The Purinergic P_{2Z} Receptor of Human Macrophage Cells

Characterization and Possible Physiological Role

Simonetta Falzoni,* Maddalena Munerati,* Davide Ferrari,* Susanna Spisani,‡ Sabrina Moretti,§ and Francesco Di Virgilio*

**Institute of General Pathology, ‡Department of Biochemistry and Molecular Biology, and §Institute of Hematology, University of Ferrara, Italy*

Abstract

We have investigated responses of human monocyte/macrophage cells to extracellular ATP (ATP_e). Freshly isolated peripheral blood monocytes showed responses linked to P_{2Y} but not P_{2Z} purinergic receptors; however, during in vitro macrophage differentiation, these cells also exhibited responses suggestive of the presence of the membrane-permeabilizing P_{2Z} receptor. In fact, in human macrophages a brief (15-min) exposure to ATP_e, but not other nucleotides, caused (1) a rapid and long-lasting plasma membrane depolarization; (2) a large increase in intracellular Ca^{2+} concentration followed by efflux of the Ca^{2+} indicator; (3) uptake of low molecular weight hydrophilic molecules such as Lucifer yellow and ethidium bromide; and (4) cell rounding, swelling, and eventual release of the cytoplasmic enzyme lactate dehydrogenase. rIFN-γ enhanced both membrane-permeabilizing and cytotoxic ATP_e effects. Membrane permeabilization and cytotoxicity were fully blocked by pretreatment of the cells with oxidized ATP, a compound recently shown to block P_{2Z} receptors covalently in macrophages. Blocking of the P_{2Z} receptor by oxidized ATP also inhibited multinucleated giant cell generation stimulated by concanavalin A or rIFN-γ without decreasing monocyte migration or membrane adhesion molecule expression. These data suggest that human macrophages express rIFN-γ–modulated purinergic P_{2Z} receptors in vitro and hint at a role for these plasma membrane molecules in the generation of macrophage polykarions. (*J. Clin. Invest.* 1995. 95:1207–1216.) Key words: ATP • inflammation • calcium • giant cell

Introduction

There is increasing evidence that extracellular ATP (ATP_e)[1] exerts important biological actions in the immune system by interacting with plasma membrane P_2 purinergic receptors (1–4). This nucleotide has been shown to modulate responses of B and T lymphocytes (4, 5), thymocytes (3), mast cells (6), neutrophils (7), and macrophages (8). However, very little information is currently available on the expression and regulation of P_2 receptors in human monocyte/macrophage cells since, except for a few recent reports (9, 10), most studies have been done in rat or murine cells.

The need for a better characterization of P_2 purinergic receptors in human immune effector cells is stressed by the recent appreciation that the various and often contradictory responses triggered by ATP_e, i.e., stimulation of cell proliferation (11) and differentiation (12), activation of the respiratory burst (7), inhibition of phagocytosis (8), and cytotoxicity (3, 4), are due to an interaction with different P_2 receptor subtypes expressed either by different immune cells or at different stages of maturation. One of the most intriguing effects of ATP_e is reversible plasma membrane permeabilization to molecules of < 900 D (13, 14), a response that is invariably associated with susceptibility to ATP_e-mediated cytotoxicity (15). Although the physiological meaning, if any, of ATP_e-mediated cytotoxicity is still obscure, it has attracted a wealth of recent interest, given the wide spectrum of normal and transformed susceptible cell types (16, 17) and the realization that this effect is not the result of a nonspecific plasma membrane perturbation, but rather a specific receptor-mediated event. In fact, (*a*) only cells expressing P_{2Z} or P_{2X} receptors are sensitive (15); (*b*) ATP_e-resistant clones can be selected from susceptible cell lines (18); (*c*) chronic treatment of mouse splenocytes with IL-2 causes resistance (3); and (*d*) pretreatment with a selective receptor blocker fully neutralizes the cytotoxic effect of ATP_e at several millimolar concentrations (19).

In murine macrophages, it has been clearly shown that ATP_e-dependent cytotoxicity is mediated by the P_{2Z} receptor, an atypical purinergic receptor that either constitutes or is coupled to the transmembrane pore responsible for the ATP_e-dependent increases in plasma membrane permeability (18). Stimulation of these cells with ATP_e causes a dramatic uptake of extracellular hydrophilic molecules such as Lucifer yellow and ethidium bromide, rounding, swelling, and lysis (18, 20). Cell death likely occurs by colloido–osmotic lysis, since it is not accompanied by nuclear condensation and degradation of genomic DNA into nucleosome-sized fragments, typical features of apoptosis (21).

Well-characterized expression of P_{2Z} receptors by murine macrophages has led to the proposal that these surface molecules might be involved in the inflammatory reaction (10, 22, 23). Although this hypothesis is appealing, the single response most clearly associated with ATP_e stimulation in murine macrophages is cell death, and it is difficult to believe that the main physiological role of P_2 receptors is to function as "suicide

Address correspondence to Dr. Francesco Di Virgilio, Institute of General Pathology, Via Borsari, 46, I-44100 Ferrara, Italy.

Received for publication 29 June 1994 and in revised form 17 October 1994.

1. *Abbreviations used in this paper:* ATP_e, extracellular ATP; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IFN-macrophage, macrophage stimulated by rIFN-γ; MGC, multinucleated giant cell; oATP, oxidized ATP; UTP_e, extracellular uridine triphosphate.

J. Clin. Invest.

0021-9738/95/03/1207/10 $2.00
Volume 95, March 1995, 1207–1216

receptors." An important clue to understanding the possible physiological meaning of purinergic P_2 receptors in the inflammatory response would be given by the demonstration that their expression can be modulated by inflammatory cytokines and that they are involved in specific macrophage responses.

In the present study, we report on the characterization of the purinergic P_2 receptor subtypes expressed by human monocytes and monocyte-derived macrophages cultured in the presence and absence of rIFN-γ. Our results show that (1) human monocytes express receptors only of the $P_{2Y/U}$ subtype; (2) in vitro culture induces expression of the P_{2Z} receptor that is upregulated by rIFN-γ; (3) blockade of the P_{2Z} receptor by the selective covalent inhibitor oxidized ATP (oATP) strongly inhibits formation of concanavalin A (Con A)-induced multinucleated giant cells (MGCs).

These observations suggest that expression of purinergic P_{2Z} receptors is greatly enhanced during in vitro monocyte/macrophage differentiation and hint at a role for this molecule in the process of cell fusion that leads to MGC formation during granulomatous inflammations.

Methods

Cells and solutions. Human monocytes were isolated from buffy coats by a one-step gradient (Percoll, Pharmacia Biotech SpA, Cologno Monzese, Italy) or by adherence on plastic petri dishes as described by Colotta et al. (24). After isolation, cells were either directly used for experiments (monocytes) or kept in culture for 5 d (macrophages) in RPMI 1640 medium containing 2 mM glutamine, 5% human serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the absence or presence of human rIFN-γ (1000 U/ml; Boehringer, Mannheim, Germany). Unless otherwise indicated, experiments were performed in saline solution containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose, 5 mM $NaHCO_3$, 1 mM $CaCl_2$ and 20 mM Hepes (pH 7.4). We will refer to this saline medium as standard saline.

Fluorimetric measurement of plasma membrane potential. Changes in plasma membrane potential were measured with the fluorescent dye bis[1,3-diethylthiobarbiturate) trimethineoxonal (bisoxonol; Molecular Probes, Inc., Eugene, OR) at the wavelength pair 540/580 nm, as previously described (25). Experiments were performed in a spectrofluorometer (model LS50, Perkin-Elmer Ltd., Beaconsfield, UK) equipped with a thermostat-controlled (37°C) cuvette holder and magnetic stirrer.

Ca^{2+} measurements. Changes in Ca^{2+} were measured with the fluorescent indicator fura-2/AM as described previously (26). Briefly, cells were loaded with 4 μM fura-2/AM and incubated in a thermostat-controlled (37°C) and magnetically stirred fluorometer cuvette (LS50, Perkin-Elmer Ltd.). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined with the 340/380 excitation ratio at an emission wavelength of 500 nm.

Changes in plasma membrane permeability. ATP_e-dependent increases in plasma membrane permeability were measured with the extracellular fluorescent tracers ethidium bromide and Lucifer yellow (Molecular Probes, Inc.) (15). For ethidium bromide uptake, cells were incubated in a fluorometer cuvette (37°C) at a concentration of 10^6 cells per ml in the presence of 20 μM ethidium bromide and challenged with various ATP_e concentrations. Fluorescence changes were monitored at the wavelength pair 360/580 nm. For Lucifer yellow uptake, cell monolayers or suspensions were incubated for 15 min at 37°C in standard saline containing 250 μM sulfinpyrazone and 1 mg/ml Lucifer yellow. After several washings to remove the extracellular dye, cells were analyzed with an inverted fluorescence microscope (Olympus IMT-2, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a 40× objective.

