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Studies of Human Granulocyte Phagocytosis Stimulation by Tuftsin

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Tuftsin (Thr-Lys-Pro-Arg), a natural immunomodulating peptide originally found to stimulate phagocytosis by polymorphonuclear leukocytes (PMNs), is now known to bind to both PMNs and monocyte-macrophages, affecting their phagocytosis and other functions. The potential roles of tuftsin in surgery-related infections have been documented using animal models. However, there have been some difficulties in demonstrating the phagocytosis-stimulating activity of tuftsin. In view of this, we have developed a suitable human PMN phagocytosis assay for tuftsin and performed preliminary kinetic studies. The assay was performed on 24-well plates between PMNs and fluorescent microspheres. The greatest effect of tuftsin over the control was observed under the following conditions: 15 min incubation at 37°C with 5 μg/ml tuftsin and a 50:1 ratio of particle to PMN. Particles bound on the surface of PMNs were removed by washing and trypsin treatment, followed by centrifugation through fetal bovine serum. This allowed us to utilize flow cytometry in this study. A flow cytometric procedure was then successfully adapted to human PMN phagocytosis that established a high correlation between microscopic evaluation and flow cytometry of phagocytosis. In addition to the above determinations of the percentage of phagocytic cells, we evaluated the effect of tuftsin on the number of particles engulfed by PMNs. Under the above optimum conditions, tuftsin has greater impact on the number of particles engulfed than on the percentage of phagocytic cells.

INTRODUCTION

Tuftsin is a natural immunomodulating factor derived from leukophilic IgG [1, 2]. We originally reported tuftsin as a phagocytosis-stimulating peptide for polymorphonuclear leukocytes (PMNs) in sera [3], isolated it, determined its chemical structure as Thr–Lys–Pro–Arg, and synthesized it using a solid-phase procedure [4]. This peptide is now known to bind to specific receptors on PMNs, macrophages, and their precursor cells, and to modulate many biological activities in addition to phagocytosis [1, 2]. In *in vivo* animal studies, Martinez *et al.* [5] reported that mice treated with tuftsin exhibited a dramatic increase in the clearance of various bacteria compared with untreated mice and that tuftsin-activated peritoneal macrophages from these mice possessed enhanced bactericidal activity.

Sepsis is a major cause of morbidity after trauma, major surgical procedures, and thermal injury [6–8]. Many investigators have, therefore, attempted to devise treatments for this complication. In those studies, the role of the reticuloendothelial system has been emphasized [9, 10]. Effect of tuftsin under such conditions has been investigated. Fox *et al.* [11] showed that the administration of tuftsin after burn injury restored immunoresponsiveness. Baker *et al.* [12] reported that tuftsin improved the rate of early survival after intraabdominal sepsis as caused by cecal ligation and puncture in rats. Ohkawa *et al.* [13] investigated the effect of tuftsin on Kupffer cell function and DNA synthesis following hepatic ischemia and partial hepatectomy. They showed that tuftsin alone and the combination of tuftsin and ATP–$MgCl_2$ are useful for hepatic support after ischemia and hepatic resection, since these agents had salutary effects on Kupffer cell function and rate of hepatic regeneration. The increased susceptibility to severe infections after splenectomy in adults [14–16] and children [17, 18] is well documented. It is then reported that splenectomized patients possess reduced levels of tuftsin in their sera [19]. We, therefore, examined the effect of tuftsin in splenectomized animals, and demonstrated that tuftsin treatment protected the splenectomized hosts against pneumococcal septic death [20].

To examine the quality of tuftsin preparations, phagocytosis stimulation has been always used as the standard *in vitro* biological assay for tuftsin. For our studies, we have used a variety of phagocytosis assays, direct microscopic determination on smears prepared from an incu-

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bation mixture of *Staphylococcus aureus* and buffy coat leukocytes [21], a microtiter assay of PMN–*S. aureus* phagocytosis [22] that was a modification of the method described by Phillips *et al.* [23], and monolayered murine peritoneal macrophages reacted with ^{51}Cr-labeled IgG-coated sheep red blood cells [24]. There appears to be two reasons why phagocytosis-stimulation activity of tuftsin sometimes cannot be detected. One reason is that many commercially available tuftsin preparations are biologically inactive due to contamination by possibly competing diastereomers of tuftsin, as we documented previously [22]. The other reason is that the phagocytosis assay conditions may not be optimal for detection of the above activity. In fact, Cooper *et al.* [25] reported that they did not detect this activity of tuftsin. Additionally, there have been no systematic kinetic studies performed on phagocytosis stimulation induced by tuftsin. In view of these reasons, we have developed a method by which various conditions were examined to detect the phagocytosis stimulation activity of tuftsin. To correlate the results, we have also adapted the flow cytometric procedure described for rat alveolar macrophages [26, 27] to human PMNs by introducing trypsin treatment. We, therefore, examined preliminary kinetic properties of PMN phagocytosis stimulation induced by tuftsin.

