

## NOTES

# Polymyxin B Stimulates Production of Complement Components and Cytokines in Human Monocytes

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**Polymyxin B (PmB), an agent often used to neutralize the effects of bacterial lipopolysaccharide (LPS), was shown to exert a dose-dependent stimulatory effect on the biosynthesis of C3, factor B, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in human monocytes. A low dose of PmB (1 to 5 µg/ml) efficiently suppressed the LPS-induced (1 or 100 ng/ml) production of IL-6, GM-CSF, and factor B, but not the C3 production induced by 100 ng of LPS per ml. A reduced level of GM-CSF may have contributed to the persisting high C3 concentrations and the apparent lack of LPS inhibition in the latter situation, since GM-CSF is an inhibitor of monocyte C3 biosynthesis.**

Polymyxin B (PmB) is a polycationic antibiotic which binds to the lipid A portion of bacterial lipopolysaccharide (LPS) and which thereby blocks many well-known biologic and toxic LPS effects (4, 13). Therefore, this antibiotic has been evaluated in the treatment of bacterial septic shock in animal models (1). PmB is also frequently added to *in vitro* cell cultures in order to neutralize possible LPS contamination (5, 6). However, studies have shown that PmB by itself is able to stimulate some cellular functions, for example, monocyte production of interleukin-1 (IL-1) (3).

Monocytes/macrophages are important contributors of extrahepatic complement factor production, and this biosynthesis is stimulated by LPS from the cell walls of gram-negative bacteria, yeasts, and streptococci (8, 11, 15). Moreover, basal and pathogen-induced complement production in monocytes is regulated by a variety of cytokines such as interferons, granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage-colony stimulating factor (9, 12). Local complement activation results in the formation of opsonins, chemotaxins, and the membrane attack complex C5b-9, factors which are all important during infection and inflammation (10).

The purpose of the investigation described here was to examine whether PmB has any effect on constitutive and LPS-induced monocyte complement factor and cytokine production. Monocytes were isolated from human buffy coats by density gradient centrifugation as described previously (12), adjusted to  $4 \times 10^6$  cells per ml in X-VIVO 10 serum-free medium (Whittaker Bioproducts, Walkersville, Md.), and seeded in cell culture plates (Costar, Cambridge, Mass.). Nonadherent cells were removed after 4 h by two vigorous washes in Hanks' balanced salt solution (Whittaker Bioproducts), and the culture medium was replaced with or without LPS (lyophilized powder prepared by phenol extraction from *Escherichia coli* serotype O111:B4; Sigma Chemical Co., St. Louis, Mo.) or PmB (Sigma) or the combination of LPS and PmB. LPS contamination of the PmB was less than 6 pg/ml for the dilutions used, as assayed by a *Limulus* amoebocyte lysate chromogen

test. Moreover, boiling of PmB efficiently suppressed its stimulatory activity (data not shown). The adherent cell population consisted of more than 85% monocytes; contaminating cells were mainly lymphocytes. Supernatants were harvested after 2 or 5 days, centrifuged, and frozen at  $-70^\circ\text{C}$ . More than 90% of the cultured cells were viable at the end of cell culture as judged by trypan blue exclusion. Quantitation of antigenic human C3 and factor B in monocyte supernatants was performed by previously described double-antibody enzyme immunoassays (12). A polyclonal goat anti-human GM-CSF antibody, a normal goat immunoglobulin G (IgG) antibody, and enzyme immunoassay kits for the detection of GM-CSF and IL-6 were purchased from R&D Systems Europe, Oxon, United Kingdom. The cytokine kits have detection limits of 1.5 and 0.35 pg/ml, respectively, and the coefficient of variation was  $<5\%$  within days and  $<10\%$  between days at low and high concentrations for both kits. Recombinant human GM-CSF was generously provided by Schering-Plough Research, Bloomfield, N.J. The statistical significance of differences between test groups was analyzed by a two-tailed Wilcoxon rank sum test.

**Effect of PmB on monocyte complement and cytokine production.** We found that PmB has a stimulatory effect on monocyte complement biosynthesis. Specifically, a dose-dependent increase in monocyte C3 production, significant at PmB doses of 5 µg/ml and higher, was observed (Table 1). Such doses are frequently used *in vitro* to neutralize possible LPS contamination (14). Also, a stimulatory effect on factor B production was found; the effect was significant at PmB doses of 5 µg/ml and higher (Table 2). Moreover, PmB exerted a dose-dependent stimulatory effect on IL-6 production (Table 3), in accordance to what has been reported previously for IL-1 (3). At a high dose of PmB (50 µg/ml), even the release of GM-CSF was augmented in some but not all donors (Table 3). Thus, PmB stimulates monocytes to produce increased amounts of both complement factors and cytokines, factors which may be essential during a local inflammatory response. A similar stimulatory effect on monocyte cytokine production has been described previously for amphotericin B (2). Both PmB and amphotericin B are surface-active agents which may intercalate themselves within cell membranes, thereby altering membrane permeability. Further studies are necessary to clarify the mech-

