TUFTSIN: ITS CHEMISTRY, BIOLOGY, AND CLINICAL POTENTIAL

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I. INTRODUCTION

Several years ago, it was shown by Najjar and co-workers[1-4] that a fraction of γ-globulin (leukokinin) binds specifically to blood neutrophilic leukocytes and monocytes. This fraction, and no other, stimulates the phagocytic activity of these cells.[5-7] This stimulatory activity was later found to reside completely in a tetrapeptide termed tuftsin (threonyl-lysyl-prolyl-arginine).[8,9] This oligopeptide represents residues 289 to 292 of the heavy chain of leukokinin. It is active only in the free tetrapeptide state. In order to achieve this active free state, the peptide is cleaved off the protein chain by two enzymes. One enzyme, tuftsin-endocarboxypeptidase, is present exclusively in splenic tissue. The other is present on the outer membrane of phagocytic cells. The latter enzyme, leukokininase, was characterized in blood neutrophilic leukocytes of human, dog, and rabbit peritoneal granulocyte. It is a highly active enzyme with a pH optimum of 6.8.[3,4,10]

Tuftsin was first prepared by solid phase synthesis[11] with good recovery of the purified tetrapeptide.[10,12] Its biological activities are varied in their manifestations. These converge into one single path, namely, the modulation of the expression and function of phagocytic cells, primarily the omnipotent macrophage. These functions are phagocytosis,[3,4,8,13-15] motility,[3,4] bactericidal[16,17] and tumoricidal activities,[18-24] restoration of age-depressed macrophage[25,26] and T-cell cytolysis, and rejuvenation of disease-depressed motility and chemotaxis,[27,28] augmentation of the immune response,[29-32] and the formation of cytotoxic oxygen, H_2O_2, $O_2^{-\cdot}$ and the $OH^{\cdot}$ radical.[33-36] This cytotoxicity is further augmented by the increase in the level of tumor necrosis factor (TNF/cachectin).[37] An important effect of tuftsin is the modulation of cyclic nucleotides and Ca^{2+} concentration in the cell.[38] These findings were the product of the effort of several groups in many countries.

The activities of tuftsin are discussed in detail and additional effects defined in the segments of this review. Because of these important properties, much interest has also been generated in the study of the effects of tuftsin on the phagocytic cell other than those listed above.

It is important to note here that the immutability of the Fc fragment underscores its biological importance. A discussion of biologically active immunoglobulin-derived peptides brings new emphasis to this aspect.[39]

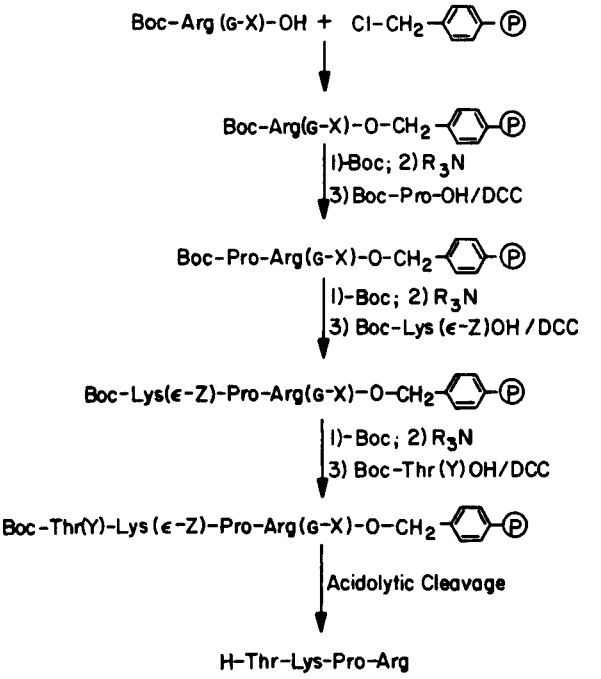


FIGURE 1. Synthesis of tuftsin following the solid-phase approach.

II. CHEMISTRY OF TUFTSIN

A. Synthesis of Tuftsin

The discovery, isolation, and structural elucidation of tuftsin[10,40] led to intensive synthesis of the peptide in various laboratories employing diverse, rather, the most available methodologies. In the following, we summarize the synthetic pathways utlized to prepare tuftsin. It is not intended to describe technical details here, but rather to outline the principles of the different synthetic approaches. The synthesis of tuftsin and its analogs has been concisely reviewed by Fridkin and Gottlieb,[41] Siemion and Konopinska,[43] and Gottlieb et al.[43]

In view of the recent glorious achievements of peptide synthesis, the preparation of tuftsin is perhaps a very modest chemical challenge. The combination, however, of three trifunctional amino acids (Thr, Lys, Arg) with proline (possessing a secondary amino group) to form the tuftsin molecule may create certain difficulties, especially when large-scale synthesis is intended.

The first synthesis of tuftsin was reported by Nishioka et al.,[10] following the solid-phase strategy. The present survey of the major synthetic tuftsin routes will start by describing this methodology.

1. Solid-phase Synthesis

Synthesis of tuftsin[10,12,43] via the solid-phase strategy (for review see Reference 44) is depicted in Figure 1. Following the attachment of a suitable carboxyl-free arginine derivative to the insoluble cross-linked polystyrene carrier, the peptide chain was elongated, through its N-terminus, in a stepwise manner. On completion of chain assembly, tuftsin was detached, along with side-chain deprotection, from the polymer by acidolysis, isolated, and purified. The *tert*-butyloxycarbonyl (*t*-Boc) group served for α-amino protection, and the ϵ-amino group of lysine was protected by the benzyloxycarbonyl group (Z), whereas the guanidine

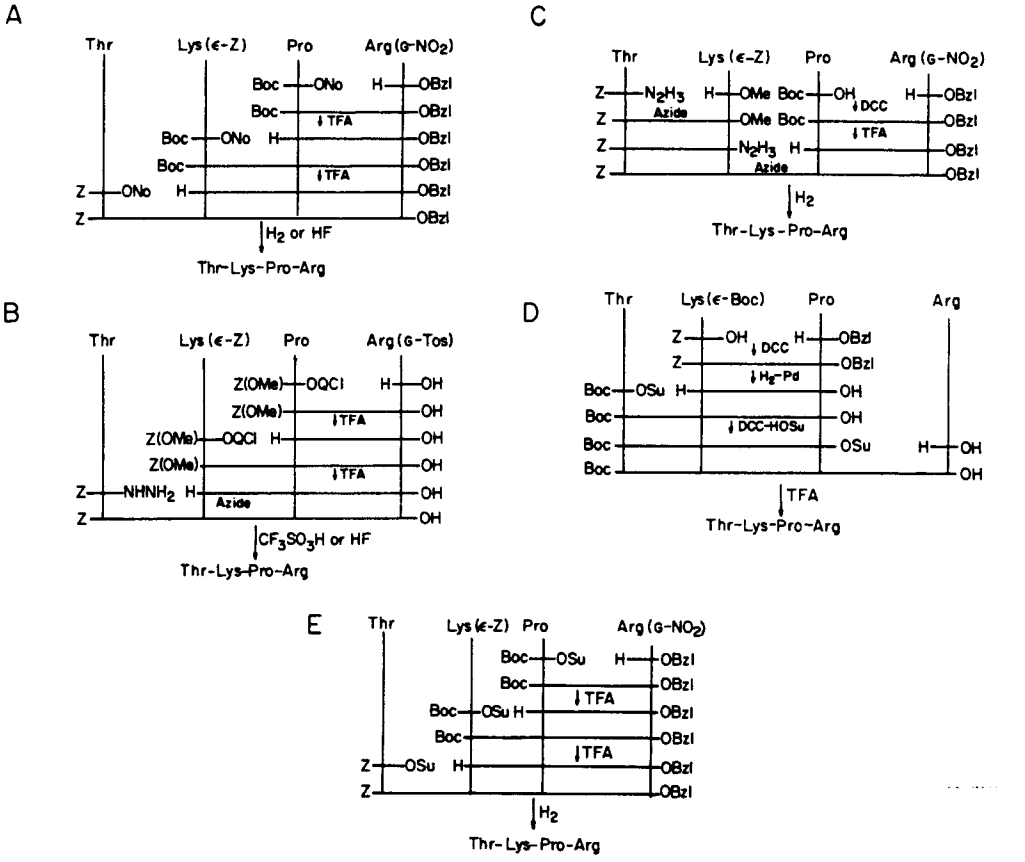


FIGURE 2. Synthesis of tuftsin following classical methodologies.

side-chain of arginine was masked (X) by the nitro (NO_2), tosyl (Tos) or mesitylene sulfonyl (Mts) group. The hydroxy moiety of threonine (Y) was left either unprotected[43] or masked by a benzyl group.[10,12] The attractive characteristics of the solid-phase method, namely, simplicity, speed, avoidance of intermediate isolation, and high reaction yields, were optimaly expressed.

2. *Classical Synthesis*

The majority of the synthetic routes to tuftsin employed the "classical strategy", i.e., synthesis in solution. Accordingly, syntheses are carried out in a homogenous phase (organic or mixed organic/aqueous). Intermediate products are purified and upon completion of the synthesis side-chain protecting groups are removed and the final products purified. Several representative syntheses are illustrated in Figure 2. As shown, synthesis can be based on the repetitive chain elongation using single, suitable N^{α}-protected/C-terminal activated amino acid derivatives (Figures 2A, B, and E; References 16, 45a, and 17, respectively) or alternatively, on coupling of fragments of two[42] (Figure 2C) or three[43] (Figure 2D) amino acid residues. Peptide bond formation was achieved using *N,N'*-dicyclohexylcarbodiimide as a coupling agent (Figures 2C, D), active esters (Figures 2A, B, C, and E) (also see, for example, Reference 45b) employing mixed anhydride methodology,[46] or with trimeric phosphonitirilic chloride.[16] Most syntheses are characterized by a single final deprotection step that leads to tuftsin.

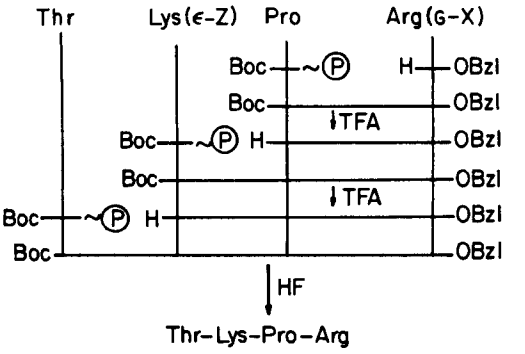


FIGURE 3. Synthesis of tuftsin using the polymeric-reagents approach. X = NO_2; Tos; Mts, P = -polymeric carrier, ~ = an active ester bond.

3. Polymeric Reagents Synthesis

Synthesis of tuftsin utilizing the polymeric reagents methodology[47] is illustrated schematically in Figure 3. Accordingly, insoluble polymeric active esters are employed to add, in a repetitive fashion, the corresponding N-blocked amino acid derivative to the free α-amino terminus of the growing tuftsin chain. As excesses of polymeric reagents can be used and easily removed at the end of the synthesis by filtration, coupling reactions are therefore markedly enhanced and are practically quantitative. Insoluble active esters derived from (4-hydroxy-3-nitro)benzylated polystyrene (PHNB) and from polystyrene-bound 1-hydroxybenzotriazole (PHBT) thus served as reagents for high overall-yield tuftsin synthesis.[13,14,43]

4. Synthesis Using N-carboxyanhydrides

Synthesis of peptides employing the *N*-carboxyanhydride methodology[48] offers several most attractive advantages: accessible reagents, i.e., *N*-carboxyanhydrides (Leuchs-anhydrides) of amino acids; minimal functional groups protection, i.e., terminal α-amino and C-carboxyl terminals and often side-chain moieties remain unprotected throughout synthesis; synthetic intermediates are not isolated; aqueous reaction medium; and exceedingly fast coupling rates. The above unique features are expressed in the synthesis of tuftsin.[43]

5. Scale-up Synthesis of Tuftsin Toward Industrial Methods

In view of the significant therapeutic potential of tuftsin as an immunomodulating, antibacterial, and antitumor drug, the need for large amounts of peptide is essential, i.e., for *in vivo* biological and, in particular, toxicological evaluation. As various derivatives and fragments of tuftsin, notably Lys-Pro-Arg ([Des-Thr^1]tuftsin),[13,14,22,41] are potent inhibitors of the parent peptide, the requirement for high purity and full bioactivity of product is vital. The efficiency and adaptability of several synthetic pathways (some of which are described in Figure 2) to large-scale synthesis of tuftsin was examined. As might have been expected, certain side reactions occurred while unmasking some protecting groups under acidic (Figure 4A) or basic (Figure 4B) conditions.[43] Two synthetic routes reported by Martinez and Winterniz[17] (Figure 2E) and by Gottlieb et al.[43] (Figure 2D) were found to be most efficient and highly reproducible in the preparation of large amounts ($\geq$ 50 g) of tuftsin. The products were purified to homogeneity (following analytical HPLC criteria) by either preparative HPLC[17] or by ion exchange chromatography[43] (Dowex-50 columns enabled purification of $\geq$ 15 g crude tuftsin in one run). The products optimally exhibited full spectra of bioactivities. Tuftsin is presently available from a rather large number of companies in small amounts, however. Abic Ltd. (Ramat Gan, Israel) has developed efficient procedures for large-scale

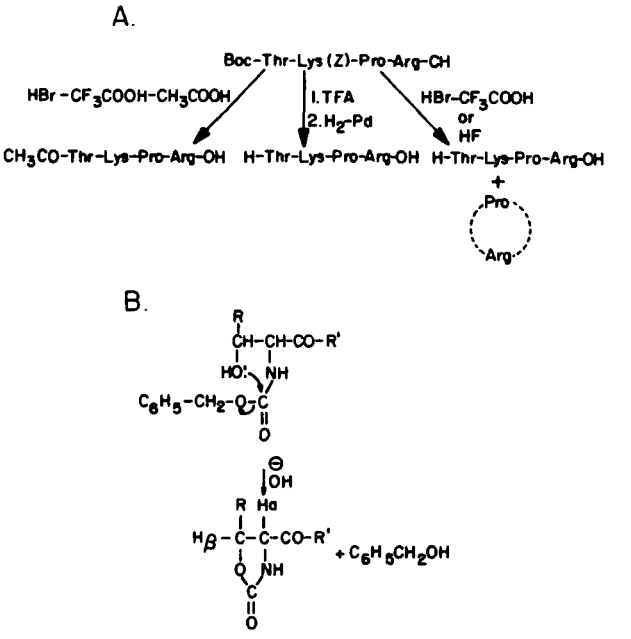


FIGURE 4. Side-reactions occurred during unmasking of protected tuftsin derivatives, employing acidic (A) or basic (B) conditions.

synthesis and purification of tuftsin, as well as for pharmaceutical preparations adequate for its clinical application.

