Modulation of Phagocytosis by P₂-Purinergic Receptors in Mouse Peritoneal Macrophages

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Abstract The effects of adenosine 5'-triphosphate (ATP) and other nucleotides on phagocytosis of peritoneal macrophages were examined by means of flow cytometry (FCM). ATP and uridine 5'-triphosphate (UTP) enhanced phagocytosis in a dose-dependent manner. Control phagocytosis was partially suppressed in Ca²⁺-free solution. Phagocytosis was suppressed by ATP in Ca²⁺-free solution but not by UTP. Agonist potency order revealed that ATP enhanced phagocytosis through P_{2u} receptors in normal external solution (NES) and inhibited it through P_{2z} receptors in Ca²⁺-free solution. The present study showed that ATP modulates the phagocytosis of macrophages, and that two types of receptors were identified by pharmacological experiments.

Key words: macrophages, P_{2u}, P_{2z}, phagocytosis, flow cytometry.

Biological responses to extracellular ATP have been documented in various tissue and cell systems, including: 1) hypotensive and vasodilator actions in vascular systems, 2) effects at neuroeffector junctions by acting directly as a neurotransmitter, by increasing or decreasing the release of other neurotransmitters, or by modulating their action, 3) Ca²⁺-dependent histamine secretion from mast cells, and 4) inhibition of antibody-dependent cytotoxicity and the activity of human or rat natural killer lymphocytes [1–3]. In macrophages, ATP has been demonstrated to induce permeabilization of the plasma membrane [4–8], an increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) [8–10], inhibition of phagocytosis [8], and superoxide generation [11].

On the basis of the order of potency of several purinergic analogues, the existence of two categories of purinergic receptors, P₁ and P₂, has been proposed [1, 12–14]. Furthermore, P₂-receptors are more sensitive to ATP and ADP than to AMP and adenosine, and can be classified into five subclasses: P_{2x}, P_{2y}, P_{2u}, P_{2t}, and P_{2z}. Their presence has been proposed in endothelial cells and platelets, smooth

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muscle, lymphocytes, mast cells, and macrophages. α,β -Methylene ATP and 2-methylthio ATP (2-MeSATP) are the most potent nucleotides at P_{2x} and P_{2y} subtypes, respectively. The P_{2x} and P_{2z} receptors mediate the proaggregatory effect of ADP in platelets and the plasma membrane permeabilization effect by ATP^{4-} , respectively. The activation of P_{2z} receptor-operated channels induces the formation of nonselective pores permeable to ions and metabolites up to the molecular weight of 1 kD. P_{2u} displays equal potency for ATP and UTP, and is insensitive to 2-MeSATP. Activation of P_{2x} receptor and P_{2y} or P_{2u} receptors evokes an increase in $[Ca^{2+}]_i$ through cation channels and phosphatidylinositol metabolism, respectively. The present study was designed to identify the involvement of these receptor subtypes on phagocytosis of macrophages.

Macrophages play a number of important regulatory and functional roles in the immune system [15]. These cells phagocytize microorganisms, process antigen, kill tumor cells, and serve as accessory cells for lymphocytes. The phagocytic and digestive capacities of macrophages are the most representative functions of these cells. In the present study, a flow cytometric assay for phagocytosis was applied to evaluate phagocytosis. This method was more time-efficient and objective [16–20] than microscopic observations which directly count the number of ingested particles per 100 macrophages under a microscope.

MATERIALS AND METHODS

Preparations of cells. Macrophages were collected from BALB/cA Jcl mice (Nihon Clea, Tokyo, Japan) of either sex (10–25 weeks old) 5 d after intraperitoneal injection of 4 ml of thioglycollate medium, according to the methods reported by Gallily and Feldman [21]. The cells were plated onto dishes (Falcon 3001, Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 5×10^5 cells per dish and cultured at 37°C in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 μ g/ml streptomycin and 100 U/ml penicillin G.

