Impaired Phagocyte Responses to Lipopolysaccharide in Paroxysmal Nocturnal Hemoglobinuria

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Bone marrow-derived cells from patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) show a defect in the expression of phosphatidylinositol-anchored membrane proteins, including the CD14 molecule. Blocking experiments with anti-CD14 monoclonal antibodies have shown that lipopolysaccharide (LPS)-induced tumor necrosis factor alpha production by monocytes depends on the interaction between CD14 and a complex formed by LPS and LPS-binding protein. We used a whole-blood model to examine the LPS-induced production of tumor necrosis factor alpha and interleukin-6 in PNH patients and healthy volunteers. At low endotoxin concentrations (1 ng/ml), PNH patients displayed a marked defect in the production of both cytokines, whereas at high LPS concentrations (100 ng/ml), cytokine production was similar to that in healthy volunteers. Using flow cytometry, we also studied the expression of the adhesion molecules Mac-1 (CD11b/CD18) and ICAM-1 (CD54) by monocytes and granulocytes after LPS stimulation. Compared with phagocytes from healthy volunteers, CD14-deficient cells showed poor Mac-1 and ICAM-1 upregulation when whole blood was stimulated with LPS (1 ng/ml), whereas their response to higher LPS doses (100 and 1,000 ng/ml) was essentially normal. The importance of the CD14 molecule in the activation of phagocytes by low LPS concentrations was confirmed by the inhibitory effect of an anti-CD14 antibody both in healthy volunteers and in PNH patients. Since these patients produce the soluble form of the CD14 molecule, these data suggest that soluble CD14 could play a role in phagocyte responses to LPS. We conclude that, in whole blood, phagocytes from PNH patients show impaired responsiveness to LPS and this phenomenon is most probably related to their defect in expression of membrane CD14.

Patients suffering from paroxysmal nocturnal hemoglobinuria (PNH) display a lack of phosphatidylinositol-anchored proteins at the surface of bone marrow-derived cells (11, 16). The CD14 membrane antigen, normally expressed on monocytes and to a lesser extent on granulocytes, is one of the molecules that are defective in PNH (9). CD14 is a high-affinity receptor for bacterial lipopolysaccharide (LPS) complexed to LPS-binding protein (21). The production of tumor necrosis factor alpha (TNF-α) by normal monocytes in response to LPS was shown to be blocked by anti-CD14 monoclonal antibodies (MAbs), suggesting that CD14 plays an important role in LPS-induced monocyte activation (19). However, other molecules at the surface of monocytes probably also function as LPS receptors (4, 8).

Phagocyte activation by LPS results in the upregulation of several membrane molecules, including complement receptor Mac-1 (also called CR3 and CD11b/CD18), a molecule also involved in leukocyte adherence to vascular endothelium (12). The increased adhesive capacity and expression of Mac-1 at the surface of LPS-stimulated granulocytes is inhibited by an anti-CD14 MAb, indicating that CD14 could also function as an LPS receptor for granulocytes (13, 20). The role of CD14 in LPS-induced upregulation of adhesion molecules in monocytes is presently unknown.

| TABLE 1. LPS-induced TNF-α and IL-6 production in whole blood from PNH patients and healthy volunteers* |
|---|---|---|
| Subject | % CD14+ monocytes | TNF-α (pg/ml) at LPS dose: | IL-6 (pg/ml) at LPS dose: |
| | | 0 | 1 ng/ml | 100 ng/ml | 0 | 1 ng/ml | 100 ng/ml |
| Patients | | | | | | | |
| 1 | 3 | 25 | 53 | 8,571 | 542 | 760 | 6,021 |
| 2 | 2 | 27 | 342 | 3,720 | 28 | 652 | 6,147 |
| 3 | 14 | 88 | 225 | 15,584 | 54 | 123 | 9,047 |
| Controls | 93-97 | 118 | 32 | 6,392 | 32 | 32 | 6,252 |
| | | 26 | 233 | 7,025 | 16 | 371 | 7,447 |
| | 54 ± 22 | 8,055 ± 1,040 | 19,450 ± 7,389 | 60 ± 41 | 4,821 ± 499 | 7,810 ± 1,353 |

* Whole blood was incubated for 2 h with medium alone or with LPS at 1 or 100 ng/ml. Cytokine values are corrected for the number of monocytes per cubic millimeter. Data for controls represent means ± SEM for eight different experiments. The results of two separate experiments are shown for patients 1 and 2.

