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SPECIFIC BINDING SITES FOR THE PHAGOCYTOSIS STIMULATING PEPTIDE TUFTSIN ON HUMAN POLYMORPHONUCLEAR LEUKOCYTES AND MONOCYTES

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SUMMARY

Highly purified and biologically active [^{3}H]tuftsin (specific activity 9 Ci/mmol) was synthesized and its binding to several types of human circulating blood cells was studied at 22°C. The binding to polymorphonuclear leukocytes and to monocytes was found to be specific, fast, saturable and reversible. Values for the dissociation constants (K_D) were derived from equilibrium experiments and are 130 and 125 nM, respectively. The number of binding sites is approximately 50,000 and 100,000 per cell, respectively. Under the same experimental conditions lymphocytes exhibited only a threshold binding capacity for [^{3}H]tuftsin whereas erythrocytes revealed no detectable binding.

INTRODUCTION

The naturally occurring basic tetrapeptide tuftsin, L-threonyl-L-lysyl-L-prolyl-L-arginine, exhibits a high activation potential for mammalian phagocytic cells, the polymorphonuclear leukocyte (PMNL) and the macrophage. Tuftsin augments both the phagocytic (1,2) and bactericidal (3) activities of these cells as well as stimulates several of their enzymatic systems (4-6). More recently it was found to trigger the immunogenic function of macrophages (7).

Our studies with human PMNL (4-6, 8) and with mice macrophages (7) have shown that altering the structure of tuftsin, drastically effects its biological activity which is usually reduced. Activity was found to be significantly impaired by: (a) amino acid substitution or deletion at the C-terminal, N-

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Abbreviations used: Standard abbreviations for amino acid and peptide derivatives are used [J. Biol. Chem., 247, 977 (1972)]. Other abbreviations include: PMNL, polymorphonuclear-leukocyte; PBS, phosphate-buffered saline; SEM, standard error of the mean.

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terminal or within the chain; (b) absence of free amino and carboxyl groups at the peptide termini; (c) substitution of the guanidine side chain at position four or altering its distance from the peptide backbone; and (d) lengthening the peptide chain by addition of amino acids at either C or N-terminus. The rather strict architectural requirements for biological activity of tuftsin suggest that in order to exert its full stimulatory effects the peptide assumes a particular three dimensional structure. Moreover, the above findings coupled with the reports of reversible inhibitory effects of certain structurally related tuftsin analogs (4-6), point to an interaction of the peptide with specific cellular binding sites. The present study was undertaken in order to examine this latter assumption.

We report below on studies of the binding of tritium-labeled tuftsin to human PMNL and several other types of circulating blood cells, as well as on its specificity. The synthesis of [^{3}H]tuftsin used in these studies, and the synthesis of [^{3}H][N-acetyl-Thr^1]tuftsin, an inactive tuftsin analog (5,6) used as a reference compound, is also reported.

MATERIALS AND METHODS

Synthesis of [^{3}H]tuftsin. N-t-Butyloxycarbonyl-L-threonyl-N$^{\epsilon}$-benzyloxycarbonyl-L-lysyl-L-proline N-hydroxysuccinimide ester (9) (60 mg) was coupled for 3 hr at room temperature with L-[5-^{3}H]arginine (1 mCi; specific activity 9 Ci/mmol; Radiochemical Center Amersham, England) in dioxane-water (1:1, v/v; 2 ml) in the presence of $NaHCO_3$ (~ 10 mg), added to achieve a weakly basic medium (pH 8.4). The reaction mixture was diluted with 90% aqueous acetic acid (50 ml) and hydrogenated catalytically (5% Pd/$BaSO_4$; Fluka AG) for 8 hr at room temperature to remove the N$^{\epsilon}$-benzyloxycarbonyl protecting group. Treatment of the product for 7 min at room temperature with trifluoroacetic acid (3 ml; Fluka AG) removed the N-t-butyloxycarbonyl group, yielding the crude tritiated tuftsin, Thr-Lys-Pro-[^{3}H]Arg, accompanied by excesses of Thr-Lys-Pro and other minor side-products. The peptide was purified by column chromatography on AG 50W-X4 cation exchange resin (1 x 30 cm; 200-400 mesh; BioRad Laboratories, Richmond, California) (5,10). The purity of the radioactive tuftsin thus obtained was > 99% as assessed by high voltage paper electrophoresis at pH 1.9 and 3.5 (6). The product was indistinguishable from unlabeled tuftsin both electrophoretically and by biological assays (the direct determination of phagocytosis and the reduction of the dye nitroblue tetrazolium by human PMNL) (6). Over-all yield, based on the initial amount of L-[5-^{3}H]arginine, was about 60%.