Assay of particle uptake. Flow cytometry has been applied to measure phagocytosis of macrophages [16–20]. The present study was performed by a modified flow cytometric assay which could evaluate phagocytosis of the cultured cells. Fluorescent beads (#09847, 2 μ m diameter, Polysciences, Warrington, PA, USA) coated with 1% bovine serum albumin (BSA) were sonicated for 2 min. Normal external solution (NES) contains (in mM); 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.5. Dulbecco's phosphate buffer solutions (PBS; 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4 , 1.5 KH_2O_4) or $0Ca^{2+} : 0Mg^{2+}$ (140 NaCl, 5 KCl, 10 HEPES, pH 7.5) are used as a Ca^{2+} -free : Mg^{2+} -free solution. There was no difference in the results of phagocytic assay in PBS(–) or in $0Ca^{2+} : 0Mg^{2+}$. Macrophage monolayers were preincubated for 20 min with NES or PBS. Macrophages were incubated with the latex beads at a density of 10^7 beads/dish for 30 min in test solution at room temperature. The monolayers were then washed five times with cold PBS to eliminate uningested beads. Each dish was then incubated in 1.5 ml PBS containing

0.25% trypsin for 3 h in an incubator at 37°C. Glutaraldehyde (40 μ l) was added to each dish to fix cells. Attached cells were scraped loose using a cell scraper (Nunc, Inc. Naperville, IL, USA). The cell-suspension was transferred to plastic tubes (Falcon 2008).

Flow cytometric analyses were performed using a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) to measure the number of particles per cell. Analysis was performed from 10,000 macrophages per tube. Data were acquired in the list mode and processed using Consort 30 software with an attached Hewlett-Packard computer. Before the data were acquired, live gates were set to obtain well-shaped cells and to exclude cellular debris and uningested free beads determined by the pattern of FSC versus FL. The fluorescence distribution was displayed as a single histogram or as a contour plot. The percentage of phagocytic cells (PP) was defined as the percentage of macrophages that ingested one or more particles within the total macrophage population (10,000 cells). The phagocytic index (PI) was defined as the average number of ingested particles per macrophage and was calculated as the total number of ingested beads divided by the number of total macrophages. The total number of ingested beads was obtained by summing (number of one-bead-ingested cells) $\times$ 1, (number of 2-bead-ingested cells) $\times$ 2, (number of 3-bead-ingested cells) $\times$ 3, (number of 4-bead-ingested cells) $\times$ 4, and (number of more than 4-bead-ingested cells) $\times$ (mean number of ingested beads in more than 4-bead-ingested cells population). The numbers of cells and means of ingested beads were analyzed and given as a histogram using a Consort 30 computer system connected to the FACStar flow cytometer.

Materials. ATP, UTP, and FBS were purchased from Boehringer Mannheim, Germany; other nucleotides and penicillin G were from Sigma, St. Louis, MO, USA; HEPES and BAPTA-AM were from Dojin, Kumamoto, Japan; streptomycin was from Meiji Seika, Tokyo, Japan; trypsin was from Difeo Lab., Detroit, MI, USA; thioglycollate medium, Dulbecco's PBS, Hanks and RPMI 1640 were from Nissui. Pharmac. Tokyo, Japan.

RESULTS

Flow cytometric analysis of phagocytosis

Figure 1a1 is a contour plot of phagocytic cells showing forward scattering (FSC) versus fluorescence intensity (FL). The left side population is the non-bead-ingested macrophage and the right population is the bead-ingested macrophage. Figure 1a2 shows that the distribution of cells depends on FL intensity from the upper plot. Figure 1b1 and b2 are the same as Fig. 1a1 and a2 except for the presence of ATP. As shown in Fig. 1c, ATP enhanced the phagocytosis in NES. On the other hand, phagocytosis was inhibited by ATP in Ca^{2+} -free and Mg^{2+} -free solution, as shown in Fig. 2. Because the amount of basic unstimulated phagocytosis is dependant on the incubation time with beads, the basic activity of macro-