B. Conformation of Tuftsin and Some Analogs

A variety of physical techniques have been used to study the conformation of tuftsin and some of the analogs, both in aqueous and nonaqueous solvents. Among these have been infrared,[49-51] circular dichroism,[52-54] and nuclear magnetic resonance (NMR).[50,55,56] Theoretical calculations have also been carried out. There has been no general agreement on the structure of tuftsin in solution. Among the structures proposed are a β-turn,[50] quasi-cyclic molecule and a hairpin with split ends.[54]

^{1}H and ^{13}C-NMR studies have been performed on tuftsin and its analog Thr-Lys-Pro-Pro-Arg.[55] It appears that in aqueous solutions, tuftsin and the pentapeptide analog exist in random conformation. By contrast, in dimethylsulfoxide, a slightly ordered structure is obtained only for tuftsin. This structure does not represent a β-turn. The pentapeptide, however, reveals no preferred conformation in either solution.[57] This view has now been contested.[51a] The presence of a proline residue in tuftsin raises the questions of *cis-trans* isomerism. However, there seems to be no evidence for the presence of the *cis* form in conformational equilibrium in water. Utilizing ^{13}C-NMR and circular dichroism (CD) for the study of tuftsin conformation, the conclusion was made that tuftsin, like other tetrapeptide analogs, possesses a strong tendency for type III β-turn in water, with proline in position i + 2 of the turn.[50,51a]

C. Metabolism of Tuftsin

As stated earlier, tuftsin must be excised off the carrier molecule leukokinin in order to exert its biological effects. The splenic enzyme tuftsin endocarboxypeptidase cleaves tuftsin distal to arginine at H···Arg-Glu···OH bond to yield a tetrapeptide attached only at the amino group of threonine, where the phagocyte membrane enzyme leukokininase cleaves the H···Lys-

Thr···OH bond to yield the free H-Thr-Lys-Pro-Arg-OH.[3,8,9] Several enzymes degrade tuftsin to yield inactive peptides.[58,59] A highly active amino peptidase removes threonine to yield Lys-Pro-Arg, an inhibitor to tuftsin activity. This tripeptide may well function in modulating tuftsin activity.[22] Finally, tuftsin is broken down to dipeptides and eventually to the constituent amino acids.[59] These enzymes have not been characterized further.

III. EFFECTS ON MACROPHAGE AND GRANULOCYTE FUNCTIONS

A. Phagocytosis

The discovery of tuftsin rested on its ability to stimulate phagocytosis by granulocyte and monocyte/macrophage cells.[1,3,4,9,60-66] Half-maximum stimulation was attained at about 100 n*M*.[66] Stimulation of phagocytosis has been documented by other groups.[13,14,16,45,52,68-71] This has now been superseded by radioimmunassay[2,13,14,22,41,72] and chemiluminescence.[26]

Stimulation of phagocytosis by tuftsin was obtained with polymorphonuclear leukocytess (PMN) cells from human, dog, rabbit,[1,9] and cow,[73] as well as with macrophages from lung and peritoneal cavity of mice and guinea pig[1] and mouse bone marrow cells. In the latter case, the stimulation of phagocytosis by P388DI was directly related to tuftsin binding.[74]

Phagocytic stimulation of PMN cells[71] from human and dog blood and other sources was inhibited by the peptide analog Thr-Lys-Pro-Pro-Arg. However, the basal activity was not inhibited. This is an important distinction and indicates that basal phagocytosis may follow a different pathway from that which follows stimulation.[75] Stimulation of pinocytosis by tuftsin is exerted only on phagocytic cells and not on L1210, $3T_{12}$, and $3T_3$.[9,71]

B. Motility and Chemotaxis

Vertical motility of neutrophils in capilalry tubes is stimulated by tuftsin, and the stimulation is also inhibited by the pentapeptide Thr-Lys-Pro-Pro-Arg.[3,4]

Chemotaxis was studied by the usual methodology at concentrations of 0.1 and 1 μg/ml. Significant chemotaxis was observed.[76] Lower concentrations yielded very slight chemotactic effects. The tuftsin analog, kentsin, Thr-Pro-Arg-Lys failed to show any stimulation. Chemotactic activity of inflamation-induced PMN cells was found to be considerably reduced. However, this activity was completely restored by pretreatment of cells with appropriate concentrations of tuftsin. Furthermore, in certain disease states, notably Hodgkin and systemic lupus erythematosus, random migration and chemotaxis of human monocytes, as assessed *in vitro*, are very depressed. The depression was completely restored by preincubation of the monocytes with tuftsin.[27,28] Chemotactic stimulation by tuftsin was compared to that of *N*-formyl-Met-Leu-Phe, which is a well-known chemotactic agent. Despite the structural difference, tuftsin was as effective as the tripeptide at concentration of 10^{-9} *M* to 10^{-7} *M*.[76-78]

An interesting *in vivo* assay for chemoattractant activity of several compounds was devised recently. Among these were several oligopeptides. The assay was based on the accumulation of certain cell types into a subcutaneously implanted device. Specific cell types were attracted to specific oligopeptides that are placed in the implant and slowly diffuse therefrom into the adjoining tissues. It was found that histamine attracted eosinophils, glycyl-histidyl-lysine attracted mast cells, and, pertinent to this review, tuftsin attracted monocytes.[79]

C. Effect on Differentiation

A significant observation has been made regarding the effect of tuftsin on the differentiation of immature bone marrow cells and the immature cell line P388D1. Bone marrow cells were tested for the development of colony-stimulating factor (CSF). Tuftsin 0.1 to 1.0 μg/ml significantly enhanced chemotaxis of PMN and macrophages, as well as colony formation.[76] The optimal concentration of tuftsin, 0.5 μg per culture of bone marrow, displayed colony-

stimulating activity to the same extent obtained with CSF. Tuftsin treatment of these cells with 0.1 to 1.0 μg per culture significantly enhanced CFU-C formation. At 10 to 100 μg/ml, there was an increase in cytotoxic activity. P388D1 represents an immature monocyte cell line that, upon differentiation, becomes cytotoxic.

D. Formation of Reactive Oxygen Compounds

During particle phagocytosis by all types of phagocytic cells, there is a clear augmentation of reactive oxygen compounds, $O_2^{-\cdot}$ and H_2O_2. Other sources of leukocyte oxidants are the chloramines. These contribute to the cytotoxic activity of the phagocytes and are formed by the interaction between HOCl and amines.[80] Put together, these highly reactive compounds serve in part to kill ingested bacteria, as well as aberrant cancer cells.[81]

It is of special interest that particles need not be ingested in order to precipitate a respiratory burst. Mere contact may at times suffice. Furthermore, certain small compounds may participate in such an oxidative burst. Among these is the naturally occurring tetrapeptide tuftsin. Tuftsin augments the formation of $O_2^{-\cdot}$ and H_2O_2 to a considerable extent without the need for particle phagocytosis.[33-36]

The production of $O_2^{-\cdot}$ has been studied in some detail.[35,36] The result showed a rapid response to various concentrations of tuftsin at 125 to 375 n*M*. Not surprising (see below) was the fact that at high concentrations, 500 and 600 n*M*, there was almost complete suppression of superoxide formation. The optimum concentration of the tetrapeptide was at 375 n*M*.[35,36] This response to tuftsin stimulation of the macrophage cell accounts for about 90% of the superoxide formed through the xanthine oxidase system. It is reasonable to assume that these highly reactive oxygen compounds along with the tuftsin augmentation of tumor necrosis factor (TNF) (see below) might well suffice to kill bacteria and tumor target cells.

E. Augmentation of Tumor Necrosis Factor

An interesting and pertinent finding was reported lately.[37] It was shown that the injection of tuftsin intraperitoneally (i.p.) increases the formation of TNF in serum and supernatants of cultured splenic and peritoneal adherant cells. This was also demonstrated *in vitro* using HL60 leukemia cells. As little tuftsin as 0.01 ng/ml stimulated TNF formation. HL60 cells were cultured in various concentrations of tuftsin (0.001 ng to 10 μg/ml in RPMI medium enriched with fetal calf serum. The highest level occurred with 1 μg/ml/24 h incubation.

F. Inmunomodulating Activity

It is generally agreed that there exists a mechanism by which the macrophage processes the antigen to render it immunogenic. The extent of the overall immune response depends on the efficiency and nature of this process. It appears that tuftsin acts at the level of antigen processing. This is so very important that an extended presentation is called for.

Antigen processing by the macrophage is allowed to take place in the presence and absence of various concentrations of tuftsin.[29] The antigen is then washed away and the adherent macrophages allowed to interact with splenic T lymphocytes *in vitro*. The lymphocytes, which now carry a signal from the macrophages, are lethally irradiated to prevent replication and injected into syngeneic mice. The draining lymph node cells were secured 6 d later. In the presence of the specific antigen, these lymphocyte take up [^{3}H]-thymidine. The uptake is markedly enhanced when the antigen was processed in the presence of tuftsin.[29,30] Maximal effects were obtained at tuftsin concentration of $5 \times 10^{-8}M$ (Figure 5). This effect proved to be highly specific and dependent on the structural integrity of tuftsin. Thus, for example, the replacement of threonine by its sister residue, serine, the derivatization of the carboxy-terminal arginine by methylation or nitration of the guanido group all resulted in the absence or dimunition of activity. However, [Ala^1]-tuftsin and tuftsinyl-glycine exhibited high po-

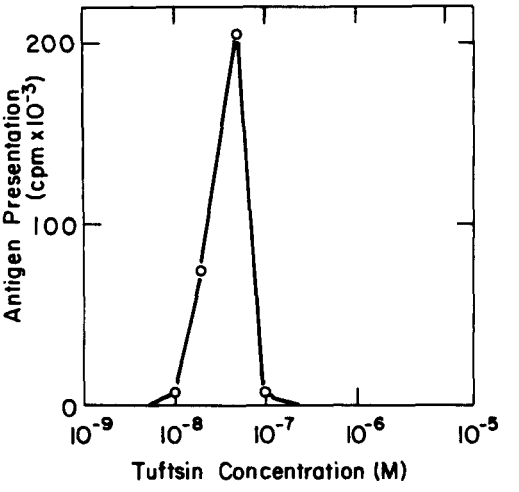


FIGURE 5. Effect of tuftsin on the immunogenic activity of antigen (KLH)-pulsed mouse peritoneal macrophages. (From Dagan, S., Gottlieb, P., Tzehoval, E., Feldman, M., Fridkin, M., Yasamura, K., Okamoto, K., and Yajima, H., *J. Med. Chem.*, 29, 1961, 1986. With permission.)

tency. Similarly, the replacement of L-Arg by D-Arg or by L-Arg-NH_2 practically did not affect activity.[29,30]

Tuftsin was coupled covalently to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) to form stable entities. Tuftsin-antigen conjugates were found to be very potent immunogens, as tested by their capacity to increase antigen presentation in culture in primary and secondary responses. Thus, monolayers of mouse peritoneal macrophages, pulsed *in vitro* with KLH-tuftsin, exerted a stronger immunogenic effect than KLH alone. BSA, which by itself was not immunogenic when applied to cells like tuftsin-conjugate, evoked a high lymphoproliferative immuno response. *In vivo*, BSA conjugated to tuftsin, when injected in aqueous solutions, significantly augmented antibody production, whereas administration of BSA alone or BSA admixed with tuftsin had no immunogenic effect. Application of the tuftsin-conjugate thus seems to lead to optimal presentation of the BSA-antigen to the same macrophage target cells. Studies conducted to elucidate the mechanisms underlying the activation of the immunogenic function of macrophages by tuftsin revealed that treatment of cells with antigen and the peptide, or with tuftsin-protein conjugates, increases IL-1 secretion and expression of cell surface Ia-encoded antigens. The effect of tuftsin on increasing the immunogenic capacity of antigens may, at least partly, be attributed to these latter effects.[82]

The effect of tuftsin on the immunogenic response is supported by results obtained from a different approach.[25,26] It was shown that the number of antigen-forming cells was increased over threefold by the injection of tuftsin T-dependent antigen. TNP-KLH was injected into mice at various time intervals following the i.v. injection of tuftsin. Maximal effects were observed when the antigen was injected several days after tuftsin administration. Similar results were obtained with T-independent antigen TNP-LPS, except that the maximum response obtained was observed in 1 to 3 d after tuftsin injections. In addition, tuftsin also enhanced the antigen-dependent cell-mediated immunity. Spleen cell cytotoxicity was also augmented to a significant degree.[25,26]

The immunomodulatory capacity of tuftsin, mediated through primary action on macrophages, was further and clearly demonstrated by Florentin et al.[82a] Thus, i.v. injection of tuftsin to mouse stimulated effector and regulatory functions, i.e., phagocytosis and IL-1

production, respectively, of macrophages and potentiated delayed-type hypersensitivity. Maximal stimulation of cells was observed 7 to 10 d after tuftsin injection. In parallel, an inhibition of lymphocyte functions, i.e., proliferative responses to mitogens; T-cell-mediated cytotoxicity; IL-2; and lymphokine production occurred. These latter immunosuppressive effects are, perhaps, attributed to the augmentation of the release of arachidonic acid metabolites from macrophages by tuftsin.

G. Restoration of Depressed Cellular Activity

It has been known for some time that cellular functions in mice are depressed with age. These can be restored by newborn thymus or adult bone marrow.[83] Thymic extracts were ineffective.[84]

The incidence of tumors in C57BL/6 mice at the age of 19 months reaches 22 to 33%. However, when these mice were given tuftsin at 6 months of age until the age of 18 months, no tumors developed.[25,26]

An equally impressive result was obtained recently in a strain of mice that develops mammary tumors after the age of 6 months at a 100% incidence. Saline injections were given to 22 control mice, and 7 mice were injected i.p. with 10 μg of tuftsin twice weekly, starting at 4 to 6 weeks old. By the age of 6 months, 21 control mice out of 22 developed tumors, but none of the injected mice showed any tumor growth[85] [$p < 0.001$ (X^2)]. These results further emphasize the relationship that exists between immunity and neoplasia, as exemplified by the greater frequency of cancer in the old mice. Restoration of disease-depressed activity has been shown *in vitro* by exposure of monocytes to tuftsin.[27,28] In Hodgkin's disease and systemic lupus erythematosus,[86] it was shown that monocyte chemotaxis is defective. Upon preincubation with tuftsin or some of its analogs there was a reversal of this process to normal levels. When chemotactic migration was measured at the leading front, the control yielded 39.08 ± 1.28 μm, while tuftsin-treated cells migrated 56.72 ± 1.35 μm. However, when measured as the total distance migrated, control cells yielded a migration distance of 585.8 ± 90.7 μm and tuftsin-treated cells migrated 1801 ± 748.9 μm. Here is an impressive *in vitro* effect. It is possible that in these diseases, an inhibitor of migration is locked into the cell to limit its motility. Tuftsin would then reverse the inhibition. Another possibility is that the mechanisms by which the cell converts metabolic energy to kinetic energy is blocked, and tuftsin removes or bypasses the block caused by the disease.

