Exogenous Cyclic AMP, Cholera Toxin, and Endotoxin Induce Expression of the Lipopolysaccharide Receptor CD14 in Murine Bone Marrow Cells: Role of Purinoreceptors

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Little is known about the mechanisms of lipopolysaccharide (LPS) signaling in immature cells that do not express the LPS receptor CD14 yet. Bone marrow granulocytes do not constitutively express CD14 but can be stimulated by low doses of LPS in the absence of serum and then express an inducible form of LPS receptor (iLpsR). We show that in addition to LPS, cholera toxin (CT) and various cyclic AMP (cAMP) analogs can also induce the expression of iLpsR, which was identified as CD14. Induction was independent of intracellular cAMP. The hypothesis that cAMP analogs act via a cell surface receptor was suggested by the unresponsiveness of trypsin-treated cells to these inducers and by the specific binding of [3H]cAMP to the cells. This binding was not inhibited by LPS or CT but was inhibited by various purine derivatives. However, the receptor involved is not a conventional purinoreceptor since both an agonist and an antagonist of such receptors were able to induce iLpsR expression. The results suggest that cAMP analogs and other purine derivatives induce iLpsR after interaction with an unconventional, trypsin-sensitive, purinoreceptor distinct from LPS and CT receptors.

Elucidating the mechanisms of cell responses to lipopolysaccharide (LPS) is an active area of investigation, owing to the severe pathological processes induced by this bacterial component. LPS acts on a wide variety of cell types by triggering the production of a number of mediators or by modulating the expression of cell surface constituents. However, most of the efforts in this area have been performed with mature, fully differentiated cells. On the other hand, the mechanisms involved in the responses to LPS of less mature cells, such as those present in the bone marrow, are poorly understood. In this connection, we reported previously that stimulation of human and murine bone marrow cells (BMC) with nanomolar concentrations of LPS triggers the expression of inducible LPS receptors (iLpsR) (19). This phenomenon of de novo receptor expression, which has been observed in other systems, should be distinguished from the upregulation of preexisting receptors which accompanies many pharmacological stimuli. Very little is known about the sequence of biochemical events associated with the phenomenon of induction of differentiation antigens and receptors, because examination of transcriptional regulation of signaling is often confined to expression of soluble mediators and other messengers. The documentation of the phenomenon of receptor induction should be of great interest, however, because of its potential implications in the production in hematopoietic tissues of cell populations more adapted to an abnormal (pathological or inflammatory) environment.

In another example of induced receptors, i.e., the expression of an intrinsically active form of opioid receptors during administration of morphine, there was strong evidence of a regulation role for the cyclic AMP (cAMP) second-messenger system (30). Furthermore, it has been shown in many systems that elevation of cAMP levels inhibits the activation of different genes in macrophages (27), the Ras-dependent activation of Raf (2), and the MAP kinase activation by GTP-binding protein-coupled receptors (25, 31). Because MAP kinase activation is involved in LPS-induced signaling in different cell types (9, 24), it is expected that increasing intracellular concentrations of cAMP would inhibit LPS-induced effects. This has indeed been observed for LPS-induced production of tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β) in human macrophages (32), for LPS-induced NO production in astrocytes (16), and for LPS-induced TNF-α production in macrophage cell lines (8).

Following up on these observations, this study was designed to analyze the role of the cAMP second-messenger system in the regulation of iLpsR expression in BMC and, more generally, to identify the key events that characterize different biochemical pathways through which the expression of iLpsR occurs.

MATERIALS AND METHODS

Animals and cell culture. Female C3H/HeOU and C3H/HeJ mice (8 to 10 weeks old) were from the Breeding Center of the Pasteur Institute. BMC were collected from mouse femurs. Culture medium (CM) was RPMI 1640 (Sigma Chemical Co., St. Louis, Mo.) containing 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 mg of streptomycin per ml. Incubations with fluorescein isothiocyanate (FITC)-labeled LPS (FITC-LPS) were performed in CM supplemented with 8% heat-inactivated (56°C, 30 min) fetal calf serum (FCS) (GIBCO, Grand Island, N.Y.).

Reagents. Adenosine, 2-chloroadenosine (2-CA), AMP, isotubyl-methylxanthine (IBMX), pentoxifylline (PTX), forskolin, and cholera toxin (CT) were from Sigma Chemical Co. 2′,5′-dideoxyadenosine (DDA) was from Calbiochem (La Jolla, Calif.). Sodium 8-bromo-cAMP (Br-cAMP), sodium dibutyryl-cAMP (db-cAMP), and sodium 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) were from Biomol Research Laboratories (Plymouth, Pa).