METHODS

Materials

Fluorescent particles (Fluoresbrite, 2.0 μm) were obtained from Polysciences, Inc. (Warrington, PA). Tuftsin was a kind gift from Takeda Chemical Ind. Ltd., Osaka, Japan. Tuftsin was further purified in our laboratory using reversed-phase high-performance liquid chromatography and P-2 gel filtration as we described previously [28]. The endotoxin levels of tuftsin solutions were routinely measured with the Limulus Ameobocyte Lysate QCL-1000 kit (Whittaker M. A. Bioproducts, Walkersville, MD) (<10 pg/ml).

Phagocytosis Assay

Human PMNs were prepared from the venous blood of healthy donors according to a protocol approved by the Institutional Review Board. Blood (30 ml) was mixed with 3 ml of 5.5% dextran (Sigma Chemical Co., St. Louis, MO) and 0.7 ml heparin (100,000 USP units/ml, Sigma Chemical Co.), and kept at 37°C for 1 hr. The leukocyte-rich plasma was collected and centrifuged at 300*g* for 10 min at 10°C. The resulting pellet was washed twice with Hanks' balanced salt solution without Ca^{2+}, Mg^{2+}, or phenol red ($HBSS^-$, Whittaker M. A. Bioproducts). The pellet was then suspended in 35 ml $HBSS^-$, layered over 10 ml lymphocyte separation medium (Organon Teknika Corp., Durham, NC) and centrifuged at 300*g* for 30 min at 10°C. The sedimented cells were washed once with $HBSS^-$. The contaminating erythrocytes were lysed by suspending the cells in 1.8 ml of sterile water for 15 sec. The isotonicity was then restored by adding 0.2 ml 10× $HBSS^-$ (GIBCO Labs., Grand Island, NY) followed by the addition of 35 ml of $HBSS^-$. After centrifuging at 300*g* for 5 min, the PMNs were washed once with Hanks' balanced salt solution (HBSS, Whittaker M. A. Bioproducts), suspended in 2 ml HBSS, counted, and adjusted to 1×10^6 viable cells/ml (viability >95% by trypan blue dye exclusion). PMNs (0.5×10^6/well) were plated in a 24-well plate (Costar Corp., Cambridge, MA) and placed in a 37°C CO_2 incubator (5% CO_2 and 95% air) for 30 min to form the PMN monolayer. The supernatant was then aspirated. Tuftsin in 250 μl HBSS and fluorescent microspheres in 250 μl HBSS were added to each well to obtain the desired final concentration of tuftsin and particle-to-PMN ratio. The plate was incubated at 37°C for the desired period of time. The supernatants were then quickly aspirated and each well was washed three times with 1 ml $HBSS^-$. For trypsin treatment, 1 ml trypsin (porcine origin, 0.25% in saline, Hazleton Biologics, Inc., Lenexa, KS) was added to each well, and the plate was incubated for 15 min. The cells were then collected, layered over 2 ml fetal bovine serum (FBS, Hyclone Labs., Inc., Logan, UT) in a 12 × 70-mm Falcon tube (Beckton–Dickinson Labware, Lincoln Park, NJ), and centrifuged at 100*g* for 10 min at 10°C. The supernatants containing free particles were removed. Each cell pellet was suspended, fixed in 0.5 ml 2% paraformaldehyde, and placed again into a well. After 10 min, PMNs were examined by microscopy, and the percentage phagocytosis (percentage of PMNs with one or more engulfed particles) or particles engulfed by PMNs were enumerated by counting 100 cells per well. The mean values from triplicates were used. For photographic documentation, fixed PMNs were placed in a 12-well plate (Costar Corp.), centrifuged at 100*g* for 1 min, and coverslipped.

Flow Cytometry

For flow cytometric quantitation, 0.5×10^6 cells/ml in paraformaldehyde were analyzed by an Epics Profile I Flow Cytometer equipped with an argon laser (Coulter Corp., Hialeah, FL) as detailed by Steinkamp *et al.* [26] and Stewart *et al.* [27]. In brief, gated analysis enabled the discrimination of whole cells from cell debris and free particles based on the amount of forward light scattering (cell size) and side scattering (cell granularity) as the cells passed through a flow cell which intersected a 488-nm line from an argon laser excitation source. Flow runs of the cell suspensions were initially monitored by forward scatter and side scatter and displayed as bitmaps (Fig. 6). Two regions, corresponding to populations of particles with different sizes, were apparent. These were identified as free particles (and debris) and whole cells based on comparison with independent analyses of particles and cells which were run individually.