Fig. 1. Effect of ATP in normal external solution (NES) on flow cytometric profiles of 10,000 cells. a1: Control contour plot based on forward scattering (FSC), which is a parameter corresponding to cell size, and fluorescence (FL). Each dot shows the cell density of 1 to 4 cells per unit square. Contour lines indicating cell densities increase by steps of 15 cells. The left and right groups are non-ingested and beads-ingested cell populations, respectively. a2: Distribution of FL intensities from the upper plot. In this histogram, non-ingested, 1-bead-ingested, and 2-bead-ingested cell population correspond to the first, second, and third peaks from the left, respectively. b1: The same plot as a1 in the presence of ATP (5×10^{-4} M). b2: Histogram from b1. c: Superimposed histogram from a2 and b2. The histograms in c were smoothed by selecting a menu of 5 times smoothing in the FACStar system. The continuous and broken lines are from a2 and b2, respectively. Note that the broken line is lower than the continuous line on the left and the broken line is above the continuous line on the right side.

phages and other experimental conditions, it is impossible to compare Fig. 1a with Fig. 2a. As shown in Fig. 11, control unstimulated phagocytotic activity in 0Ca^{2+} : 0Mg^{2+} is lower than that in NES within the same trial.

Effects of ATP in NES and PBS

ATP enhanced PP and PI in a dose-dependent manner in NES, as shown in Fig. 3 (closed circles). On the other hand, ATP markedly suppressed the phagocytosis in PBS, as shown by the open circles. Because it has been reported that ATP inhibited phagocytosis at more than 5 mM [8], the effects of ATP at low and high concentrations (10^{-4} and 10^{-2} M) were examined within the same trial. As shown

Fig. 2. Effect of ATP in PBS solution on FCM profiles. a1: Control contour plot. a2: Distribution of FL intensities from the upper plot. b1: The same plot as a1 in the presence of ATP (5×10^{-4} M). b2: Histogram from b1. c: Superimposed smoothed histogram from a2 and b2. The continuous and broken lines are from a2 and b2, respectively. Note that the broken line and the continuous line were reversed, compared with Fig. 1c.

in Fig. 4, ATP (10 mM) inhibited phagocytosis. This result is consistent with the report [8].

Effect of other nucleotides

In NES, purine nucleotides, inosine 5'-triphosphate (ITP), and guanosine 5'-triphosphate (GTP) slightly enhanced phagocytosis, as shown in Fig. 5. Pyrimidine nucleotides, thymidine 5'-triphosphate (dTTP), and cytidine 5'-triphosphate (CTP) slightly enhanced the phagocytosis at a concentration of 10^{-3} M. Adenosine 5'-O-(2-thiodiphosphate) (ADP β S) at concentrations of 2×10^{-4} and 10^{-3} M slightly enhanced phagocytosis.

In PBS, ITP induced a slight suppression only at 10^{-3} M, as shown in Fig. 6. GTP had no effect up to 10^{-3} M. CTP and dTTP also showed less suppressive effects than did ATP. ADP β S had little effect.

Effects of ATP analogues

To identify the subtypes of purinergic receptors, ATP analogues were examined in NES and PBS. Adenosine (2×10^{-4} M, $n=2$; 10^{-3} M, $n=4$), 2-chloro adenosine (2×10^{-4} M, $n=2$), and adenosine 5'-monophosphate (AMP, 2×10^{-4} M, $n=2$) had no obvious effect in NES. UTP enhanced phagocytosis as effectively as

Fig. 3. Effects of ATP concentrations on phagocytosis based on two parameters, percentages of phagocytic cells (PP) in the left graph and phagocytic index (PI) in the right graph. Data are means expressed as percentage of control phagocytosis. Closed and open circles are data in NES and PBS, respectively. Bar in each circle shows standard deviation (SD, $n=8$).