Measurement of enzymatic activity. Lactate dehydrogenase activity was measured according to standard methods (27). Hydrolysis of ATP_e and oATP by plasma membrane ecto-ATPases was measured as described by Ames (28).

MGC formation. Monocytes were placed in flat-bottomed 96-well microtiter plates at a concentration of 10^5 cells per 0.2-ml well and incubated for 24–72 h in the presence of the various stimulants (Con A, 10 μg/ml; rIFN-γ, 1000 U/ml; or Con A plus rIFN-γ) (29). Plates were dried, stained in the microtiter plate with May-Grunwald-Giemsa, and viewed at × 400. MGC (> 2 nuclei per cell) formation was quantitated by counting the number of nuclei in at least 100 cells (mono- or polynucleated) in three different microscopic fields. Fusion index% was calculated according to the following equation: fusion index% = (number of nuclei within MGC)/(total number of nuclei counted) ×100.

Random locomotion. Random locomotion was evaluated with a 48-well microchemotaxis chamber (BioProbe, Milano, Italy) equipped with a 8-μm pore size (Millipore S_PA, Milano, Italy) filter by estimating the distance, in micrometers, migrated by the leading front of the cell suspension (2×10^5 cells per ml) during a 90-min incubation, as described by Zigmond and Hirsch (30).

Chemotaxis. Chemotaxis was measured in the chamber previously described containing the chemoattractant in the bottom compartment. The chemotactic factors used were as follows: casein "Hammarsten" (Merck, Darmstadt, Germany) at a concentration of 2 mg/ml in Krebs-Ringer phosphate containing 0.1% (wt/vol) glucose and 0.5 mg/ml BSA, pH 7.4; fMet-Leu-Phe (FMLP) at a concentration of 10^{-8} M; and pooled fresh serum (15%) activated with LPS (25 μg/ml; *Escherichia coli*). Cells were preincubated with oATP for 2 h, washed, and tested for chemotaxis toward all the stimulants.

Measurement of LFA-1 and ICAM-1 expression. Integrin expression was evaluated with a cytometer equipped with a single argon ion laser (FACS an, Becton Dickinson, San Jose, CA) as previously described (31). Surface markers were analyzed by direct immunofluorescence using mAbs directed against CD11a (anti-human LFA-1a-FITC, clone G-25.2; Becton Dickinson), CD18 (anti-human LFA-1-FITC, clone BL-5; Immunotech S. A., Marseille, France), and CD54 (anti-human ICAM-1-PE, clone LB-2; Becton Dickinson). Controls were performed by using nonreactive isotypic mAbs (also purchased from Becton Dickinson and Immunotech). Individual cells samples were incubated for 30 min with each mAb and then washed twice in PBS. 10,000 events were acquired for each condition and analyzed with computer software (LYSYS II; Becton Dickinson). The percentage of positive cells was obtained by comparison with negative controls. The monocyte population analyzed was > 96% pure, as judged by lack of expression of the specific lymphocyte markers CD3 (anti-human CD3, clone SK 7-FITC; Becton Dickinson) and CD19 (anti-human CD19, clone 4G7-PE; Becton Dickinson).

Results

An early response observed upon ATP_e stimulation of several cell types is a change in plasma membrane potential, either hyperpolarization or depolarization, depending on the P_2 receptor subtype expressed (23). Fig. 1 shows that in freshly isolated monocytes, ATP_e triggered a fast and transient hyperpolarization followed by a slight depolarization (trace *a*). A subsequent addition of KCl caused a large depolarization, thus showing that failure to detect a depolarizing response to ATP_e was not due to lack of a normally polarized plasma membrane. On the contrary, macrophages (Fig 1, trace *b*) and macrophages stimulated by rIFN-γ (IFN-macrophages) (trace *c*) were readily and near completely depolarized by ATP_e. Depolarization was specifically triggered by ATP_e, as other nucleotides were ineffective (data not shown).

Most cells possess P_{2Y}/P_{2U} receptors, which can be functionally discriminated from other P_2 receptors as their activation induces a biphasic $[Ca^{2+}]_i$ increase: a fast and transient early spike followed by a second, lower, slowly decaying rise (23).

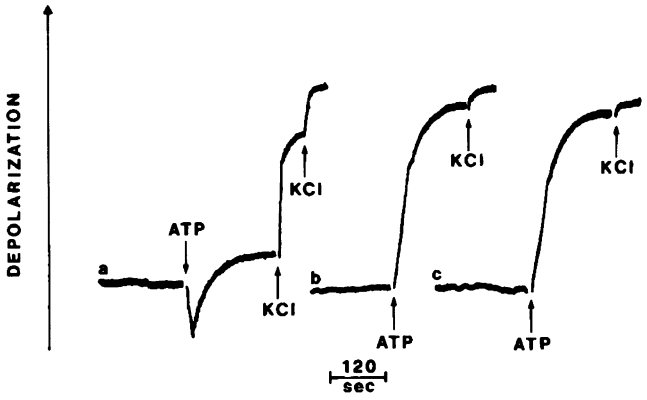

Figure 1. ATP_e depolarizes macrophage but not monocyte plasma membrane. Monocytes (trace *a*), macrophages (trace *b*), and IFN-macrophages (trace *c*) (2×10^5 per ml) were incubated at 37°C in standard saline supplemented with 100 nM bisoxonol and challenged with ATP_e (5 mM). Each KCl addition was 30 mM.

The first phase is due mainly to Ca^{2+} release from intracellular stores; the second reflects Ca^{2+} influx across the plasma membrane, probably via the newly identified but still poorly characterized Ca^{2+} release-activated plasma membrane Ca^{2+} channels (32). As shown in Fig. 2 *A* (*dotted line*), ATP_e triggered in monocytes a fast $[Ca^{2+}]_i$ increase, followed by the slowly declining plateau typical of P_{2Y}/P_{2U} receptor activation. A second ATP_e pulse caused a slower and prolonged $[Ca^{2+}]_i$ increase. Macrophages, (Fig. 2 *A, broken line*) responded to the first ATP_e pulse similarly to monocytes, but underwent a much larger $[Ca^{2+}]_i$ rise in response to the second addition of the nucleotide. IFN-macrophages (Fig. 2 *A, continuous line*) gave a strikingly different response: the first pulse caused a sustained $[Ca^{2+}]_i$ increase to a level about twofold higher than that observed in monocytes and macrophages, whereas the second pulse increased $[Ca^{2+}]_i$ to nearly saturation of fura-2 fluorescence. These results suggest that ATP_e-dependent Ca^{2+}-mobilizing mechanisms are more efficient in macrophages and IFN-macrophages than in monocytes. However, since the experiments reported in Fig. 2 *A* were done in the presence of extracellular Ca^{2+}, we cannot exclude that the large and sustained $[Ca^{2+}]_i$ increase was apparent and, at least in part, due to extracellular leakage of fura-2. This was in fact the case, because addition of EGTA (Fig. 2 *A*) to chelate extracellular Ca^{2+} caused an abrupt decrease of fura-2 fluorescence, a clear indication of dye release into the extracellular milieu.

As previously shown by Greenberg et al. (33), the most obvious interpretation of these data is that ATP_e triggered in macrophages and IFN-macrophages two qualitatively different responses: (1) Ca^{2+} release from intracellular stores and influx across specific plasma membrane channels, likely via activation of a P_{2U}/P_{2Y}-type receptor, and (2) a nonspecific increase in plasma membrane permeability, which on the one hand enhanced Ca^{2+} influx and on the other caused fura-2 release, probably via activation of a P_{2Z} receptor. Consistent with this interpretation is full activation of the permeabilization response only after the second ATP_e addition; in fact, plasma membrane permeability changes are known to require ATP_e concentrations much higher than those needed to trigger Ca^{2+} release from intracellular stores (14, 33). Fig. 2 *B*, shows that in the absence of extracellular Ca^{2+}, ATP_e caused in the three different cell types only the fast transient due to Ca^{2+} release from intracellular stores, thus confirming the presence of P_{2U}/P_{2Y} receptors.

