THE CHARACTERISTICS, ISOLATION AND SYNTHESIS OF THE PHAGOCYTOSIS STIMULATING PEPTIDE TUFTSIN†

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Summary: The phagocytosis stimulating peptide present in γ-globulin has been isolated and its amino acid sequence shown to be L-threonyl-L-lysyl-L-prolyl-L-arginine. It was synthesized by the solid phase technic.

INTRODUCTION

In previous communications it was shown that only one particular fraction of freshly prepared human γ-globulin obtained on phosphocellulose (PC) chromatography, PC IV, contains a specific cytophilic γ-globulin, leucokinin. This binds specifically and reversibly to the autologous polymorphonuclear leucocyte (PMN) and stimulates its phagocytic activity 2-2.5 fold.[1-3]

A study of the mechanism of action of leucokinin showed that a highly active small fragment was responsible for the full effect of the whole molecule. It was expected, early in this study, that the mere binding of leucokinin to the receptor sites on the PMN cell membrane was all that was required for the expression of a high level of phagocytic activity. This level would be maintained while leucokinin was bound to the cell. This was not the case. The high stimulatory effect was not maintained and

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the rate of phagocytosis fell off in time to control levels despite the continuous presence of leucokinin. Further addition of leucokinin effected an immediate stimulation which eventually subsided. Thus leucokinin seems to be inactivated during the process. A corolary experiment was performed in which leucokinin was incubated with fresh, thoroughly washed, intact PMN cells for 30 minutes at 37°. The reisolated leucokinin preparation showed complete loss of biological activity. It failed to stimulate phagocytosis in a fresh preparation of PMN cells. However, there was no measurable alteration in its molecular weight, immunochemical and solubility properties. One reasonable interpretation of this phenomenon is that the interaction of leucokinin with an enzyme on the outer surface of the cell membrane resulted in the cleavage of a small active fragment which the cell utilized to enhance its biological activity. On this basis, cell membrane preparations were made from glass purified PMN neutrophils.[4] Such preparations indeed showed a highly active leucokininase. The enzyme cleaves a peptide that is extractable in 80% ethanol. This peptide, tuftsin, proved to be solely responsible for the full activity of the leucokinin molecule yielding the same level of activity obtained with the parent molecule.

MATERIALS AND METHODS

The materials used were: chloromethyl resin (SBR), silica gel-G (Anal-Tech), palladium on barium sulfate 5% (Engelhard), N^{α}-tertiary butyloxycarbonyl (BOC)-L-amino acids (Fox Chemical), amino acid analyzer (Beckman-Spinco 121).

A typical biological assay is detailed in the legend to Table I. Essentially, tuftsin is liberated from PC IV leucokinin either by incubation with leucokininase or trypsin at the appropriate pH at 37° for 1 hour. The liberated tuftsin is then extracted in ethanol and dried. It is then taken up in Krebs-Ringer buffer and assayed for its ability to stimulate phagocytosis of Staphylococcus aureus by either PMN cells from human or dog buffy coat preparations, PMN cells from guinea pig peritoneal exudates, as well as macrophages from mouse peritoneal and rabbit lung washings.

RESULTS AND DISCUSSION

Leucokininase releases tuftsin only from PC IV after which the peptide becomes readily dialyzable. The enzyme activity varies directly with its concentration and has a pH optimum at 6.7. It is insoluble, heat labile, and deteriorates on freeze-thawing and prolonged storage. Most preparations are contaminated by tuftsin destroying enzymes, consequently, the yield of the peptide is always low. However, it was possible to define some of the properties of this peptide. It is retained on Sephadex G-10, and is resistant to trypsin, chymotrypsin, carboxypeptidase A, clostripain, phosphatase, DNAse and RNAse. It is destroyed by carboxypeptidase B (CP-B), leucine aminopeptidase (LAP) and pronase. Trypsin also releases all the tuftsin present in the parent PC IV fraction. Further treatment with leucokininase fails to release any more tuftsin activity. The peptide released by trypsin has all the physicochemical and biological characteristics of that released by leucokininase (Table I). These include behavior in several chromatographic systems, susceptibility to various enzymes and biological activity in various PC fractions in normal and disease states.[5,6] Thus the identity of the two products biologically and physicochemically was strongly indicated. Consequently, the isolation of the tryptic peptide was undertaken.

