ADP-ribosylation of tuftsin suppresses its receptor-binding capacity and phagocytosis-stimulating activity to murine peritoneal macrophages

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Abstract Arginine-specific ADP-ribosyltransferase present in granules of chicken polymorphonuclear leukocytes (so-called heterophils) is released into the extracellular space by stimulus of calcium ionophore A23187 or opsonized zymosan [Terashima et al. (1996) J. Biochem. 120, 1209–1215]. In the present work, we examined extracellular targets of the released transferase and identified tuftsin, a phagocytosis-stimulating tetrapeptide derived from leukokinin, as a preferential substrate of the enzyme in chicken plasma. Specific binding of FITC-tuftsin to murine peritoneal macrophages, observed under a fluorescent microscope, was impaired by ADP-ribosylation of the labelled peptide. Phagocytic assay analyzed by flow cytometry revealed that ADP-ribosylation of tuftsin decreased its phagocytosis-stimulating activity towards the macrophages. Thus, the ADP-ribosylation of tuftsin apparently decreases its biological activity and ADP-ribosylation may possibly be involved in inflammatory processes through alterations in tuftsin activity.

Key words: ADP-ribosyltransferase (EC 2.4.2.31); ADP-ribosylation; Tuftsin; Phagocytosis; Macrophage

1. Introduction

Arginine-specific mono ADP-ribosylation is a post-translational modification, in which the ADP-ribose moiety of NAD is transferred to the guanidine group of the arginine residue of various peptides and proteins. Cholera toxin, a well-known example of arginine-specific ADP-ribosyltransferase, ADP-ribosylates an arginine residue of $G_s\alpha$ to inhibit its GTPase activity [1,2]. Activity of the ADP-ribosyltransferase had been also detected in animal tissues or cells including avian blood cells [3–5] and mammalian skeletal muscle [6,7]. Since cells contain ADP-ribose donor NAD inside, the transferase has been thought to regulate functions of intracellular proteins such as $G_s\alpha$ or actin [8,9] via the modification of arginine residues of these proteins.

Recent studies have revealed that some arginine-specific ADP-ribosyltransferases are expressed on the surface of murine T lymphocytes [10] or myoblasts [11], as GPI-anchored proteins, and can modify cell surface proteins including integrins [11–13]. This suggests an extracellular ADP-ribosylation and involvement of the modification in control of cell adhesion. We identified an arginine-specific ADP-ribosyltransferase in cytoplasmic granules of chicken heterophils [4]. From primary structure of the transferase and expression experiments of its cDNA in eukaryotic cells, the transferase seemed to be a secretable protein [5]. We demonstrated that chicken ADP-ribosyltransferase is released from the granules by the stimulus of calcium ionophore A23187 with calcium or serum-opsonized zymosan [14].

We attempted to search for extracellular substrates for the released transferase in the fresh chicken plasma, and found that the tetrapeptide tuftsin (Thr-Lys-Pro-Arg) is a preferential substrate for the transferase. Tuftsin is a naturally occurring tetrapeptide produced by leukokininase at residues 289–292 of the C_H2 domain in the leukokinin, a fraction of immunoglobulin [15,16]. Tuftsin binds specific cell surface receptors of polymorphonuclear neutrophils and macrophages and stimulates the functions of these cells, including phagocytosis, mobility and chemotaxis [17], thereby exerting anti-microbial and anti-tumor activities to play a crucial role in host defense mechanisms [18,19].

We show here that arginine-specific ADP-ribosyltransferase released from the heterophils modifies tuftsin in situ and that this modification suppressed its phagocytosis-stimulating activity towards murine peritoneal macrophages, presumably by inhibiting the binding of tuftsin to the cell surface receptors.

2. Materials and methods

2.1. Materials

Chickens were obtained from a local slaughterhouse, and BALB/c mice were from Charles River Co. [adenylate-^{32}P]NAD (29.6 TBq/mmol) was purchased from New England Nuclear. Tuftsin, EDTA and PMSF were from Sigma Co. Ltd. Fluorescent latex beads (CAT #09719, 1.5 μm diameter) was from Polyscience, Inc. and FITC was from Wako Chemical Co. Ltd.