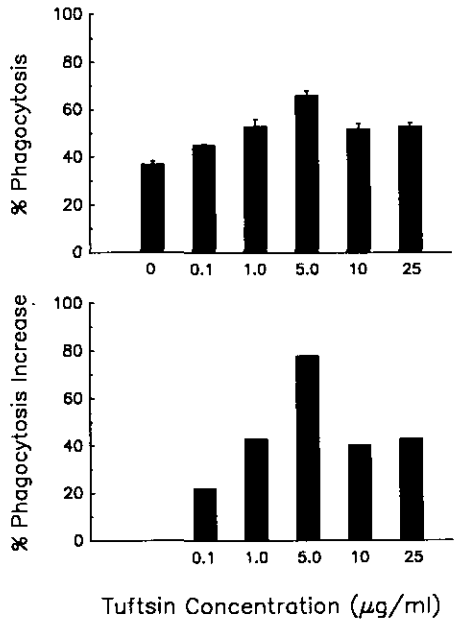

FIG. 1. Optimum concentration for tuftsin. Phagocytosis assays were performed as described under Methods employing different concentrations of tuftsin at 37°C for 15 min with a ratio of particle to PMN of 50:1. Percentage phagocytosis enumerated by microscopy is expressed by mean ± standard deviation.

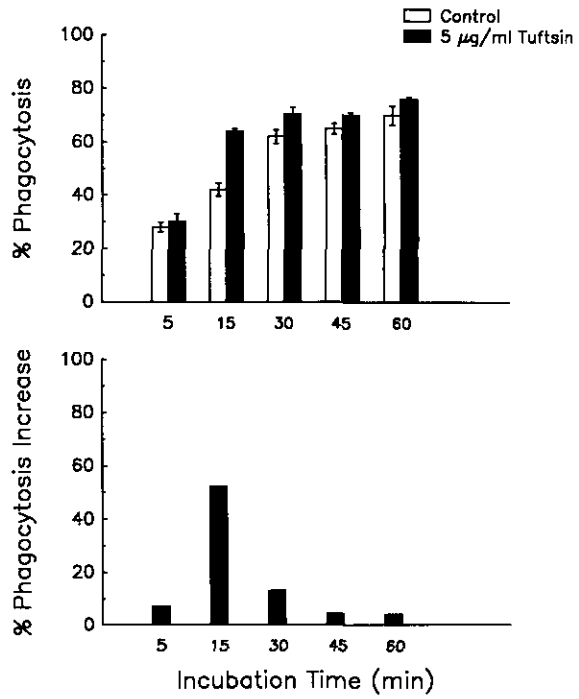

FIG. 2. Optimum incubation time for tuftsin. Phagocytosis assays were performed as described under Methods with different periods of incubation time at 37°C with and without tuftsin (5 μg/ml) and a ratio of particle to PMN of 50:1. Percentage phagocytosis enumerated by microscopy is expressed by mean ± standard deviation.

Fluorescence measurements were consequently made with appropriate gate adjustment in order to ensure that only events corresponding to whole cells were analyzed for particle load. It was observed that the region of the scatter plot corresponding to whole cells became somewhat broader with increasing particle load per cell obtained at the higher particle-to-cell ratios. As a result, the gating was adjusted for each run as necessary. The fluorescence was plotted in frequency-distribution histograms as number of cells vs fluorescence (channel number). The mean number of phagocytized particles per cell was determined from the weighted mean fluorescence for all of the cells counted. A total of 5000 gated events were counted per run.

RESULTS

In order to optimize this tuftsin phagocytosis assay procedure using human PMNs and microspheres, we first examined the optimum concentration of tuftsin using microscopic enumeration from 0.1 to 25 μg/ml. As shown in Fig. 1, 5 μg/ml gave the greatest stimulation of phagocytosis. We then examined incubation times at 37°C from 5 to 60 min. The incubation time of 15 min gave the greatest ratio of phagocytosis stimulation, relative to the control without tuftsin, as shown in Fig. 2. We then varied the ratio between particles and PMNs up to 100 to 1. The greatest effect of tuftsin relative to the control was observed at the ratio of 50 to 1 as shown in Fig. 3. These observations indicate that the best conditions under which to observe the effect of tuftsin in this assay are to perform the phagocytosis assay with 5 μg/ml of tuftsin for 15 min at 37°C with the ratio of particle to