Depressed chemotactic activity was also observed with PMN cells harvested following an inflammatory induction. However, preincubation of these cells with tuftsin concentrations of 0.001, 0.01, and 0.1 μg/ml completely restored chemotactic activity to normal values.[26]

H. Effect on Cell Cytotoxicity

Considerable effort has been directed in recent years to the attempted enhancement of antitumor immunoresponse by immunomodulators capable of stimulating reticuloendothelial and T-cell-mediated tumor destruction. Thus, the effect of tuftsin on augmentation of cellular cytotoxicity was evaluated.

1. In Vitro Studies

The effect of tuftsin on the cytotoxicity of mice (DBA/2) peritoneal macrophages against L1210 keukemia target cells was investigated by Nishioka,[18] using [^{3}H]proline release assay. Tuftsin (10 μg/ml) significantly enhanced (32%) macrophage cytotoxicity. Alveolar macrophages, collected from DBA/2 mice were significantly stimulated by tuftsin toward ^{51}Cr-labelled Cloudman S-91 melanoma cells.[24]

Tuftsin-treated purified human PMN leukocytes were found to exhibit rather low, but significant, enhanced cytotoxicity against established human melanoma cell lines, but not toward normal embryonic lung cell line, WI-38.[18,24]

The cytotoxic response of human monocytes (from 57% of healthy donors) was substantially enhanced by tuftsin. The peptide, at doses of 5×10^{-3} to 5×10^{-1} μg/ml, augmented cell's cytotoxicity against a myeloid cell line (K562), but not toward a solid epidermoid tumor cell line (A431).[87] On the other hand, natural killing activity of human lymphocytes against the above two target cells was not affected by tuftsin.[87]

These latter results contradict the findings of Phillips et al.,[88] who reported the enhancement by tuftsin (maximally at concentration of 50 to 100 μg/ml) of mouse splenic natural killer cell cytotoxicity against T-cell lymphoma Yac-1. This tuftsin induced stimulation was not strain-specific and affected cells obtained from CBA; J, C57BL/10; and DBA/2 mice.

2. In Vivo Studies

Florentin et al.[25] reported that peritoneal macrophages isolated from tuftsin-treated mice exhibited elevated tumor-cytostatic capacity and augmented antibody-dependent cell-mediated cytotoxicity. In accordance with this Bruley-Rosset et al.[31,32] have studied the effect of prolonged administration of tuftsin in the prevention of spontaneous tumors in aged mice (C57BL/6). Restoration of impaired responses of cells (macrophages, cytotoxic T cells, K cells, and natural killer cells) associated with enhanced immunodefeciency was particularly investigated. Thus, aged-decreased cytotoxic capacity of macrophages was practically restored to normality by tuftsin. Similarly, the peptide-stimulated cytotoxic T-cell activity and antibody-dependent cell-mediated cytotoxicity. On the other hand, depressed natural killing activity of spleen cells (in line with certain *in vitro* studies[87] and contradictory to other findings *in vitro*[88]) was not affected by tuftsin. Impaired splenic K-cell was similarly unaffected by tuftsin.

Catane et al.[20] reported the enhancement, by tuftsin, of mouse (C3H/eb) spleen cell cytotoxicity against ^{51}Cr-labeled T-cell lymphoma-derived cell line (RL 1) as targets. The effect of tuftsin was most pronounced when injected into animals in doses of 50 to 500 μg/kg weight.

Following single i.v. injection of tuftsin (at doses of 2.5 μg to 0.96 mg/kg body weight) to human cancer patients, a dramatic leukocytosis was observed. A parallel significant enhancement (>100%) of lymphocyte cytotoxicity against K-562 myeloid cell line target cells was manifested. This was dose-dependent and particularly evident at tuftsin levels of 0.24 to 0.48 mg/kg weight.[20]

I. Nontoxicity in Animals and Humans

Studies on the acute lethal toxicity in mice revealed that the LD_{50} of intravenously injected tuftsin was about 2.5 g/kg body weight. Upon i.p. injection of the peptide, up to 2.5 g/kg, mice survival was 100%. Augmentation of white blood counts was observed following both i.v. and i.p. administration.[20] Injection of tuftsin (i.v. up to 25 mg/kg body weight) to rats had no effect on blood pressure, heart and respiratory rates, and electrocardiogram patterns.[3]

Similarly, i.v. injection of tuftsin (1 mg/kg) to beagle dogs did not change their respiratory rate, pulse, or behavior. Monitoring of various serum parameters (e.g., complete blood count, electrolytes, creatinine, glucose, cholesterol, globulin, alkaline phosphatase, and tranaminase), following i.v. administration of tuftsin, also revealed unmodified levels. However, extensive leukocytosis was prominent.[20]

In a phase I study, tuftsin proved to be nontoxic in human adult patients (15) with advanced cancer, when injected once i.v. (preparations of Abic Ltd., Ramat Gan, Israel), up to a dose of 0.96 mg/kg body weight. Extensive augmentation of white blood counts and enhanced cytotoxicity of lymphocytes were notable at levels of 0.04 to 0.96 mg peptide per kg.[20] No detectable tuftsin-related toxicity was noticed in human patients during a phase II study, where the peptide was administered i.v. twice a week at total doses of 5 mg per injection. Marked leukocytosis was observed.[21]

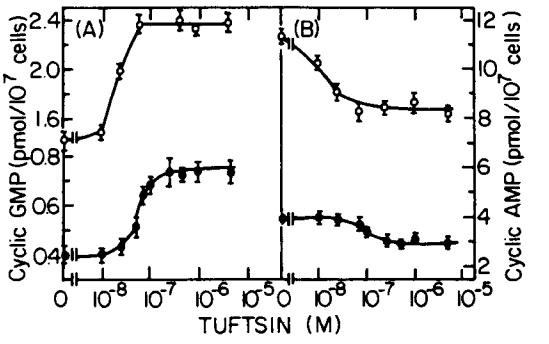


FIGURE 6. Tuftsin-dependent modulation of intracellular cGMP (A) and cAMP (B) levels in human polymorphonuclear leukocytes (●) and thioglycollate-stimulated mouse peritoneal macrophages (○). (From Stabinsky, Y., Bar-Shavit, Z., Fridkin, M., and Goldman, R., *Mol. Cell Biochem.*, 30, 71, 1980. With permission.)

J. Modulation of Cellular Cyclic Nucleotides and Ca^{2+}

The conceivability that the specific tuftsin-phagocytes association is translated into modulation of intercellular levels of cyclic nucleotides was investigated. This was done with the purpose of shedding light on the mechanisms involved in potentiating immuno and other cellular responses by tuftsin. Thus, Stabinsky et al.[38] reported that incubation of human neutrophils or thioglycollate-sensitized mouse peritoneal macrophages with tuftsin (200 to 250 n*M* at 37°C) enhanced intracellular cGMP levels by 80 to 90%, with a concomitant decrease of 20 to 25% in intracellular cAMP levels (Figure 6). Substantial modulations of both cyclic nucleotides were briefly notable (4 min) after tuftsin-cell interaction were maximal at 10 to 20 min and lasted for at least 60 min. The similarity in the dose-dependencies of these events and that of tuftsin-mediated phagocytosis by human neutrophils and by mouse peritoneal macrophages suggests a cause-and-effect relationship between the two responses. The above findings are in accordance with observations, in granulocytes, that elevation of intracellular cGMP or cAMP levels are associated with enhanced or inhibited cell locomotion, respectively (e.g., see References 89 to 93). Dagan et al.[94] reported that tuftsin (at concentration of 50 n*M*) elicited a marked decrease in cAMP levels in both mouse peritoneal macrophages and in a macrophage-hybridoman cell line (E2-7.7) (up to 60%) accompanied by simultaneous elevation of cAMP levels (>50% in both cells).

Modulation of cyclic nucleotide levels by tuftsin was also observed in preparations of purified plasma membranes obtained from both cells. Thus, tuftsin (at 50 n*M*) evoked an increase of cGMP (~40%) and a parallel decrease of cAMP (up to 65%).[94]

Roch-Arveiller et al.[95] reported that tuftsin did not alter intracellular cyclic nucleotide levels in rate polymorphonuclear leukocytes, but did increase cGMP levels in inflammatory cells.

As elevation of cellular cGMP may be due to activation of guanilate cyclase,[96] and via modulation of intracellular Ca^{2+} levels,[97] the effect of tuftsin on calcium ion mobilization was investigated.[38] The peptide (at concentration of 2.5×10^{-7} *M*) did not affect $^{45}Ca^{2+}$ influx into human PMN leukocytes or mouse macrophages, but did, however, specifically affect the efflux of $^{45}Ca^{2+}$ from preloaded cells.

IV. ASSAY OF TUFTSIN

Ever since the isolation of tuftsin and the observation that the peptide, as well as its

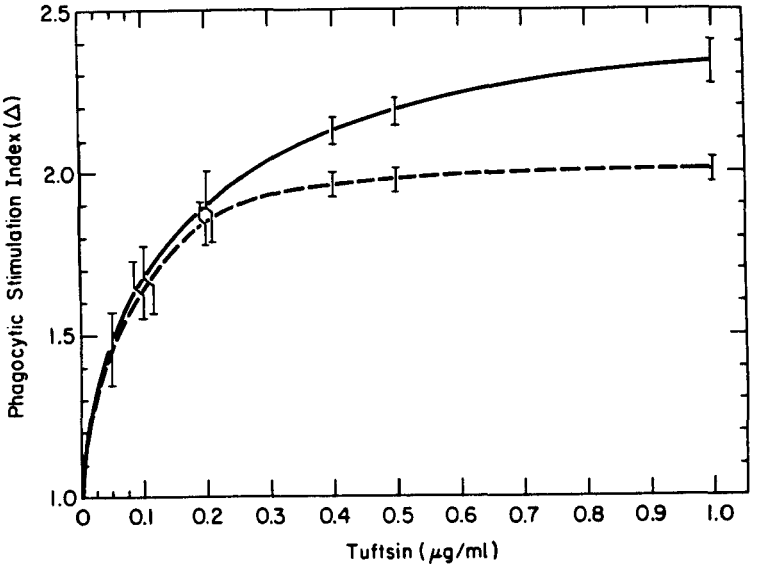


FIGURE 7. The stimulatory effect of tuftsin on phagocytosis of heat-killed yeasts by human polymorphonuclear leukocytes.[14] Phagocytosis index based on number of phagocytizing cells (- -) or a number of ingested yeasts (-).

parent-protein molecule leukokinin, exert a potent stimulatory effect on the phagocytosis capacity of phagocytic cells,[1,40] the scope of its biological activities has been expanded widely. Accordingly, methods for the quantitative evaluation of tuftsin and its analogs and of tuftsin-like peptides have been developed. In the following, some of the most applicable assays for tuftsin are discussed briefly.

A. Phagocytosis

Evaluation of the stimulatory effect of tuftsin and tuftsin-related peptides on phagocytosis was performed with several types of phagocytic cells isolated from different mammalian species. Thus, for example, PMN leukocytes was obtained from guinea pig,[1,10,15,98] dog,[4,10] rabbit,[1,62] cow,[73] and human.[1,14,51,99] Macrophages were obtained from mouse peritoneum,[1,16,100] spleen and liver,[16] and from rabbit lung.[1] Recently, the stimulation of phagocytosis by retinal pigment epithelium[101] and by the unicellular Tetrahymena[102] was reported. Various particular materials were utilized as swallowing targets for the phagocytes: opsonized bacteria such as *Staphylococcus aureus* (originally used by Najjar and associates [e.g., References 3,4,10,45,73,98]), opsonized glycine-1-^{14}C *S. aureus*,[103] heat-killed *Saccharomyces oviformis* yeast cells,[14,100,104,105] immunoglobulin G-coated sheep erythrocytes (IgG-coated SRBC),[100] and ^{51}Cr-IgG-coated SRBC.[105,106a]

The stimulatory effect that tuftsin exerts on the capacity of phagocytic cells to phagocytize is dose-dependent. Thus, with neutrophils, following a typical saturation pattern, maximal enhancement (generally two fold, or higher, of basal levels) is prevailed at concentrations of about 200 to 400 n*M* tuftsin (Figure 7). However, maximal stimulation of macrophages requires lower concentrations of tuftsin (~50 n*M*). Furthermore, at higher doses of peptides, a typical inhibitory pattern is often observed (Figure 8). The different phagocytosis assays were most efficiently employed in evaluating various synthetic routes to tuftsin, as well as in studying numerous analogs.[41,42,85,106]

B. Radioimmunoassay

Tuftsin was rendered antigenic through coupling of its activated derivative, *p*-diazonium

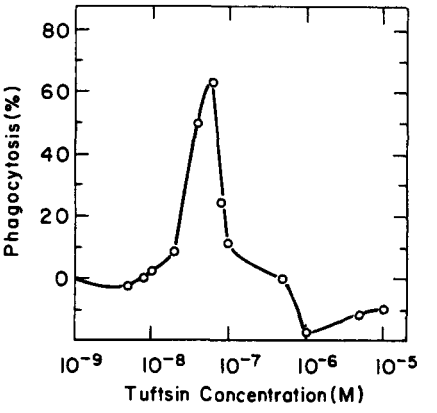


FIGURE 8. Tuftsin-dependent stimulation of phagocytosis (of ^{51}Cr-labeled IgG-coated sheep red blood cells) by mouse peritoneal macrophages. (From Dagan, S., Gottlieb, P., Tzehoval, E., Feldman, M., Fridkin, M., Yasamura, K., Okamoto, K., and Yajima, A., *J. Med. Chem.*, 29, 1961, 1986. With permission.)