2.2. Analysis of ADP-ribosylated peptide in chicken plasma

Fresh chicken plasma (100 μl) collected with 1 mM EDTA and 0.2 mM PMSF was incubated with 50 mM Tris-HCl (pH 9.0), 5 mM dithiothreitol, 50 μM [adenylate-^{32}P]NAD (55 kBq/tube) and 100 ng purified chicken heterophil ADP-ribosyltransferase [4] for 1 h at 25°C in a total volume of 200 μl. After the incubation, the mixture was added by 10% PCA (v/v) and centrifuged at 15000×g for 10 min. The obtained supernatant was applied to a reverse-phase HPLC column (C_{18}, Nakalai AR) and developed with a linear gradient of 0–60% CH_3CN in 0.1% TFA for 60 min at a flow rate of 0.3 ml/min. The effluent was monitored by absorbance at 214 nm, and collected every 0.3 ml. The radioactivity of each fraction was measured. One highly radioactive fraction eluted after NAD was collected and was further purified with re-chromatography, under the same conditions described above. Amino-acid sequence analysis of the radioactive material was done by automated Edaman degradation, using a model-477 gas-phase peptide sequencer and a model 120 PTH amino-acid analyzer (Applied Biosystems).

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Abbreviations: GPI, glycosyl-phosphatidylinositol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; FITC, fluorescein isothiocyanate; PCA, perchloric acid; TFA, trifluoroacetic acid; TKPR, tuftsin

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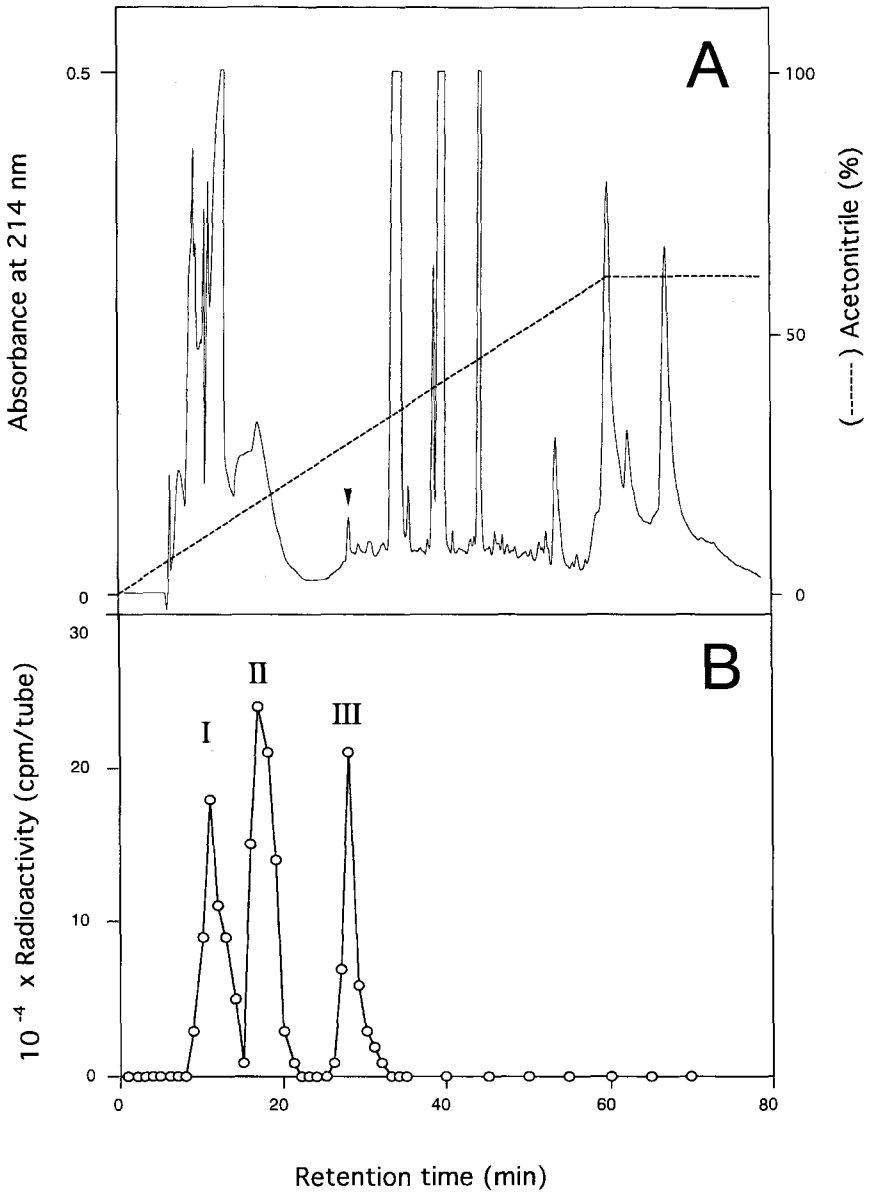