Fig. 4. Comparison among effects of ATP (10^{-4} M), ATP (10^{-2} M), and UTP (10^{-4} M) on phagocytosis in the simultaneous trial in NES. Left graph is PP and right is PI. Data are means $\pm$ SD ($n=4$) expressed as a percentage of control phagocytosis.

Fig. 5. Effects of various kinds of nucleotides in NES. The left graph is PP and the right is PI. Data are means expressed as a percentage of control phagocytosis. Bar on each column shows SD ($n=4$).

Fig. 6. Effects of various kinds of nucleotides in PBS. The left graph is PP and the right is PI. Data are means expressed as a percentage of control phagocytosis. Bar on each column shows SD ($n=4$).

Fig. 7. Effects of ATP analogues in NES. The left graph is PP and the right is PI.

Data are means expressed as a percentage of control phagocytosis. Bar on each column shows SD ($n=4$).

ATP, as shown in Figs. 4, 7, and 8. Adenosine 5'-diphosphate (ADP) and β , γ -methylene ATP (meATP) had no effect, as shown in Fig. 7. Adenosine 5'-O-(3-thiotriphosphate) (ATP γ S) at concentrations from 10^{-6} to 10^{-3} M slightly enhanced phagocytosis.

A selective agonist for P_{2z} purinergic receptors, 3'-O-(4-benzoylbenzoyl)-adenosine 5'-triphosphate (BzATP) was more suppressive than ATP in PBS, as shown in Figs. 9 and 10. Even in NES, BzATP (1 mM) suppressed phagocytosis. ADP and meATP at concentrations from 10^{-5} to 10^{-3} M had no effect. ATP γ S at concentrations from 10^{-6} to 10^{-3} M was less effective in suppression than ATP. UTP did not induce a suppressive effect, as shown in Fig. 8.

Effect of Ca^{2+} and Mg^{2+}

To compare the effect of ATP in NES with that in $0Ca^{2+} : 0Mg^{2+}$ solution and to evaluate the effect of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$, effects of ATP in various concentrations of $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ were examined within the same trial. As shown in Fig. 11, unstimulated phagocytosis (PP, $61.7 \pm 4.5\%$; PI, 1.33 ± 0.15 particles/cell, mean $\pm$ SD, $n=4$) in $0Ca^{2+} : 0Mg^{2+}$ solution was smaller than that (PP, $69.5 \pm 0.7\%$; PI, 1.63 ± 0.03) in NES ($2Ca^{2+} : 1Mg^{2+}$). This suggests that almost one-tenth of the phagocytosis in NES is dependent on $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$. This figure clearly showed that ATP enhanced phagocytosis in NES and inhibited it in $0Ca^{2+} :$

Fig. 8. Effects of different concentrations of UTP in NES (closed circles) and PBS (open circles). The left graph is PP and the right is PI. Bar on each circle shows SD ($n=4$).

Fig. 9. Effects of ATP analogues in PBS. The left graph is PP and the right is PI. Data are means expressed as a percentage of control phagocytosis. Bar on each column shows SD ($n=4$).

Fig. 10. Effects of BzATP concentrations in PSS (closed circles) and PBS (open circles) on PP and PI. Bar on each circle shows SD ($n=4$).

Fig. 11. Effects of Ca^{2+} and Mg^{2+} on unstimulated and ATP-stimulated phagocytosis in the same experimental trials. Left and right graphs show absolute values of PP and PI. Bar in each column shows SD ($n=4$). Concentrations of Ca^{2+} and Mg^{2+} are designated by the numbers in mM.

Fig. 12. Effect of suramin (SRN, 10^{-4} M) with or without ATP (10^{-4} M). Data are means $\pm$ standard error of means ($n=4$) expressed as a percentage of control phagocytosis. The left and right graphs show absolute values of PP and PI. In each graph, cross-hatched columns are in NES and hatched column are in PBS. The third column in each pair indicates stimulation by ATP in the presence of suramin.