Purification and Sequence: PC IV, 50 mg, were incubated with 2.5 mg of 3 × crystallized trypsin in 12 ml of 0.1 M Tris-HCl buffer pH 8.1 for 1 hour at 37°. TCA was then added to 6% concentration. The supernatant was freed of TCA by ether extraction and the aqueous phase lyophilized. It was chromatographed on Sephadex G-10, 1.8 × 116 cm in 0.1 M acetic acid. The only active fraction, 114-160 ml, was separated on Aminex AG 50 W-X4, 0.6 × 56 cm, 60°, linear pyridine acetate gradient, starting buffer 100 ml, pyridine 1.2 M pH 4 and limiting buffer 100 ml, pyridine 2.5 M pH 6. The activity was located only in one small ninhydrin positive peak, at 142 ml and 2.2 molar pyridine. Analysis yielded a ratio of Lys 1.0, Arg 1.05, Thr 0.84, Pro 1.05.

Table I

BIOLOGICAL AND PHYSICOCHEMICAL CHARACTERISTICS OF THE PHAGOCYTOSIS STIMULATING PEPTIDE

Characterization	Releasing Enzyme	
	Trypsin	Leucokininase
1. Aminex AG-50, elution molarity and volume	2.2 M, 142 ml	2.2 M, 143 ml
2. Silica gel-G, Rf of the dansylated peptide		
(a) Chloroform:methanol:acetic acid	0.23	0.22
(b) *n*-butanol:acetic acid:water	0.11	0.11
(c) Methylacetate:isopropanol:ammonia	0.44	0.45
3. Susceptibility to degradative enzymes		
(a) Trypsin	resistant	resistant
(b) Chymotrypsin	"	"
(c) Carboxypeptidase A	"	"
(d) Clostripain	---	"
(e) Phosphatase	"	"
(f) Ribonuclease	"	"
(g) Deoxyribonuclease	"	"
(h) Leucine aminopeptidase	destroyed	destroyed
(i) Carboxypeptidase B	"	"
(j) Pronase	"	"
4. Biological activity		
(a) PC IV from normal subjects $\frac{1}{2}$ maximal assay activity derived from	30 μg	30 μg
(b) PC I, PC II, PC III	inactive	inactive
(c) PC IV from splenectomized subjects	"	"
(d) PC IV from patients with tuftsin deficiency	"	"

Legend to Table I: Biological activity[5,6] is routinely measured by the stimulation of the phagocytic activity of thoroughly washed blood PMN leucocytes in Krebs-Ringer medium pH 7.4, using opsonized Staphylococcus aureus as the target particle.

Tuftsin is liberated from PC IV by digestion for 1 hr at 37° with 4 μg of leucokinin or 50 μg of trypsin per mg of PC IV per ml in 0.1 M phosphate buffer pH 6.7 or 8.1 respectively. Controls with enzymes alone are run concurrently. Four volumes of ethanol are added and the supernatant evaporated to dryness. The precipitate is taken up in 0.25-1.0 ml of Krebs-Ringer medium as required.