Fig. 1. Separation of [^{32}P]ADP-ribosylated peptide in chicken plasma. Fresh chicken plasma (100 μl) incubated with the heterophil ADP-ribosyltransferase in the presence of [^{32}P]NAD was fractionated with reverse-phase HPLC, as described in Section 2. The elution profile was monitored by the absorbance at 214 nm (A) and the radioactivity (B). An arrowhead in panel A indicates the fraction containing the radiolabelled peptide.

2.3. ADP-ribosylation of tuftsin

Tuftsin (100 nmol) was incubated with 50 mM Tris-HCl (pH 9.0), 5 mM dithiothreitol, 1 mM NAD and 50 ng purified chicken arginine-specific ADP-ribosyltransferase in a total volume of 500 μl for 2 h at 25°C. After the incubation, the reaction mixture was applied to a reverse-phase HPLC column (C_{18}, Nakalai MS) and eluted with a linear gradient of 0–10% CH_3CN in 0.1% TFA for 30 min at a flow rate of 0.3 ml/min. The elution profile was monitored by absorbance at 254 nm. The fraction containing ADP-ribosylated tuftsin, eluted at 27.6 min was collected and lyophilized.

2.4. Preparation of peritoneal macrophages

Macrophages obtained from peritoneal exdates of mice that had been given 4 ml of 3.5% solution of thioglycollate broth intraperitoneally 5 days before killing [20], were washed twice with Hanks' balanced saline solution (HBSS) and plated at the concentration of 5×10^5 cells per 35 mm culture dishes with medium RPMI 1640 containing 10% fetal calf serum (FCS), 100 μg/ml streptomycin and 100 unit/ml penicillin G. After incubation for 2 h at 37°C in a CO_2 incubator, the cells were washed twice with phosphate-buffered saline (PBS), and cultured in medium RPMI 1640 containing 10% FCS.

2.5. Binding of FITC-tuftsin and FITC-tuftsin-ADP-ribose to murine peritoneal macrophages

Fluorescent labelling and subsequent purification of tuftsin were done according to Amoscato et al. [21] but with some modifications. Briefly, FITC (1 mg) was reacted with 500 μg tuftsin in 0.1 M $NaHCO_3/Na_2CO_3$ (pH 8.3) for 4 h at room temperature in a total volume of 300 μl. The reaction mixture was sequentially applied to the cation-exchanger (Radial-PAK^{TM} 8P SCX, Waters) and reverse-phase column (C_{18} Nakalai AR) connected to the HPLC system (Waters, model 510, scanning fluorescence detector 474, multiwavelength M-490). The amino-acid composition and concentration of purified FITC-tuftsin were determined using amino-acid analyzer (Hitachi 835) monitored at 570 nm and 440 nm using ninhydrin as a detecting reagent, and intact tuftsin as a control. The ratio of amino-acids analyses of FITC-tuftsin was 0.16:0.95:1.0:1.0 from the N-terminus, much the same ratio as described previously [21]. The obtained FITC-

tuftsin was ADP-ribosylated and further purified with the reverse-phase HPLC as described in Section 2.3.

The binding of FITC-tuftsin and ADP-ribosylated FITC-tuftsin towards murine peritoneal macrophages was observed under a fluorescent microscope (Nikon, Japan).

