**Summary**

Microglial cells express a peculiar plasma membrane receptor for extracellular ATP, named P2Z/P2X7 purinergic receptor, that triggers massive transmembrane ion fluxes and a reversible permeabilization of the plasma membrane to hydrophilic molecules of up to 900 dalton molecule weight and eventual cell death (Di Virgilio, F. 1995. Immunol. Today. 16:524–528). The physiological role of this newly cloned (Surprenant, A., F. Rassendren, E. Kawashima, R.A. North and G. Buell. 1996. Science (Wash. DC). 272:735–737) cytolytic receptor is unknown. In vitro and in vivo activation of the macrophage and microglial cell P2Z/P2X7 receptor by exogenous ATP causes a large and rapid release of mature IL-1β. In the present report we investigated the role of microglial P2Z/P2X7 receptor in IL-1β release triggered by LPS. Our data suggest that LPS-dependent IL-1β release involves activation of this purinergic receptor as it is inhibited by the selective P2Z/P2X7 blocker oxidized ATP and modulated by ATP-hydrolyzing enzymes such as apyrase or hexokinase. Furthermore, microglial cells release ATP when stimulated with LPS. LPS-dependent release of ATP is also observed in monocyte-derived human macrophages. It is suggested that bacterial endotoxin activates an autocrine/paracrine loop that drives ATP-dependent IL-1β secretion.

**Materials and Methods**

Cell Culture and Cytokine Measurement. N9 and N13 microglial cell lines were a kind gift of Dr. Paola Ricciardi-Castagnoli (University of Milano, Italy) and were grown in RPMI 1640 medium (PAA, Linz, Austria) supplemented with 2 mM glutamine and 10% (heat-inactivated) FCS (Life Technologies Ltd., Paisley, Scotland), 100 U/ml penicillin, and 100 μg/ml streptomycin as described previously (2). Human monocytes were isolated from buffy coats by one-step gradient (Percoll; Pharmacia Biotech SpA, Cologno Monzese, Italy) or by adherence on plastic Petri dishes. After isolation, cells were kept in culture for 5 d in RPMI medium containing 2 mM glucose, 5% (heat-inactivated) FCS (Life Technologies Ltd., Paisley, Scotland), 100 U/ml penicillin, and 100 μg/ml streptomycin. IL-1β and IL-6 in the supernatant of LPS (Sigma Chemical Co., St. Louis, MO) treated cells were measured with the Intertext-1β mouse IL-1β ELISA kit and Intertext-6X mouse IL-6 ELISA kit, respectively (Genzyme srl, Cinisello Balsamo, Italy). All reagents used were dissolved in endotoxin-free water (Sigma) and checked for endotoxin contamination. Periodate-oxidized ATP (oATP) was synthesized by Dr. S. Hanau as described in reference 13.
Measurement of Extracellular ATP. Microglial cells (25 × 10^3/well) were plated in microtiter plastic wells in culture medium and incubated in a CO₂ incubator at 37°C in the absence or presence of LPS for 24 h. At the end of this incubation, the monolayers were thoroughly rinsed with saline solution and supplemented with 100 μl of a special diluent buffer (FireZyme Ltd., San Diego, CA) to stabilize extracellular ATP and directly placed in the test chamber of a luminometer (FireZyme). Then, 100 μl of luciferin-luciferase solution (FireZyme) was added, and light emission was recorded. As a control, total cellular ATP content was also routinely monitored. Under resting conditions, extracellular ATP amounted to ~10–15% of total.

Results and Discussion

Fig. 1 shows that a 24-h incubation in the presence of 10 μg/ml LPS triggers release of IL-1β and that this is blocked by pretreatment with the selective P2Z/P2X₇ inhibitor (13) oATP. To show that the effect of oATP is not due to a nonspecific inhibition of cell responses, we have also monitored IL-6 release, which is much less affected. A further proof that oATP does not have nonspecific effects, we show that IL-1β release is restored in LPS-treated, oATP-inhibited cells by the K⁺ ionophore nigericin, an agent known to cause IL-1β release through a receptor-independent pathway (4, 10).

Autocrine/paracrine stimulation of purinergic receptors can also in principle be prevented by exogenously added ATP-consuming enzymes such as apyrase or hexokinase. Fig. 2A shows that apyrase completely inhibits LPS-dependent IL-1β release (the inactivated enzyme has no such effect). Surprisingly, hexokinase does not inhibit but rather stimulates IL-1β release. The main difference between apyrase and hexokinase is that the first hydrolyzes ATP and ADP, thus generating AMP, whereas hexokinase uses ATP as phosphorus donor to phosphorylate glucose, thus generating glucose 6 phosphate and ADP. It is known that ADP is an agonist at P2Z/P2X₇ receptor, though less potent than ATP (12). Thus we checked whether the potentiating ef-
The effect of hexokinase is mediated by stimulation of the P2Z/P2X$_7$ receptor by accumulated ADP. This seems to be the case because pretreatment with aATP blocks IL-1$\beta$ secretion due to the combined addition of LPS and hexokinase (Fig. 2 A), and more importantly, exogenous ADP (ADP$_\text{e}$) is a much more potent stimulus than ATP (Fig. 2 B). These experiments suggest that IL-1$\beta$ release could be modulated by ATP$_\text{e}$ and ADP$_\text{e}$, probably released by the inflammatory cells themselves under LPS stimulation.