Labeled reagents. The LPS from Salmonella enterica serovar cholerasuis (serotype serotype 6,14, prepared by the phenol-water extraction procedure, was labeled with FITC (Sigma Chemical Co.) as described previously (19). The fluorescent LPS derivative (FITC-LPS) was stored in the dark (4°C) until used. The rat anti-mouse CD14 monoclonal antibody (mAb5-3) was from Pharmingen (San Diego, Calif.). Biotin-labeled and FITC-labeled goat-anti-rat immunoglobulin antibodies and peroxidase-labeled streptavidin were from Southern Biotechnologies Associates (Birmingham, Ala.). 2,6-[3H]cAMP (monosodium salt) (1.4 TBq/ mmol) was from ICN Pharmaceuticals (Irvine, Calif.).

Expression of iLpsR and CD14 in BMC. BMC (5 × 10^5 cells) were incubated (18 to 24 h, 37°C) with the inducer (usually 10 ng of LPS per ml) in CM (400 μl)
in the absence of serum. The cultures were then maintained for 1 h at 4°C. For analysis of the expression of iLpsR, the cells were then incubated (18 h, 4°C) with FITC-LPS (0.2 μg/ml in 500 μM of CM containing 8% FCS). For detection of membrane receptors, the cells were incubated first (30 min, 0°C) with the rat anti-mouse CD14 monoclonal antibody (rmC5-3) and stained by reincubation (30 min, 0°C) with an FITC-labeled anti-rat immunoglobulin antibody. In each case, stained cells were layered on a 50% FCS solution and centrifuged, and the cell pellet was resuspended in 0.5 ml of staining buffer (phosphate-buffered saline [PBS]), 5% FCS and 0.02% sodium azide containing propidium iodide (0.2 μg/ml) to stain dead cells. Fluorescent cells were detected by analysis (5000 cells per sample) on a fluorescence-activated cell sorter (FACS) flow cytometer (FACScan; Becton-Dickinson Electronic Laboratories, Mountain View, Calif.), using Cellquest Software. Dead cells, which incorporated propidium iodide, were gated out of analysis. Cells with a fluorescence intensity higher than the autofluorescence level (10 arbitrary fluorescence units) were scored as fluorescent cells.

**RESULTS**

**Influence of CT and cAMP analogs on iLpsR expression.** To examine the influence of cAMP on the expression of LPS receptors in BMC, we used three membrane-permeable cAMP analogs. We found that Br-cAMP, db-cAMP, and CPT-cAMP mimicked the LPS-induced effect on BMC (Fig. 1). On the other hand, membrane-permeable cGMP analogs (db-cGMP and 8-parachlorophenylthio-cGMP) did not induce the expression of LPS receptors (data not shown). In contrast to LPS, the three cAMP analogs were active in both LPS-responsive (C3H/HeOU) and LPS-hyporesponsive (C3H/HeJ) mouse strains.

Comparison of the kinetics of iLpsR expression on cells incubated for different times with LPS and CPT-cAMP indicated a similar time course with the two inducers. In both cases, a rather long exposure to the inducer (more than 5 h) was required for significant expression of the receptor (data not shown).

We can see in Fig. 1 that CPT-cAMP was the most active analog and Br-cAMP was the least active. It should be noted that the arrangement of the cAMP analogs in the order of their ascending abilities to induce iLpsR expression (Br-cAMP < db-cAMP < CPT-cAMP) parallels their resistance to degradation by phosphodiesterases (23). In this connection, as expected, we observed that PTX, a known inhibitor of phosphodiesterases which should enhance the stability of the cAMP analogs, also enhances their abilities to stimulate BMC (Table 1). However, LPS-induced stimulation of BMC was not enhanced by PTX, thus suggesting that endogenous cAMP is not produced after LPS treatment or is less sensitive to degradation than exogenously added cAMP analogs. Because CT has been demonstrated to modulate adenylyl cyclase (via the ADP-ribosylation of G-proteins), we examined the effect of CT on iLpsR expression. In line with the results mentioned above, we observed that CT (10 nM) can mimic the effect of 100 μM CPT-cAMP (70% iLpsR+ cells) in BMC from C3H/HeOU and C3H/HeJ mice.