Synthesis of [^{3}H][N-acetyl-Thr^1]tuftsin. [^{3}H]tuftsin (200 µCi) was dissolved in a solution of 45% HBr in glacial acetic acid (2 ml; Fluka AG) and allowed to react for 8 hr at room temperature to give [^{3}H][O-acetyl-Thr^1]tuftsin. The solvent was then evaporated under reduced pressure, the residue was taken into an aqueous solution of NH_4OH (2 ml; 0.03 N; pH 7.8), and the homogeneous

mixture obtained was allowed to stand for 2 hr at room temperature. Under these conditions O→N shift of the acetyl group occurred (11) to give the desired product, [^{3}H][N-acetyl-Thr^1]tuftsin. Yield was 82%. The peptide was purified on a AG 50W-X4 column and found to be electrophoretically pure and identical with authentic unlabelled marker (5).

Media. Dulbecco's phosphate-buffered saline (PBS) (pH 7.4) was prepared from instant tissue culture powder medium (Grand Island Biological Company, New York) using double distilled water. Sucrose buffer contained 0.27M sucrose (B.D.H. Chemicals) in $5x10^{-3}$M phosphate buffer (pH 7.4). The phosphate-buffered saline and the sucrose buffer were sterilized by millipore (0.22μ) filtration.

Cell Preparations. The cell preparations studied were obtained from heparinized peripheral blood of healthy human individuals. For preparation of polymorphonuclear leukocytes, the mononuclear cell fraction and purified lymphocytes, the blood was initially fractionated by Ficoll-Hypaque centrifugation according to Böyum (12), and the specific cells isolated essentially as detailed by Williams et al. (13).

Polymorphonuclear leukocytes were washed once more with PBS and then resuspended in PBS at a concentration of $3x10^7$ cells per ml. The preparation consisted at least 98% PMNL as counted with a hemocytometer.

The mononuclear cell fraction that was obtained from Ficoll-Hypaque centrifugation contained 79% lymphocytes and 21% monocytes as determined by morphological criteria. The cells were suspended in PBS at a concentration of $4.9x10^7$ cells per ml.

The lymphocyte preparation was purified after the Ficoll-Hypaque gradient by two sequential passages over a nylon mesh column (13). It then contained more than 97% lymphocytes by morphological criteria. The cells were suspended in PBS at a concentration of $4.9x10^7$ cells/ml.

Erythrocytes. Heparinized blood was used within 1 h after drawing. Cells were sedimented at 1000 x g, washed three times with PBS and resuspended in PBS at concentration of 10^8 cells/ml.

Binding Assay. All binding studies were performed in PBS at 22°C. [^{3}H]tuftsin (at the indicated concentrations; see Figs 2,4) and cells were incubated with gentle agitation for 45 min (unless otherwise specified). The incubation was begun by addition of cells (in 100μl PBS) and terminated by dilution with 0.5 ml of ice-cold sucrose buffer, followed by centrifugation. The supernatant was discarded by aspiration and the cell pellet was then washed with 0.5 ml ice-cold sucrose buffer and separated again by centrifugation. The cell pellet was then dissolved in aqueous sodium dodecyl sulphate (250μl;0.1%) and the solution obtained transferred to counting vials containing 15 ml of Triton/toluene scintillation mixture. Radioactivity was measured in a liquid scintillation spectrophotometer at an efficiency of 40%. Binding to PMNL ($3x10^6$ cells/tube) was performed in small plastic tubes at a final volume of 250μl. Cell pellets were isolated by centrifugation for 5 min at 600 x g. Erythrocytes (10^7 cells/tube) were assayed similarly. Binding to the mononuclear cell fraction and purified lymphocytes ($4.9x10^6$ cells/tube) was performed in Eppendorf polyethylene microtubes at a final volume of 500μl. Cell pellets were isolated by centrifugation for 2 min at room temperature at 10,000 x g (Eppendorf centrifuge model 5412). Non-specific binding was defined as the amount of binding not inhibited by 20μM unlabelled tuftsin and was usually equal to about 30-40% of the total counts bound. Specific binding was defined as the total amount of [^{3}H]tuftsin bound minus the non-specific binding. Values of binding given in all figures refer to specific binding.