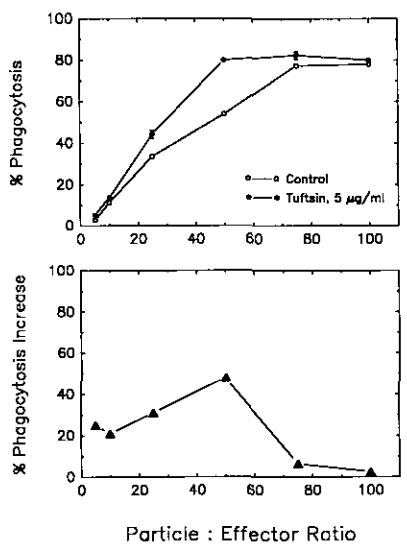

FIG. 3. Optimum ratio of particle to PMN for tuftsin. Phagocytosis assays were performed as described under Methods with different ratios of particle to PMN from 5:1 to 100:1, for 15 min at 37°C, with and without tuftsin (5 μg/ml). Percentage phagocytosis enumerated by microscopy is expressed by mean ± standard deviation.

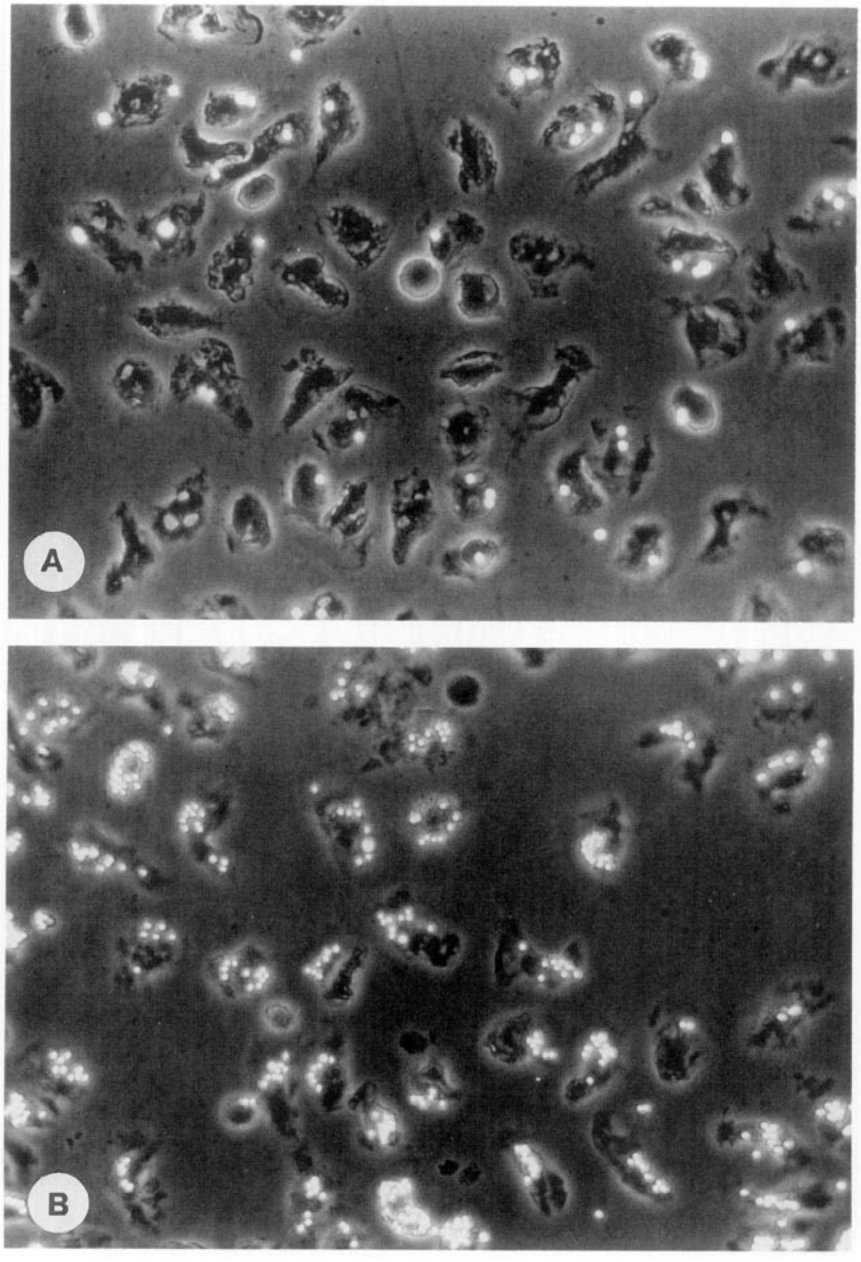

FIG. 4. Photomicrographs of phagocytosis reactions. Phagocytosis assays were performed as described under Methods without (A) and with (B) tuftsin (5 μg/ml) at 37°C for 15 min with a ratio of particle to PMN of 50:1. The pictures were taken under phase-contrast with fluorescence.