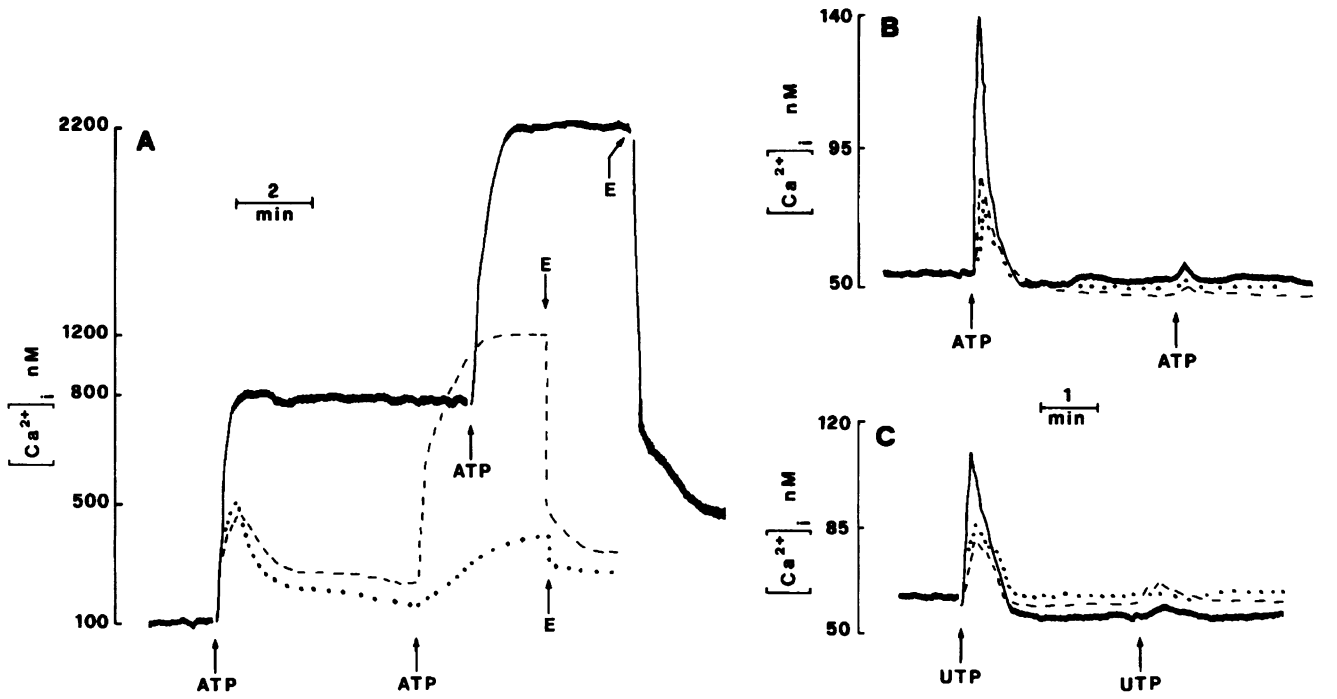

Figure 2. rIFN-γ enhances $[Ca^{2+}]_i$ changes triggered by ATP_e. Monocytes (*dotted line*), macrophages (*broken line*), or IFN-macrophages (*continuous line*) were incubated in standard saline (*A*) or Ca^{2+}-free, 500 μM EGTA-supplemented saline (*B* and *C*) at a concentration of 5×10^5 cells per ml. ATP (*A* and *B*) and UTP (*C*) were added at a concentration of 1 mM (first addition) or 2 mM (second addition). EGTA (*E*, panel *A*) was added at a concentration of 2 mM.

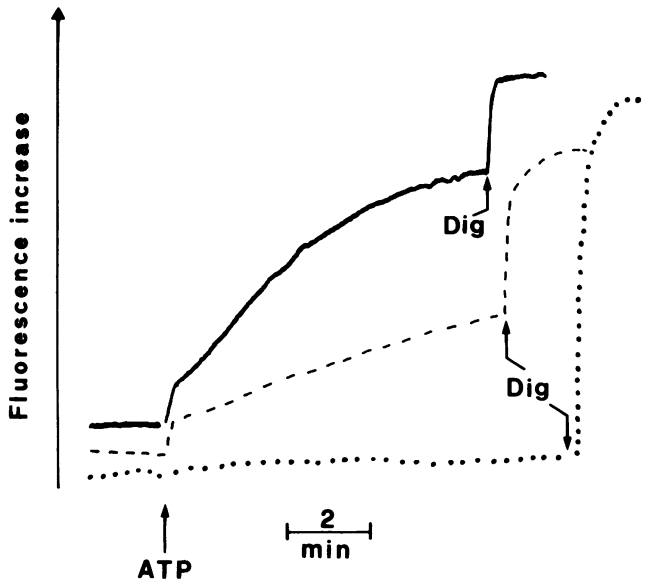

Figure 3. ATP_e causes ethidium bromide uptake by macrophages but not monocytes. Monocytes (*dotted line*), macrophages (*broken line*), and IFN-macrophages (*continuous line*) (10^6 cells per ml) were incubated in standard saline supplemented with 20 μM ethidium bromide and challenged with 1 mM ATP. Digitonin (*Dig*) was added at a concentration of 100 μM.

Rather interestingly, IFN-macrophages (Fig. 2 *B*, continuous line) showed a much higher response to ATP_e compared with macrophages (*broken line*) and monocytes (*dotted line*). In all three cell types, a second addition of the nucleotide was ineffective, thus providing further indication that the sustained $[Ca^{2+}]_i$ increase observed in Fig. 2 *A* was due exclusively to influx across the plasma membrane. Similarly to ATP_e, extracellular uridine triphosphate (UTP_e) caused a larger Ca^{2+} mobilization in IFN-macrophages than in macrophages and monocytes (Fig. 2 *C*).

More direct proof of the membrane-permeabilizing activity of ATP_e is offered by the experiments reported in Fig. 3, which shows ethidium bromide uptake by ATP_e-pulsed cells. Ethidium bromide is a 360-D, slowly permeant hydrophylic dye that greatly increases its fluorescence when bound to DNA. In the absence of plasma membrane permeabilization (monocytes; Fig. 3, *dotted line*), ethidium bromide fluorescence did not change after ATP_e addition. On the contrary, a steady increase in fluorescence was observed in macrophages (Fig. 3, *broken line*) and IFN-macrophages (*continuous line*), which is clear evidence of membrane permeabilization.

Besides ethidium bromide, other low molecular hydrophylic fluorescent tracers such as Lucifer yellow (molecular weight 457) can be used to monitor ATP_e-dependent changes in plasma membrane permeability (18). Fig. 4 shows the effect of ATP_e on Lucifer yellow uptake in monocytes and macrophages. Macrophages showed cytoplasmic uptake of the fluorescent dye in the presence (Fig. 4 *B*) but not in the absence of ATP_e (*A*), whereas monocytes exhibited some pinocytic dye uptake without cytoplasmic staining (*C*). It was not possible to monitor Lucifer yellow uptake by IFN-macrophages, as these cells were rapidly lysed by ATP_e and did not reseal after removal of the nucleotide, thus forbidding cytoplasmic dye trapping.

Blanchard et al. (10) have recently reported that incubation in the presence of rIFN-γ enhances susceptibility of macrophages to the lytic effects of ATP_e. In the experiments reported in Fig. 5, we confirm their observation by measuring release of the cytoplasmic enzyme lactate dehydrogenase and provide evidence for involvement of the P_{2Z} receptor in the cytotoxic process. In fact, Fig. 5 shows that oATP, the 2′,3′-dialdehyde derivative of ATP recently reported to block P_{2Z} receptor of murine macrophages covalently, also prevents ATP_e-dependent release of lactic dehydrogenase in human macrophage cells.

Although these experiments are strong indication for a rIFN-γ–modulated expression of the P_{2Z} receptor during in vitro macrophage culture, the possibility that enhanced ATP_e susceptibility of macrophages and IFN-macrophages was due to a reduced ability to hydrolyze ATP_e cannot be excluded. In fact, it is known that almost all cell types possess very active plasma membrane ecto-ATPases (or ecto-nucleotidases) that rapidly destroy extracellular nucleotides (23). This hydrolytic activity seems to be responsible for the resistance of human Langerhans' cells to ATP_e (34) and has also been proposed as a protective mechanism in cytotoxic T lymphocytes (35). However, the increased sensitivity to ATP_e of IFN-macrophages does not seem to be due to a lesser ability to destroy ATP_e, as ATP hydrolytic activity was very similar in macrophages and IFN-macrophages, i.e., 16 and 19 nmol of Pi per 10^6 cells per min, respectively.