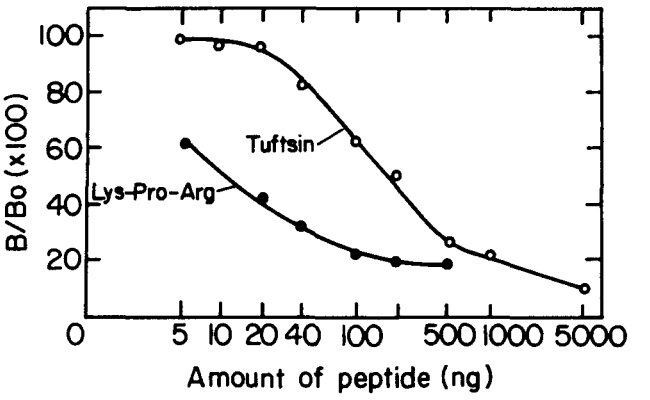


FIGURE 9. Displacement of ^{125}I-labeled *p*-aminophenylacetyl-tuftsin binding to antiserum to tuftsin by tuftsin and by [Des-Thr^1]tuftsin (H-Lys-Pro-Arg-OH).

phenylacetyl-tuftsin, to BSA. The BSA-tuftsin conjugate was employed to immunize rabbits.[107] Anti-tuftsin antibodies thus obtained bound ^{125}I-labeled *p*-amino-phenylacetyl-tuftsin that could be displaced by unlabeled tuftsin, as well as by analogs bearing the tripeptide Lys-Pro-Arg ([Des-Thr^1]tuftsin) moiety (Figure 9). Utilizing the antibodies, a highly reproducible method (with an interassay correlation index of 0.98) was developed to evaluate levels of tuftsin in human blood. Thus, tuftsin concentrations in trypsinized serum of normal subjects were found to be about 260 ng/ml, whereas lower levels were observed in humans with various pathological situations.[107-110] Specific rabbit anti-tuftsin antibodies were also generated by immunization with BSA-azo-phenylacetyl-Gly-Gly-tuftsin conjugate.[72,111] Conjugates of BSA-tuftsin and of IgG-tuftsin were utilized to elicit generation of antibodies in White Leghorn roosters.[112]

C. Enzyme Immunoassay (EIA)

Tuftsin was cross-linked (e.g., by dialdehydes) and attached to KLH, and the tuftsin-KLH conjugate was used to immunize rabbits. The anti-tuftsin antibodies thus obtained were

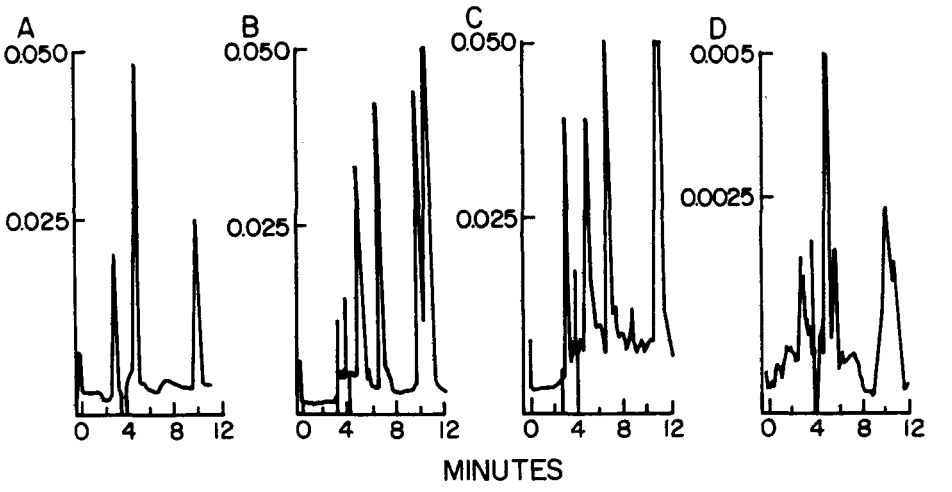


FIGURE 10. RP-HPLC separation of tuftsin from human serum. (A) Synthetic tuftsin (elution time 10 min); (B) untrypsinized serum sample with added synthetic tuftsin; (C) control serum; (D) tuftsin fraction isolated from trypsinized serum.

utilized to develop a kit for the solid-phase enzyme (alkaline phosphatase) immunoassay of tuftsin. The sensitivity of the method, which was used to monitor the peptide levels in serum is of 5 to 10 ng/ml (Abic Ltd., Ramat Gan, Israel; Pat. Appl.).

D. Reverse-Phase HPLC and Mass Spectrometry

Anti-tuftsin antibodies might also cross-react (depending on the particular tuftsin-carrier conjugate used for immunization) with tuftsin-related sequences. This may result in certain limitations while determining tuftsin levels in biological fluids. A method for unambiguous identification, and hence evaluation of tuftsin in trypsinized human serum was reported recently.[113] Accordingly, tuftsin is first separated from other blood components by reverse-phase HPLC (Figure 10), and then subjected, following conversion to its methyl ester derivative, to mass spectrometry analysis. Using this technique, normal human serum was found to contain tuftsin levels similar to those determined by the radioimmunoassay method.[107]

E. Radioreceptor Assay

Displacement of radiolabeled-tuftsin from its specific receptor sites on phagocytic cells is a valuable tool in both qualitative and quantitative evaluation of various tuftsin preparations, as well as of tuftsin-related peptides. The synthesis of the [^{3}H-Arg^4]tuftsin and the [^{3}H-Pro^3]tuftsin ligands is schematically summarized in Figures 11A and B. Both tritiated peptides were purified to homogeneity and possessed full tuftsin-like bioactivity. [^{3}H-Arg^4]tuftsin preparations (specific activity 9 to 24 Ci/mmol)[104,114] are primarily employed in the binding-competition studies. However, application of [^{3}H-Pro^3]tuftsin (specific activity 5 to 15 Ci/mmol)[114] lead to nearly identical results. Binding assays are usually performed on cell (mouse macrophages or human PMN leukocytes) monolayers at 22°C (e.g., References 100, 104), or at 0°C (e.g., Reference 66) at pH 7.4. Cell suspensions have also been used widely (e.g., References 106, 106a).

Different tuftsin preparations and numerous analogs of tuftsin have been assayed, employing the radioreceptor assay (e.g., References 104, 115). As a rule, a direct relation exists between the ability of tuftsin derivatives to inhibit the specific binding of tritiated tuftsin to its cellular receptors and their capacity to stimulate the cells, or to inhibit tuftsin-mediated stimulation. Some binding-competition patterns are illustrated in Figures 12A, B, and C.

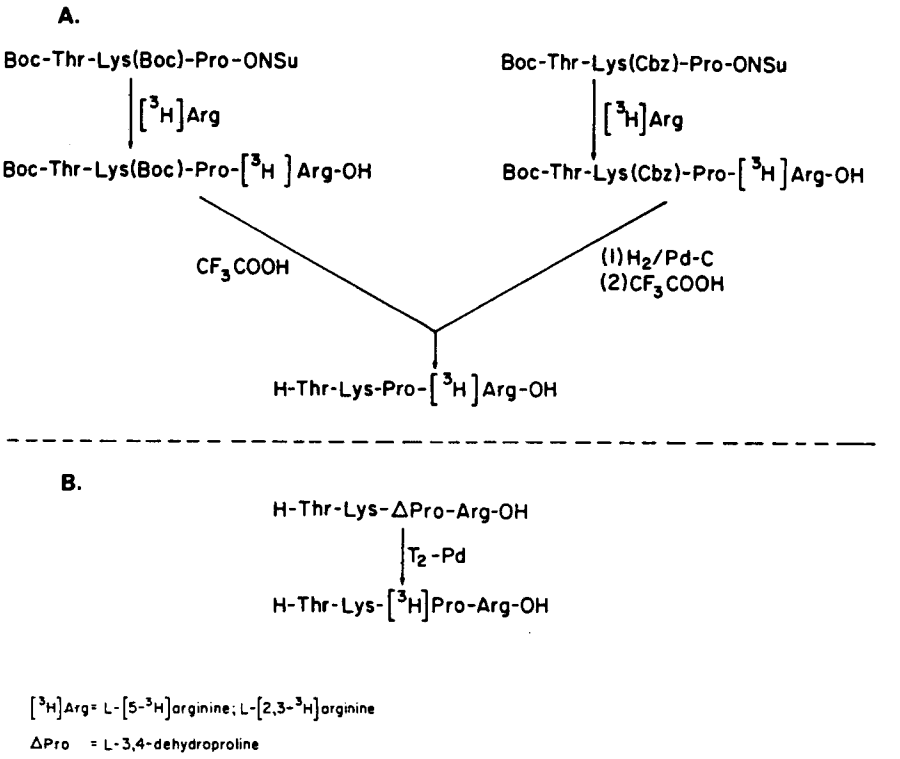


FIGURE 11. Synthetic routes to [^{3}H-Arg^4]tuftsin (A) and to [^{3}H-Pro^3]tuftsin (B).

Nair et al.[112] reported on the synthesis of three radiolabeled derivatives of tuftsin: tuftsin[^{14}C]methyl ester (specific activity 17.9 mCi/mmol), [^{125}]tuftsin-Tyr^5, and $N^{\alpha,\epsilon\text{-}125}$I-Bolton-Hunter-labeled tuftsin. The actual application of these compounds in a radioreceptor assay was not described.

F. Nitro Blue Tetrazolium Reduction and $^{14}CO_2$ Release

The enhanced respiratory burst of stimulated phagocytic cells, which directly reflects augmented phagocytic response,[116,117] can serve as a basis for evaluating tuftsin and tuftsin-related compounds. Thus, methods available for monitoring hexose monophosphate shunt (HMPS), a prominent metabolic oxidatory event, activity of cells were utilized for this purpose.

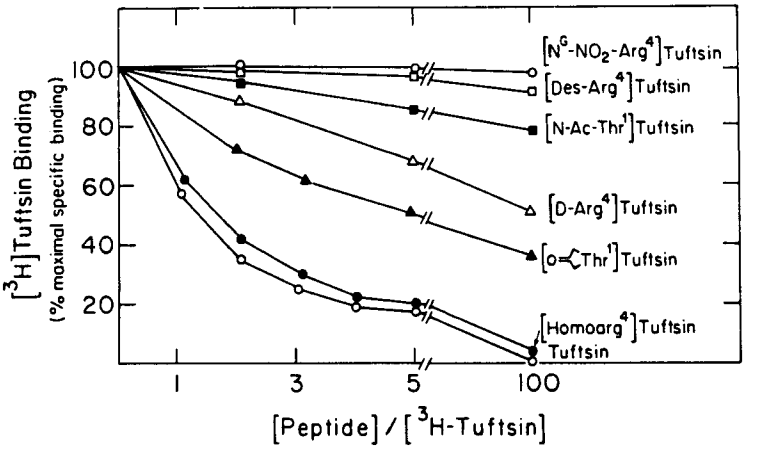
Stimulated phagocytes are capable of reducing the yellow water-soluble dye nitro blue tetrazolium, which enters the cell through phagocytosis while complexes to fibrinogen or haparin.[118] Spectrophotometric determination of the insoluble blue formazan reduced product (λ_{max} = 515 nm) is a measure of HMPS, i.e., cell activation.[119]

Oxidation of glucose via the HMPS generate CO_2, originating from carbon-1 of the hexose.[116,117] Analogously, oxidation of [^{14}C-1-glucose] will result in $^{14}CO_2$, which can be easily utilized in monitoring shunt activity. Both methods, the NBT-reduction assay[14,120,121] and the $^{14}CO_2$-release assay,[41] were applied most successfully to the semiquantitative evaluation of tuftsin and many of its analogs.

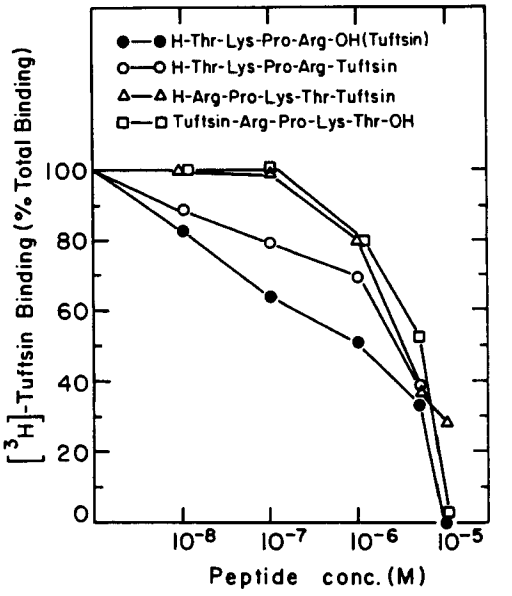
V. RECEPTOR STUDIES

A. Receptor-Mediated Internalization of Tuftsin

What are the immediate events on phagocytic cell surfaces following tuftsin-receptor

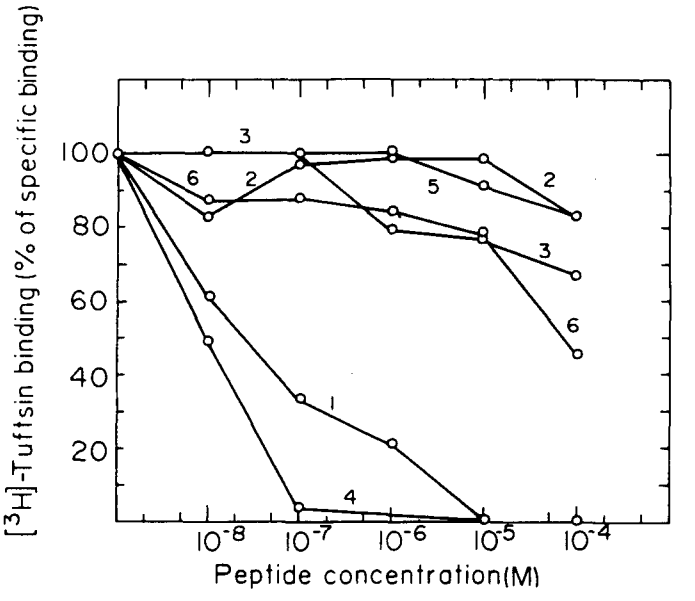


A



B

FIGURE 12. (A) Effect of unlabeled tuftsin and some of its synthetic analogs on the binding of [^{3}H-Arg^4]tuftsin to human PMN leukocytes. (From Stabinsky, Y., Gottlieb, P., Zakuth, V., Spirer, Z., and Fridkin, M., *Biochem. Biophys. Res. Commun.*, 83, 599, 1978. With permission.) (B) Displacement of [^{3}H-Arg^4]tuftsin binding to mouse peritoneal macrophages by unlabeled tuftsin and various tuftsin analogs. (From Dagan, S., Gottlieb, P., Tzehoval, E., Feldman, M., Fridkin, M., Yasamura, K., Okamoto, K., and Yajima, A., *J. Med. Chem.*, 29, 1961, 1986. With permission.) (C) Displacement of [^{3}H-Arg^4]tuftsin binding by tuftsin, rigin, and different tuftsin-sugar derivatives. 1, tuftsin; 2, N^{α}-gluconyl-tuftsin; 3, H-Thr[*O*-glucosyl)-Lys-Pro-Arg-OH; 4, H-Thr[($\alpha+\beta$)-D-glucosyl]-Lys-Pro-Arg-OH; 5, H-Lys-Pro-Arg-NH-Glc; 6, rigin. (From Rocchi, R., Biondi, L., Cavaggion, F., Filira, F., Gobbo, M., Dagan, S., and Fridkin, M., *Int. J. Peptide Protein Res.*, 29, 262, 1987. With permission.)