PMN at 50:1. The example depicted in Fig. 4 clearly demonstrates the enhanced effect of tuftsin on PMN phagocytosis under these conditions.

Since one of the potential problems associated with flow cytometric determination of phagocytosis is that PMNs with surface-bound fluorescent particles without engulfed particles are counted as phagocytized cells, we have examined by microscopy our PMNs treated with the fluorescent microspheres after the following procedures: (1) washing alone, (2) washing and trypsin treatment, or (3) washing and trypsin treatment followed by FBS centrifugation, to see if we can detect significant percentages of PMNs with surface-bound particles. As shown in Fig. 5, although washing alone left large numbers of PMNs with bound particles (42.3 and 45.0% for control and tuftsin-treated PMNs, respectively), trypsin treatment significantly reduced the numbers (control, 13.7%; tuftsin treatment, 16.3%). Trypsinization followed by FBS centrifugation further reduced PMNs with surface-bound particles (control, 3.7%; tuftsin treatment, 6.3%). We then examined the percentage of PMNs having only surface-bound particles without en-

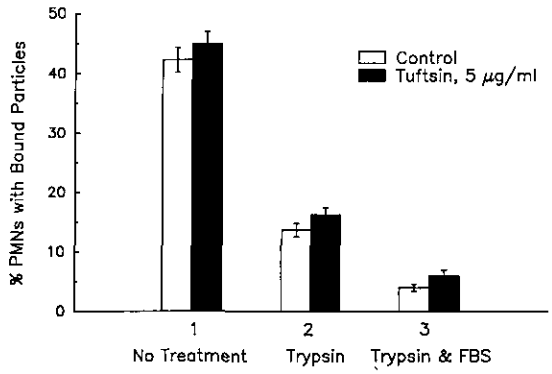

FIG. 5. PMNs with surface-bound microspheres. Phagocytosis assays were performed under the optimum conditions for tuftsin as described in the legend for Fig. 4. In group 1, after phagocytosis reaction, PMNs in each well were washed three times and fixed with 2% paraformaldehyde without any further treatment. Group 2 wells were treated with trypsin after phagocytosis and washing, and all PMNs detached. After fixation, PMNs were placed back in the wells. Group 3 PMNs were, in addition, centrifuged through FBS after trypsin treatment, fixed, and placed back in the wells. All wells were evaluated for PMNs with surface-bound particles under microscopic observation. Percentage PMNs with bound particles are expressed by mean ± standard deviation.

gulfed particles after the trypsin treatment followed by the FBS centrifugation and found 1.25% for controls and 1.40% for tuftsin-treated cells, indicating that the majority of PMNs with surface-bound particles already had engulfed particles. Since these figures representing this potential source of error were negligibly small, we examined the correlation between flow cytometry and microscopic evaluation of phagocytosis. As shown in Fig. 6, gated analysis clearly enabled us to discriminate PMNs from cell debris and free particles based on the

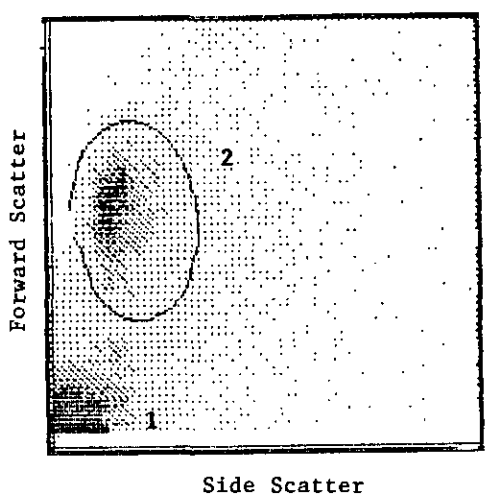

FIG. 6. Particle-size distribution of PMNs after incubation with fluorescent microspheres based on forward and side light scatter. Region 1 corresponds to free particles and debris; region 2 corresponds to phagocytic and nonphagocytic PMNs.