Increased expression of P_2 receptors during in vitro macrophage culture and modulation by an inflammatory cytokine such as IFN-γ raise the possibility that these surface molecules could have an until now unsuspected role in the inflammatory reaction, maybe under conditions in which intimate communication between macrophage cells is needed. To test this hypothesis, we explored the effect of the specific P_{2Z} receptor inhibitor oATP on MGC formation, a typical feature of granulomatous formations. Fig. 6 *B*, shows that, as recently demonstrated (29), a 72-h incubation of monocyte-derived macrophages in the presence of Con A is a powerful stimulus for MGC formation. Quite interestingly, this process was strongly inhibited by oATP (Fig. 6 *C*). A similar inhibitory effect of oATP was observed when MGC formation was induced by rIFN-γ or Con A plus rIFN-γ (data not shown). Fig. 7 reports a measurement of the inhibitory effect of oATP made by evaluating the fusion index in several experiments performed with different cell preparations. Con A stimulation caused an average MGC formation of about 70%, which was reduced to 20% in the presence of 300 μM oATP, a concentration that we have previously shown to block P_{2Z} receptor fully in murine macrophages (19). Polykarion formation was also inhibited, although to a lesser extent, when oATP was added during the last 24 h of Con A stimulation.

Investigation of polykarion formation required a long incubation in the presence of cells at high density. Thus, it is likely that oATP was rapidly hydrolyzed by serum or plasma membrane ATPases. Monocyte monolayers hydrolyzed oATP at a rate of about 10 nmol/10^6 cells per min; nonetheless, monocytes were still partially refractory to ATP_e stimulation 3 d after oATP addition, suggesting that the oxidized nucleotide bound to the receptor was a weak substrate for ecto-nucleotidases. Fig. 8 shows that whereas ATP_e caused a clear Lucifer yellow uptake in control 3-d monocyte cultures (*A*), very little dye was taken up by monocytes incubated for the same time in the presence of an initial oATP concentration of 300 μM (*C*). It is interesting to note that oATP-treated cells showed minor morphological changes (rounding and swelling), which on the contrary were

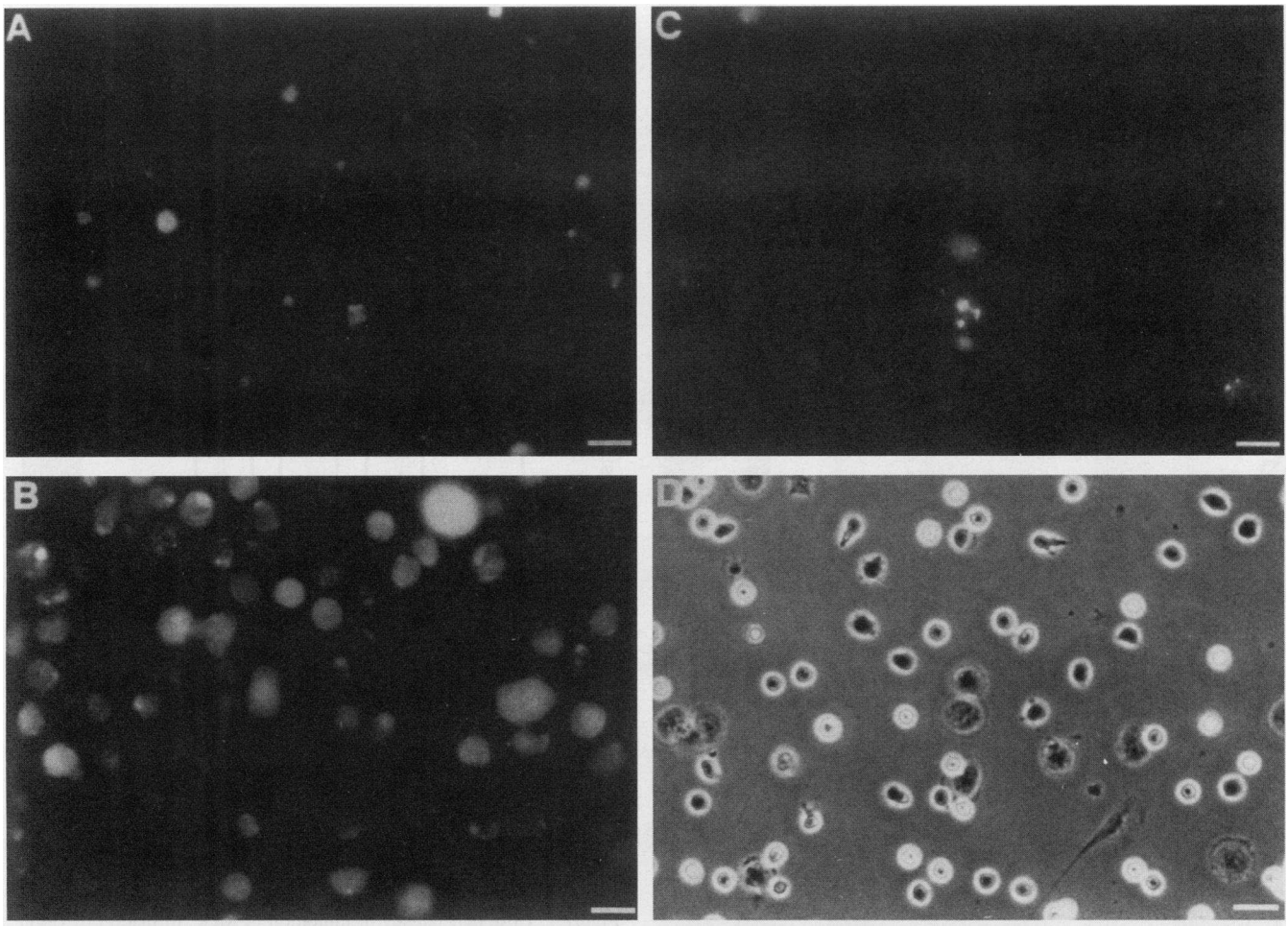

Figure 4. ATP_e causes Lucifer yellow uptake by macrophages but not monocytes. Cell monolayers were incubated in the absence (*A*) or presence (*B, C,* and *D*) of 5 mM ATP_e for 10 min in standard saline containing 1 mg/ml Lucifer yellow, rinsed with RPMI containing 10% horse serum, and examined under phase contrast (*D*) or fluorescent light using a fluorescein filter (*A, B,* and *C*). (*A* and *B*) macrophages; (*C* and *D*) monocytes. Cells were observed with a 40× objective. Bars, 24 μm.

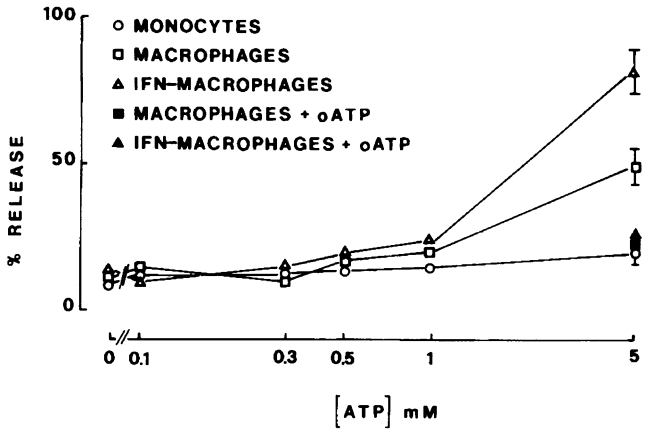

Figure 5. ATP_e causes macrophage cell lysis. Monocyte, macrophage, or IFN-macrophage monolayers (10^5 cells per ml) were incubated at 37°C in standard saline in the presence of increasing concentrations of ATP_e. In some experiments, macrophages and IFN-macrophages were also preincubated for 2 h in the presence of 300 μM oATP before being challenged with ATP_e. Lactic dehydrogenase release is shown as a percentage of total cell content.

present in control monocytes (Fig. 8 *B, arrows*). Thus, morphological evidence clearly supports the suggestion that the ATP_e receptor was still blocked 3 d after the initial challenge with oATP. A more sensitive index of the functional presence of the P_{2Z} receptor is plasma membrane depolarization. The experiment reported in Fig. 8 *E* shows that even after 3 d of incubation, oATP-treated monocytes (trace *b*) are partially refractory to ATP_e-triggered depolarization. Rather interestingly, in oATP-inhibited 3-d macrophages, ATP_e triggered first a hyperpolarization, reminiscent of that observed in freshly isolated monocytes (see Fig. 1, trace *a*), and then a slow depolarization.