RESULTS AND DISCUSSION

The specific binding of [^{3}H]tuftsin to human polymorphonuclear leukocytes

is a time-dependent process. At a peptide concentration of 130 nM it was rapid, with a half time of less than 5 min at 22°C (Fig. 1). Binding reached a plateau value at about 15 min. The reversibility of [^{3}H]tuftsin binding was examined by adding a large excess (20μM) of unlabelled tuftsin to equilibrium mixtures of [^{3}H]tuftsin and PMNL after 15 min incubation. The results (not shown in Fig.1) clearly indicated that 15 min after addition of unlabelled tuftsin, specific binding of the tritiated tuftsin was reduced by more than 90% of its initial value.

The concentration dependence of [^{3}H]tuftsin binding to PMNL at equilibrium was determined by varying the concentration of peptide added to a fixed number of cells ($3x10^6$) (Fig. 2A). Specific binding was found to reach a saturation value at a peptide concentration of about 280 nM. Scatchard analysis of the data gave a straight line (Fig. 2B), indicating that tuftsin binds to a single kind of site. The equilibrium dissociation constant, K_D, calculated from the Scatchard plot was $1.3x10^{-7}$M; very close to the concentration of tuftsin required to elicit a half-maximal phagocytic stimulation response in human PMNL (5,6). At saturation, the $3x10^6$ PMNL present in the incubation mixture are capable of binding 0.26 pmoles of [^{3}H]tuftsin (Fig. 2B). This value corresponds to about 50,000 binding sites per PMNL cell, assuming an equimolecular ligand-receptor complex. Specific binding was linear with cell concentration over the range of 0 to $5x10^6$ cells.

The specificity of PMNL binding sites for [^{3}H]tuftsin was also tested by binding competition experiments with unlabelled tuftsin (6,9), with three structurally related peptides, and with L-arginine. As shown in Fig. 3, [Des-Arg^4] tuftsin, an inactive analog of tuftsin (Stabinsky and Fridkin, to be published), was unable to compete with tritiated tuftsin even at concentrations as great as 100 times that of the [^{3}H]tuftsin. On the other hand, [D-Arg^4]tuftsin, an analog with a rather low (~35%) though distinct tuftsin-like activity (Stabinsky and Fridkin, to be published), and to a much lesser extent [N-acetyl-Thr^1]tuftsin, an inactive analog that is capable of somewhat inhibiting tuftsin's activity (6), partially competed with the binding of [^{3}H]tuftsin. When the molar ratio of

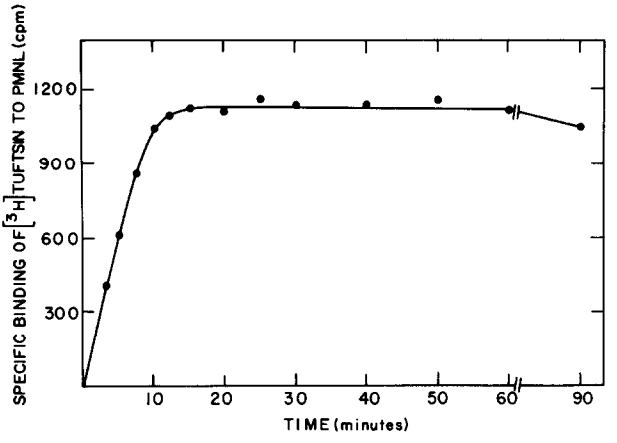

Fig. 1 . Time course of [^{3}H]tuftsin specific binding to human PMNL. [^{3}H] tuftsin (130 nM) was incubated with human PMNL for the indicated time intervals at 22^{o}C and specific binding was assayed. Each point is the mean of determinations from two separate incubation mixtures with SEM smaller than 10 per cent.