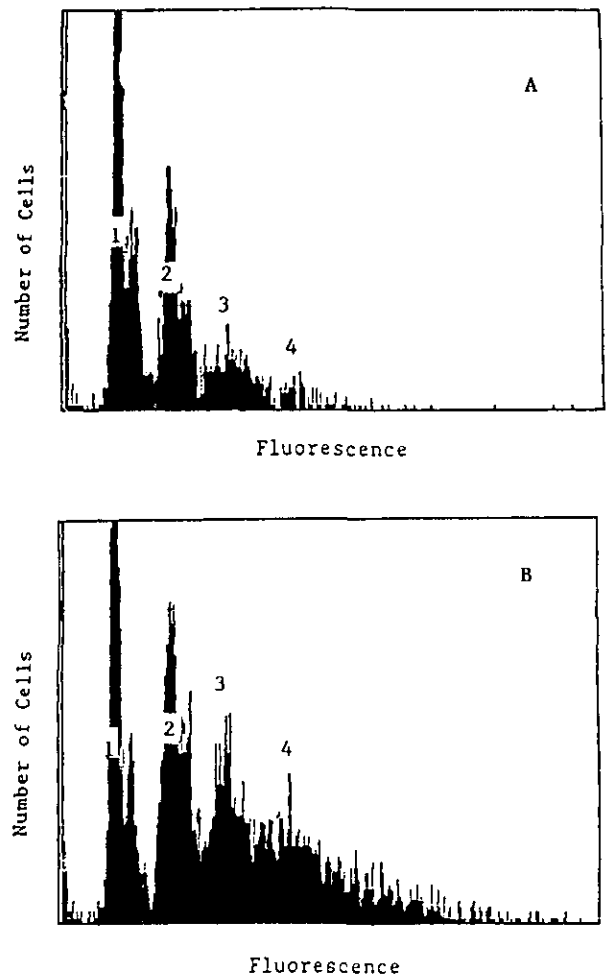

FIG. 7. Fluorescence distribution of PMN-phagocytized particles. Only fluorescence signals corresponding to light scatter from PMN region 2 (Fig. 6) are displayed. Phagocytosis assays were performed as described under Methods without (A) and with (B) tuftsin (5 μg/ml) at 37°C for 15 min with a ratio of particles to PMNs of 50:1. Peaks 1 through 4 represent the number of PMNs which have phagocytized one, two, three, or four fluorescent particles, respectively.

amount of forward light scattering and side scattering. Figures 7A and 7B demonstrate the effects of tuftsin, resulting in greater numbers of high particle-containing PMNs. Figure 8 is the result of plotting all the assays from 55 samples determined by concurrent microscopic and flow cytometry methods under different tuftsin concentrations, incubation times, and ratios of particle to PMN. Our linear regression analysis gave a correlation coefficient of 0.98, demonstrating a very high correlation between these two methods.

Although the effect of tuftsin on percentage of phagocytic PMNs is relatively modest, the effect of tuftsin on engulfed particle number appeared more dramatic as seen in Fig. 4. Therefore, we examined the number of engulfed particles by PMNs in the presence and absence of tuftsin in our time course experiment. In this particular experiment, we examined both percentage of phago-

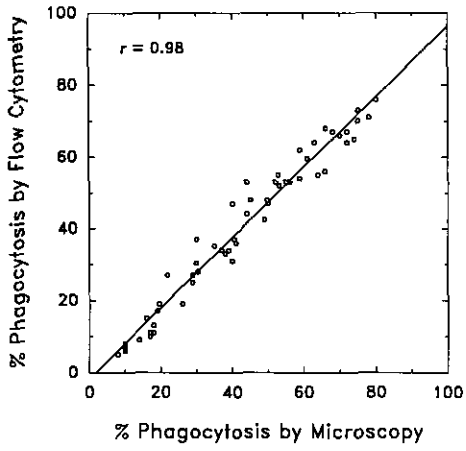

FIG. 8. Correlation between flow cytometry and microscopic evaluations of percentage phagocytic PMNs. Phagocytosis assays were performed under a variety of conditions as described under Methods, and percentage phagocytosis of each sample was determined by both flow cytometry and microscopic evaluations.

cytosis and engulfed particle number in the same experiment. As shown in Fig. 9, 15 min incubation also gave the greatest effect of tuftsin on particles engulfed over the control without tuftsin. At 15 min incubation time, while the percentage of phagocytosis showed a 58.4% increase over the control, the particles engulfed gave a 174% increase over the control, indicating a more dramatic effect of tuftsin in the engulfment of particles. We then examined the correlation of particle load between flow cytometry and microscopic evaluations by plotting the results of 20 samples. As shown in Fig. 10, our linear regression analysis gave a correlation coefficient of 1.00 displaying an excellent correlation between these two methods.