2.6. Phagocytosis assay and flow cytometry

Fluorescent latex beads were incubated with 1% bovine serum albumin for 30 min at 37°C followed by ultrasonication. The macrophages were washed twice with HEPES balanced bath solution (10 mM HEPES, pH 7.5, 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$) and preincubated for 30 min at room temperature. After adding the fluorescein-labelled latex beads (2×10^7/dish) and indicated concentrations of tuftsin, the cells were further incubated for 15 min at room temperature, washed five times with PBS and then incubated with PBS containing 0.2% trypsin (Difco) and 0.5 mM EDTA for 15 min at 37°C. Each macrophage preparation was fixed with 0.5% glutaraldehyde, collected with pipetting and scraping and centrifuged at $400\times g$ for 5 min. The precipitated cells were applied to flow cytometric analyses using a FACStar (Becton Dickson Immunocytometry Systems) [22–24]. Analyses were made on 10^4 cells per tube. The percentage of phagocytosis (PP) was defined as the percentage of macrophages that ingested one or more particles. The phagocytic index (PI) was defined as the average number of particles ingested per macrophage and calculated by dividing the total number of ingested beads by the total number of macrophages. Under these conditions, the estimated K_m value of tuftsin for phagocytosis was 0.1 μM, as described by Najjar [15] and maximal phagocytosis-stimulating activity of tuftsin occurred at 10 μM concentration and 15 min incubation, as described by Nishioka et al. [25,26].

3. Results and discussion

To detect extracellular substrates for the arginine-specific ADP-ribosyltransferase released from chicken heterophils, we incubated chicken plasma with purified heterophil transferase in the presence of [^{32}P]NAD, and analyzed PCA-soluble material of the plasma on reversed-phase HPLC (Fig. 1). Radioactive peaks I and II were degradative products of [^{32}P]NAD and [^{32}P]NAD itself, respectively. Peak III seemed to be [^{32}P]ADP-ribosylated peptide. When heterophils (5×10^6 cells) suspended in plasma were incubated with 5 μM calcium ionophore A23187 with 1 mM calcium and 50 μM [^{32}P] NAD [14], much the same results were obtained (data not shown). When a fraction of peak III was subjected to amino-acid sequence analysis, the sequence of the peptide in the fraction was revealed to be Thr-Lys-Pro-Arg, known as tuftsin (Table 1). The same four amino acids were obtained with a low yield of arginine by amino-acid composition analysis of the labelled peptide (data not shown). When synthetic tuftsin was ADP-ribosylated with the heterophil transferase and [^{32}P]NAD, the modified peptide was eluted at the same retention time at that of peak III in Fig. 1, on the reverse-phase HPLC, and analyses of the peptide sequence or amino-acid composition also demonstrated a low yield of arginine (data not shown). These results indicate that peak III is ADP-ribosylated tuftsin, and that the tetrapeptide is preferentially ADP-ribosylated by extracellularly released ADP-ribosyltransferase with NAD in plasma. The low yield of arginine may be due to the disrupted determination caused by the covalent modification of ADP-ribose, as described previously [9]. ADP-ribosylation of synthesized tuftsin with purified heterophil transferase exhibited one mol ADP-ribose incorporation into one mol tuftsin (data not shown). When the heterophils (5×10^7/ml) were stimulated with 5 μM A23187 and 1 mM $CaCl_2$ in the presence of 1 μM tuftsin (estimated concentration in chicken plasma) and 10 μM [adenylate-^{32}P]NAD at 37°C for 5 min, about 50% of added tuftsin was ADP-ribosylated (data not shown).

Table 1
Amino-acid sequencing of the radiolabelled peptide

Cycle	Amino acid	Yield (pmol)
1	Thr	44.5
2	Lys	25.8
3	Pro	29.8
4	Arg	3.1
5	N.D.	–

Sequence analysis of the radiolabelled peptide obtained from the fraction indicated by arrowhead in Fig. 1, was made as described in Section 2.