Although oATP is a specific ligand for ATP-binding sites, inhibition of MGC formation could be due to reasons other than blockade of plasma membrane purinergic receptors. For example, macrophage fusion could be blocked as a consequence of a nonspecific inhibition of Con A–dependent stimulation, of cell adhesion molecule expression, or of cellular chemotaxis. However, Fig. 9 shows that Con A–stimulated $[Ca^{2+}]_i$ changes were not reduced in oATP-treated (trace *a*) as compared with control (trace *b*) monocytes. Rather interestingly, the lag preceding the $[Ca^{2+}]_i$ rise was shorter in oATP-treated cells. Furthermore, Fig. 10 reports a FACS analysis of expression of LFA-1 (CD11a, CD18) and ICAM-1 (CD54), two surface structures involved in cell adhesion that have also been pro-

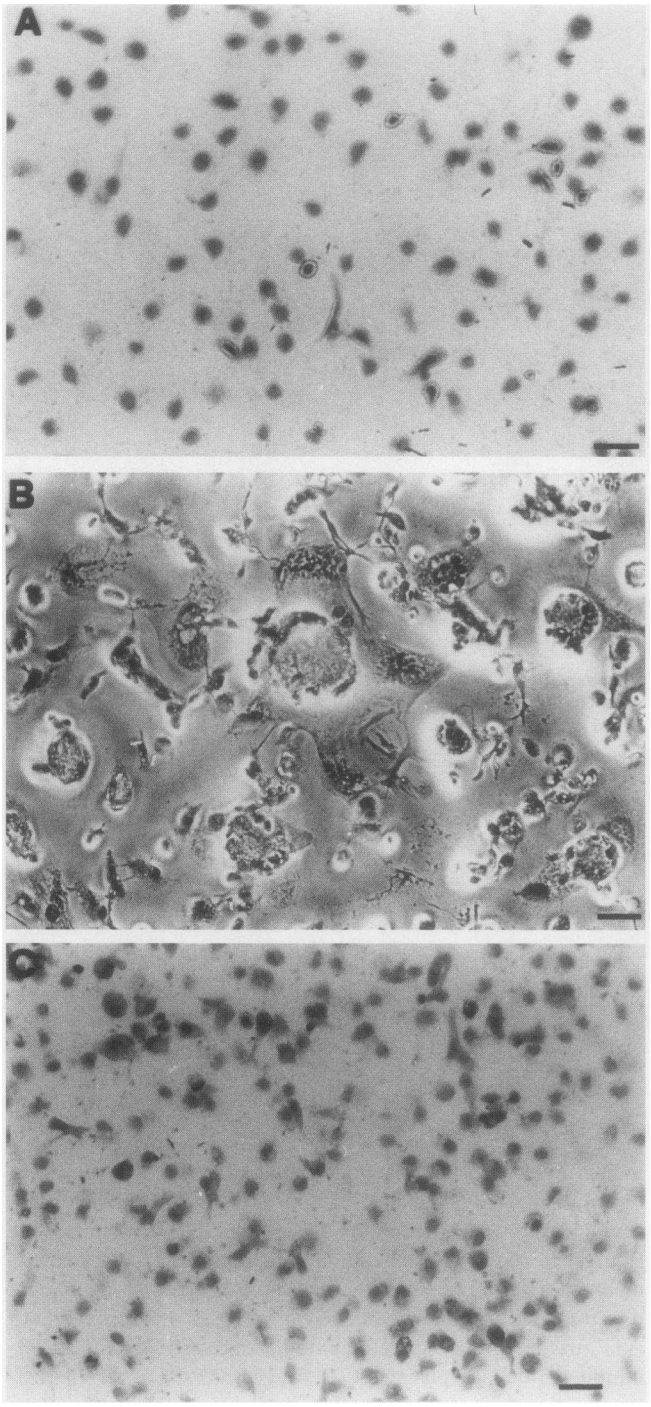

Figure 6. oATP inhibits MGC formation. Monocytes were suspended in RPMI medium supplemented with 5% human serum, seeded in 96-well microtiter plates at a concentration of 10^5 cells per well, and stimulated with Con A (10 μg/ml) in the absence (*B*) or presence (*C*) of 300 μM oATP. Control monocytes are shown in *A*. After 3 d, cells were rinsed, fixed, stained, and observed with a 40× objective. Bars, 24 μm.

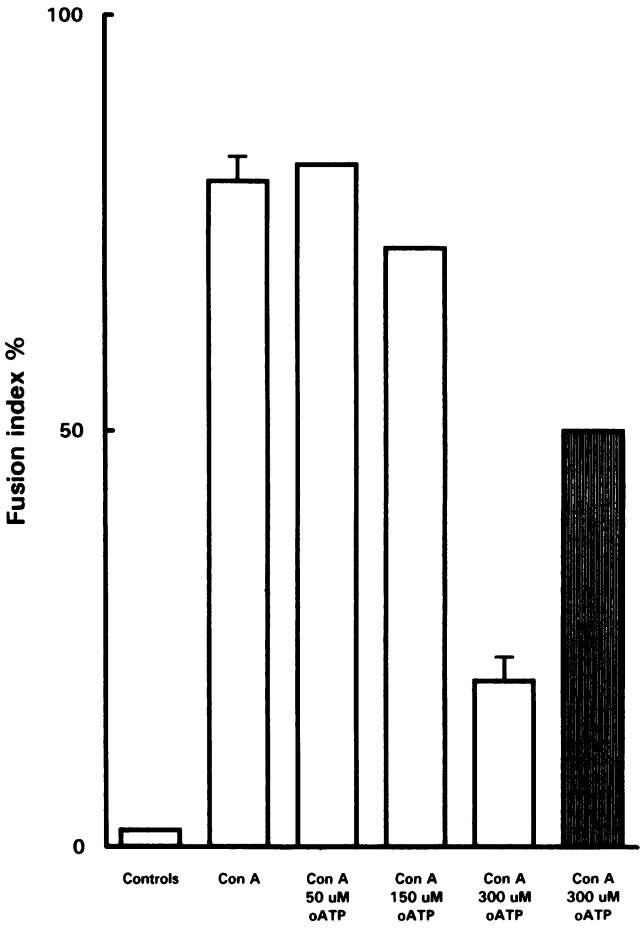

Figure 7. Dose dependency of inhibition of MGC formation by oATP. Monocytes were incubated as described in Fig. 6 and evaluated for MGC formation after a 3-d incubation. oATP was added at concentrations of 50, 150, and 300 μM, and Con A was added at a concentration of 10 μg/ml. In the experiments indicated by hatched bar, oATP (300 μM) was added during the last 24 h of incubation in the presence of Con A.

posed to play a role in giant cell formation. Con A stimulation caused an increase in the expression of CD11a, CD18, and CD54 that was only weakly altered by oATP. To our surprise, the oxidized nucleotide slightly increased the level of expression of CD54 in resting monocytes. Experiments to explain this effect are in progress in our laboratory. Finally, chemotaxis of oATP-treated monocytes stimulated with three different chemoattractants was investigated (Table I) and found to be unchanged with respect to control cells.

Discussion

Purinergic receptors are known to be expressed on the plasma membrane of most human and animal cell types. According to pharmacological and functional criteria, five purinergic receptor subtypes are known, P_{2T}, P_{2Y}, P_{2U}, P_{2X}, and P_{2Z} (23), although recent cDNA evidence suggests that the P_{2U} receptor may bind both ATP and UTP (36, 37).

Among these receptors, one of the most intriguing is P_{2Z}, a molecule that is thought to coincide or be associated with a nonselective membrane pore permeable to hydrophilic molecules with molecular masses up to 900 D (2, 6, 18). This receptor is specifically activated by ATP, and a few other ATP analogs, at concentrations that are at least 1 order of magnitude higher than those needed to activate other P_2 receptors (2, 15). This apparent low affinity for ATP_e may be due to the fact that