DISCUSSION

The physiological significance of tuftsin was obtained by the demonstrations of various clinical conditions particularly in patients with congenital tuftsin abnormality [29]. As mentioned above, tuftsin improved the survival rate after intraabdominal sepsis in rats [11] and after injection of bacteria into splenectomized mice [20], indicating that tuftsin may be useful for septic patients and to prevent infection after splenectomy. Under these conditions, phagocytosis by PMNs constitutes a critical mechanism of host defense. To evaluate such significance of tuftsin on PMNs, systematic kinetics studies on phagocytosis stimulation by tuftsin are necessary. However, the kinetic studies have not been carried out to date.

Tuftsin is known to bind specifically to PMNs and then becomes quickly internalized [30], indicating that tuftsin acts on PMNs, but not on the particles in this system. To confirm this, we pretreated the PMN mono-

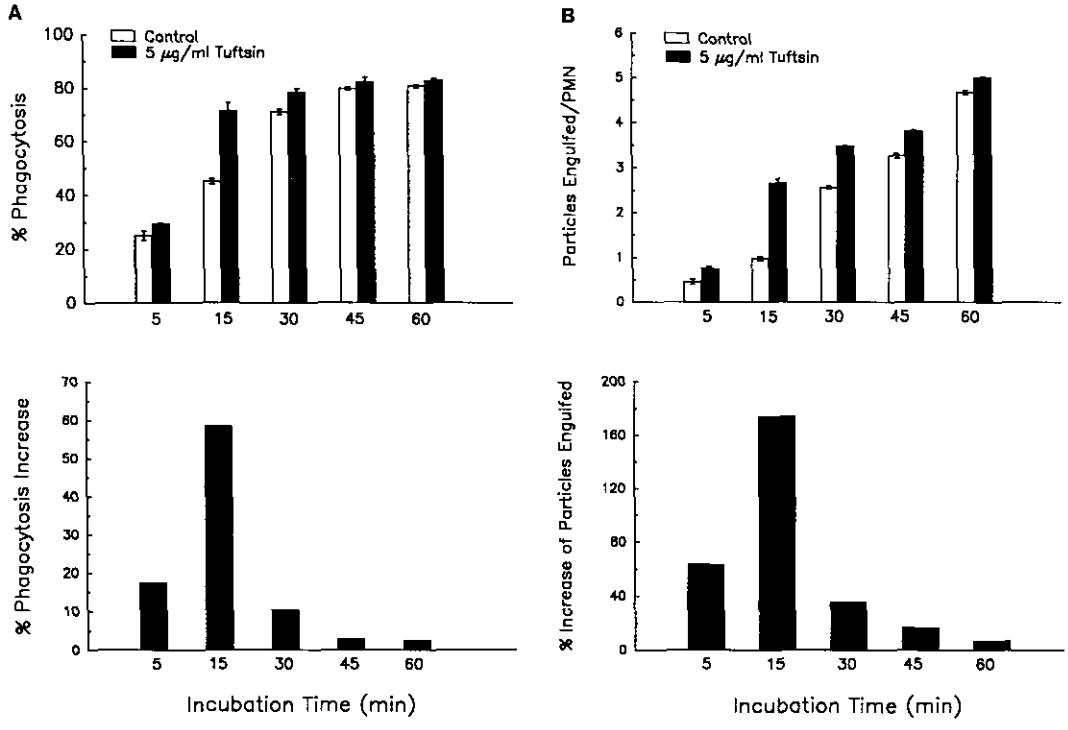

FIG. 9. Comparative effect of tuftsin on percentage phagocytosis and number of particles engulfed. Phagocytosis assays were performed under the optimum conditions for tuftsin as described in the legend for Fig. 4. Both percentage phagocytosis (A) and mean number of particles engulfed (B) were determined by microscopic evaluation and expressed by mean ± standard deviation.