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In vivo, the activation of circulating phagocytes by LPS probably depends both on its direct effect on these cells through specific receptors and on the induction of soluble mediators, such as cytokines and complement activation products. These different pathways of activation are operative in vivo when human whole blood is stimulated by LPS. To get insight into the role of CD14 in LPS-induced phagocyte activation, we therefore examined TNF-α and interleukin-6 (IL-6) production as well as Mac-1 expression by phagocytes from CD14-deficient PNH patients in a whole-blood model. In addition, we took advantage of these experiments to study the effect of LPS on the expression of monocyte ICAM-1, an adhesion molecule functioning as a ligand for LFA-1 (CD11a/CD18) and Mac-1 (6, 12).

**MATERIALS AND METHODS**

**Patients.** The experiments involved three patients affected by PNH and eight healthy volunteers. None of the PNH patients experienced hemolytic crisis or required blood transfusion during the 3 months preceding the study. In one of them (patient 3), normal peripheral blood cells (CD14+ monocytes and CD16+ granulocytes) were still present in addition to the abnormal cell population. All PNH patients and volunteers had normal values for leukocyte and differential counts, as determined with an automated cell counter (Coulter STKS; Coulter Electronics, Hialeah, Fla.). In every experiment, at least one healthy volunteer was studied together with a PNH patient.

**Reagents.** RPMI 1640 medium was purchased from Gibco (Grand Island, N.Y.). Heparin was obtained from Novo Industry A/S, Bagsvaerd, Denmark. The endotoxin content of RPMI medium and heparin was less than 2 pg/ml, as determined by a Limulus assay (LAL-QCL-1000; Whittaker M. A. Bioproducts). LPS from *Escherichia coli* O111:B4 was obtained from Sigma Chemical Co., St. Louis, Mo. Phycoerythrin-conjugated anti-CD11b (Mac-1) MAb Leu-M5, fluorescein isothiocyanate-labelled anti-CD14 MAb Leu-M3, and anti-ICAM-1 MAb CD54 were purchased from Becton Dickinson (Mountain View, Calif.). Anti-CD14 blocking MAb IOM2 was obtained from Immunotech, Marseille, France. Immunoglobulin G (IgG) isotype-matched control antibodies were obtained from Becton Dickinson, Coulter, and Immunotech.

**Whole-blood stimulation.** Blood obtained from PNH patients and healthy volunteers was collected in heparinized (10 U/ml) syringes. Whole blood was then incubated in polystyrene tube (Falcon 2051; Becton Dickinson) for 2 h at 37°C with 50 μl of RPMI medium alone or RPMI medium containing LPS (final concentration, 1 to 1,000 ng/ml) per ml. In some experiments, whole blood was incubated with anti-CD14 blocking MAB IOM2 (20 μg/ml) or an isotype-matched irrelevant MAb for 30 min before LPS stimulation.

**Flow cytometry analysis.** After incubation, aliquots (200 μl) of whole blood were suspended in phosphate-buffered saline supplemented with 0.5% bovine serum albumin and centrifuged at 2,000 rpm for 5 min. The pellets were then incubated either simultaneously with 10 μl of anti-Mac-1 (CD11b) or anti-ICAM-1 (CD54) MAB and 10 μl of anti-CD14 MAB or with fluoresceinated anti-CD14 MAB for 30 min at 4°C in the dark. In every experiment, cells were also incubated with IgG isotype-matched control antibodies. After lysis of erythrocytes (FACS Lysing Solution; Becton Dickinson), leukocytes were washed and fixed in 1% paraformaldehyde solution. Analysis with a FACScan flow cytometer (Becton Dickinson) was performed with forward light scatter and side scatter properties to acquire data for monocytes and granulocytes only (10^4 events). Specific cell fluorescence was then studied by side scatter properties to gate monocytes and granulocytes. This procedure gave results similar to those obtained by gating based on CD14 positivity. Changes in CD14 expression or side or forward scatter properties during experiments did not interfere with the gating of cells.