C

specific association? To gain insight into these processes, fluorescent-labeled derivatives of tuftsin were synthesized to allow direct visualization of peptide-cell encounter employing image intensification microscopy.[114,122-124]

Gottlieb et al.[114,122] have synthesized an extended-chain analog of tuftsin carrying the highly fluorescent prosthetic group tetramethylrhodamine (excitation at 550 nm and maximal emission at 573 nm) at its C-terminus. This fluorescent derivative, H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-tetramethylrhodamine)OH, was highly pure and capable (identical to tuftsin) of specifically binding to tuftsin receptor on thioglycolate-stimulated mouse peritoneal macrophages and of stimulating the phagocytic response of these cells toward opsonized, ^{51}Cr-labeled sheep red erythrocytes.[122] Figure 13 illustrates the surface-associated events occurring upon incubation of the fluorescent-labeled peptide (200 n*M*) with mouse peritoneal macrophages, at 37°C. Uniform dispersion of fluorescence around the cells commence concomitantly with cell-peptide interaction, and is most evident after 5 min (Figure 13A). Fluorescent clusters were apparent on cells after a period of about 5 to 10 min (Figure 13C). Endocytosis of clusters into cells started after about 10 min and practically terminated within less than 30 min (Figure 13D). Rates of the above events were markedly enhanced with increased concentrations of fluorescent-tuftsin derivative. Specificity of fluorescent peptide-macrophage binding was indicated by inhibition by unlabeled tuftsin (20 μ*M*) (Figure 13B).

Interaction of the fluorescent peptide with macrophages at 22°C led to similar, though rather slower, cell surface-related events. At 4°C, however, no specific peptide-cell association could be observed, even after prolonged incubations.

Amoscato and colleagues[123,124] have synthesized a fluorescein-labeled derivative of tuftsin [(N^{α}-Thr^1-fluorescein)tuftsin]. This highly pure derivative possessed full-tuftsin-like activity, as demonstrated by its capacity in identical manner to tuftsin to augment both the phagocytic and bactericidal response of human neutrophils. The association of cells with the fluorescent peptide at 37°C evoked receptor-mediated events (Figure 14) identical, though rather accelerated, to those observed with the tetramethylrhodamine-tuftsin derivative. The processes of uniform distribution of fluorescence around the neutrophils and clustering of peptide-receptor complexes were accomplished after about 1 min and again at 4 to 8 min, respectively. Endocytosis, which was already apparent after 8 min, was terminated within 20 min. In

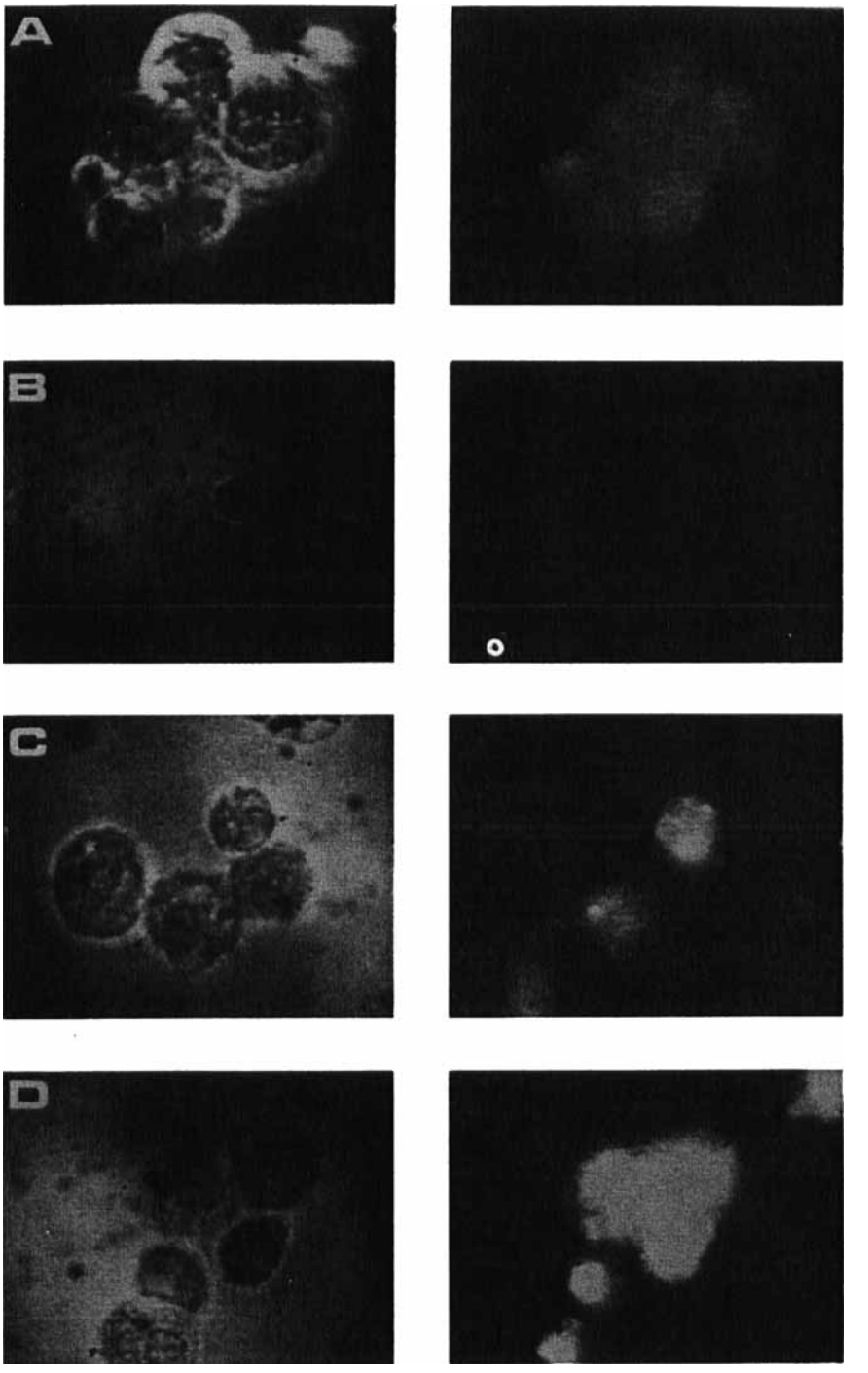


FIGURE 13. Fluorescent distribution of H-Thr-Lys-Pro-Arg-Gly-Lys(N^{ϵ}-tetramethyl-rhodoamine)-OH, following its association at 37°C with mouse macrophages. Cells were observed by phase contrast (left) and fluorescence (right) microscopy. (From Gottlieb, P., Stabinsky, Y., Hiller, Y., Beretz, A., Hazum, Z., Tzehoval, E., Feldman, M., Segal, S., Zakuth, V., Spirer, Z., and Fridkin, M., *Ann. N.Y. Acad. Sci.*, 419, 93, 1983, and Gottlieb, P., Hazum, E., Tzehoval, E., Feldman, M., Segal, S., and Fridkin, M., *Biochem. Biophys. Res. Commun.*, 119, 203, 1984. With permission.)

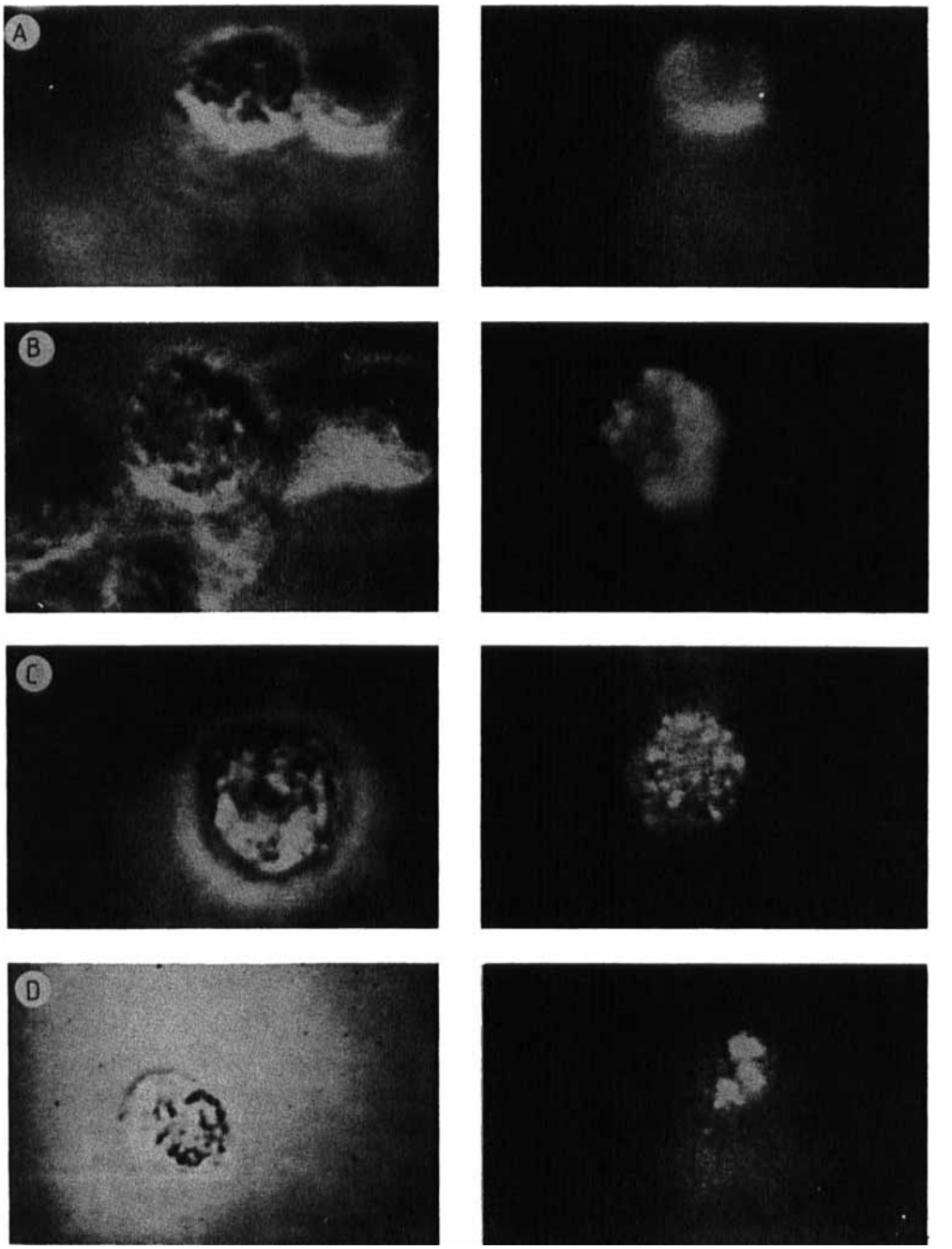


FIGURE 14. Fluorescent distribution of [N^{α}-Thr^{1}-fluorescein]tuftsin following its interaction at 37°C with human PMN leukocytes. Cells were observed by phase contrast (left) and fluorescence (right) microscopy. The incubation times with the fluorescent tuftsin derivative (2.6×10^{-7} *M*) were 1, 4, 8, and 15 min for A, B, C, and D, correspondingly. (From Amoscato, A. A., Da Vies, P. S. A., Babcock, G. F., and Nishioka, K., *J. Reticuloendothelial Soc.*, 34, 53, 1963. With permission.)

contrast to the studies with tetramethylrhodamine-tuftsin, however, the fluorescein-labeled tuftsin derivative was able to bind to cells (though human neutrophils) at 4°C.

Are the membrane-associated processes described above of any relevance to the mode of cell activation by tuftsin or perhaps merely a means of peptide clearance through internalization into the cell? Chemically stable insoluble conjugates of tuftsin-Sepharose were shown to significantly stimulate the phagocytic response of mouse peritoneal macrophages.[94] This finding, coupled with the fact that rabbit PMN leukocytes were shown to contain enzymes capable of digesting tuftsin,[59] may indicate, though rather preliminary, that clustering and internalization of receptor complexes are not required for eliciting functional responses to cell triggering by tuftsin.

B. Isolation and Characterization of Receptors

For the expression of its biological activity, tuftsin must interact with specific receptors. Membrane receptors are mostly, if not all, glycoproteins. Consequently, they have their share of neuraminidate at the end of carbohydrate chains. It was, therefore, appropriate to determine if, in any manner, this moiety is involved in the activity of the tuftsin receptor complex. To that end, PMN granulocytes of rabbit peritoneal exudate were treated with affinity-purified neuraminidase and subjected to a phagocytosis assay. The ability of tuftsin to stimulate phagocytosis was completely abolished.[62] However, the capacity to bind [^{3}H]-tuftsin appears not to have been altered.[190] This means that a productive tuftsin receptor complex should involve the neuraminidate residue as one of its components. In this case, the effect may be due to the lack of binding. It may also be the result of the formation of nonproductive conformation of the receptor-tuftsin complex. In this connection, several tuftsin analogs bind the receptor even more strongly than tuftsin.[111] This can be interpreted in the same manner, namely, that these analogs failed to yield a proper and productive conformation of the receptor complex, even though they bind the receptor more tightly than tuftsin.

This concept is further strengthened by the isolation of a (macrophage-myeloma) hybridoma clone, E2-7-7.[125] This clone possesses many attributes of the present macrophage, such as morphology, adherance to plastic, esterase activity, lysosome secretion, and binding of tuftsin, and manifests an antigen-presenting capacity to primed T cells. However, its phagocytic activity is poor. It is possible that in this hybridoma cell, a productive receptor complex with the proper conformation was not obtained. The absence of a neuraminidate residue in the receptor could also account for this discrepancy.

Further work on cell receptor showed a dissociation constant of 13×10^{-8} *M*, along with 50,000 receptor per human PMN cell and twice that number on human monocytes.[100,104] The binding of tritiated tuftsin to mouse peritoneal macrophages revealed K_D of approximately 5.3×10^{-8} *M* and about 75,000 sites per macrophage cell.[100] Similar values were obtained in rabbit and mouse peritoneal neutrophils 6×10^{-8} *M*, with 40,000 receptors per cell and an equivalent number of mouse peritoneal cells.[22,66,85,126,127]

Studies on lymphocyte binding to radioactive derivatized tuftsin, [^{14}C]-methylester and [^{125}I]-tuftsinyl-tyrosine showed some binding, which may well be significant. Several tuftsin derivatives have been synthesized for receptor studies that promise to be quite useful.[128] Such derivatized tuftsin may well have binding properties similar to tuftsin, while some analogs bind more tightly than tuftsin.[111]

Purification of tuftsin receptor to homogeneity has been reported recently.[128] This was done quite effectively by affinity chromatography. Affigel, activated as the *N*-hydroxysuccinimide ester was coupled at pH 7.9 to the pentapeptide analog Thr-Lys-Pro-Pro-Arg primarily at the amino group of threonine, which has an appropriate pK of 7.[55]

Affigel affinity column (0.8 × 4 cm) was washed with ammonium carbonate pH 7.9 and ammonium acetate pH 5.0, and the CHAPS-solubilized rabbit peritoneal PMN membrane

run through the column. The column was washed again and eluted with either 20 n*M* tuftsin or the pentapeptide analog. After removing the eluting oligopeptide with Dowex 50, the eluted material was assayed for [^{3}H]-tuftsin binding by precipitation with polyethylene glycol.