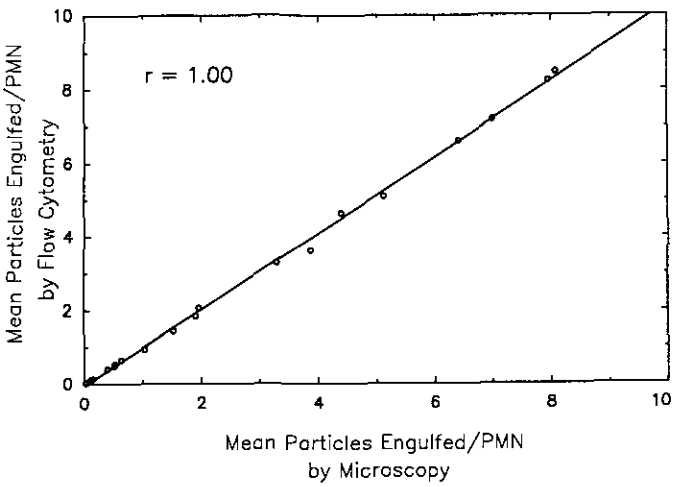

FIG. 10. Correlation between flow cytometry and microscopic evaluations of particles engulfed per PMN. Phagocytosis assays were performed under a variety of conditions as described under Methods, and particles engulfed per PMN were determined by both flow cytometry and microscopic methods.

layer with tuftsin for 5 min at 37°C, washed the PMNs to remove free tuftsin, and added the particles. This experiment gave similar results to those obtained by simultaneously adding both tuftsin and particles to the PMN monolayer.

In separate experiments, we have used different tuftsin concentrations, incubation times, and particle-to-PMN ratios. The optimum conditions we obtained were very reproducible. For instance when we varied the particle-to-PMN ratios 30:1, 40:1, 50:1, 60:1, and 70:1, we still obtained the greatest effect of tuftsin at 50:1.

Since PMN monolayers are subjected to many different treatments in the assay, we were concerned about the recovery rate of PMNs following trypsin treatment. Thus we examined the yield of PMNs. The result showed that PMN recovery rates were over 90% of the 0.5×10^6 PMNs plated on trypsin treatment, and there were no significant differences between control and tuftsin-treated wells.

As shown in Fig. 1, we observed significantly lower phagocytosis stimulation at 10 and 25 μg/ml tuftsin than at 5 μg/ml tuftsin. This was not unexpected as tuftsin has exhibited a similar trend in *in vitro* tumor cytotoxicity assay [31, 32]. One explanation for this phenomenon was offered by Nagaoka and Yamashita [33] who demonstrated the presence of an ectoenzyme, leucine aminopeptidase, on the plasma membrane of PMNs. This enzyme could produce [Des-Thr]tuftsin which is known to be inactive in stimulating phagocytosis and yet is capable of competing with tuftsin for the receptor.

As shown in Fig. 5, trypsin not only detaches PMNs quantitatively from wells, but also efficiently removes surface-bound particles from PMNs. The PMNs with surface-bound particles have been a serious concern to us, as these cells can be a source of error in flow cytometry. Oda and Maeda [34] have used a similar method except that after washing the phagocytized PMNs the cells were directly solubilized by sodium dodecyl sulfate for measurement of the fluorescence intensity. They established linearity of phagocytosis up to 60 min with a 50:1 ratio of particle to PMN. However, when compared with microscopic evaluation, fluorescence measurement gave the higher values. This result might have been caused by cells with surface-bound particles. We have examined the effect of tuftsin on PMNs from different individuals using this phagocytosis assay. There was some variation among individuals tested (30–70% phagocytosis increases). Within individuals, however, similar responses were always obtained. In one case, we tested one individual repeatedly over 1 year with very reproducible results [35]. The differences seen among different individuals may be due to different endogenous tuftsin levels of individuals. We have also noted a variation in phagocytosis among different batches of the fluorescent particles. In any case, the microscopic and flow cytometric methods work equally well for determination of phagocytosis.

Based on the flow cytometric procedure described by Steinkamp *et al.* [26] and Stewart *et al.* [27] for rat alveolar macrophages, we have attempted to separate PMNs with different numbers of engulfed particles by flow cytometry. We were reliably able to separate PMNs containing up to four particles. We have found it difficult to separate any PMNs with more than four particles. With 15 min incubation time, while we have found PMNs with less than four particles in the absence of tuftsin, in the presence of tuftsin (5 μg/ml) we have constantly observed PMNs with markedly higher particles (Fig. 7). Thus it is not feasible to apply flow cytometry to quantitate numbers of PMNs with particles greater than four, although it is possible to determine mean numbers of particles engulfed per PMN.

This study demonstrates that tuftsin not only increases number of phagocytic PMNs, but also augments numbers of particles engulfed. This effect, however, can be observed under relatively restricted conditions. This activity of tuftsin may help a host at the onset of the infection by quickly facilitating phagocytes to attack bacteria. This may also be the case in antimicrobial activity of tuftsin demonstrated in *in vivo* animal experiments [5, 20]. In this respect, Fig. 3 suggests that tuftsin facilitates this phenomenon by lowering K_m or increasing affinity between PMNs and particles, although confirmation of this observation requires further study.

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