**Flow cytometry standardization microbeads.** (Alignment Microbead Standards; Flow Cytometry Standards Corporation, Research Triangle Park, N.C.) coated with a defined amount of fluorescein were used to establish that the mean fluorescence channels measured related linearly to the number of fluorescein molecules bound per cell (18). This al-

**FIG. 1.** LPS-induced monocyte Mac-1 and ICAM-1 upregulation in whole blood is defective on CD14-negative cells. Whole blood from healthy volunteers (●) and PNH patients (○) was incubated for 2 h with medium alone or with LPS (1 to 1,000 ng/ml). Monocytes were stained with phycoerythrin-conjugated anti-Mac-1 or fluoresceinated anti-ICAM-1 MAb, and fluorescence was measured by flow cytometry. Results are expressed as the change in MESF units compared with the value for monocytes incubated with medium alone (mean ± SEM for one representative experiment with three different donors).
allowed us to transform the mean fluorescence channels into units of mean equivalent soluble fluorescence (MESP).

**Determination of cytokine and soluble CD14 levels.** TNF-α and IL-6 levels in plasma were determined by an enzyme immunoassay with a sensitivity of 15 pg/ml (Medgenix, Fleurus, Belgium). Results were then corrected for monocytes and calculated for 500 monocytes per mm³. Monocytosis levels in PNH patients and healthy volunteers were 266 ± 68 and 333 ± 84 monocytes/mm³ (mean ± standard error of the mean [SEM]), respectively. Soluble CD14 (sCD14) levels were determined by an enzyme immunoassay with a sensitivity of 3 ng/ml (Immuno Biological Laboratories, Hamburg, Germany).

**Statistical analysis.** Statistical analysis was performed with the two-tailed Wilcoxon's rank-sum test on unpaired samples.

**RESULTS**

LPS-induced production of IL-6 and TNF-α is impaired in PNH. The production of IL-6 and TNF-α in whole blood was studied after 2 h of incubation with two different concentrations of LPS (Table 1) in two independent experiments. Compared with healthy individuals, PNH patients displayed a pronounced defect in the secretion of both IL-6 (318 ± 142 pg/ml versus 4,821 ± 499 pg/ml in healthy volunteers, \( P < 0.001 \)) and TNF-α (177 ± 59 pg/ml versus 8,055 ± 1,040 pg/ml in healthy volunteers, \( P < 0.001 \)) at low LPS concentrations (1 ng/ml). In contrast, when whole blood from PNH patients was incubated with LPS at 100 ng/ml, the production of TNF-α and IL-6 was similar to that in the healthy controls.

**Impaired upregulation of Mac-1 and ICAM-1 molecules on CD14-deficient monocytes.** The expression of the adhesion molecules Mac-1 and ICAM-1 on monocytes was measured after incubation of whole blood for 2 h in the presence and absence of LPS. As shown in Fig. 1, the upregulation of these adhesion molecules induced by low doses of LPS (1 ng/ml) at the surface of CD14-negative monocytes was lower than that observed in healthy individuals. This difference, observed in two independent experiments involving three different donors in each group, was statistically significant (\( P < 0.01 \) for both Mac-1 and ICAM-1). Figures 2a and b display histograms of monocyte Mac-1 and ICAM-1 induction by LPS (1 ng/ml) at the surface of monocytes from patient 2 and from a representative control. When whole blood was incubated with higher LPS concentrations (100 and 1,000 ng/ml), adhesion molecule upregulation on CD14-deficient monocytes was similar to that of CD14-positive cells from healthy controls (Fig. 1). The persistence of a normal bone marrow-derived cell population in PNH patient 3 allowed us to study the modulation of Mac-1 and ICAM-1 on CD14-positive and CD14-negative monocytes incubated in the same conditions. As shown in Fig. 3, CD14-positive monocytes showed normal upregulation of both Mac-1 and ICAM-1 after stimulation with LPS at 1 ng/ml, whereas CD14-negative cells behaved like monocytes from PNH patients 1 and 2. In control experiments, LPS did not increase the binding of an isotype-matched control MAb.