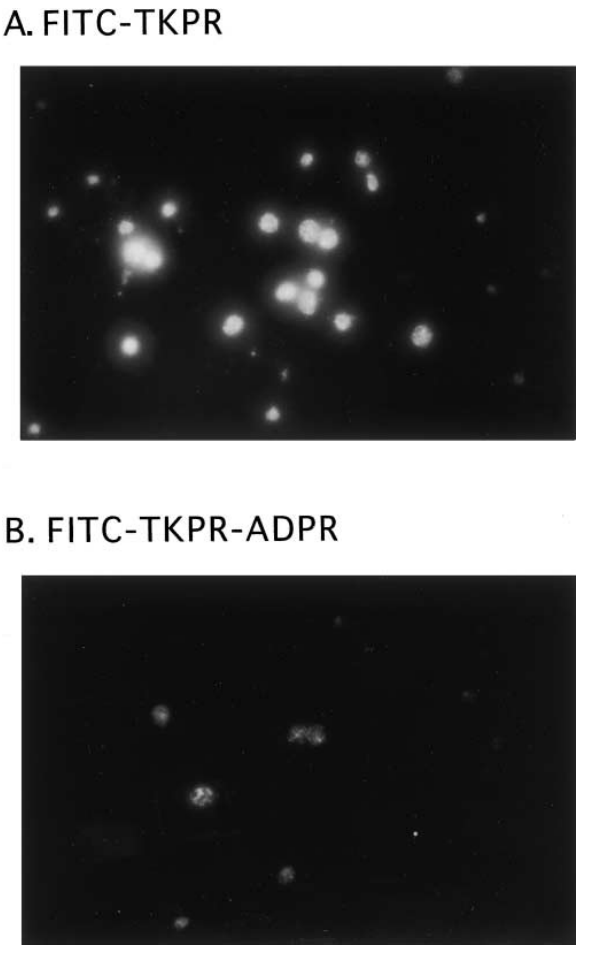

Fig. 2. Binding of FITC-TKPR (A) and FITC-TKPR-ADP-ribose (B) to murine peritoneal macrophages. Murine peritoneal macrophages monolayered on a slide glass were incubated with each FITC-conjugate for 5 min at room temperature. After a wash in PBS, photographs of the cells were taken using a fluorescent microscope. FITC-TKPR and FITC-TKPR-ADP-ribose mean FITC-labelled tuftsin and ADP-ribosylated FITC-labelled tuftsin, respectively.

Tuftsin, a plasma peptide derived from leukokinin, stimulates the function of neutrophils or macrophages, such as phagocytosis, mobility or chemotaxis [17]. To examine how ADP-ribosylation affects the function of tuftsin, we investigated the effect of ADP-ribosylation on the receptor-binding of the peptide, using FITC-labelled tuftsin. FITC-tuftsin bound well to the cell-surface receptor of murine peritoneal macrophages, whereas ADP-ribosylated FITC-tuftsin scarcely

