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RECEPTOR-MEDIATED ENDOCYTOSIS OF TUFTSIN BY MACROPHAGE CELLS

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SUMMARY: A fluorescent analog of the phagocytosis stimulating peptide tuftsin was prepared by coupling tetramethyl rhodamine isothiocyanate to a C-terminal elongated derivative of tuftsin. This analog, Thr-Lys-Pro-Arg-Gly-Lys(N^{ε}-tetramethyl rhodamine)-OH, was used to visualize tuftsin receptors on mice macrophage cells by fluorescent image intensification. Fluorescent labelling was carried out at 37°C, using a concentration of 200 nM and 2 µM of the fluorescent tuftsin derivative. The formation of peptide-receptor clusters and their subsequent internalization, as discerned by image intensification, were rapid processes, 5 min and 5-30 min, respectively. Preincubation of macrophages with tuftsin for various time intervals, followed by quantification of the tuftsin receptor using radiolabelled tuftsin, suggest that tuftsin receptors are initially increased in amount (5-7 min) and subsequently reduced (after 10-15 min) as judged by sites available for tritiated tuftsin. The binding studies are rather complementary to the fluorescence observations and support the assumption that the tuftsin receptor on the membrane of the mice macrophage cell is rapidly mobilized.

Tuftsin, Thr-Lys-Pro-Arg, is an immunologically active peptide which stimulates, both *in-vivo* and *in-vitro* a number of phagocytic cell functions (1,2). The effect of tuftsin is initiated by its binding to distinct plasma membrane receptors which were found on polymorphonuclear leukocytes (PMNL) and monocytes (3,4), and on macrophages (MØ) (5). Structure-function studies of tuftsin indicate that its binding to phagocytes and their consequent activation is dependent upon rather strict conservation of its molecular structure. Thus, modification of the peptide at its N-terminus or within the chain, lead to a significant reduction or even loss of activity and the ability of binding to PMNL (2,6). Our recent study (7), however, has demonstrated the feasibility of extending the C-terminus of tuftsin while still preserving biological activity and the capacity to significantly inhibit the specific binding of tritiated tuftsin to macrophage cells.

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We have reported on some features related to the interaction between tuftsin and its specific binding sites on the phagocytic cell surface, e.g. equilibrium dissociation constants, population of receptors (2,3,5) and possible relevant evoking of second messengers (2,8). In order to further characterize the tuftsin receptor, and to shed more light on events connected with its association with the peptide, we have prepared a highly fluorescent analog of tuftsin by convalently adding tetramethyl rhodamine to its C-terminus. This analog was utilized in the present study for the visualization of tuftsin's binding sites on mice macrophage cells, and to follow the various stages of receptor endocytosis on these cells. In parallel, quantification of the receptor with tritium labelled tuftsin was performed.

MATERIALS AND METHODS

Synthesis: Tuftsin and tritiated tuftsin ([^{3}H-Arg^{4}]tuftsin, specific activity 20.2 Ci/mmol) were synthesized as previously described (3,9).

Thr-Lys-Pro-Arg-Gly-Lys(N^{ε}-tetramethyl rhodamine: Boc-Thr-Lys(Boc)- Arg-Gly-Lys-OH (7) (0.003 mmoles, 2.5 mg) was coupled with tetramethyl rhodamine β-isothiocyanate (Research Organics, Ohio, USA) (0.003 mmoles, 1.5 mg) in N,N'-dimethylformamide (200 µl) containing triethylamine (0.007 mmole, 1 µl) for 3 hrs at room temperature in the dark. Solvents were removed *in vacuo* and the residue dried in a dessicator over P_2O_5. Removal of t-butyloxycarbonyl protecting groups (t-Boc) was achieved by treating the product with 2 ml of a mixture of trifluoroacetic acid:dichloromethane (1:1, v/v) for 15 minutes at room temperature. After solvent removal at 30°C *in vacuo*, the product was dried in a dessicator over NaOH pellets. Purification of the crude product was achieved chromatographically on a carboxylmethylated cellulose ion exchange column (CM-23; 1x20 cm), using a gradient of ammonium acetate pH 6.5. The column was equilibrated with 1 mM buffer and salt molarity increased to 0.5 M in a linear gradient with constant pH. An additional purification step was carried out by gel filtration on Sephadex G-15 equilibrated with phosphate buffered saline (PBS; pH 7.4). Amino acid analysis, after exhaustive acid hydrolysis (6 N HCl, 110°C, 18 hrs in evacuated sealed tubes) gave: Thr, 0.98; Lys, 1.85; Pro, 1.08; Arg, 1.00; Gly, 1.00. The peptide was kept frozen, in the dark, in PBS.

