboxymethylcellulose.

has four less residues than the peptide produced by the macrophage enzymes, it too shows only one phenylalanine residue and generally resembles leukokinin-M. It has a molecular weight of 2416. The observation that bradykinin is not part of the leukokinin molecule raised the question as to the substrate which the white cell enzymes attack to form the leukokinins. In order to answer this question, pure bovine bradykininogen I[10] was incubated with macrophage enzyme. No kinin liberation could be detected, indicating two other possibilities: (1) that human kininogen which is used in our laboratory as the usual substrate differs considerably from the bovine variety, or (2) that white cell kinin-forming enzymes (in the main) attack a different substrate than bradykininogen, e.g. a leukokininogen (see Discussion).

Other chemical properties of the leukokinins. The sensitivity to proteases of leukokinin-PMN obtained from PMN enzymes and of leukokinin-M obtained from macrophage enzymes is the same. In both cases, it was found that exposure to chymotrypsin or carboxypeptidase B destroyed the activity. Trypsin and carboxypeptidase A had no effect on the activity. The kininases present in leukocytes destroy leukokinin-M at neutrality, as is the case with leukokinin-PMN.[5]

Partial sequence of leukokinin-M. The amino acid sequence of leukokinin-M was studied by a modification of the method of Gray[8] (see Materials and Methods). This procedure uses the Edman method for removing, in sequence, the amino acids from the N-terminal position of peptides and determines the new end-group by means of dansylation. The partial sequence of the peptide in peak IV was thus found to be: NH_2-Arg^1-Ala^2-Ser^3-?^{4}-?^{5}-Lys^6-.

Two typical chromatograms indicating that the terminal amino acids are arginine and alanine are seen in Figs. 3 and 4. As may be noted, there was difficulty in identifying amino acids 4 and 5 from the amino terminal position. It is suspected that these positions may be occupied by proline or glutamine residues. In the former case, DNS-proline is known to be labile to acid hydrolysis, while glutamine may form pyrolidone carboxylic acid during the chemical procedures and will not react with dansyl chloride. It should be noted that bradykinin (containing three proline residues) could be completely sequenced by the above procedures. During the sequencing, only one spot (or none at cycles 4 and 5) was observed after each cycle, which further substantiates the purity of the peptide being analyzed.

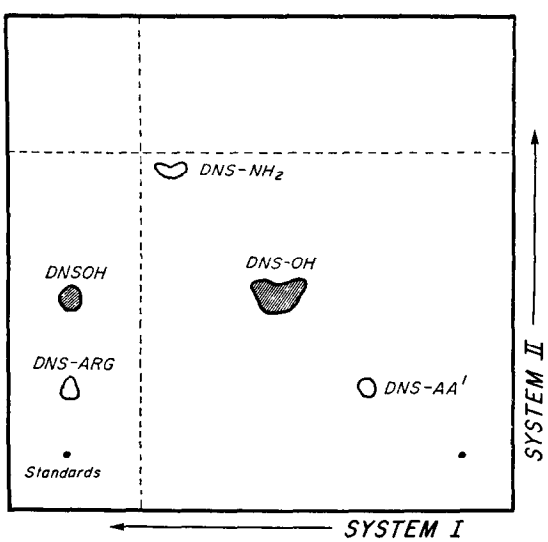

FIG. 3. Two-dimensional thin-layer chromatography (TLC) of dansyl end-group or peak IV on Silica gel H. Seven μg of bradykinin equivalents (rat uterus assay) of peak IV was dansylated and degraded as described in text. The free dansylamino acid of the end-group of peak IV (DNS-AA^1) was then spotted on a Silica gel H plate (20 × 20 cm) and developed two-dimensionally by ascending chromatography. The first dimension was carried out in solvent system I and the second dimension in solvent system II (see text). Standard DNS-arginine (DNS-ARG; 1 nmole) was spotted on the same plate after first dimension and chromatographed in system 2. DNS-NH_2 (1-dimethylamino naphthalene-5-sulfonamide) is a by-product with yellow fluorescence.