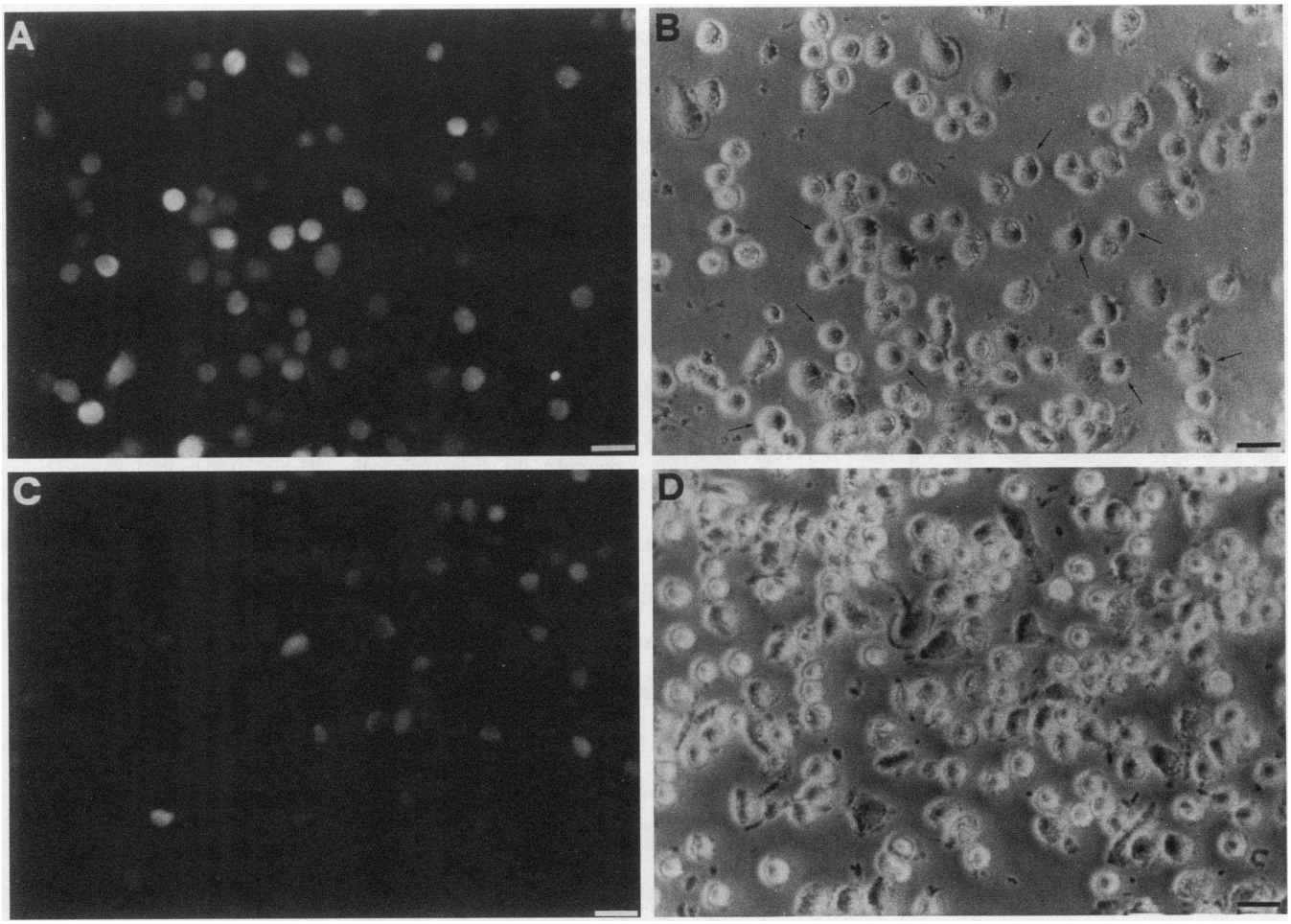

Figure 8. Inhibition of the P_{2Z} receptor by oATP in 3-d macrophages. Monocytes were seeded in a 96-well microtiter plate in RPMI supplemented with 5% human serum at a concentration of 10^5 cells per well. In *C* and *D*, 300 μM oATP was also added. Cells were incubated for 3 d in a thermostatted (37°C) CO_2 incubator and tested for ATP_e-mediated Lucifer yellow uptake (*A, B, C,* and *D*) or plasma membrane depolarization (*E*). For Lucifer yellow uptake, cells were transferred to standard saline containing 5 mM ATP_e and 1 mg/ml Lucifer yellow. After 10 min, cells were rinsed with RPMI containing 10% horse serum and examined under phase contrast (*B* and *D*) or fluorescent light (*A* and *C*). Cells were observed with a 40× objective. Bars, 24 μm. For measurement of plasma membrane depolarization (*E*), cells were rinsed and suspended in Ca^{2+}-free standard saline containing 100 nM bisoxonol. ATP_e addition was 500 μM and KCl was 30 mM. Trace *a*, control 3-d monocytes; trace *b*, oATP-treated monocytes.

the active nucleotide form recognized by the receptor is the fully dissociated species (ATP^{4-}), which under normal physiological conditions of pH and divalent cation concentration, amounts to a small fraction of total ATP_e (14).

Activation of the P_{2Z} receptor causes a large Na^+ and Ca^{2+} influx accompanied by plasma membrane depolarization and loss of endogenous ions and metabolites. Quite interestingly, this nonspecific increase in plasma membrane permeability is reversible if ATP_e is removed within a few minutes of the addition (14). Long exposures to ATP_e cause irreversible cell damage and death. The "point of no return" depends largely on the given cell type, as some cells are exceedingly sensitive to the membrane-perturbing activity of ATP_e and die within 5–10 min, whereas others are more resistant and tolerate longer stimulations (38). Furthermore, sometimes ATP_e-pulsed cells apparently recover their morphological and functional integrity only to die several hours later (39). The membrane-permeabilizing effect is specific since it is not observed in cells lacking the P_{2Z} receptor and can be inhibited by selective P_{2Z} receptor blockers (19).

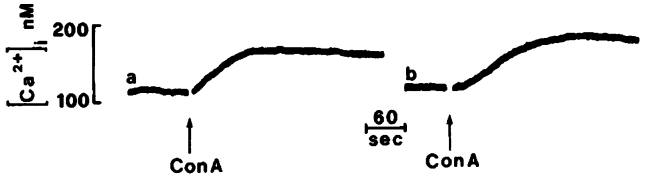

Figure 9. oATP does not inhibit Con A–triggered $[Ca^{2+}]_i$ changes. Monocytes were incubated for 2 h at 37°C in the presence (trace *a*) and absence (trace *b*) of 300 μM oATP. They were then centrifuged, suspended in standard saline, loaded with fura-2/AM as detailed in Methods, and placed in a fluorometer cuvette at a concentration of 5 × 10^5 cells per ml. Con A was added at a concentration of 10 μg/ml.

Although murine peritoneal macrophages and the macrophage-like J774 murine cell line express P_{2Z} receptors, thus suggesting an important role for this molecule in the immune response, little information is available on human macrophages. A recent paper reports on the cytotoxic effect of ATP_e on human monocyte-derived macrophages (10), but given that at least two P_2 receptors (P_{2X} and P_{2Z}) have been linked to the cytotoxic response, this is not per se evidence for the expression of P_{2Z} receptors by human macrophages. Furthermore, no attempt was made to explain the ATP_e-dependent cytotoxic mechanism. Characterization of purinergic receptors expressed by human macrophages can also be relevant in view of the possible exploitation of ATP_e-mediated reversible plasma membrane permeabilization as a tool for manipulating the composition of the cytoplasm.

Table I. oATP Does Not Inhibit Monocyte Chemotaxis

	Control	oATP 50 μM	oATP 150 μM	oATP 300 μM
Random locomotion*	41±3	46±2	45±2	43±2
Chemotaxis‡				
Casein	1.05±0.1	0.85±0.7	0.89±0.8	0.97±0.1
FMLP (10^{-8} M)	1.00±0.1	1.10±0.8	0.97±0.8	1.10±0.1
LPS	0.98±0.8	1.10±0.8	0.83±0.1	0.96±0.8

Monocytes, at a concentration of 2 × 10^5 cells per well, were placed in the top compartment of a chemotaxis chamber, as described in Methods, and challenged during a 90-min test with the various stimulants. * Results are expressed in micrometers (±SE) covered by the leading front during incubation in the chemotaxis chamber. ‡ Results are expressed as the chemotactic index, which is given by the following ratio: (migration toward test attractant − migration toward the buffer)/migration toward the buffer.

Our results suggest that expression of the P_{2Z} receptor occurs during in vitro monocyte to macrophage differentiation. In fact, freshly isolated monocytes, upon stimulation with ATP_e, exhibited responses typical of the presence of P_{2Y}/P_{2U} receptors, i.e., an increase in $[Ca^{2+}]_i$ largely dominated by release from intracellular stores and paralleled by a transient hyperpolarization. ATP_e did not cause changes in plasma membrane permeability in these cells. Accordingly, monocytes were resistant to ATP_e-dependent cytotoxicity (10 and this work).