Macrophages: Peritoneal exudate cells were aseptically collected from thioglycollate-stimulated BALB/C strain male mice (6-8 weeks old) as previously described (5). The cell preparations consistently contained >90% macrophages of which more than 87% were viable cells.

Image Intensification Fluorescent Microscopy: Image intensification-microscopy system (10) of Dr. J. Schlessinger, Department of Chemical Immunology, The Weizmann Institute of Science, was used. The distribution and binding of fluorescent tuftsin was visualized by using a Zeiss Universal microscope equipped with epifluorescence, an oil emersion planapo 63/1.25 N.A. Lens, and an RCA silicon intensifier target TV camera (TC 1030H). Video output was recorded on a panasonic recorder (VTR-NV-8030) and displayed on a TV monitor (NV-5300) from which polaroid photographs were taken.

Fluorescent Studies: Macrophage cells were incubated, in plastic tubes (Nunc), at 37°C in PBS, with various concentrations (ranging from 200 nM to 2 μM) of the fluorescent derivative of tuftsin. At specified times, the reaction was terminated by centrifugation and pelleting of cells. The cells were washed twice with PBS (4 ml, 22°C), isolated by centrifugation and then fixed by treatment with 1% formaldehyde solution in PBS (1 hr at 4°C). Cells were washed twice with PBS and inspected by fluorescence image intensification. Parallel incubations and fluorescent studies were carried out in which an excess of tuftsin (20 μM) was added to displace the fluorescent analog.

Competitive Binding Studies: Macrophage cells ($0.5\text{-}1.0\times10^6$) were incubated in PBS with tritiated tuftsin (50 nM) and varying concentrations of tuftsin or its fluorescent analog in plastic tubes (NUNC); 0.5 ml final volume, 30 min, indicated temperature. Binding assay was terminated by the addition of PBS (3 ml, 22°C), centrifugation and aspiration of supernatant. Cells were subsequently washed similarly once more. The cell pellets were dissolved in 0.3 ml sodium dodecyl sulfate (0.2%) and the solutions obtained were collected and added into 4 ml Triton-toluene scintillation cocktail. Radioactivity was measured in a Beckman 7500 scintillation spectrophotometer with 60% efficiency for tritium. Each point of the resulting binding plots derived from triplicate tubes with standard error of the mean (SEM) of less than 5%. Specific binding is defined as the amount of labelled tuftsin displaceable by the addition of 20 μM tuftsin. All figures refer to specific binding.

RESULTS AND DISCUSSION

A highly fluorescent analog of tuftsin was synthesized by coupling tetramethyl rhodamine to the extended C-terminus of tuftsin (Fig. 1). The method of attachment and the fluorescent group location in the molecule was carefully designed to preserve the bioactivity of the peptide and the integrity of the prosthetic fluorescent moiety. Particular care was taken in this regard

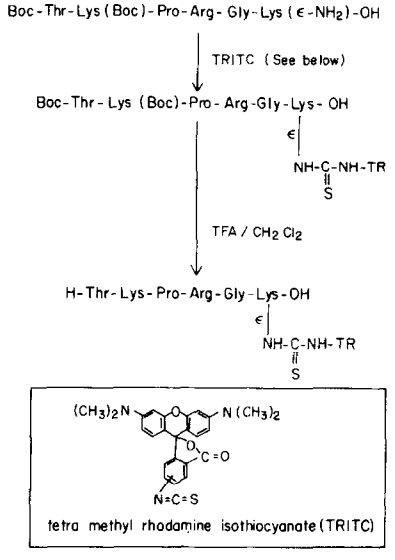

Figure 1: Synthetic scheme for the preparation of tetramethyl rhodamine analog of tuftsin.

to achieve mild conditions for the removal of the t-butyloxycarbonyl protecting group (7).