**Granulocyte Mac-1 upregulation induced by low doses of LPS is impaired in PNH.** Granulocytes from PNH patients are known to display, like monocytes, a defect in CD14 expression (11). These cells were also studied for Mac-1 expression after LPS stimulation in whole blood. In patient 3, as with monocytes, a normal granulocyte population was still represented, as demonstrated by the presence of CD16-positive cells (another membrane protein defective in PNH [11]) among CD15-positive cells (a marker of granulocytes [3]) (data not shown). The intensity of CD14 staining did not allow us to differentiate normal and abnormal cells from this patient. Therefore, only granulocytes from patients 1 and 2 were studied for LPS-induced Mac-1 upregulation. Figure 4 shows that Mac-1 upregulation at the surface of normal granulocytes was already maximal after stimulation with LPS at 1 ng/ml, whereas granulocytes from PNH patients showed only marginal upregulation by this low LPS dose. This difference, observed in two independent experiments, was statistically significant (\( P < 0.05 \)). Stimulation of whole blood from PNH patients with 100 and 1,000 ng of LPS per ml induced granulocyte Mac-1 upregulation similar to that in blood from healthy controls. Figure 2c displays histograms of granulocyte Mac-1 induction by LPS (1 ng/ml) in samples from patient 2 and from a representative control. In control experiments, LPS did not increase the binding of an isotype-matched control MAb.

**Anti-CD14 blocking MAB inhibits phagocyte activation by low doses of LPS.** To further study the role of the CD14
molecule in the activation of phagocytes by various doses of LPS, we evaluated the effects of an anti-CD14 blocking MAb on LPS-induced Mac-1 upregulation and cytokine production in whole blood. We first verified that staining of phagocytes with MAb IOM2 gave the same results as MAb Leu-M3 in both healthy individuals and PNH patients (data not shown). The data presented in Table 2 indicate that, at a high LPS concentration (100 ng/ml), Mac-1 upregulation and TNF-α production were not reduced by addition of the anti-CD14 MAb in either healthy volunteers or PNH patients, while a slight inhibition of IL-6 production was observed. In contrast, at an LPS dose of 1 ng/ml, the anti-CD14 MAb clearly inhibited the responses of normal phagocytes, confirming the major role of CD14 in this setting. Interestingly, the low phagocyte activation induced by LPS at 1 ng/ml in PNH patients was also inhibited by the addition of anti-CD14 MAb, as assessed by Mac-1 expression in all three patients and by cytokine production in patient 1 (the cytokine production induced by LPS at 1 ng/ml in patients 2 and 3 was too low to quantitate the inhibition by the anti-CD14 MAb). This effect of the anti-CD14 MAb on CD14-deficient phagocytes might be related to the production of soluble sCD14 molecules by these cells (10). Indeed, we observed the presence of sCD14 in the plasma of these

**FIG. 3.** Differential Mac-1 and ICAM-1 upregulation on CD14-negative and CD14-positive monocytes from the same PNH patient. Whole blood from PNH patient 3 was incubated for 2 h with various concentrations of LPS. Monocytes were stained as described in the legend to Fig. 1. Mac-1 and ICAM-1 expression on CD14-positive (●) and CD14-negative (○) monocytes was studied. Results are expressed as the change in MESF units compared with the value for monocytes incubated with medium alone.

**FIG. 4.** LPS-induced granulocyte Mac-1 upregulation in whole blood is defective in PNH patients. Whole blood from healthy volunteers (●) and PNH patients (○) was incubated for 2 h with medium alone or with LPS (1 to 1,000 ng/ml). Granulocytes were stained with phycoerythrin-conjugated anti-Mac-1 MAb, and fluorescence was measured by flow cytometry. Results are expressed as the change in MESF units compared with the value for granulocytes incubated with medium alone (mean ± SEM for two independent experiments, each involving two different donors).

**TABLE 2.** Inhibition of LPS-induced phagocyte activation by the anti-CD14 blocking MAb

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<thead>
<tr>
<th>Group and LPS dose (ng/ml)</th>
<th>Mean % inhibition ± SEM</th>
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<tbody>
<tr>
<td></td>
<td>Monocyte Mac-1</td>
</tr>
<tr>
<td>Controls</td>
<td>≤0</td>
</tr>
<tr>
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<td>68 ± 20</td>
</tr>
<tr>
<td>PNH patients</td>
<td>8 ± 4</td>
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<tr>
<td>1</td>
<td>70 ± 43</td>
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*Whole blood was stimulated for 2 h with LPS (1 or 100 ng/ml) after no treatment and after 30 min of incubation with anti-CD14 MAb IOM2 (20 μg/ml). Results are expressed as percent inhibition by the anti-CD14 MAb of Mac-1 upregulation or cytokine release induced by LPS (mean ± SEM for data obtained from three different donor patients). A value of ≤0% indicates no inhibition or stimulation. In these experiments, an irrelevant isotype-matched MAb did not inhibit phagocyte responses to LPS (data not shown).