Gel filtration of the purified or the solubilized receptor was done on Sephacryl S-300. The column was equilibrated in 0.1 *M* ammonium acetate pH 5.0 containing 4 m*M* CHAPS. The receptor in the filtrate was assayed by [^{3}H]-tuftsin binding as above. One large tuftsin-binding fraction, comprising the bulk of the receptor, and a smaller fraction were obtained. Their motility in the filtration column gave M_r values of 500 and 250 kDa, respectively.

The affinity fractions that bound tuftsin were concentrated and dialyzed. These were reduced with 2-mercaptoethanol and alkylated with idoacetamide. They were then subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis (PAGE) and stained with silver. Two clear bands, α and β subunits, were obtained at 62 and 52 kDa. This contrasted with the high M_r obtained with gel filtration in Sephacryl S-300 of 500 and 250 kDa. These findings indicate that, in solution, the two monomers associate to form much larger oligomers. Electron microscopic studies were supportive of this and showed a uniform sphere, which was calculated to have a molecular weight of about 500 ± 40 kDa ($M_r/PN = 4/3\pi r^3$, P = Protein density, M_r = relative molecular weight, and N = Avogadro's number). The peaks from the gel electrophoresis were transferred to nitrocellulose paper by electroblotting,[129] overlaid with [^{3}H]-tuftsin after appropriate blocking. Fluorography revealed two adjacent radioactive bands that correspond in mobility to the silver-stained bands.

The concentration of receptor in the gel filtration medium was vanishingly small. Despite that fact the presence of a predominant 500 kDa indicates that the dissociation constant of the subunits must be very low. Assuming that an equal number of α and β subunits compose the oligomer, it appears likely that the receptor is an octamer $\alpha_4\beta_4$.

It is of special interest that treatment of the native membrane receptor with 2 mercapto-ethanol or dithiothreitol diminished tuftsin-binding capacity to a maximum of approximately 50% of the untreated control.[66,130] This is in harmony with the possibility that one of the binding subunits, in a multisubunit receptor, possesses a sensitive disulfide bond that is necessary for tuftsin binding.

A similar study was made on the human cell line HL60.[37,131] Again, two subunits were isolated with M_r 66 ± 1.8 kDa and 57 ± 2.5 kDa. In solution, an oligomer of relatively low concentration (see above) exists with a molecular mass of 550 ± 50 kDa.

VI. TUFTSIN ANALOGS

A. Naturally Occurring Tuftsin and its Analogs

Tuftsin sequence appears in all four classes of IgG. However, only leukokinin, a small fraction of IgG1, displays tuftsin activity that encompasses the various facets of this review. What functions this tetrapeptide has or potentially may have in other classes remains to be determined. Recently, one possible function for tuftsin has been suggested.[132] Thus, the binding of antigen IgG complex to C1 of complement involves four basic residues — His 285, Lys 288, Lys 290, and Arg 292 of the Fc portion: Val-Gln-Val-His-Asn-Ala-Lys-Thr-Lys-Pro-Arg #292. These residues, located on the outer surface of the $C_\gamma 2$ domain, interact with complementary sites of C1q to bind to human IgG. It is obvious from this sequence that tuftsin contributes two of the four basic residues and may play a significant role in this binding. In fact, tuftsin inhibits C1-mediated immune hemolysis, albeit at a concentration range of 200 to 800 μ*M*. The occurrence of tuftsin sequence in various IgG classes has been tabulated along with some analogs.[132] In addition to its presence in the Fc portion of the four classes of IgG, tuftsin occurs in guinea pig G2 exactly in the same location as in human γ-globulin 289 to 292.[133] In mouse strain G1 (MOPC21), the analog sequence Thr-Gln-Pro-Arg also appears at the same location. Tuftsin sequence appears also at 9 to 12 residues

from the amino terminal of P12 protein of Rauscher murine leukemia virus. A very close analog where Lys and Arg exchange places, Thr-Arg-Pro-Lys, is found in yet another virus protein residue 214-217 of the influenza hemagglutinin JAP HA (H_2'',57). Finally, dog tuftsin represents a replacement of Arg by Lys, Thr-Lys-Pro-Lys.[75] It should be stressed that at the level of phagocytic activity, such analogs as Thr-Arg-Pro-Arg, Thr-Lys-Pro-Lys, and Thr-Arg-Pro-Lys are on the whole as active as tuftsin Thr-Lys-Pro-Arg.[15,41,68-70,134-136]

As was mentioned above, the pentapeptide Thr-Lys-Pro-Pro-Arg is a potent inhibitor of tuftsin[111] and was used for affinity purification of the tuftsin's receptor.[128] The tetrapeptide Lys-Pro-Pro-Arg (postin) was also found to efficiently inhibit phagocytosis, superoxide anion production, and chemotaxis of both human and rat PMN leukocytes and monocytes.[133a] Postin is released from the slow form of the protein cystatin C, which can inhibit various PMN cell functions. This activity of the protein is suggested to reside in postin.[133a]

The tripeptide Thr-Lys-Pro [(Des-Arg^4)tuftsin], which corresponds to positions 289 to 291 of the CH_2 domain of IgG, was found to exert considerable regulatory effect on several macrophage functions. Thus, it inhibits phagocytosis, cell locomotion, and superoxide anion production,[133b] as well as IgE-dependent cellular cytotoxicity, β-glycuronidase release, and IL-1 production.[133c]

Certain remote homologies have been detected between the acute phase reactant, C-reactive protein (CRP) and the CH_2 region of IgG, the molecular origin of tuftsin. CRP contains three tuftsin-related sequences: Lys-Pro-Arg, Thr-Lys-Pro, and Thr-Lys-Arg. In view of the involvement of CRP in phagocytic processes, and that proteolytic degradation of CRP by neutrophil-derived enzymes leads to a mixture of peptides capable of modulating neturophil functions, several related analogs of tuftsin were synthesized. The compounds prepared are [Leu^4]tuftsin, [Gly^1]tuftsin, and [Gln^4]tuftsin,[133d] as well as longer segments encompassing these sequences.[133e,f] The peptides were found to possess substantial tuftsin-like characteristics. The results may shed light on the important role of CRP as a possible immunomodulator during inflammation. It is worth mentioning, however, that the elongated peptides failed to bind to the cellular tuftsin receptors.[133e,f]

B. Synthetic Analogs and Derivatives

Following the chemical synthesis of tuftsin and the initial exploration of the scope of its biological activity, a multitude of analogs (>100) were synthesized for the study of structure-function relations (for review see References 41, 42, 137). This family of peptides included structural modifications, e.g., amino acid substitutions or deletions, throughout the tuftsin's chain, blocking of N and C terminals, and chain extensions. Elongation of tuftsin in accordance with its location and origin in the CH_2 domain of immunoglobulin G,[114,132,138-141] of several tuftsin and tuftsin-like dimers,[23,106a,140,141] and of tuftsin derivatives bearing sugar moieties at its N and C terminals[115,143] is particularly noteworthy. The synthesis of the various analogs and derivatives of tuftsin essentially followed the procedures described above for tuftsin and primarily employing solid-phase and classical methodologies.

From extensive structure-activity studies of tuftsin analogs, it can be rather ambiguously concluded that the entire peptide sequence is vital for manifestation of its full spectra of activities. Structural modifications, often minute, considerably effect activity and may result in an intense decrease, total loss, or even inhibition of action. Thus, for example, of all the many synthetic analogs of tuftsin studied, only (Leu^1) tuftsin,[144] (Homoarg^4)-tuftsin, and derivatives in which Lys^2 and Arg^4 exchange or replace each other[41] augment phagocytosis to the same extent as tuftsin. Some analogs, however, possess certain greater activities than tuftsin; for example, (Ala^1)-tuftsin was found capable of augmenting, *in vitro*, the immune response of mouse macrophages stronger than tuftsin,[29] whereas the dimer-peptide tuftsinyl-tuftsin exhibited antitumor effects in mice considerably greater than tuftsin.[23] The analog (Gly^1)tuftsin augmented phagocytosis-induced chemiluminescence to the same extent as

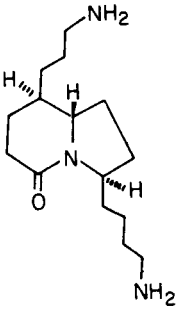


FIGURE 15. Nonpeptide indolizidine mimicking [Des-Thr¹]tuftsin.

tuftsin, whereas two formylated derivatives, *N*-α-formyl tuftsin and formyl-methionyl tuftsin, stimulated macrophage functions quite similarly to tuftsin, but did not concomitantly induce depression of lymphocyte functions.[82a] In view of the wide spectrum of tuftsin's activities and from the substantial data obtained through structure-function studies, two questions of relevance arise: (1) are the tuftsin receptors, on phagocytes, multifunctional homogeneous or perhaps heterogeneous species; (2) even though the occurrence of a human mutation, Thr-Glu-Pro-Arg[2,63] showing symptoms of immune deficiency, what then is the exact physiological role of tuftsin? The answer to both is as yet open.

Computational calculations,[54] as well as spectroscopical studies (e.g., References 50, 51), suggest that a low-energy conformation of tuftsin may exist as a β-turn or a hairpin with a two-split-ends structure. Based on this data, an idolizidinone (Figure 15), a nonpeptidic molecule that mimics the tuftsin's inhibitor Lys-Pro-Arg ([Des-Thr¹]tuftsin), was synthesized with the aim of obtaining an immunosuppressant.[145] The compound was found to block the stimulatory effect of tuftsin on phagocytosis by macrophages.

VII. ANTIMICROBIAL EFFECTS

In general, the various effects of tuftsin are exerted through the stimulation of phagocytic cells, principally the macrophage. However, some effects are the result of direct action on the bacterial organisms. At the high concentration of around 60 μg/ml, tuftsin was shown to thoroughly destroy several microoorganisms. Among these is the medically important Pneumococcus.[146] This concentration is quite high when compared to normal tuftsin serum levels of 250 to 500 ng/ml.[13,43,107,108] Given the LD_{50} dose of 2.4 g/kg,[20] it may be feasible to attain a serum level of 60 μg/ml in a therapeutic attempt without any untoward effects.

Further studies relative to activation of the phagocyte were made.[16,16a] Tuftsin was injected with 10 or 20 mg/kg bacteria (Listeria monocytogenes) into mice. Shortly thereafter, macrophages with internalized bacteria were harvested and incubated for various times. Control mice were injected with saline along with bacteria. It was shown that after 15 min of incubation, tuftsin-treated mice showed 10 times the killing ability of the control.

Other experiments by the same group were also impressive. Mice were injected i.v. with one of four bacterial species, followed immediately by tuftsin 10 or 20 mg/kg. Blood samples were withdrawn at various times and the number of bacteria assessed. There was a remarkable augmentation of blood clearing of bacteria with tuftsin. Further it was shown[147] that a measured number of *Pneumococci* injected into mice yields only 10% survival, while in those injected with tuftsin 20 mg/kg, the survival rate was 50%.

Keratitis caused by *Pseudomons aeruginosa* is an extremely serious disease. In experiments designed to alleviate this condition, 50 organisms were injected into the midstroma of each

eye. After 18 h, each eye received two subconjunctional injections of 0.5 mg of tuftsin alone or combined with 20 mg of Gentamicin, and Gentamicin alone. Saline, 0.5 ml, was injected in the controls. The number of eyes were 14, 12, 19, and 12, respectively. The number of surviving bacteria were plated on nutrient agar and counted after 24 h of growth. Tuftsin alone had no effect. The best results were obtained with Gentamicin and tuftsin combined with a $p < 0.03$ significance compared to Gentamicin alone.[148]

A similar study with tuftsin alone, given at the time of corneal inoculation, prevented the development of keratitis in 50% of the eyes and significantly reduced corneal colony counts. Bacterial colony counts, represented by log final count minus inoculum counts, were for saline control 5.4 ± 0.7 and for tuftsin 3.0 ± 0.6 with a significance of $p < 0.02$.[149]

The main difference between these two reports is the time of inoculation. In the first study, tuftsin was injected 18 h after the bacterial injection. By contrast, in the second report showing good tuftsin effect, bacterial and tuftsin injections took place at the same time. It was thought that tuftsin is more effective when used earlier in the course of the disease.

An interesting effect on *Candida albicans* infection has recently been reported with good results.[150]

VIII. ANTINEOPLASTIC EFFECTS

A. Virus-Induced Tumors

1. Rous Sarcoma

Rous sarcoma virus preparations were injected into the thighs of newborn mice C57B1/10 SN. After 1 or 7 d, mice were injected with 10 and 25 μg tuftsin per mouse three times a week. After 120 d of the experiment, controls that were given saline injections instead of tuftsin showed a tumor incidence of 65%, when compared with 25 to 44% incidence in tuftsin-treated mice.[24]

2. Friend Leukemia

Friend leukemia uniquely produces a massive enlargement of the spleen with prominent erythroblastosis and, as such, it can be readily recognized. Here, tuftsin was given early by i.p. injection of 10 and 20 μg per mouse. Five days later, a preparation of the virus, carefully pretitered to give an LD_{50}, was injected i.p. The controls, as expected from the titered virus, yielded 45% survival, whereas tuftsin-treated mice showed 75% survival. On the other hand, tuftsin failed to afford any protection when given on the same days as the virus,[131] again emphasizing the importance of an immunological stimulus.

3. Rauscher Leukemia

This is another virus-induced leukemia that responded to tuftsin treatment.[151] Here the result was time- and dose-dependent. The best results were obtained in mice injected with 25 μg per mouse 4 d prior to virus infection, below 5 and above 30 μg per mouse, the yield had little if any effect. Control mice, injected with saline, survived 27.7 ± 4.3 d. Tuftsin-treated mice, 25 μg per mouse, survived 56 ± 11.3 d. This is another predictable instance where high doses of tuftsin give negative results. This is true in almost all expressions of tuftsin activity.