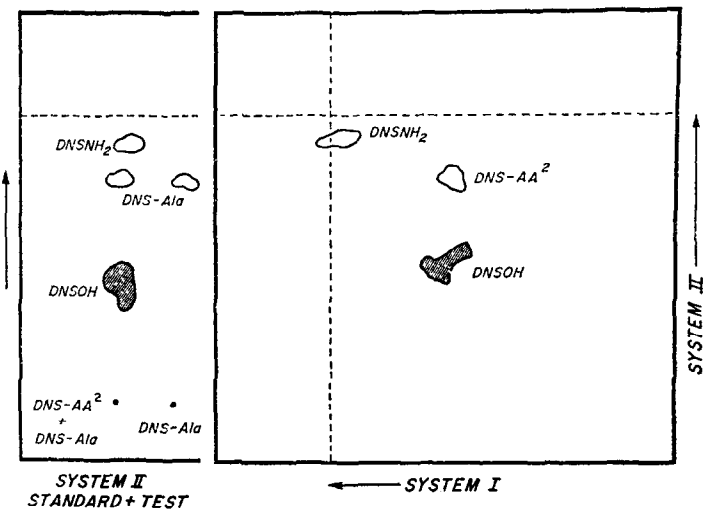

FIG. 4. Thin-layer chromatography (TLC) of dansyl amino acid[2] (the second amino acid of peak IV) on silica gel H. Two to three nmoles of peak IV with one amino acid residue less was dansylated. The dansyl peptide was treated with acid to free the dansyl amino acid (DNS-AA[2]) as described in the text. DNS-AA[2] was developed two-dimensionally by ascending chromatography on Silica gel H with system I followed by system II (right). The hatched spot is the blue fluorescence of 1-dimethylamino naphthalene-5-sulfonic acid (DNSOH). The open spots represent the yellow fluorescence of 1-dimethylamino naphthalene-5-sulfonamide (DNS-NH_2) and DNS-AA[2] whose R_f value is the same as that of standard DNS-alanine (left).

DISCUSSION

The results obtained show that enzymes in macrophages and polymorphonuclear leukocytes can catalyze the formation of potent pharmacologically active peptides from human plasma proteins which differ from bradykinin. The amino acid analysis of the two leukokinins isolated definitively indicates that bradykinin *per se* is not part of these higher molecular weight polypeptides, since the amount of phenylalanine in both leukokinin-M and leukokinin-PMN is not sufficient (only 1 mole) to include bradykinin as part of the molecule. The other amino acids of bradykinin, including three residues of proline, are present. One possibility is that the amino acid sequence of bradykinin is present in the leukokinins with some other amino acid replacing the phenylalanine residue. Further experiments are obviously necessary to elucidate this point. The partial sequence obtained from the amino-terminal end did not show any bradykinin-like sequence. In fact, the sequence obtained is unlike any other known by the authors.

In comparing the amino acid analyses of leukokinin-M and leukokinin-PMN, it will be noted that the two peptides differ from one another in that leukokinin-M is larger than leukokinin-PMN by four amino acids (a single residue of alanine, arginine, glutamic acid (amine) and lysine respectively). The additional basic amino acids are the probable reason why the leukokinins PMN and M (peaks III and IV respectively) can be separated on carboxymethylcellulose. One additional difference found is that, while leukokinin-M has two valines and one tryosine, the leukokinin-PMN polypeptide has one valine and two tyrosine residues. Overall, these differences no doubt account for the different pharmacological activities of these peptides (*Biochem. Pharmac*, **21**, 3107 (1972)).

The finding that leukokinin-PMN and leukokinin-M have been isolated from incubates of human kininogen with neutrophil and macrophage enzymes, respectively, does not mean that these white cells specifically make one leukokinin or another. Our observations seems to indicate that macrophages and other white cells probably can make one or more of these peptides under the appropriate conditions. Thus the macrophage incubates can produce both leukokinin-M and leukokinin-PMN (Fig. 2, Table 3). Furthermore, the pattern of leukokinins obtained by the macrophages is the result of total protease action on the substrate used, which gives no idea of the selectivity of leukokinin formation or if the final materials isolated are the initial peptides formed.