* Determined only for patient 1 because of the very poor stimulation achieved with LPS at 1 ng/ml in patients 2 and 3.
PNH patients at concentrations similar to or even higher than those in healthy volunteers (7,219 ± 725 in PNH patients versus 3,985 ± 23 ng/ml in healthy volunteers, mean ± SEM for three individuals in each group).

DISCUSSION
The present study indicates that CD14-negative phagocytes from PNH patients display impaired responsiveness to LPS in a whole-blood model. First, the induction of TNF-α production by LPS at a concentration of 1 ng/ml was markedly reduced in whole blood from PNH patients compared with that in blood from healthy volunteers, whereas at a higher dose of LPS (100 ng/ml), TNF-α production was normal. Together with the inhibitory effect of the anti-CD14 MAb on TNF-α production elicited by a low dose of LPS in normal individuals (4, 21; also this study), these data support a crucial role for CD14 in LPS-induced TNF-α production. Our experiments also showed that, as for TNF-α, IL-6 production induced by LPS at 1 ng/ml is low in PNH patients but reaches normal values after stimulation with LPS at 100 ng/ml. In addition, IL-6 production in normal individuals was also inhibited by the anti-CD14 MAb, suggesting that, at low concentrations, LPS stimulates the production of both cytokines through the same receptors. Recent experiments performed with isolated cells support this hypothesis (5).

The adhesion molecule Mac-1 plays a central role in the adhesion of both neutrophils and monocytes to vascular endothelium (12). Upregulation of Mac-1 expression at the surface of monocytes and granulocytes can be induced in vitro on isolated cells by stimulation with LPS, TNF-α, and complement system activation product C5a (2, 14). Our results demonstrate that, in a whole-blood model, monocyte Mac-1 upregulation by low LPS concentrations is dependent on the presence of CD14 molecules on the surface of these cells. The data obtained from studies with the patient with both normal and abnormal monocyte populations show that the defect in Mac-1 induction observed in PNH patients after stimulation with LPS at 1 ng/ml is not related to the reduced production of secondary mediators such as TNF-α, since CD14-positive monocytes showed normal upregulation. Interestingly, granulocytes from PNH patients also display a defect in Mac-1 upregulation in response to low doses of LPS. This defect could be directly related to the lack of CD14 molecules at the granulocyte surface or could be a consequence of the decreased TNF-α production. Since the anti-CD14 blocking MAb has been shown to inhibit Mac-1 upregulation by LPS on isolated granulocytes (13), we favor the first hypothesis.

We also observed that LPS increased ICAM-1 expression on the surface of monocytes and that this phenomenon is dependent on the presence of CD14 molecules on these cells. Monocyte ICAM-1, a ligand for the LFA-1 and Mac-1 adhesion molecules (6, 12), has been shown to be involved, together with class II major histocompatibility complex molecules, in the presentation of antigens to T cells (1). Thus, LPS could modulate monocyte antigen-presenting function through its binding to CD14 molecules.

Although we cannot exclude the involvement of other phosphatidylinositol-anchored proteins, our data support the concept that CD14 molecules play a major role in phagocyte responses to concentrations of LPS within the range commonly found in patients suffering from gram-negative sepsis (17). At higher LPS concentrations, other receptors are clearly sufficient to trigger maximal release of TNF-α and adhesion molecule upregulation. Among these receptors, the recently described 73-kDa LPS-binding molecule on the surface of human peripheral blood cells could be important (8). As far as IL-6 is concerned, the slight inhibition induced by the anti-CD14 MAb at an LPS dose of 100 ng/ml suggests that the production of this cytokine remains partially CD14 dependent even at this high LPS concentration.

The ability of the anti-CD14 MAb to further reduce the low-grade activation of CD14-deficient phagocytes induced by LPS at 1 ng/ml could be related to the production of sCD14 molecules by these cells. Indeed, sCD14 levels in PNH patients were similar to those observed in healthy volunteers, confirming the capacity of cells from PNH patients to produce unanchored CD14 proteins (10). sCD14 was found in other models to mediate LPS-induced activation of CD14-negative cells (7, 15). Further investigations should clarify the role of sCD14 in phagocyte responses to LPS.

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J. Duchow and A. Marchant contributed equally to this study.

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