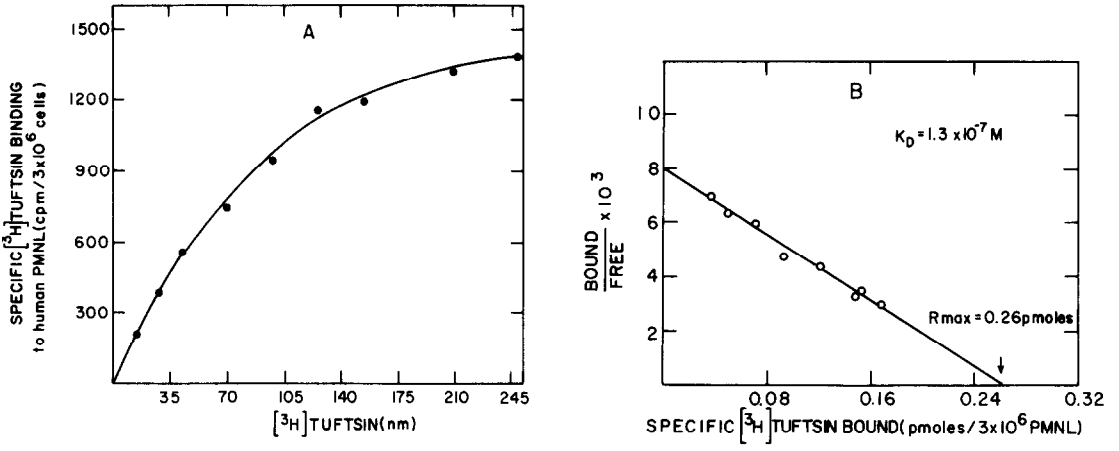

Fig. 2. Specific binding of [^{3}H]tuftsin to human PMNL as a function of concentration of [^{3}H]tuftsin.(A) [^{3}H]tuftsin at the indicated concentrations was incubated with human PMNL for 45 min at 22^{o}C, and specific binding was assayed. Each point is the mean of duplicate determinations with a SEM smaller than 10%. (B) The data of specific binding are plotted according to Scatchard analysis (bound/free v. bound).

unlabelled to tritiated tuftsin was one to one, about 55% inhibition was observed. This result was expected if the binding of tuftsin is indeed specific. The direct binding of [^{3}H][N-acetyl-Thr^1]tuftsin to PMNL was also examined at a

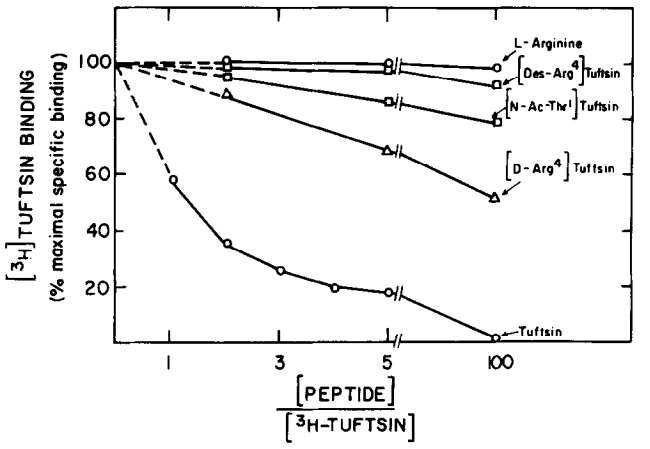

Fig. 3. Effect of unlabelled tuftsin and its analogs on the binding of [^{3}H] tuftsin to human PMNL. [^{3}H]tuftsin (200 nM) and PMNL (3 x10^6) were incubated for 45 min at 22°C in the presence of increasing amounts of tuftsin or its analogs and specific binding was assayed. The specific [^{3}H]tuftsin bound under equlibrium conditions was expressed as a percentage of the maximum specific binding measured in the absence of any added peptide.

wide concentration range. The results clearly revealed that the peptide failed to bind specifically to the cells. Binding-competition experiments of many other synthetic tuftsin's analogs (6-9) and determinations of binding-biological activity correlations are currently under way in our laboratory.