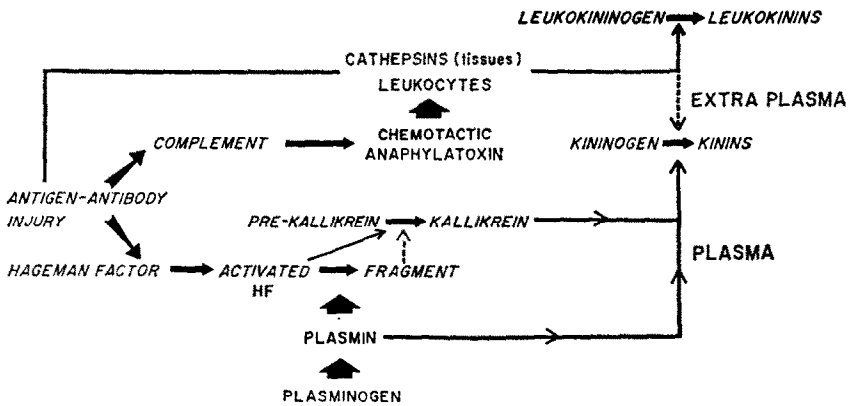

FIG. 5. Interrelationship of the leukokinin system with other systems in the formation of mediators of inflammation. The scheme reflects our interpretation of the inter-relationships of six systems in inflammation: blood clotting, fibrinolysin, complement, antigen–antibody, kallikrein–bradykinin, and leukokinin. Injury causes activation of Hageman factor, which is acted upon by plasmin to yield an activator of pre-kallikrein.[12–15] Kallikrein acts on plasma kininogen to yield bradykinin. Cathepsin-like proteases from tissues act on plasma proteins to yield leukokinins.[16] Antigen–antibody reactions can set off the complement system which liberates leukocyte chemotactic factors and also mast cell histamine-releasing mediators such as anaphylatoxin. Leukocytes emigrating to the sites of injury release leukokinin-forming enzymes which act on leukokininogen substrate(s) in plasma or fluids to yield leukokinins (see Discussion). The dotted line indicates the possibility that enzymes from leukocytes also may release some bradykinin.[5] The phrases "extra plasma" and "plasma" appearing on the right of the diagram refer to the source of the enzymes forming kinins and leukokinins.

If the leukokinins do not contain bradykinin as part of their molecule, this could mean that leukokinins are being cleaved from a substrate that is different from bradykininogen. The result obtained, indicating that pure bradykininogen (albeit bovine) does not serve as a substrate for white cell enzymes, supports evidence for this view. Additional support for this conclusion is derived from evidence that human plasma (which is the source of this laboratory's partially purified kininogen preparation) has been found to contain such leukokininogens which are chromatographically separable from bradykininogens.[11]

Our current view of kinin formation in injury, inflammation and anaphylaxis is seen in Fig. 5. Injury results in the activation of Hageman factor in plasma which, either directly or through the intervention of plasmin,[12–15] activates kallikrein to act on bradykininogen to yield bradykinin-like peptides. This is an "immediate" type of

system. The "delayed" leukokinin generating system is brought into play after white cells are brought to the site by chemotactic factors liberated by leukocytes including lymphocytes. Whether or not leukokinins themselves have chemotactic activity is now under study. Also under investigation is the mechanism by which white cell enzymes come into contact with leukokininogen substrates. As indicated in the Results, a good part of the activity resides in the membrane-debris fraction. This could mean that leukokinin-forming enzymes are present in releasable form in the cell membrane of white cells. To support this contention are our recent findings that guinea pig lymphocytes, while completely viable, release leukokinin-forming enzymes into a culture medium. If these enzymes are released at a more rapid rate from sensitized lymphocytes challenged with antigen than from normal cells, a direct link between anaphylaxis and leukokinin formation would be established.

The leukokinin-forming enzymes are not a property of white cells alone. The formation of kinins at acid pH by enzymes from spleen and other tissues,[16] as well as from malignant cells,[5,17] is no doubt the result of the activity of these enzymes. While acid pH seems to be a "nonphysiological" environment and leaves room to question the significance of these systems, it should be noted that we are dealing with highly potent pharmacological agents which need be liberated only in small amounts at discrete locations to be effective. Localized acidic pH changes with immediate release of leukokinins at the site certainly can take place. The significance of white cells, cancer cells, and tissues containing enzymes which liberate leukokinins that have potent "inflammatory" properties is impressive and should not be overlooked because conditions *in vitro* do not match with predicted conditions *in vivo*, especially when pathological conditions prevail.

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