The tuftsin analog synthesized was shown to be pure as judged by high voltage paper (Whatman No. 3 paper) electrophoresis (pyridine-acetate buffer, pH 3.5; formic-acetic acid mixture, pH 1.9), a fluorescent species which stained positively with Sakaguchi and ninhydrin reagents, and according to its amino acid composition. In addition, the fluorescent segment of this molecule retains similar spectrophotometric characteristics reported earlier (11,12) for bound-rhodamine with excitation at 550 nm and maximal fluorescent emission at 574 nm, and with maximal absorptions at 360 nm ($\varepsilon \simeq 12,000\ M^{-1}\ cm^{-1}$), and at 550 nm ($\varepsilon \simeq 80,000\ M^{-1}\ cm^{-1}$).

The ability of the fluorescent analog to inhibit the binding of tritiated tuftsin to tuftsin's specific binding sites on macrophage cells is shown in Fig. 2. Scatchard analysis (not shown) reveals that the binding affinity of the fluorescent analog to the receptor site is somewhat lower ($\sim$100 nM) than tuftsin itself (50 nM). This finding agrees with a similar type of extended analogs synthesized and tested previously (7). The fluorescent analog was capable of stimulating the macrophage cell to phagocytize opsonized sheep red blood cells (5) practically to the same extent as unmodified tuftsin. This and the above finding confirm the fact that the peptide is a tuftsin-like active material.

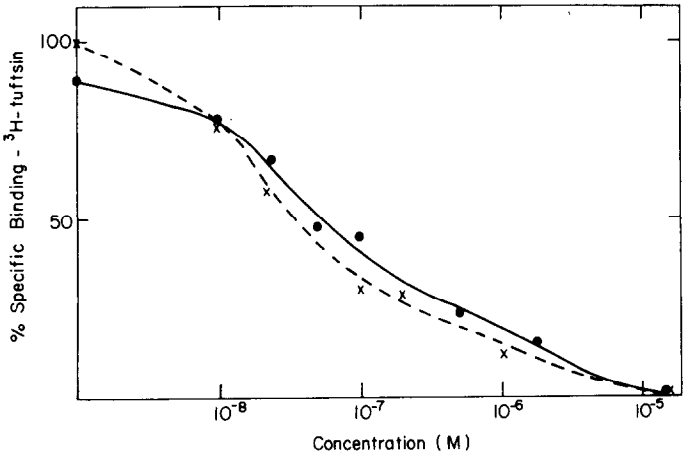

Figure 2: Binding competition study of tuftsin (x---x) and fluorescent tuftsin analog (●---●). Tritiated tuftsin (50 nM) was incubated with macrophages (1.0×10^6 cell per tube) in the presence of varying concentrations of indicated peptides. Results are displayed in terms of specific binding.

In order to gain additional insight into the tuftsin receptor, the tuftsin-fluorescent analog was incubated with macrophage cells, harvested from mice, and the association was monitored by image intensification. Figure 3 displays the fluorescent distribution of the peptide at various time intervals at 37°C. Thus, when macrophages were incubated with the peptide, at a concentration of 200 nM, uniform distribution of fluorescence was apparent after 5 minutes (Fig. 3A). Further, fluorescent clusters were observed on cells after a period of 10 minutes (Fig. 3C). These assemblies began to internalize into cells after about 10 minutes, and the process was apparently accomplished within less than 30 minutes (Fig. 3D). Excess unlabelled tuftsin (20 μM) practically abolished the capacity of the fluorescent analog (200 nM) to attach to cells (Fig. 3B), pointing thus at the specificity of binding sites.

Identical patterns of slightly less rapid events was observed when incubation of cells and fluorescent peptide was carried out at 22°C (not shown). At 4°C however, no specific peptide-cell association occurred even after a few hours of incubation. Endocytosis of the tuftsin-receptor complex was also found to be a concentration dependent event. Thus, fluorescent visualization by image intensification over a concentration range of 200 nM to 2 μM of Thr-Lys-Pro-Arg-Lys(N^{ε}-tetramethyl rhodamine) (not shown) reveals that the rate of formation of receptor-peptide clusters and their subsequent internalization increase substantially with concentration, i.e. 5-10 min at 2 μM as compared with 10-30 min at 200 nM.

The rapidity of internalization of tuftsin-receptor complexes is not surprising in light of the known high activity of the macrophage membrane. The recent report that receptors for the chemotactic peptide on other phagocytic cells, i.e. polymorphonuclear leukocytes can internalize in less than 1 minute when associated with the respective peptide (13) lent credence to the possibility that tuftsin receptor may also undergo radip internalization. This latter assumption is strongly supported by the fact that tuftsin and the chemotactic peptide evoke similar events in phagocytic cells (14).