**TABLE 1. Influence of PTX on iLpsR expression induced by LPS or cAMP analogs**

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Conc</th>
<th>Fluorescent cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without PTX</td>
<td>100 μM PTX</td>
</tr>
<tr>
<td>None</td>
<td>2.0 ± 0.5</td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>LPS</td>
<td>0.1 ng/ml</td>
<td>11.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>1 ng/ml</td>
<td>36.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10 ng/ml</td>
<td>48.9 ± 2.8</td>
</tr>
<tr>
<td>CPT-cAMP</td>
<td>10 μM</td>
<td>5.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>17.5 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>81.2 ± 2.5</td>
</tr>
<tr>
<td>db-cAMP</td>
<td>250 μM</td>
<td>36.4 ± 0.5</td>
</tr>
<tr>
<td>Br-cAMP</td>
<td>500 μM</td>
<td>27.9 ± 0.2</td>
</tr>
</tbody>
</table>

*Brachyuryctymin (Br-cAMP) was the least active.*

*After preincubation (1 h, 37°C) with (100 μM) or without PTX in CM in the absence of serum, BMC from C3H/HeOU or C3H/HeJ mice were incubated for 24 h at 37°C with various concentrations of CPT-cAMP, db-cAMP, or Br-cAMP. Expression of LPS receptors was then detected by incubation (18 h, 4°C) with FITC-LPS (0.2 μg/ml) in medium containing 8% FCS. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic means ± standard deviations of duplicate samples.

The horizontal broken lines represent the cell response to LPS (10 ng/ml).

**FIG. 1. Influence of cAMP analogs on LPS receptor expression in BMC.** BMC (5 × 106 cells) from C3H/HeOU, or C3H/HeJ mice were incubated for 24 h at 37°C with various concentrations of CPT-cAMP, db-cAMP, or Br-cAMP. Expression of LPS receptors was then detected by incubation (18 h, 4°C) with FITC-LPS (0.2 μg/ml) in medium containing 8% FCS. The percentage of fluorescent cells was determined by FACS analysis of the gated granulocyte population. Values represent the arithmetic means ± standard deviations of duplicate samples.

The horizontal broken lines represent the cell response to LPS (10 ng/ml).
Induction of CD14 expression with iLpsR inducers.

We have previously established that after exposure to LPS, iLpsR and CD14 are concomitantly expressed on the cell surface in human (19) and murine (18) bone marrow granulocytes. It was therefore important to determine whether the other iLpsR inducers identified above (cAMP analogs and CT) also induce the expression of CD14. The results in Fig. 2 show that this is indeed the case. After incubation (24 h, 37°C) of BMC from C3H/HeOU mice with LPS (20 ng/ml), CPT-cAMP (100 μM), and CT (0.1 μg/ml), CD14 was detectable with the rat anti-mouse monoclonal antibody rmC5-3 by SDS-PAGE analysis of cell lysates (Fig. 2A) and by FACS analysis of intact cells (Fig. 2B, C, and D). In the following experiments, we will restrict our analyses to LPS receptors detectable with the fluorescent ligand FITC-LPS.

Endogenous cAMP is not involved in iLpsR expression.

To analyze the role of intracellular cAMP on iLpsR expression, we examined the influence of agents able to modulate directly the endogenous production of cAMP via activation or inhibition of adenyl cyclase. Using the cell-permeable adenyl cyclase activator forskolin, we found that intracellular concentrations of cAMP reached substantial levels (47 μM) in BMC exposed to 500 μM forskolin (Fig. 3A). However, the same concentration of forskolin alone did not induce iLpsR expression (data not shown) and did not enhance the LPS-induced effect (Fig. 3B). This shows that despite the stimulatory activities of the cAMP analogs, an increase in the intracellular levels of cAMP cannot induce the expression of LPS receptors in BMC. Furthermore, we found that the adenyl cyclase inhibitor DDA did not inhibit the expression of iLpsR induced by LPS (30.6 ± 0.1, 28.1 ± 0.1, and 35.9 ± 0.9 fluorescence units with 0, 250, and 1,000 μM DDA, respectively). Therefore, expression of iLpsR is not correlated with the intracellular level of cAMP. This conclusion was substantiated by the results of the estimation of intracellular levels of cAMP after exposure to LPS and CT. We found that exposure to LPS (10 ng/ml) had no effect on the level of intracellular cAMP. After exposure to CT, the intracellular concentration of cAMP (4.3 μM) was significantly higher than in unstimulated cells (1.0 μM) but remains much lower than that required for cell activation with CPT-cAMP (50 μM).