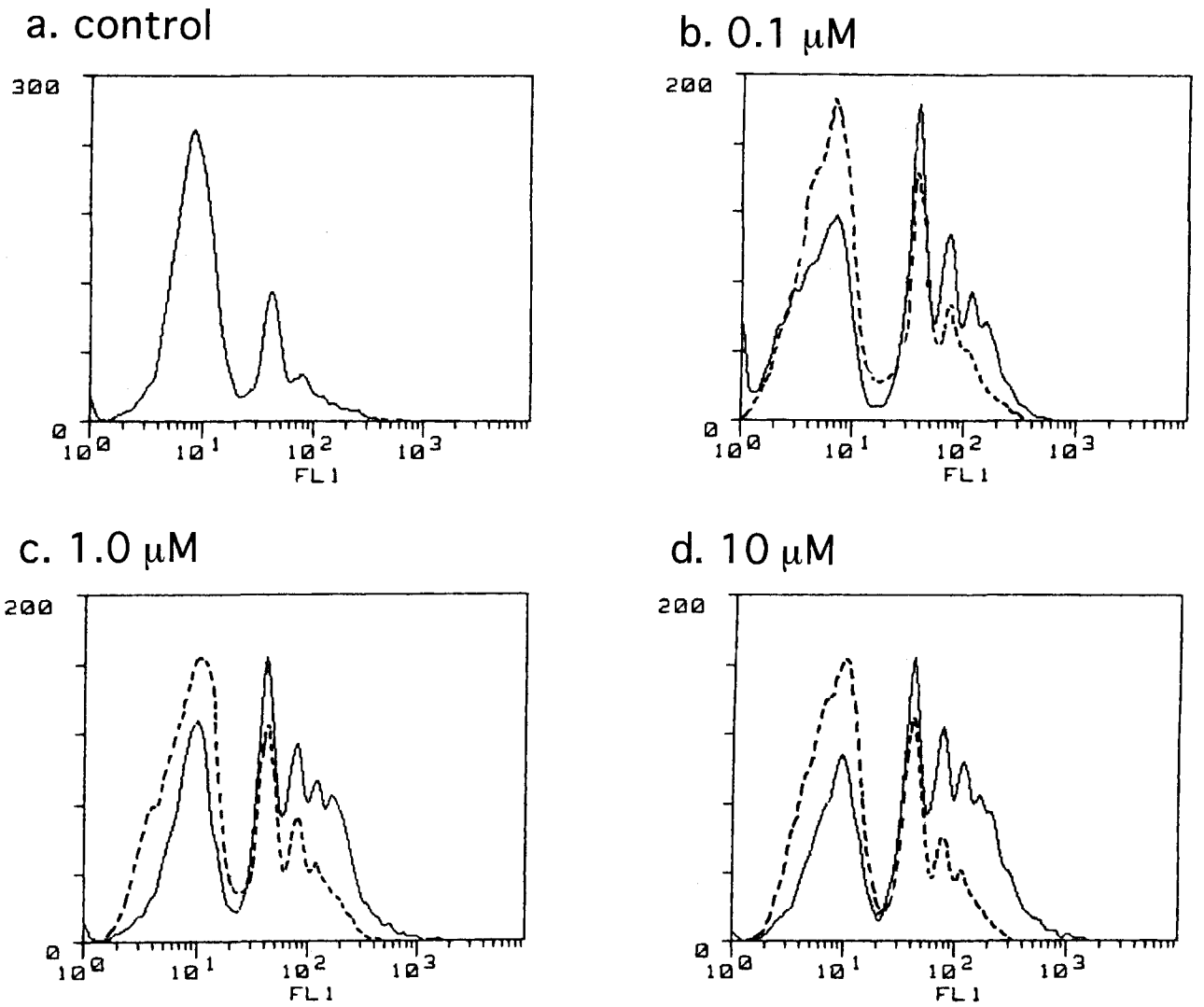

Fig. 3. Flow cytometric profiles for the effect of ADP-ribosylation on tuftsin-induced phagocytosis. Phagocytosis assay and flow cytometric analysis were performed as described in Section 2 with 0 (a), 0.1 (b), 1.0 (c) or 10 (d) μM of tuftsin (solid line), or ADP-ribosylated tuftsin (broken line). The data are representative of four separate experiments.

did so (Fig. 2). Similar results were obtained when binding assays were carried out at 4°C for 20 min (data not shown). Excess amounts of free tuftsin added simultaneously to the FITC-tuftsin eliminated the cell-associated fluorescence, thereby indicating FITC-tuftsin specifically binds to its own cell surface receptor [21,27]. These results are interpreted to mean that ADP-ribosylation of tuftsin on the carboxyl-terminal arginine suppresses its binding to the specific receptor on macrophages.

Next, we investigated the stimulation of phagocytosis by intact or ADP-ribosylated tuftsin. Murine peritoneal macrophages were incubated with 0.1–10 μM tuftsin or ADP-ribosylated tuftsin in the presence of fluorescent latex beads, and the cell-engulfed fluorescent particles were analyzed by the flow cytometry (Fig. 3). Smoothed histograms revealed the distribution of non-ingested, one-bead-ingested, two-beads-ingested, and more-beads-ingested cell populations. Tuftsin-induced phagocytosis increased with increasing concentrations of the peptide while ADP-ribosylated tuftsin caused no remarkable stimulation of phagocytosis, even at 10 μM. These differences were clearly expressed in the % of phagocytosis (PP) and phagocytic index (PI) as shown in Fig. 4. Tuftsin enhanced PP and PI, in a dose-dependent manner at the maximum of 67.4% and 1.82, respectively, although no increase in PP and PI was observed in the case of ADP-ribosylated tuftsin. These results indicate that ADP-ribosylation of tuftsin on the arginine residue impairs its stimulatory effect on phagocytosis of macrophages.