Dynamical alterations of the tuftsin receptor on macrophage cells was also demonstrated by the use of radiolabelled tuftsin ([^{3}H]tuftsin). Thus, tuftsin

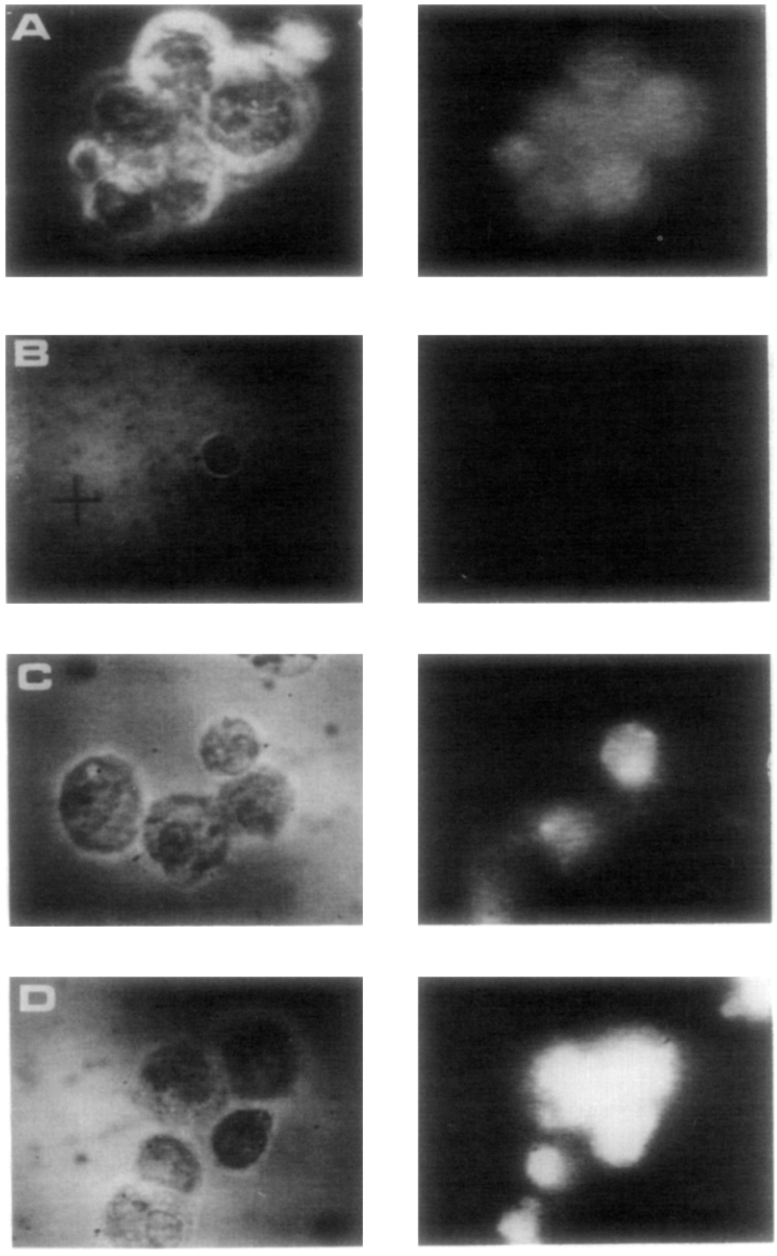

Figure 3: Fluorescent visualization of tetramethyl rhodamine tuftsin-analog binding to mice macrophages at 37°C. On the left, phase-contrast micrographs; on the right, fluorescent micrographs of the same field. (A) Uniform distribution of fluorescent-tuftsin (200 nM) after incubation period of 5 min. (B) Non-specific binding of fluorescent-tuftsin. Cells were incubated with 200 nM fluorescent peptide in the presence of 20 μM tuftsin; (C) Cluster formation on cells following incubation with fluorescent-tuftsin (200 nM); (D) Internalized clusters after 30 min incubation period with fluorescent-tuftsin (200 nM).