BMC stimulation with exogenous cAMP.

We showed above that cAMP analogs stimulated LPS receptor expression on BMC, but activation or inhibition of adenyl cyclase did not influence this expression. Thus, endogenously produced cAMP is ineffective, whereas exogenously administered cAMP analogs are active. This was confirmed by the observation that cAMP, which is not cell permeable, is nonetheless able to induce LPS receptor expression in BMC (25% iLpsR⁺ cells after exposure to 20 mM cAMP). The activity of cAMP is low compared to its analogs, but this is probably because of its higher sensitivity to phosphodiesterases, since its activity was...
markedly increased (two times) in the presence of the phosphodiesterase inhibitor PTX.

A cAMP receptor that cross-reacts with purine derivatives. The ability of a cell-impermeant agent (cAMP) to induce a cellular effect implies the existence of a cell surface receptor for this agent. The existence of a cell surface cAMP-binding protein on some mammalian cells has already been reported (14), and cross-reactivities between adenosine and cAMP and their respective receptors have been observed in Dictyostelium discoideum (13, 28). To determine whether a cell surface receptor for cAMP is present on BMC, we used tritium-labeled cAMP. To avoid degradation of the ligand by cell phosphodiesterases, all incubations were performed in the presence of 100 μM PTX, a phosphodiesterase inhibitor. In accord with our hypothesis, we found a specific (Fig. 4A), saturable (Fig. 4B), and reversible (Fig. 4C) binding of [3H]cAMP to BMC.

The affinity of the interaction between cAMP and the receptors was determined by fitting the data in Fig. 4B to the four-parameter Hill function with computer software. We found that at the temperature used (0°C), the apparent Kd was 2.7 × 10−5 M. Based on this Kd value and on the data in Fig. 4A applied to the Michaelis-Menten function, the calculated number of receptors was 1.8 × 106 molecules/cell. The binding of [3H]cAMP to BMC was also inhibited by the cAMP analog CPT-cAMP and by three other purine derivatives: adenosine, its 2-chloro derivative 2-CA, and IBMX (54% ± 2%, 36% ± 4%, 42% ± 4%, and 42% ± 2% inhibition with 13.5 μM concentrations of the agents, respectively). It should be noted that the binding was not inhibited by LPS or CT (data not shown), thus indicating that these agents do not stimulate the cells via an interaction with this cAMP receptor.

Involvement of a trypsin-sensitive unconventional purinoreceptor. To check that receptors for cAMP present on the cell surface are actually required for iLpsR expression induced by cAMP analogs, we examined the influence of pretreatment of the cells with trypsin. After incubation in the presence or absence of trypsin, BMC were exposed to LPS, CPT-cAMP, or CT. The expression of the LPS receptor CD14 induced by these agents was analyzed by Western blotting with the anti-mouse CD14 antibody rmC5-3. The results in Fig. 5 show that trypsin treatment abolished the expression of CD14 induced by CPT-cAMP. This demonstrates that a signaling receptor for cAMP analogs is present on the cell surface. The trypsin treatment also blocked the response to LPS. This is consistent with a previous study (7) showing that the constitutive LPS receptor of BMC is trypsin sensitive. In contrast, the responsiveness of the cells to CT was not modified by a trypsin treatment, as expected, since the unique receptor for CT is the ganglioside GM1 (6), which is trypsin resistant.

Because cAMP and three other purine derivatives (adenosine, 2-CA, and IBMX) can all bind to BMC, the idea that a purinoreceptor plays an important role in the induction of iLpsR expression becomes attractive. This receptor could interact with cAMP or with one of its degradation products. In this connection, it is known that extracellular cAMP can be sequentially converted into AMP (via cAMP phosphodiesterase) and adenosine (via 5’ nucleotidase). Thus, each cAMP...
induced expression of iLpsR in BMC, we examined the influence that cell-permeant cAMP analogs, like LPS, induced the expression of LPS receptors detectable with the labeled ligand FITC-LPS and with a specific anti-CD14 antibody. This result was unexpected, since elevation of intracellular cAMP levels usually inhibits LPS-induced effects (8, 16, 32). Only one LPS effect has been reported so far to be enhanced by cAMP analogs: the induction of NO production in macrophages (8, 16, 26). However, in BMC, LPS alone cannot induce NO production, and LPS-induced iLpsR expression is not reduced by inhibitors of NO biosynthesis (data not shown). It should be noted that the triggering of LPS receptor expression by cAMP analogs in BMC does not completely mimic the effect of LPS, since in contrast to the latter, the effect of cAMP was observed in both LPS-responsive (C3H/HeOU) and LPS-hyporesponsive (C3H/HeJ) mouse strains. Because CT has been reported to increase cAMP levels in different cell types via activation of adenylyl cyclase, we examined its effect on iLpsR expression. We found that, like cAMP analogs, CT induces iLpsR expression in BMC from C3H/HeOU and C3H/HeJ mice. Therefore, CT and cAMP analogs bypass the requirement of the functional Lpsn gene of C3H/HeOU mice by direct downstream activation of the signaling pathway.