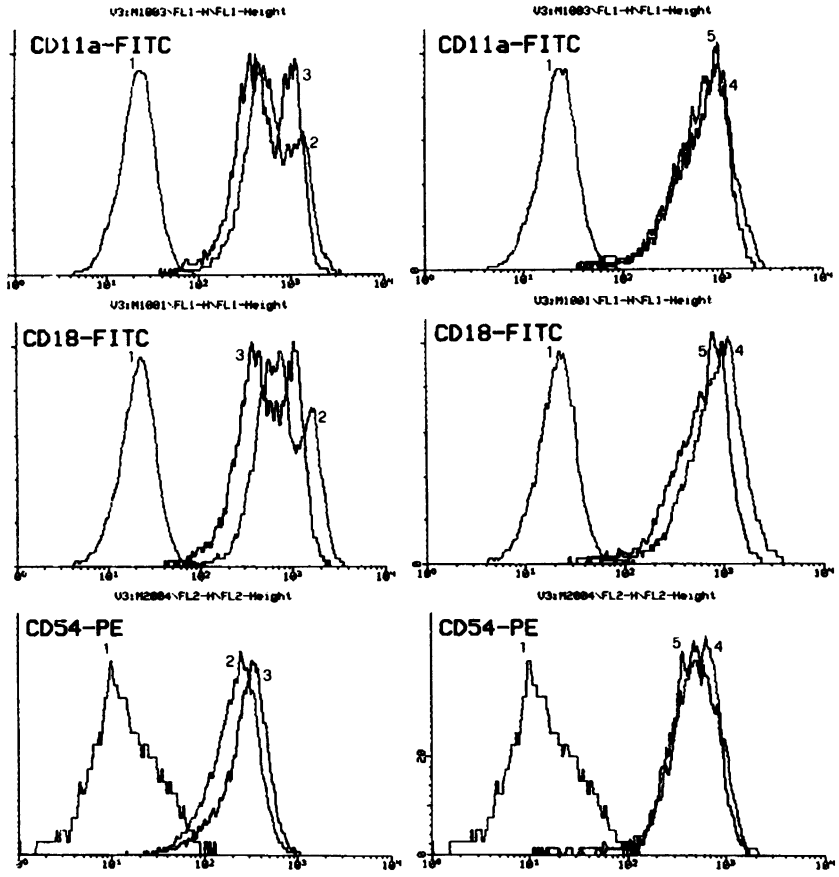

Figure 10. FACS analysis of CD11a, CD18, and CD54 expression in monocytes stimulated with Con A in the presence and absence of oATP. Monocytes were separated by adherence on plastic (> 96% pure), incubated for 24 h under the different experimental conditions, and analyzed for expression of the various surface adhesion molecules. Con A and oATP were included at 10 μg/ml and 300 μM, respectively. Data are from a single experiment performed with similar results on three different occasions. Trace *1*, isotypic controls; trace *2*, controls; trace *3*, oATP-treated cells; trace *4*, Con A–treated cells; trace *5*, oATP, Con A–treated cells.

Macrophages after 5 d in vitro retained the Ca^{2+}-mobilizing response associated with the P_{2Y}/P_{2U} receptor, and became very sensitive to the membrane-permeabilizing effect of ATP_e. Sensitivity to ATP_e was strongly potentiated by treatment with rIFN-γ, a cytokine that has a pivotal role in monocyte/macrophage activation during the inflammatory reaction. An increased sensitivity to the membrane-perturbing effect of ATP_e could also be due to a reduced ecto-ATPase activity of macrophage with respect to monocytes. In fact, it has been reported that human Langerhans' cells are protected against the permeabilizing effects of ATP_e by their powerful ecto-ATPase activity (34). However, we found that ATP_e hydrolytic activity was very similar in monocytes, macrophages, and IFN-γ–treated macrophages, thus ruling out an involvement of plasma membrane ecto-ATPases.

Expression of the P_{2Z} receptor during macrophage differentiation and its upregulation by an inflammatory cytokine such as IFN-γ suggest that this receptor may have an important role in macrophage physiology. During inflammatory and immune responses, macrophages are known to establish close contact with each other and with different cell types, such as helper or cytotoxic lymphocytes, and to release several cytokines, often via unknown mechanisms (e.g., IL-1). Therefore, during the inflammatory reaction, the P_{2Z} receptor/pore could be involved either in the establishment of cell–cell communication or in the extracellular release of inflammatory mediators generated by macrophages.

A common feature of granulomatous inflammations is MGC formation. It is well accepted that MGCs originate from the fusion of monocytes and not from endomitosis (i.e., nuclear division without cytoplasmic division) (29, 40), but the mechanism and the membrane molecules involved are still unknown. IFN-γ TNF-α, IL-3, and IL-4 have been reported to be required for or to induce MGC formation, but available data are contrasting and no clear-cut conclusion about the identity of the putative fusogenic cytokine has been reached (29, 40–42). The identity of the putative fusogenic macrophage plasma membrane molecules is similarly unknown. It has been proposed that LFA-1 family molecules might be involved (42), but no definitive evidence has been provided.

The P_{2Z} receptor is still a molecule in search of a function. Macrophages are among the few cell types in which this receptor has been thoroughly characterized, but even in these cells, its physiological role is completely unknown (2). We have proposed that this ATP_e-gated membrane pore might be a kind of "suicide receptor" involved in cytotoxic reactions (16, 17), but it is difficult to envisage why macrophages should express such a dangerous membrane structure. An alternative view would be that the P_{2Z} receptor, which can be functionally assimilated to a gap junction (43), is involved in allowing close cytoplasmic communication between cells that differentiate into multicellular structures. Preliminary experiments by our group (Falzoni S., and F. Di Virgilio, unpublished data) show that susceptibility to ATP_e-mediated permeabilization in Con A–stimulated monocytes closely parallels formation of MGCs. Therefore, we hypothesize that the ATP_e-gated pore makes macrophages competent for a fusion event that also needs other factors (e.g., release of cytokines and/or expression of adhesion molecules) stimulated by fusogenic agents. According to this view, the main role of this receptor would be in intercellular communication, whereas cytotoxicity, one of the most striking cellular responses to ATP_e, would be a side effect observed under extreme conditions.

However, an unresolved issue is how the high ATP_e levels needed for P_{2Z} receptor activation could be achieved under physiological conditions. ATP_e concentration in the extracellular fluids is very low (5–20 μM) (44), although several events may generate large localized increases. Probably the most relevant source of ATP_e is the cytoplasm, from which ATP can be released following hypoxia, stress, or membrane damage—conditions that are likely to occur at sites of inflammation. In addition, protected compartments may be formed at sites of close cell–cell contact, in which ATP_e released from adjacent cells may accumulate. Thus, it is likely that tissue macrophages are exposed to local ATP_e concentrations that may be sufficient to activate the P_{2Z} receptor.

In conclusion, our data suggest that human macrophages express rIFN-γ–modulated purinergic receptors of the P_{2Z} subtype and that these plasma membrane molecules may be involved in the process of MGC formation.

Acknowledgments

We wish to thank Dr. Paola Chiozzi for helpful suggestions.

This work was supported by grants from the National Research Council of Italy (target projects Biotechnology & Bioinstrumentation [BTBS] and Clinical Application of Cancer Research [ACRO] and special project Biology and Pathology of Calcium), the Ministry of Scientific Research (MURST), the Italian Association for Cancer Research (AIRC), the VII AIDS Project and Telethon of Italy.

References

1. Schmidt, A., J. R. Ortaldo, and R. B. Herberman. 1984. Inhibition of human natural killer cell reactivity by exogenous adenosine 5′-triphosphate. *J. Immunol.* 132:146–150.

2. Steinberg, T. H., H. P. Buisman, S. Greeenberg, F. Di Virgilio, and S. C. Silverstein. 1990. Effects of extracellular ATP on mononuclear phagocytes. *Ann. NY Acad. Sci.* 603:120–129.

3. Di Virgilio, F., V. Bronte, D. Collavo, and P. Zanovello. 1989. Responses of mouse lymphocytes to extracellular adenosine 5′-triphosphate (ATP). Lymphocytes with cytotoxic activity are resistant to the permeabilizing effects of ATP. *J. Immunol.* 143:1955–1960.

4. Filippini, A., R. E. Taffs, and M. V. Sitkovsky. 1990. Extracellular ATP in T-lymphocyte activation: possible role in effector function. *Proc. Natl. Acad. Sci. USA.* 87:8267–8271.

5. Padeh, S., A. Cohen, and C. M. Roifman. 1991. ATP-induced activation of human B lymphocytes via P2 purinoceptors. *J. Immunol.* 146:1626–1632.

6. Cockcroft, S., and B. D. Gomperts. 1980. The ATP^{4-} receptor of rat mast cells. *Biochem. J.* 188:789–798.

7. Ward, P. A., T. W. Cunningham, K. K. McCulloch, and K. J. Johnson. 1988. Regulatory effects of adenosine and adenine nucleotides on oxygen radical responses of neutrophils. *Lab. Invest.* 58:438–447.