ADP-ribosylation of tuftsin on the arginine residue decreased its stimulatory activity on phagocytosis of macrophages as well as binding of the peptide to the receptor on the cells, findings consistent with the documented importance of the positive charge on the arginine side chain for its biological activity [21,28]. In physiologically active peptides containing tuftsin-related domain, such as neurotensin or substance P, an arginine residue in the domain is probably crucial to exert biological effects [29,30]. These peptides may possibly serve as targets of the transferase. In this work, we examined possible physiological targets of arginine-specific ADP-ribosyltransferase secreted to extracellular space from cytoplasmic granules of chicken heterophils, in case of stimulation of the cells with calcium ionophore or opsonized zy-

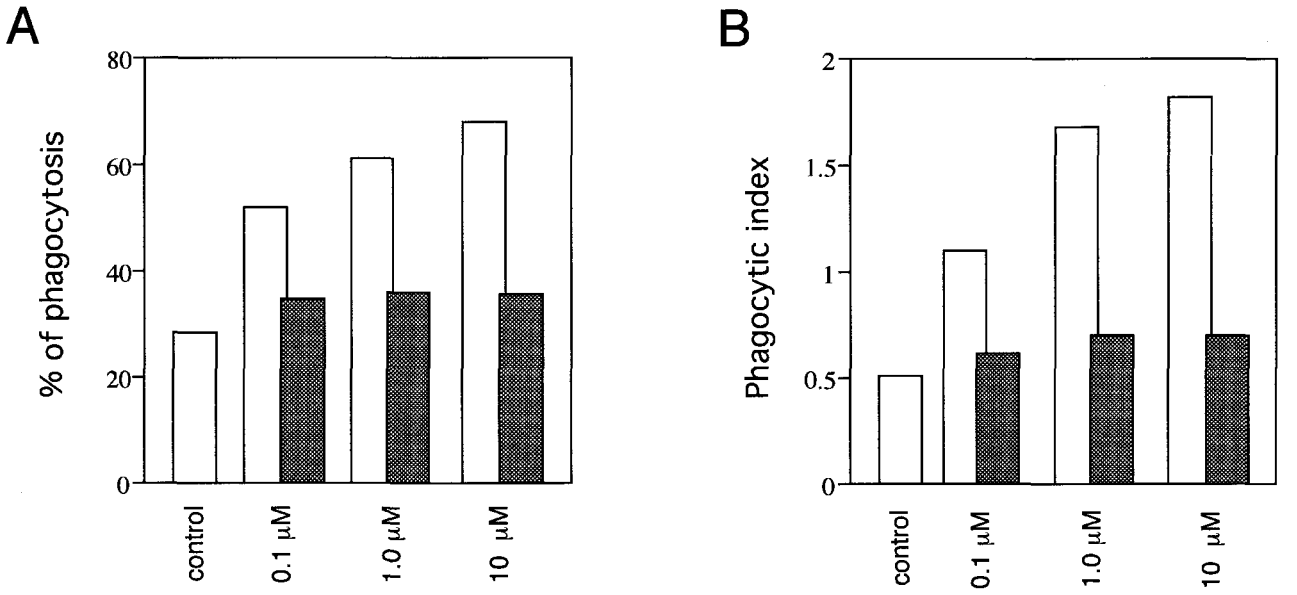

Fig. 4. Effect of ADP-ribosylation of tuftsin on percentage of phagocytosis (PP) and phagocytic index (PI). PP (A) and PI (B) were calculated from the flow cytometric analysis of Fig. 2 as described in Section 2. Open and dotted columns show the effect of tuftsin and ADP-ribosylated tuftsin, respectively.

mosan. We found that tuftsin, a tetrapeptide stimulator of neutrophils or macrophages, was specifically ADP-ribosylated in situ, and that ADP-ribosylation of tuftsin by the transferase inhibited stimulatory effects of the peptide on phagocytosis of murine peritoneal macrophages, probably via suppressing specific receptor-binding activity of the peptide. In light of these findings, together with the presence of ADP-ribosyltransferase in mammalian neutrophils [31,32], in the site of inflammation, ADP-ribosyltransferase released from granulocytes might inhibit phagocytosis-stimulating activity of tuftsin, through modification of the arginine residue with an NAD supply. Thus, the released transferase would modulate the inflammatory process by terminating the signal of tuftsin to stimulate phagocytosis in vivo. NAD may be released during the exocytosis of the leukocyte granules since nucleotide ATP is reported to be secreted into the extracellular space by some stimuli [33], or it may be derived from damaged or dead cells. We are now examinating whether these possible mechanisms can provide the extracellular NAD.

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