Because CT catalyzes the ADP-riboseylation of the stimulatory G-protein Gsα, which in turn stimulates adenylyl cyclase and enhances cAMP levels, we wished to determine whether adenylyl cyclase is involved in the expression of iLpsR induced by the different activators. Using specific modulators, we found that adenylyl cyclase and intracellular cAMP are not involved in the phenomenon.

The expression of iLpsR independently of the intracellular production and level of cAMP suggested that the cAMP analogs can activate the cells at another location. An attractive possibility was that cell surface receptors for cAMP can play an important role in the induction of iLpsR expression. The observations that unmodified cAMP (which is cell impermeant) can also induce iLpsR and that trypsin-treated cells were unresponsive to CPT-cAMP (Fig. 5) supported this hypothesis. The existence of cell surface receptors for cAMP was assessed by the specific, saturable, and reversible binding of tritium-labeled cAMP (Fig. 4). This binding was not inhibited by CT or LPS, thus indicating that the binding site of cAMP is distinct from those of the CT receptor and the LPS receptors.

The observation that CPT-cAMP is more potent than cAMP for induction of iLpsR and for inhibition of [3H]cAMP binding suggests that cAMP is not the physiological agonist of the receptor involved in the biological response of the cell. Other agents, such as adenosine, its 2-chloro derivative 2-CA, and the methylxanthine derivative IBMX, inhibited the binding of [3H]cAMP. Because all these inhibitors are actually purine derivatives, this cross-reactivity suggests that cAMP and its analogs mediate their effects through the binding to a purino-receptor.

This family of receptors has been divided into two classes: adenosine receptors (P1 purinoreceptors) and adenine nucleotide (ADP and ATP) receptors (P2 purinoreceptors). P1 receptors have been subdivided into four families (A1, A2a, A2b, and A3), and P2 receptors have been subdivided into six families (P2X1, P2X2, P2X3, P2Y1, P2Y4, and P2Y6). Some of these purino-receptors have been shown to play a role in inflammation and in LPS-induced effects. For instance, adenosine analogs (17) and inhibitors of adenosine metabolism (5) were shown to protect mice against endotoxic shock. The potent anti-inflammatory properties of adenosine (3, 4) and its ability to enhance LPS-induced IL-10 secretion (11) and to inhibit LPS-induced production of TNF-α, IL-6, and IL-8 in human monocytes (1, 20) are mediated in part by P1 purinoreceptors. Occupancy of A1 (15), A2 (21), and A3 (22) receptors and even currently unknown adenosine receptors (11) has been suggested to play a role in these LPS-induced effects. To determine whether purinoreceptors also play a role in iLpsR expression, we used agonists and antagonists of these receptors. We found that...
both an agonist (2-CA) and an antagonist (IBMX) of P$_i$ receptors were able to induce iLpSR expression (Fig. 6). This paradoxical result may suggest that an unconventional purinoceptor is involved in this effect. The existence of such receptors has been suggested by different researchers (10–12).

Taken together, the results obtained in this study suggest that CAMP analogs, CT, and LPS induce the expression of iLpSR in BMC after interaction with different receptors: CAMP analogs induce this effect after interaction with a cell surface, trypsin-sensitive, CAMP binding site, which is probably cAMP analogs induce this effect after interaction with a cell receptor in BMC after interaction with different receptors: that cAMP analogs, CT, and LPS induce the expression of receptor is involved in this effect. The existence of such receptors of low affinity are constitutively expressed on mouse bone marrow cells. Immunology 1994. Induction of the P2x/P2X7 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma. Immunology 1997. Lipopolysaccharide and the glycosylated of tumor necrosis factor induce CD14 expression on bone marrow granulocytes by different mechanisms. Mol. Pharmacol. 52:692–700.