8. Sung, S. S., J. D.-E. Young, A. M. Origlio, J. M. Heiple, H. R. Kaback, and S. C. Silverstein. 1985. Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic Ca^{2+} and inhibits phagocytosis in mouse macrophages. *J. Biol. Chem.* 260:13442–13449.

9. Cowen, D. S., H. M. Lazarus, S. B. Shurin, S. E. Stoll, and G. R. Dubyak. 1989. Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. *J. Clin. Invest.* 83:1651–1660.

10. Blanchard, D. K., S. McMillen, and J. Y. Djeu. 1991. IFN-gamma enhances sensitivity of human macrophages to extracellular ATP-mediated lysis. *J. Immunol.* 147:2579–2585.

11. Gregory, S., and M. Kern. 1978. Adenosine and adenine nucleotides are mitogenic for mouse thymocytes. *Biochem. Biophys. Res. Commun.* 83:1111–1116.

12. Cowen, D. S., M. Berger, L. Nuttle, and G. R. Dubyak. 1991. Chronic treatment with P2-purinergic receptor agonists induces phenotypic modulation of the HL-60 and U937 human myelogenous leukemia cells. *J. Leukocyte Biol.* 50:109–122.

13. Steinberg, T. H., and S. C. Silverstein. 1989. ATP permeabilization of the plasma membrane. *Methods Cell Biol.* 31:45–61.

14. Di Virgilio, F., and T. H. Steinberg. 1993. ATP permeabilization. *In* Cell & Tissue Culture: Laboratory Procedures. Wiley & Sons Ltd., Chichester, UK. p. 5B:4.1–4.5.

15. Pizzo, P., M. Murgia, A. Zambon, P. Zanovello, V. Bronte, D. Pietrobon, and F. Di Virgilio. 1992. Role of P2Z purinergic receptors in ATP-mediated killing of tumor necrosis factor (TNF)-sensitive and TNF-resistant L929 fibroblasts. *J. Immunol.* 149:3372–3378.

16. Di Virgilio, F., P. Pizzo, P. Zanovello, V. Bronte, and D. Collavo. 1990. Extracellular ATP as a possible mediator of cell-mediated cytotoxicity. *Immunol. Today.* 11:274–277.

17. Di Virgilio, F., P. Zanovello, and D. Collavo. 1993. Cell-permeabilizing properties of extracellular ATP in relation to lymphocyte-mediated cytotoxicity. *In* Cytotoxic Cells M. Sitkovsky and P. Henkart, editors. Birkhauser, Boston, MA. 314–320.

18. Steinberg, T. H., A. S. Newman, J. A. Swanson, and S. C. Silverstein. 1987. ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.* 262:8884–8888.

19. Murgia, M., S. Hanau, P. Pizzo, M. Rippa, and F. Di Virgilio. 1993. Oxidized ATP. An irreversible inhibitor of the macrophage purinergic P2z receptor. *J. Biol. Chem.* 268:8199–8203.

20. Murgia, M., P. Pizzo, T. H. Steinberg, and F. Di Virgilio. 1992. Characterization of the cytotoxic effect of extracellular ATP in J774 mouse macrophages. *Biochem. J.* 288:897–901.

21. Zanovello, P., V. Bronte, A. Rosato, P. Pizzo, and F. Di Virgilio. 1990. Responses of mouse lymphocytes to extracellular ATP. II. Extracellular ATP causes cell type-dependent lysis and DNA fragmentation. *J. Immunol.* 145:1545–1550.

22. Freyer, D. R., L. A. Boxer, R. A. Axtell, and R. F. Todd. 1988. Stimulation of human neutrophil adhesive properties by adenine nucleotides. *J. Immunol.* 141:580–586.

23. Dubyak, G. R., and C. El-Moatassim. 1993. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.* 265:C577–C606.

24. Colotta, F., F. Re, M. Muzio, R. Bertini, N. Polentarutti, M. Sironi, J. G. Giri, S. K. Dower, J. E. Sims, and A. Mantovani. Interleukin-1 type II receptor: a decoy target for interleukin-1 regulated by interleukin-4. *Science (Wash. DC).* 261:472–474.

25. De Togni, P., G. Cabrini, and F. Di Virgilio. 1984. Cyclic AMP inhibition of f Met-Leu-Phe-dependent metabolic responses in human neutrophils is not due to its effects on cytosolic Ca^{2+}. *Biochem. J.* 224:629–635.

26. Di Virgilio, F., C. Fasolato, and T. H. Steinberg. 1988. Inhibitors of membrane transport system for organic anions block fura-2 excretion from PC12 and N2A cells. *Biochem. J.* 256:959–965.

27. Bergmeyer, H. U. 1983. Methods of Enzymatic Analysis, Vol. III. Academic Press, London. 118–133.

28. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8:115–121.

29. Takashima, T., K. Ohnishi, I. Tsuyuguchi, and S. Kishimoto. 1993. Differential regulation of formation of multinucleated giant cells from concanavalin A-stimulated human blood monocytes by IFN-gamma and IL-4. *J. Immunol.* 150:3002–3007.

30. Zigmond, S. H., and J. G. Hirsch. 1973. Leukocyte locomotion and chemotaxis: new method for evaluation and demonstration of a cell-derived chemotactic factor. *J. Exp. Med.* 137:387–395.

31. Lanza, F., F. Fagioli, R. Gavioli, S. Spisani, F. Malavasi, G. L. Castoldi, and S. Traniello. 1991. Evaluation of CR1 expression in neutrophils from chronic myeloid leukemia: relationship between prognosis and cellular activity. *Br. J. Haematol.* 77:77–82.

32. Penner, R., C. Fasolato, and M. Hoth. 1993. Ca^{2+} influx and its control by Ca^{2+} release. *Curr. Opin. Neurobiol.* 3:368–370.

33. Greenberg, S., F. Di Virgilio, T. H. Steinberg, and S. C. Silverstein. 1988. Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. *J. Biol. Chem.* 263:10337–10343.

34. Girolomoni, G., M. L. Santantonio, S. Pastore, P. R. Bengstresser, A. Giannetti, and P. C. Cruz, Jr. 1993. Epidermal Langerhans cells are resistant to the permeabilizing effects of extracellular ATP: *in vivo* evidence supporting a protective role of membrane ATPases. *J. Invest. Dermatol.* 100:282–290.

35. Filippini, A., R. E. Taffs, T. Agui, and M. V. Sitkovsky. 1990. Ecto-ATPase activity in cytolytic T lymphocytes. Protection from the cytolytic effects of extracellular ATP. *J. Biol. Chem.* 265:334–340.

36. Lustig, K. D., A. K. Shiau, A. J. Brake, and D. Julius. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA.* 90:5113–5117.

37. Erb, L., K. D. Lustig, D. M. Sullivan, J. T. Turner, and G. A. Weisman. 1993. Functional expression and photoaffinity labeling of a cloned P2u purinergic receptor. *Proc. Natl. Acad. Sci. USA.* 90:10449–10453.

38. Murgia, M., P. Pizzo, P. Zanovello, A. Zambon, and F. Di Virgilio. 1992. *In vitro* cytotoxic effects of extracellular ATP. *Alternatives to Laboratory Animals.* 20:66–70.

39. Hogquist, K. A., M. A. Nett, E. R. Unanue, and D. D. Chaplin. 1991. Interleukin-1 is processed and released during apoptosis. *Proc. Natl. Acad. Sci. USA.* 88:8445–8450.

40. McInnes, A., and D. M. Rennick. 1988. Interleukin 4 induces cultured monocyte/macrophages to form giant multinucleated cells. *J. Exp. Med.* 167:598–605.

41. Weinberg, J. B., M. M. Hobbs, and M. A. Misukonis. 1984. Recombinant human gamma-interferon induces human monocyte polykarion formation. *Proc. Natl. Acad. Sci. USA.* 81:4554–4560.

42. Most, J., H. P. Neumayer, and M. P. Dierich. 1990. Cytokine-induced generation of multinucleated giant cells *in vitro* requires interferon-gamma and expression of LFA-1. Eur. *J. Immunol.* 20:1661–1667.

43. Beyer, E. C., and T. H. Steinberg. 1991. Evidence that the gap junction protein connexin-43 is the ATP-induced pore of mouse macrophages. *J. Biol. Chem.* 266:7971–7974.

44. Born, G. V. R., and M. A. A. Kratzer. 1984. Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. *J. Physiol.* 354:419–429.