Several circulating blood cell types other than PMNL were tested with [^{3}H] tuftsin, for the presence of specific tuftsin binding sites. Purified lymphocyte preparations were found to bind less than 5% of tritiated tuftsin per cell as compared with PMNL. The binding capacity was, in fact, too low to be accurately determined. This minimal binding may stem from the absence of specific binding sites, but may also be due to low affinity of interaction. Mononuclear cell preparation (79% lymphocytes and 21% monocytes), however, showed a specific [^{3}H]tuftsin binding per cell, 41% of that of the PMNL preparation. This binding capacity was therefore attributed mainly to the monocytes present in the mixture. The saturability curve of the monocyte binding sites for [^{3}H]tuftsin was found to be similar to that obtained for the PMNL (Fig. 4A), with K_D equal to 125 nM and approximately 100,000 binding sites per cell as derived from a Scatchard plot

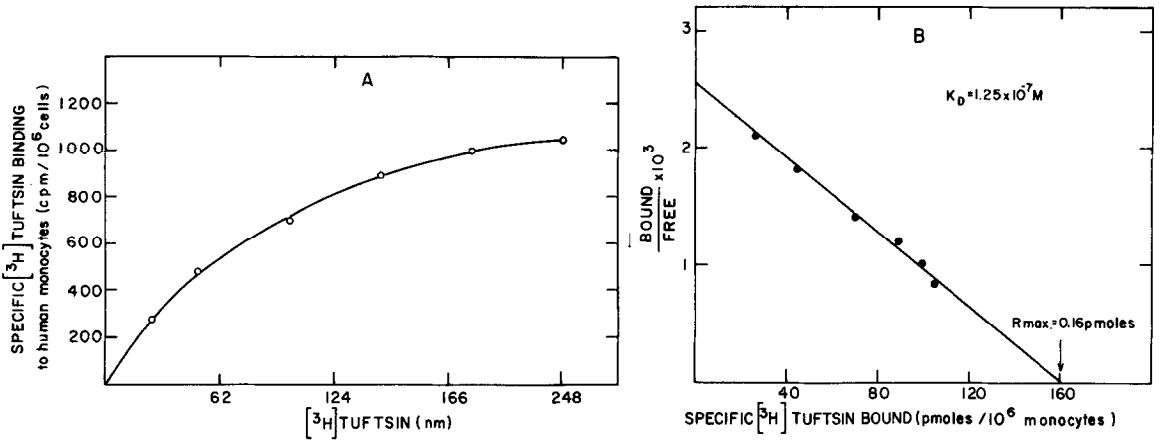

Fig. 4. Specific binding of [^{3}H]tuftsin to human monocytes as a function of concentration of [^{3}H]tuftsin. (A) [^{3}H]tuftsin at the indicated concentrations was incubated with mononuclear cell fraction containing 21% monocytes (10^6 cells) for 45 min at 22^{o}C and specific binding was assayed. Each value shown represents the mean of duplicate determinations with SEM smaller than 10%. (B). The data of specific binding are plotted according to Scatchard analysis.

(Fig. 4B). Experiments with erythrocytes revealed no specific binding of [^{3}H]tuftsin.

We have directly demonstrated that [^{3}H]tuftsin binding to human polymorphonuclear leukocytes and monocytes is a saturable, reversible and a time-dependent process. At 22^{o}C at least 60-70% of the binding appears to be specific. Experimental evidence indicates no specific binding sites on human erythrocytes nor, most likely, on human lymphocytes. We cannot yet, at this stage, comment either on the positioning of tuftsin binding sites on the cells or on their character. As tuftsin is a part of (14), and probably orginates from (15,16) the Fc portion of the immunoglobulin molecule, the possibility that it binds to Fc-receptors should be taken into consideration. Moreover, this assumption seems also to be logical in view of the direct involvement of Fc-receptors in the phagocytosis event (e.g. 17.18) and the fact that tuftsin stimulates this process. We are currently investigating these questions in our laboratory.

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