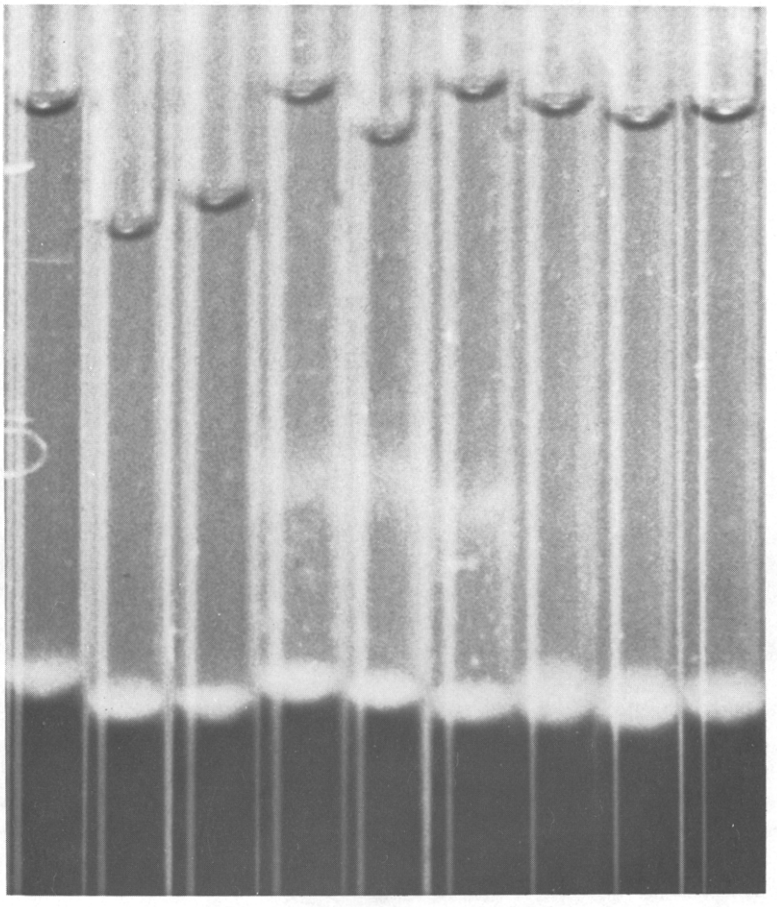
The phagocytosis reaction mixture is prepared by the rapid addition of the following: (a) 100 μl of PMN leucocytes washed in Krebs-Ringer medium, containing 2×10^6 PMN and 0.3 mg of bovine serum albumin. (b) 100 μl of ethanol extract of trypsin or leucokininase digest. (c) 100 μl of an 18 hr culture of Staph. aureus containing 4×10^6 bacteria to start the reaction. The mixture, in siliconized 1 ml glass stoppered tubes and controls containing enzyme digests lacking PC IV, are incubated at 37° for 30 min in a vertical rotor and tumbled at 8 cycles per minute. Smears are then stained and cells containing bacterial particles are recorded per 100 PMN. Usually 200-400 PMN leucocytes are counted. All samples are run in duplicates.

The dansyl-peptide[7] with an Rf 0.11 on silica gel-G in *n*-butanol:acetic acid:water, 50:1:50. was hydrolyzed and chromatographed in chloroform : methanol : acetic acid, 15 : 4 : 1. Two fluorescent residues were identified, N^{α}dansyl-Thr, Rf 0.61 and N^{ϵ}dansyl-Lys, Rf 0.15. Incubation of 30 *n* moles with 30 μg of LAP for 4 hours in barbital buffer pH 8.5 at 40° liberated 32% of the threonine and 13% of the lysine. An aliquot of the LAP digest was dansylated and chromatographed without hydrolysis on TLC in *n*-butanol:acetic acid:water. Two fluorescent residues were obtained corresponding to the dansyl markers, N^{α}dansyl-Thr, Rf 0.47 and N^{α}, N^{ϵ}-didansyl-Lys, Rf 0.57. The sequence thus far is Thr-Lys (Pro Arg).

Treatment of the peptide with CP-B in phosphate buffer pH 7.4 yielded arginine which was identified in the amino acid analyzer. On thin layer cellulose (Avicel) in phenol:water, 3:1, it yielded a Sakaguchi positive spot Rf 0.36. The carboxy-terminal arginine was further confirmed by tritium labeling. Here 8 *n* moles were dissolved in pyridine 0.2 ml, acetic anhydride 50 μl and $^{3}H_2O$ 50 μl according to Matsuo.[8] Arginine alone incorporated tritium, 153 cpm, whereas Thr, Lys, Pro and controls yielded 9-13 cpm. The sequence thus far is Thr-Lys (Pro) Arg.