0Mg²⁺ solution. Furthermore, ATP-stimulated phagocytosis was increased by increasing [Mg²⁺]_o from 0 to 1 with 1 mM [Ca²⁺]_o, or from 0 to 2 with 2 mM [Ca²⁺]_o. ATP-stimulated phagocytosis was also increased by increasing [Ca²⁺]_o from 1 to 2 mM in the presence of 0 or 1 mM [Mg²⁺]_o. The enhancement of phagocytosis by increasing [Mg²⁺]_o at a constant [Ca²⁺]_o might be caused by reducing the concentration of ATP⁴⁻ which is specific for P_{2z} receptors [1, 9].

Effect of suramin, a known ATP antagonist

Suramin (10^{-4} M) by itself suppressed phagocytosis (PP, 74%; PI, 61%) compared with control phagocytosis in NES and also inhibited phagocytosis (PP, 87%; PI, 77%) in PBS (Fig. 12). Though phagocytosis was enhanced (PP, 111%; PI, 116%) by ATP in NES, phagocytosis was suppressed (PP, 91%; PI, 82%) in the solution containing ATP and suramin. Phagocytosis was suppressed (PP, 91%; PI, 82%) by ATP in PBS. The suppressive effect was greater (PP, 86%; PI, 74%) in ATP and suramin containing PBS.

DISCUSSION

Flow cytometric assay for phagocytosis of fluorescent latex particles was originally applied to rat pulmonary macrophages [16]. A linear relationship between the number of fluorescent latex particles and the fluorescent intensity was observed [17]. This method defined the role of calcium and characterized the immune opsonin-independent phagocytosis by pulmonary macrophages [18]. Furthermore, phagocytosis of monocytes mediated via Fc-receptors and complement-receptors was evaluated by this method by using IgG-coated or complement-coated latex particles [19]. Because this improved assay system is suitable to detect phagocytosis of peritoneal macrophages, the effect of ATP on phagocytosis was examined.

The present flow cytometric assay revealed that ATP enhanced the phagocytosis of mouse peritoneal macrophages in NES and inhibited it in PBS at concentrations ranging from 10^{-6} and 10^{-3} M. FCM can easily analyze 10,000 macrophages within 1 min and can obtain more objective data than direct visual observation in which the number of particles phagocytized per 100 cells are counted. Sung and co-workers [8] reported that ATP inhibited phagocytosis at concentrations higher than 5 mM by using the direct visual counting method; they did not detect the augmentation of phagocytosis by ATP. Therefore, the present result demonstrates that the present method is more sensitive to changes in phagocytosis than the visual counting method.

Because adenosine and AMP had no effect on phagocytosis, the receptors responsible for the present study are P_2 receptors. According to the rank order of potencies of different ATP analogues and their specificity, the P_2 receptors are subdivided into five classes: P_{2x} , P_{2y} , P_{2u} , P_{2z} , and P_{2t} [1, 12–14]. P_{2x} receptors are characterized by the following rank order of potency; $\text{meATP} > \text{ATP} = \text{ADP} > \text{AMP}$. The order for P_{2y} receptors is $\text{ADP}\beta\text{S} = 2\text{-MeSATP} > \text{ATP} = \text{ADP} > \text{meATP}$. That for P_{2u} receptors is $\text{UTP} = \text{ATP} > \text{ATP}\gamma\text{S} > \text{ADP} > \text{meATP}$. P_{2z} receptors are specific for ATP^{4-} and the agonist potency order is $\text{BzATP} > \text{ATP} = \text{ATP}\gamma\text{S} \gg \text{ADP} = \text{AMP}$. P_{2t} receptors are found only on platelets which respond to ADP only (ATP being an antagonist). Because meATP and ADP had no effect in NES and PBS, P_{2x} and P_{2t} receptors are not involved. Furthermore, P_{2y} receptors are not involved because ADP β S is less effective than ATP. Phagocytosis was enhanced by ATP as well as UTP and slightly by ATP γ S. This shows that enhancement in NES is mediated by P_{2u} . Conversely, P_{2z} receptors are involved in phagocytic suppression, because the agonist potency order is $\text{BzATP} \gg \text{ATP} > \text{ATP}\gamma\text{S}$ and a marked effect was observed in the solution of PBS where ATP was largely ionized to ATP^{4-} .