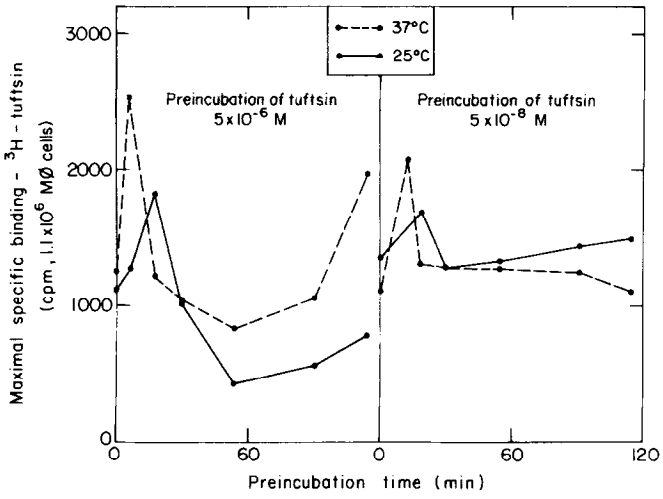

Figure 4: The effect of preincubation of macrophages with unlabelled tuftsin on the binding of [^{3}H]tuftsin. Macrophage cells were preincubated with tuftsin for various time periods and checked for their ability to bind tritiated tuftsin. The left section represents maximal specific binding, at each indicated time, of tritiated tuftsin (50 nM) subsequent to incubation with 5 μM of tuftsin. Parallel incubations were carried out at 37°C (•---•) and at 22°C (o——o). Prior to binding studies, cells were washed extensively with PBS to remove unlabelled ligand. The right section demonstrates the binding pattern of tritiated tuftsin (50 nM) after preincubation with 50 nM tuftsin. Identical conditions were used in both binding studies.

and macrophage cells were incubated at various periods, at both 22°C and 37°C. Cells were then washed extensively with PBS to dissociate bound peptide and binding competition studies with tritiated tuftsin were performed. As shown in Fig. 4 marked fluctuations in the ability of tuftsin to competitively inhibit binding of labelled peptide to its receptor site were apparent at both temperatures. The effect was more evident when macrophages were preincubated with 5 μM of unlabelled tuftsin, but rather similar at a much lower concentration of tuftsin, 50 nM. In both cases, one can discern an initial increase in the ability of the macrophage cells to bind tuftsin, although at higher temperatures the effect is faster and more pronounced. A little later (after ~10 min at 37°C) a noticable drop in the specific binding of tuftsin was observed. After 90 min preincubation of tuftsin with macrophages, there was another increase in binding, signifying perhaps a regeneration of the tuftsin receptor. This increase was saturable (not shown) and does not indicate transport of labelled tuftsin into the cell. Scatchard analysis of the binding data (not shown) suggests that there is practically no change in the affinity of

tuftsin's attachment to cell, but a drop of up to 60% in the number of sites available for its binding.

In light of these receptor visualization and binding studies, the tuftsin-receptor appears to follow a pattern of dynamic events previously described for other peptide hormone-receptor complexes (10, 11, 13, 15-17). Thus, it is first uniformly distributed on the macrophage cell, whereupon there is a formation of clusters and internalization of complex. A rather similar pattern of events was reported recently by Amoscato et al (18) when using N-terminal fluorescein-labelled derivative of tuftsin and polymorphonuclear leukocytes. Two additional features of interest were gleaned from our experiments. The first is the initial increase-decrease in tuftsin receptors. At present, we have no explanation of the physiological significance of this phenomenon. The second is the quick regeneration of tuftsin's receptor site. It is not clear whether there is recycling of internalized receptor or the emergence of stored receptor sites in the cell.

Several recent studies have indicated that in some systems (e.g. insulin, and gonadotropin releasing hormone) receptor cross-linking at the cell surface is by itself sufficient to trigger the subsequent biological events of hormonal action (for review see 19), although ligand internalization occurs. On the other hand, in some other systems (e.g. low density lipoprotein), internalization and degradation of protein-receptor complexes is required for evoking of biological activity (19). A question may arise as to the connection between the mobility of the tuftsin's receptor and the biological function of tuftsin. The concentration of the fluorescent analog used in this study (200 nM) is practically identical to that needed to elicit maximal phagocytic activity in macrophages (2,5). Moreover, the time schedule of the receptor-related events described herein is very similar to that of several cellular occurrences which follow the interaction of tuftsin with macrophages, e.g. changes of intracellular levels of cyclic nucleotides or redistribution of Ca^{++} ions (2,8). Furthermore, the stimulatory effect of tuftsin on the phagocytosis by macrophages is very prominent after about 10 minutes at 37°C

(5). Yet, whether clustering or internalization of tuftsin receptors have any relevance to the biological functions of tuftsin is currently not known.

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