4. Sarcoma Virus

[Lys^4]-Tuftsinyltuftsin was administered to mice, 10 μg per mouse 1 d before and 1 d after the injection of virus. On the fourth day, tumor growth in treated and untreated mice was apparent. Growth proceeded till the sixth day, after which treated mice showed a rapid reduction in the size of the tumor. The untreated controls showed a continuous and progressive growth by the eighth day, then decreased slightly thereafter.[139]

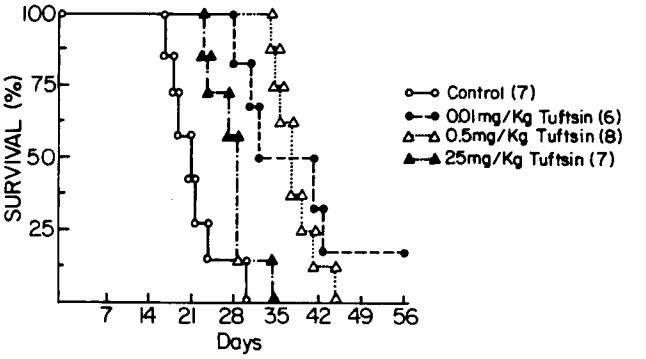


FIGURE 16. The effect of i.p. tuftsin administration on the survival of C3H mice received 2 × 10^4 fibrosarcoma cells, i.p. on day 0. In parenthesis is the number of mice in each group. (From Catane, R., Schlanger, S., Weiss, L., Penchas, S., Fuks, Z., Treves, A. J., Gottlieb, P., and Fridkin, M., *Ann. N.Y. Acad. Sci.*, 419, 251, 1983. With permission.)

5. Abelson Leukemia

The Ableson leukemia virus is an unusually virulent virus that causes blood dyscrasia during the first few days of life. Several lines of evidence indicate that the antineoplastic effect of tuftsin is perhaps totally the result of immunogenic stimulation, both cellular, such as the activation of the macrophage, and humoral, through antibody augmentation, among other effects. In view of this, it was of interest to investigate the effect of tuftsin on the Abelson virus. Since the virus is given immediately after birth, it grows and kills the animal by the age of 6 to 8 d, long before maturation of the immune system. Indeed, the result of this experiment was expected to be negative, and so it was.[191]

Tuftsin was given 10 μg per mouse i.p. a few hours after birth, along with the virus. Both control and experimental mice developed tumors within a week, much as predicted. This again reinforces the immunological role in tuftsin activity.

B. Chemically Induced Sarcoma

1. Murine Fibrosarcoma

Catane et al.[19-21] reported on the effective antitumor activity of tuftsin in a murine fibrosarcoma model. A transplantable fibrosarcoma, originally induced to C3H/eb mice by the carcinogen 3-methylcholanthrene and maintained by serial intramuscular passages in syngeneic mice, was used for the studies. Thus, for example, administration of tuftsin (i.p. three times per week) significantly prolonged the lifespan of C3H/eb mice that received (i.p.) fatal inoculum of fibrosarcoma cells (2 × 10^4). Doses of 10 μg tuftsin per mouse were most effective (Figure 16). It is worth noting that several analogs of tuftsin and, in particular, Ala^1-tuftsin (a potent stimulator of macrophage immunopotency), were found inactive.[152] Tuftsin entirely abolished tumor development when the mice received a smaller tumor inoculum (1 × 10^4 cells) and treated three times per week with tuftsin (0.5 mg/kg weight) (Figure 17). As shown, 80% of the control group of mice did not survive the fibrosarcoma. The finding that tuftsin exhibited substantial antitumor effect in splenectomized mice inoculated with fibrosarcoma (1.5 × 10^4 cells) (Figure 18) deserve special attention.[152] The activity of tuftsin was also manifested when tumor cells (0.5 × 10^6) were injected subcutaneously into the footpad of mice. Tumor growth, as measured by its size, was substantially inhibited by tuftsin (i.p. 500 μg per dose twice a week).[152]

The antitumor effect of tuftsin is mediated, seemingly by macrophages. Coinjection of carrageenan, a macrophage inhibitor, with tuftsin resulted in complete neutralizing of the activity of the peptide.

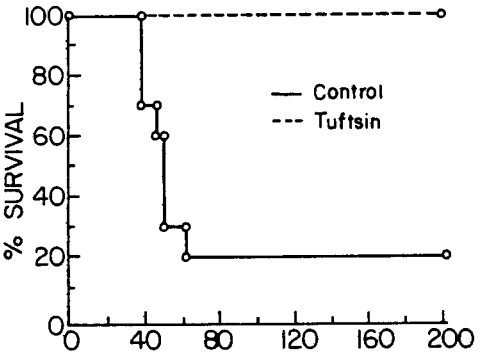


FIGURE 17. Effect of tuftsin (0.5 mg/kg) on the development of murine fibrosarcoma (inoculum of 10^4 viable tumor cells (ten mice in each group). (From Catane, R., Sulkes, A., Uziely, B., Gez, E., Isacson, R., Treves, R. A., and Fridkin, M., *Int. J. Immunotherapy*, 11, 81, 1986. With permission.)

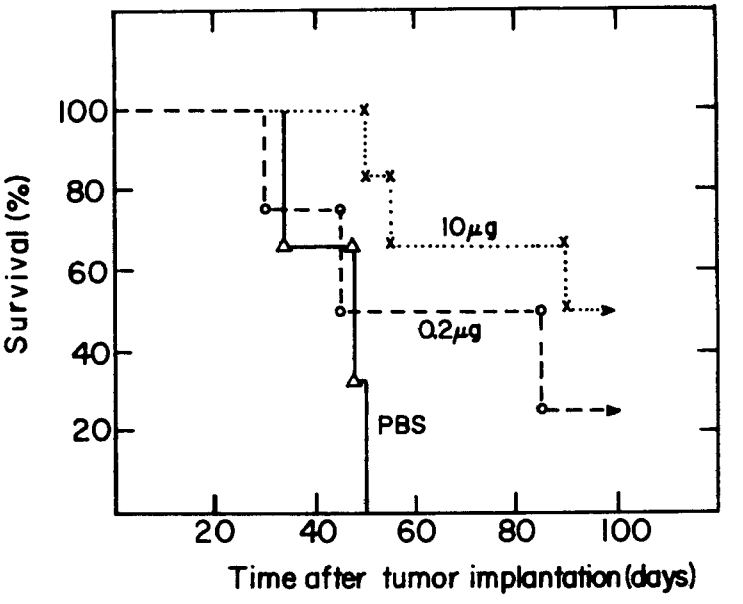


FIGURE 18. The effect of i.p. tuftsin administration on the survival of splenectomized (3 weeks prior to tumor inoculum) C3H mice received 1.5×10^4 fibrosarcoma cells, i.p. on day 0. (From Schlanger, S., M.D. thesis, The Hebrew University of Jerusalem, 1982. With permission.)

2. Direct Chemical Induction

Fibrosarcoma was directly induced in BALB/C mice through injection (i.m.) of a suspension of 3-methylcholanthrene in sesame oil. The effect of tuftsin (when injected into mice i.p. at doses of 0.2, 10, and 500 μg once a week) was evaluated. Only high doses of peptide (500 μg per mouse) had an effect, though rather mild, on the survival (control, 138 d; tuftsin-treated, 153 d) of the animals.[152]

Banks et al.[153] also reported on the ability of tuftsin to inhibit the development of primary murine fibrosarcoma. Accordingly, 3-methylcholantrene was injected i.m. into mice (C57BL/10), followed by administration of tuftsin (i.p. 10 or 25 μg three times a week). It was

observed that fewer incidents of tumor development occurred initially in tuftsin-treated mice when therapy began at either d 1 or d 60 after inoculation with the carcinogen. Differences, however, between experimental and control groups became gradually marginal with time.

C. Murine Leukemia L1210

Murine leukemia L1210 was used to assess the effect of tuftsin on the implantation of neoplastic cells. DBA/2 mice were injected with these cells i.p.[18,22,24,127] All experimental mice were given i.p. 10 μg of tuftsin several days before the L1210 cells, and this dosage was continued throughout the experiment. The tumoricidal activity of tuftsin was found to be rather small. Other experimental mice were given tuftsinyltuftsin. This dimer, Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg, was assumed to be cleaved in the body of the animal to yield the monomer tuftsin at a steady-state low level. This approach was dictated by the desire not to cause an unduly great elevation of tuftsin level, as would happen with injection of relatively large amounts of tuftsin. High doses would allow the accumulation of Lys-Pro-Arg through the action of a highly active membrane and cytosol aminopeptidase.[58,59] The objection to the accumulation of the tripeptide is that it is a strong inhibitor of tuftsin activity[120] and would, therefore, antagonize any positive effect by tuftsin. This inhibitory property[14,100] may well render it a regulator of tuftsin activity.[22,72,75] Tuftsinyltuftsin was found more effective than tuftsin in increasing significantly the lifespan of mice innoculated with the tumor.

D. Murine Melanoma

Murine melanoma cells were a subject of study. Cloudman S-91 melanoma cells were injected into syngeneic mice as before. Tuftsin-treated recipient mice showed a distinct suppression of growth of the cells, whether injected subcutaneously or i.v.[6,7,18,24,154]

Melanoma B16/5B was similarly studied in syngeneic C57BL/6 mice.[22,72,75,106b] Mice were injected subcutaneously with tuftsin 20 or 30 μg per mouse. These were started 7 d before and 7 d after melanoma cell injection and continued twice weekly. The result was very impressive. All controls receiving saline developed tumors after 8 to 10 d. However, 50% of tuftsin-treated mice showed no tumors for 60 to 80 d thereafter, at which time the experiment was terminated. Similar results were obtained in a similar experiment, except that the dimer tuftsinyl-tuftsin was used at 2 ng or 2 μg per mouse in the treated experimental animals. All such treated animals did not develop any tumors, while all the controls on saline exhibited tumor growth. In another approach, C57 BL/6J mice were treated with 25 μg per mouse of tuftsin along with α-difluoromethylornithine, an ornithine decarboxylase inhibitor in drinking water. In these experiments, the inhibitor alone resulted in 80% inhibition of tumor growth, and tuftsin alone yielded 23 to 26% inhibition of growth. However, the combination of the two agents showed an inhibition of tumor growth amounting to 90 to 96%.[155]

E. Lymphoma

Cells were tested for tuftsin activity by injecting tumor cells i.v. into syngeneic mice as usual. 1×10^6 cells were given to each mouse. Little or no effect was obtained with peptide doses of 0.1 and 1.0 μg per mouse. However, animals on 10 and 100 μg per mouse suppressed growth of the tumor for 150 d.[24] Using the same protocol, but with tuftsin given i.p., no effect was noted. A surprising result in view of the many positive effects noted above.

F. Comparative Effects of Tuftsin and Tuftsinyl-Tuftsin

One of the major differences between the tetrapeptide and the linear dimer octapeptide is that the latter binds the receptor approximately ten times more avidly than tuftsin. Further-

more, the tetrapeptide is active in the *in vitro* phagocytosis system, whereas the octapeptide is not. However, *in vivo,* the antineoplastic effect of tuftsinyl-tuftsin is more pronounced than tuftsin itself.[23,139] It is possible that the activity of the octapeptide resides in the slow production of tuftsin from the dimer such that little if any tripeptide (Lys-Pro-Arg) would accumulate. In this case, the tripeptide would inhibit tuftsin activity. In actual fact, this is the reason why the octapeptide was synthesized.

G. Human Tumors

Initial clinical studies in which the capacity of tuftsin to inhibit development of human tumors was evaluated were performed by Catane and colleagues.[21,156] Twenty five adult human patients with various advanced malignancies (primarily nonsmall cell carcinoma of the lung), 18 of whom were previously subjected to chemotherapy, were treated with tuftsin. The peptide (sterile pharmaceutical preparations, Abic Ltd, Ramat Gan, Israel) was administered i.v. twice a week at doses of 5 mg each. Evaluation of therapy was possible in 16 patients, following at least 6 injections of tuftsin over a period of 3 weeks. Thus, two patients responded, as judged by decreased blast counts and appearance of normal granulocytes. The condition of five patients remained stable, whereas the diseases progressed in nine patients. As previously noted, no toxic side effects were observed during therapy. Application of higher doses of tuftsin is currently under investigation. The considerable tuftsin-mediated enhancement of leukocytosis and mononuclear cell cytotoxicity may suggest coadministration of tuftsin with other therapies. Indeed, preliminary observations in mice indicated that the tuftsin reduces cyclophosphamide-related myelotoxicity.[21]

H. Mechanism of Tumoricidal and Bactericidal Activity

The production of active oxygen compounds by macrophages and PMN cells has been known for some time.[80,81,116] The effect of tuftsin has been shown to augment considerably the production of superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2),[33-36] presumably formed by dismutation of $O_2^{\cdot}$ by superoxide dismutase. H_2O_2, together with $O_2^{\cdot}$, give rise to hydroxyl radical ($OH^{\cdot}$). All three oxygen compounds are highly reactive. These, in addition to hydroperoxides and hypochlorites, may take part in the demise of the bacterial or neoplastic target cells.[157] It appears that $O_2^{\cdot}$ is formed by NADPH oxidation and by the metabolic breakdown of purines. Adenosine is deaminated to inosine, which leads to hypoxanthene through the action of purine nucleoside phosphorylase. The oxidation of hypoxanthene to xanthene yields $O_2^{\cdot}$, and further oxidation of xanthene to uric acid also yields $O_2^{\cdot}$.[35,36,81] Tuftsin stimulation of the macrophage produces increased amounts of $O_2^{\cdot}$. This appears to be the source of at least 90% of the superoxide anion.[33-36] The increased production of TNF stimulated by tuftsin may also play an important role in cytotoxicity of phagocytic cells.[37]

IX. TUFTSIN DEFICIENCY SYNDROMES

Only one type of congenital tuftsin deficiency was identified out of several possible and phenotypically similar inherited deficiences. However, two types of acquired deficiency have been described. One type appears in patients who had undergone splenectomy or had suffered severe curtailment of splenic function of disease.[62,63,110,158] The other deficiency involves patients with leukemia.