Proline as the penultimate carboxy-terminal was identified also by tritium labeling after cleaving arginine with CP-B using Matsuo's modification.[8] It was clear that CP-B removal of arginine had exposed proline as the carboxy-terminal of the resulting tripeptide. Proline incorporated 170, Thr 29, Arg 44 and Lys 39 cpm per *n* mole. The full sequence is therefore established as Thr-Lys-Pro-Arg.

Synthesis of the Tetrapeptide: The Merrifield solid phase technic was used for the synthesis.[9] N^{α}-*t*-butyloxycarbonyl (BOC) N^{G}-nitro-L-arginine was esterified to the chloromethyl copolystyrene-2% divinylbenzene resin containing 2.3 *m* moles of chloride per gram. One gram of resin esterified 0.32 *m* mole of BOC nitro-arginine. The amino protecting group was cleaved by trifluoroacetic acid 50% in methylene chloride. After



Legend to Figure 1

Fresh heparinized dog blood was washed three times with four volumes of Hank's solution. 0.1 ml of packed cells was mixed with 0.1 ml of Hank's solution containing tuftsin. The mixture was drawn into microhematocrit tubes, sealed, centrifuged, and incubated openside up at 37°. After two hours the migration in tuftsin tubes was demonstrably greater than in the control tubes with upward swarming of PMN cells. This picture was taken after eighteen hours' incubation. The first three tubes on the left are control tubes, the next three and the last three tubes to the right contain 25 and 5 n moles per ml of tuftsin respectively. Note that the PMN cell front, in tubes containing 25 n moles per ml, swarmed a distance of 3.3 mm. The tubes containing 5 n moles per ml showed no swarming but a considerable number of cells, not visible in the figure, moved 1.2 mm. PMN cells in control tubes leveled off early at 0.3 mm in three hours.

washing the resin and neutralization with triethylamine, N^{α}-BOC-L-proline 0.9 *m* mole was added to the resin and the α amino group of nitro-arginine coupled to the carboxyl group of proline by dicyclohexylcarbodiimide 0.9 *m* mole in methylene chloride. The procedure was repeated for each of the incoming two other residues N^{α}-BOC-N^{ϵ}-carbo benzoxy-L-lysine and N^{α}-BOC-O-benzyl-L-threonine respectively. Finally, the peptide was released from the resin by hydrogen bromide in trifluoroacetic acid and exposed to catalytic hydrogenation at 40 PSI with palladium on barium sulfate as catalyst. The tetrapeptide was then purified on Aminex as before. The effluent peak coincided exactly with that of natural tuftsin with an effluent volume of 145 ml at 2.2 M pyridine yielding an amino acid ratio of Lys 1.0, Arg 0.94, Thr 1.1, Pro 0.99. It also gave identical Rf values before and after dansylation in the various systems described above. High voltage electrophoresis, 40 volts per cm at 5°, gave identical mobilities. Both synthetic and natural peptides at pH 8.9 in 0.1 M ammonium carbonate for thirty minutes, remained at the origin, at pH 8.4 for ninety minutes, migrated 8.5 cm and at pH 1.9 in 0.75 M formic acid for sixty minutes, migrated 24 cm to the cathode. Both peptides were equally susceptible to LAP, CP-B and pronase. Their specific biological activity was identical, reaching maximum at 0.1 μg per ml of the mixture.

In addition to the stimulation of particle ingestion, bacteria and latex, the peptide also stimulates pinocytosis of ^{131}I-albumin. The cells survive much longer in the presence of 5-25 *n* moles of the peptide. Motility studies in capillary tubes[10] showed that in the presence of small concentrations of the peptide PMN migration was stimulated for a prolonged period of time. This is shown in Figure 1. The only synthetic analog tested, Thr-Lys-Pro-Pro-Arg, failed to exhibit any stimulation of PMN motility.

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