Phagocytosis of pulmonary macrophages was enhanced by Ca^{2+} ionophore A 23187 at concentrations lower than 10^{-6} M but was inhibited at higher concentrations [22]. In other reports, A23187 inhibited phagocytosis at 2×10^{-5} M [18], but

it was also used as a stimulator for phagocytosis at 10^{-7} M [23]. UTP is bound to P_{2u} receptors and then leads to a long lasting increase in $[Ca^{2+}]_i$ in NES by activating phospholipase C [1, 2, 12, 14]. This sustained increase in $[Ca^{2+}]_i$ may lead to the enhancement of phagocytosis in NES. Because ATP (10^{-6} – 10^{-3} M) enhanced phagocytosis, the P_{2u} -mediated effect might be larger than the P_{2z} -mediated effect in the concentration range. At higher concentrations than 5 mM, P_{2z} might be more effective than P_{2u} . P_{2z} -stimulation induces the formation of non-selective pores in the plasma membrane which induces Ca^{2+} influx from extracellular solution, resulting a large increase in $[Ca^{2+}]_i$. A large $[Ca^{2+}]_i$ might inhibit phagocytosis like high concentrations of A23187, as reported by Parod and Brain [18, 22].

In PBS, UTP had no effect. Though a transient increase in $[Ca^{2+}]_i$ might be induced by P_{2u} stimulation in PBS [9], it might be impossible to induce long lasting Ca^{2+} mobilization in the cells. This may be one reason why UTP is unable to enhance phagocytosis in PBS. Because an increase in $[Ca^{2+}]_i$ induced by P_{2z} stimulation in PBS cannot be induced, increase in $[Ca^{2+}]_i$ may not explain the suppression of phagocytosis in PBS. Because ATP^{4-} induces the formation of non-selective channel pores which admit molecules smaller than 831 daltons into the cytoplasmic matrix [7], pore formation in PBS might induce Ca^{2+} efflux from cytoplasm and/or disturb the phagocytic process in the cell membranes, leading to the suppression of phagocytosis.

Though suramin is known as a P_{2x} and P_{2y} antagonist [24], suramin did not antagonize the ATP effect. Suramin was originally prepared for treatment of trypanosomiasis and has other actions [25]. Suramin induces a Ca^{2+} release from junctional but not longitudinal sarcoplasmic vesicles in skeletal muscle and an inhibition of Ca^{2+} pump [26]. Reduction of Ca^{2+} concentration in some intracellular storage site, which is essential for phagocytic processes, may induce suppression of phagocytosis. In fact, reduction of $[Ca^{2+}]_i$ by the intracellular Ca^{2+} chelating agent, BAPTA-AM reduces phagocytic activity [27].

Macrophages exhibit several activities such as phagocytosis and secretion of inflammatory mediators [15]. Expression of these activities is regulated in part by $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ is induced either by release of Ca^{2+} from intracellular stores via the activation of P_{2u} receptors or by influx from extracellular medium via the activation of P_{2z} receptors [9, 10]. The present study clarified that P_{2u} receptors enhanced phagocytosis in NES and that P_{2z} receptors inhibited phagocytosis in Ca^{2+} - and Mg^{2+} -deficient solution. These observations demonstrate that macrophage function may be regulated by the local release of ATP from neurons, endothelial cells, platelets and damaged cells.

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