A. Congenital

This syndrome was discovered before the isolation and structure determination of tuftsin. It was considered early on to be a mutation of the normal compound, because in every case peptide extracts from the serum of these patients strongly inhibited phagocytosis stimulation by peptide extracts from the serum of normal individuals.[63,159-163] This was finally verified

by comparative studies of the activities of the isolated mutant tuftsin, Thr-Glu-Pro-Arg. The synthetic mutant peptide was as effective an inhibitor per mol as that of the peptide extracted from the patients' serum. There have been 20 cases of this deficiency studied in a few years search in the U.S.[2,8,9,159-162,164] and 2 cases in Japan.[67] A very significant property of the mutant peptide is that its inhibitory activity parallels its greater affinity to the receptor, which amounts to four times that of tuftsin. In all cases studied so far, only one parent of the affected patient had the same phenotypic deficiency with recurrent infections.[2]

The deficiency is not uncommon, since so many patients have been recruited with little effort. This is done by selecting patients who give a history of severe and repeated infections that respond rather dramatically to γ-globulin injections. γ-Globulin contains leukokinin, which would supply tuftsin to the patient. In these cases, complement components, all IgG types, are normal. In light of these telltale findings, an assay for tuftsin usually clinches the diagnosis. The symptoms are much more severe during infancy, presumably because of the parallel immaturity of the immune system. The usual signs in infants are *Pneumococcus* or *Streptococcuss* pneumonia, infected eczematous dermatitis with draining lymph nodes, otitis, and sinusitis. These are the most severe and most common manifestations. In addition to the signs and symptoms common to all, the peptide extracts in each case inhibited the extracts from the normal controls. One such patient (CGK) was studied in detail. The inhibitory peptide was isolated using the same detailed procedure employed for identifying tuftsin.[3,4] In the final step of Dowex-50 column chromatography, the elution volume of tuftsin peak was traced with [^{3}H]-tuftsin. There was a complete absence of ninhydrin positive peak at the tuftsin locus. However, a much earlier peak showed inhibitory capability. Sequence analysis showed it to be Thr-Glu-Pro-Arg, a typical transition mutation where AAA or AAG coding for lysine was replaced by GAA or GAG coding for glutamate. This mutant peptide was synthesized and inhibited tuftsin activity at the same level of the naturally mutant peptide. It is significant in this study that no trace of the normal tuftsin was obtained. Therefore, it appears that the trait is passed on in a phenotypically dominant heterozygote with allelic suppression. Radioimmunoassay of serum tuftsin of such patients inevitably yields an expected high false value of 1200 to 1500 ng/ml, simply because of the strong avidity of the mutant peptide toward the specific cell receptors.[192] Normal values are ~250 ng/ml.[107]

B. Acquired

1. After Spenectomy and in Splenic Disease

We indicated earlier that one of the enzymes necessary for the generation of tuftsin is a splenic-endocarboxypeptidase. Consequently, in the absence of the spleen or in the event that its function is highly comprised because of infiltration or sclerosis, as in leukemia, and in severe splenic involvement in sickle cell disease, the tetrapeptide remains attached to the parent carrier heavy chain and as such is inactive. Recently, the loss of splenic function in sickle cell disease has been emphasized[107-109,158,163,165,166] along with the prevalence of *Pneumococcus* infections, much as is encountered following splenectomy. It has been known for some time that splenectomy results in lowered resistance to infection with occasional fulminating bacterial invasions that result in the death of the subject.[64,65,167-170] It has since been shown that following splenectomy, the injection of tuftsin into infected mice augments considerably the survival of these animals.[171] Splenectomized mice subjected to *P. sepsis* and treated with tuftsin had a significantly improved survival rate, much like those receiving autotransplant of splenic tissue.

2. Functional Deficiency in Granulocyte Leukemia

Seven patients with acute granulocytic leukemia and six patients with myelofibrosis were studied. Their blood neutrophils failed to show stimulation with synthetic tuftsin or with the natural precursor, serum leukokinin. Their serum level of tuftsin activity was minimal or

absent. Five of these patients were in remission with normal-looking granulocytes and normal white blood count. In all respects, all these patients were essentially tuftsin-deficient, because their neutrophils could not respond to either tuftsin or serum stimulation. In contrast, two patients with myelomonocytic leukemia showed vigorous unstimulated phagocytic activity and responded normally to synthetic tuftsin stimulation. Their serum contained normal levels of tuftsin.[65]

X. OTHER FUNCTIONAL EFFECTS OF TUFTSIN

A. Effect on Sepsis in Splenectomized Mice

Mice splenectomized 3 months before the initiation of the experiment were subjected to *P. sepsis* by injecting 200 organisms per mouse i.v. Tuftsin 25 to 100 μg per mouse were administered at various times before administering the bacteria. Tuftsin-treated mice showed considerably more survivals than the splenectomized nontreated controls with a $p < 0.001$. On the second and seventh day, respectively, sham-operated mice and autotransplanted mice both showed 100 and 95% survival, respectively, and one group receiving 25 μg tuftsin per mouse, showed 90 and 79%. Two groups receiving 50 and 100 μg per mouse showed 74% survival on days 2 and 7, respectively.[172] From the preceding section it is expected that high doses of tuftsin would be, to some extent, counterproductive.

B. Effect on Burn Injury

Following a burn injury, a depression of the immune response and the appearance of potent suppressor T-cell results. Here, tuftsin injected i.p., 20 μg per mouse, to burned mice on various post-burn days, restored splenic T-cell proliferative capacity. This was done in an allogeneic mixed lymphocyte reaction (MLR). Burned mice not receiving tuftsin exhibited about 3.5% of the normal response.[173]

C. Hormonal Effect

Following the i.v. administration of tuftsin, 300 μg per kg, to rats, the hypothalamic thyrotropin-releasing hormone (TRH) was found to be significantly decreased in the hypothalamus, whereas its plasma level showed little if any increase. However, plasma thyrotropin (TSH) levels increased significantly above 200 μg/kg in a dose-related manner. Tuftsin appears to stimulate thyrotropin secretion in rats.[174,175] In order to exert this effect, tuftsin must itself pass the blood brain barrier, rather than a byproduct thereof, stimulated by tuftsin.

XI. OTHER PERTINENT OBSERVATIONS

A. Tuftsin Liposomes

It was indicated earlier that tuftsin binds to receptors of PMN leukocytes, monocytes, and macrophages, but not on lymphocytes or erythrocytes. This fact was utilized to determine whether liposomes charged with derivatized tuftsin in their bilayers could bind to tuftsin receptors, particularly on PMN cells. The tetrapeptide was derivatized at the carboxyterminal by covalent incorporation of long hydrocarbon reagents. Small unilamellar liposomes were prepared from the derivatized tuftsin molecules, egg phosphatidylcholine (PC), egg (^{14}C)-PC and cholesterol. The appropriate concentrations of the derivatized tuftsin, phosphatidylcholine (PC), [^{14}C]-PC, 30 μCi/μmol, and cholesterol. The liposomes so prepared led to significantly enhanced binding to PMN leukocytes at 37°C, but not at 0°C. No such enhanced binding was obtained with erythrocytes or lymphocytes. The results may be of value when tuftsin is used for human therapy. This type of liposome would specifically recognize PMN cells, where it would deliver its contents for more effective activation.[176]

It was later shown independently[177] that tuftsin could be entrapped in liposomes made up of cholesterol and PC in equimolar concentrations. Such liposomes were injected into the peritoneal cavity of mice. Controls were injected i.p. with liposomes alone or liposomes and tuftsin without entrapment. Liposomes containing tuftsin caused significantly greater mobilization of intraperitoneal macrophages and leukocytes than controls. Similarly, there was markedly increased phagocytosis of latex particles in mice treated with liposomes-entrapped tuftsin. The latter was more effective than tuftsin alone, without liposomes, in prolonging median survival of Balb/c mice inoculated with BCL leukemia cells.

B. Augmentation of White Blood Cell Counts

The level of white blood cells in the blood is increased following tuftsin treatment. This was documented in man, dog, and mouse. The augmentation in all cases was nonselective, but affected all elements of white blood cells within normal proportions. There were no changes in the red cell or platelet counts. The increase in the level of white blood cells in man was about twice the control and lasted 4 d to 1 week following tuftsin administration of 1.0 mg/kg. In mice, 0.5 and 5 mg of tuftsin i.v. per kg consistently produced leukocytosis after a few days and lasted about 1 week.[20] It is possible that this resulted from stimulation of differentiation discussed above or due to longer survival of the leukocytes.

C. Central Effects of Tuftsin

Tuftsin and its two analogs, [D-Arg^3]-tuftsin and [D-Arg^4]-tuftsin, have significant anti-nociceptic effect following their injection into the cerebral ventricles of rats at a dose of 200 μg. This effect is not counteracted by naloxone, 2.5 mg/kg, injected i.p. 30 min before tuftsin and its analogs. This indicates that this effect is not mediated by opiate receptors.

Exploratory and locomotor activities were also affected by tuftsin in a biphasic manner, starting with depression and followed by stimulated behavior.[178] In another laboratory, tuftsin, 500 mg/kg injected i.p., enhanced locomotor activity with induction of aggressiveness. Furthermore, with i.p. dosage of 20 to 250 mg/kg, there was an increase in the exploratory activities of rats.[179,180] It appears, therefore, that similar central effects were produced both by intraventricular and i.p. injections of tuftsin. In view of this, it would be of great interest to explore the possibility of tuftsin crossing the blood brain barrier, as these results suggest.

Other central effects were also observed after i.p. tuftsin injection. It was reported recently that withdrawal behavior of morphine-dependent rats induced by naloxone was attenuated after tuftsin injection, but only poorly with tuftsin analog [Lys^4]-tuftsinyl-tuftsin.[181] However, the tetrapeptide fragment 1-4 of substance P was completely inert.

D. Enhanced Virus Expression

The phosphoprotein p12, one of the gag gene products, on Rauscher leukemia virus encompase the tuftsin sequence at position 9-12 from its amino terminal.[182] Luftig et al.[183] reported that the addition of tuftsin, in concentration of up to 100 μ*M*, to an RLV-infected culture of mouse cells, increases threefold virion-associated reverse trancriptase, as well as virus budding.

Increased expression of an endogenous xenotropic retrovirus was described by Suk and Long,[184,185] following treatment of highly transformed fibroblasts with tuftsin. The increased expression was concentration-dependent at 0.001 to 1000 μg/ml of tuftsin. This result was achieved within 3 to 4 h of incubation reaching a maximum at 16 to 18 h. The enhancement was proportional to the spontaneous release of virus and required *de novo* RNA synthesis, since actinomycin D inhibited the induction of virus. During a 16-h incubation, tuftsin enhanced DNA, RNA, and protein synthesis in K-Balb cells.

The increased expression of the virus seems to be specific since Gly-Leu-Gly-Leu, Leu-

Tyr-Leu, Thr-Lys-Phe, Lys-Lys-Lys-Lys, and Tyr-Gly-Gly-Phe-Met did not increase virus expression.

E. The Inhibitory Effects of High Doses of Tuftsin

In many of the systems that are stimulated by tuftsin, relatively high doses cause inhibition of the system. Thus, the effect of tuftsin in stimulating the antibody response is significant at 25 μg per mouse and absent at 100 and 400 μg per mouse.[26]

HL60 cells can be stimulated by tuftsin to secrete TNF. However, the stimulation at 10 μg/ml was less than that at 1 μg/ml.[37] The immunogenic response of macrophages to tuftsin is optimal at 5×10^{-8} *M* and not at 10^{-8} or 10^{-7} *M*.[106a] Superoxide (O_2^-) formation by mouse macrophages is stimulated considerably by tuftsin. Maximal stimulation occurred at around 350 n*M*, but no stimulation was obtained at 500 and 600 n*M*.[36]

Tuftsin-stimulated colony formation from bone marrow *in vitro* maximally at 0.5 μg per culture. This stimulation of CFU-C formation was equivalent to that obtained with colony-stimulating factor. However, at higher concentration of tuftsin, there was a suppression of colony formation and at 1.5 μg, the number of CFU-C was less than half that obtained at 0.5 μg. The effect of tuftsin on monocyte chemotaxis was greater at 10 than at 100 μg/ml.[76]

The effect of tuftsin on biosynthetic pathways was assayed by incorporation of [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-leucine into k-Balb cells. More incorporation of thymidine was obtained with 1 than with 100 μg/ml, more incorporation of uridine with 0.01 than with 1 μg/ml, and of leucine with 1 than with 100 μg/ml.[184,185] It appears that the tuftsin requirement for uptake varies among the three major pathways.

Intracellular levels of cAMP were more effectively reduced at a tuftsin concentration of 10^{-6} *M* than at 10^{-8} *M* for both human PMN and thioglycolate-induced macrophages.[38,74]

F. Possible Favorable Effects on Acquired Immune Deficiency Syndrome (AIDS)

The macrophage-granulocyte system is not manifestly incumbered in early AIDS, as is the T-cell system. In the latter, the defect is demonstrable by the presence of severe leukopenia, a low helper-suppressor T-cell ratio, reduced blast transformation by lectins, and severe opportunistic infections.[193] Thus, it is possible to alleviate the symptoms of AIDS by stimulating the macrophage-granulocyte system by a modulator such as tuftsin. This system plays an important role in processing the antigen and therefore modulates antibody levels, as well as cytolysis of tumor cells and bacteria.

The first case of AIDS in Israel showed typical manifestations of the disease. The patient was subjected to various therapeutic regimens,[186] such as amphortericin B, metronidazole, ketoconazole, cimetidine, and zinc, with no avail. The administration of tuftsin, 5 mg per week, when the patient's condition was desperate (extreme cachexia, profuse diarrhea, personality changes, and septic shock due to Gram-negative bacteria) induced a dramatic improvement in his clinical condition. The improvement was noted 1 week after the institution of this treatment. The perianal vesicles, fever, and diarrhea receded completely.

G. Molecular Mimicry

It was shown by two groups that an antibody to a particular epitope on an enzyme may be patterned after the active site among other epitopes, since substrate dramatically inhibits the reaction of the specific antibody with the enzyme.[187,188]

Because of these results, it was postulated that in such antibodies the conformation of the antibody site would be complementary to the enzyme site in the manner of the substrate. Furthermore, if an antibody is induced by the anti-enzyme antibody (idiotype), the induced antibody (anti-idiotype) might have a reactive site similar enough to that of the enzyme so as to acquire some enzyme-like properties. Such appeared to be the case. The antibody to

the anti-lecithinase antibody exhibited lecithin-binding property.[111,189] A follow-up of this postulate is that the active site of an antibody to the ligand tuftsin would be patterned after tuftsin in a manner similar to tuftsin-receptor binding site. This molecular mimicry was readily shown. Antibodies to BSA-tuftsin, generated according to published reports,[13,41,107] exhibited binding properties to various tuftsin peptides almost identical to the binding properties of tuftsin receptor. Thr-Lys-Pro, Ala-Lys-tuftsin-Glu-Ala_3 showed no binding to either protein sites. Ala-Lys-tuftsin bound less avidly than tuftsin in both cases. Thr-Lys-Pro-Pro-Arg and Thr-Glu-Pro-Arg bound receptor and antibody about four times as avidly as tuftsin. Finally, the octapeptide analog Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg showed the strongest binding with equal avidity to receptor and antibody.[111] Whether the active site of either binding proteins, one induced and the other constitutive, represents the involvement of similar amino acid residues remains to be determined.

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