An obvious sine qua non of this hypothesis is that microglial cells must release ATP in response to LPS. Fig. 3 A shows that microglial cells chronically stimulated with LPS release ATP. Because the incubation medium is changed right before ATP determination, extracellular ATP measured in this experiment is very likely not accumulated in the bulk phase but continuously generated by the microglial cells. In support of this interpretation, we consistently found very little extracellular ATP in the cell-free supernatant (not shown). This observation is consistent with that previously reported by Filippini et al. (14) in T lymphocytes. The LPS dose-response curve for ATP release closely matched that for IL-1$\beta$ release, as shown in Fig. 3 B. It has been shown previously that ATP is a powerful stimulus for IL-1$\beta$ secretion from macrophages (10, 11), thus suggesting that this nucleotide might also have a role in autocrine/paracrine stimulation of these cells. In support of this hypothesis, Fig. 4 shows that ATP is released by human macrophages isolated from three different subjects after stimulation with LPS.

The mechanism of IL-1$\beta$ processing and release is a key issue in immunology (5–9, 15). Rather surprisingly, recent evidence points to a decrease in cytoplasmic K$^+$ as a pivotal stimulus for ICE activation and IL-1$\beta$ maturation (4, 10). However, LPS itself does not directly activate plasma membrane K$^+$ channels, and mouse microglial cells express inwardly but not outwardly rectifying K$^+$ channels (16), thus raising the issue of the mechanism responsible for lowering the cytoplasmic K$^+$ concentration. It has been suggested that this might be achieved by a LPS-dependent increase in the number of voltage-dependent K$^+$ channels in the macrophage plasma membrane (4), but typical K$^+$ channel inhibitors blocked IL-1$\beta$ release only at concentrations far above those necessary to inhibit these channels (4). The P2Z/P2X$_7$ receptor is a good candidate to mediate cytoplasmic K$^+$ depletion. This receptor is typically expressed in macrophages and macrophage-like cells (2, 17, 18), and it is modulated by inflammatory cytokines (17, 19). A brief stimulation with ATP$_\text{e}$ triggers massive K$^+$ efflux (12) and release of processed IL-1$\beta$ (2, 10, 11), whereas a sustained activation causes cell death (1, 17, 20). Our data suggest that IL-1$\beta$ release from microglial cells requires a double simulation: first, LPS-dependent transcription of the IL-1$\beta$ gene and cytoplasmic accumulation of proIL-1$\beta$; second, paracrine/autocrine activation of the P2Z/P2X$_7$ receptor that causes release of the mature cytokine. A denile nucleotides can originate from many different sources: (a) the microglial cells themselves can release ATP$_\text{e}$ either spontaneously or under LPS stimulation; (b) injured or damaged cells certainly release significant amounts of this nucleotide, a process likely to occur in vivo at sites of inflammation; (c) in

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**Figure 3.** LPS dose response for IL-1$\beta$ and ATP release. Microglial cells were plated in 24-well plates as described in Fig. 1 for IL-1$\beta$ secretion or microtiter plastic wells as described in Materials and Methods for ATP release and stimulated with LPS for 24 h in a CO$_2$ incubator at 37°C. For measurement of ATP, release samples were processed as follows: monolayers were rinsed and 100 $\mu$L of diluent buffer (Firezyme) were added (see Materials and Methods). Accumulation of extracellular ATP was measured by the luciferin/luciferase assay. Data for IL-1$\beta$ release are duplicates from a single experiment repeated with similar results with three different batches of microglial cells. Data for ATP release are means of quadruplicate determinations ± SD from a single experiment repeated in three different occasions.

**Figure 4.** Stimulation with LPS triggers ATP release from human macrophages. Macrophages were isolated from three different donors (A–C) as described in Materials and Methods and plated in microtiter plastic wells at a concentration of 50 × 10$^6$/well. After plating, cells were stimulated for 24 h with LPS and ATP release measured as detailed in Fig. 3. Data are mean ± SD from quadruplicate determinations.
the central nervous system, ATP, can be released by neurons that establish close contact with the microglial cells.

It might seem paradoxical that ADP is a better IL-1β-releasing agent than ATP, although notoriously it is a less potent stimulus at the P2Z/P2X7 receptor. However, this is not unexpected because ADP, in contrast with ATP, is devoid of cytotoxic activity, and data from our laboratory show that release of IL-1β is optimal in response to a submaximal, noncytotoxic stimulation of the P2Z/P2X7 receptor, such as that due to ADP (D. Ferrari and F. Di Virgilio, manuscript in preparation).

Involvement of the P2Z/P2X7 purinergic receptor in LPS-dependent IL-1β release may allow the development of new pharmacological antagonists (i.e., oATP and derivatives) to modulate the in vivo production of this cytokine in pathological conditions such as septic shock or chronic inflammatory diseases.

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