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TABLE 1. Effect of PmB on basal and LPS-induced C3 production<sup>a</sup>

LPS concn (ng/ml)	PmB concn (μg/ml)	C3 concn (ng/ml)	
		Day 2	Day 5
0	0	1.8 (0.4–3.3; 7)	7.0 (1.2–10.3; 13)
0	1	3.3 (1.1–3.9; 4)	6.1 (<0.4–12.6; 10)
0	5	3.1 (1.9–7.5; 7) <sup>b</sup>	14.0 (2.1–20.4; 13) <sup>c</sup>
0	20	6.4 (3.6–17.0; 7) <sup>b</sup>	21.6 (9.9–40.5; 13) <sup>c</sup>
0	50	6.6 (3.2–20.1; 7) <sup>b</sup>	31.8 (8.3–55.3; 13) <sup>c</sup>
1	0	6.8 (1.1–15.8; 7) <sup>b</sup>	29.6 (12.7–56.2; 11) <sup>c</sup>
1	1	2.3 (<0.4–3.7; 5)	8.8 (2.6–15.5; 9) <sup>d</sup>
1	5	3.3 (1.3–7.4; 7) <sup>d</sup>	13.5 (9.5–19.2; 7) <sup>d</sup>
1	20	6.7 (3.1–16.0; 7)	24.8 (16.6–34.4; 7) <sup>d</sup>
1	50	5.4 (1.5–19.8; 7)	30.2 (18.3–75.6; 11)
100	0	4.6 (1.3–20.7; 6) <sup>b</sup>	26.0 (8.8–39.8; 10) <sup>c</sup>
100	1	5.5 (0.7–11.0; 5)	30.4 (17.3–77.5; 9)
100	5	7.7 (1.8–16.2; 6)	29.2 (19.7–67.2; 8)
100	20	8.5 (3.0–22.6; 6)	34.7 (25.2–56.5; 8)
100	50	4.9 (2.7–30.9; 6)	35.0 (22.7–63.5; 10)

<sup>a</sup> Monocytes were cultured with PmB or LPS alone or in combination from the initiation of cell culture. Supernatants were harvested after 2 or 5 days and were analyzed for their C3 contents. Data are median (range; number of experiments).

<sup>b</sup> Significantly increased ( $P < 0.05$ ) compared with untreated control cells.

<sup>c</sup> Significantly increased ( $P < 0.01$ ) compared with untreated control cells.

<sup>d</sup> Significantly decreased ( $P < 0.05$ ) compared with LPS-treated cells without PmB.

agents of a stimulated cytokine production by these two different antimicrobial agents.

**Effect of PmB on LPS-induced cytokine and complement production.** LPS is known to be a potent stimulator of monocyte production of cytokines and complement factors (4, 12,

TABLE 2. Effect of PmB on basal and LPS-induced factor B production<sup>a</sup>

LPS concn (ng/ml)	PmB concn (μg/ml)	Factor B concn (U/ml)
		Day 5
0	0	2.1 (<1.1–12.3; 9)
0	1	3.1 (<1.1–14.2; 8)
0	5	4.0 (1.5–13.5; 9) <sup>b</sup>
0	20	9.0 (2.4–21.4; 9) <sup>c</sup>
0	50	9.5 (1.6–46.5; 9) <sup>b</sup>
1	0	9.8 (3.2–36.5; 11) <sup>c</sup>
1	1	4.7 (1.4–11.1; 10) <sup>d</sup>
1	5	3.1 (2.4–18.0; 6) <sup>c</sup>
1	20	7.2 (5.0–48.2; 6)
1	50	13.3 (1.1–44.4; 11)
100	0	20.6 (6.2–56.3; 11) <sup>c</sup>
100	1	8.2 (1.9–30.6; 10) <sup>d</sup>
100	5	8.3 (3.4–18.8; 10) <sup>d</sup>
100	20	13.3 (6.0–38.0; 10) <sup>c</sup>
100	50	14.7 (1.7–41.1; 11) <sup>c</sup>

<sup>a</sup> Monocytes were cultured with PmB or LPS alone or in combination from the initiation of cell culture. Supernatants were harvested after 5 days and were analyzed for their factor B contents. Data are median (range; number of experiments).

<sup>b</sup> Significantly increased ( $P < 0.05$ ) compared with untreated control cells.

<sup>c</sup> Significantly increased ( $P < 0.01$ ) compared with untreated control cells.

<sup>d</sup> Significantly decreased ( $P < 0.01$ ) compared with LPS-treated cells without PmB.

<sup>e</sup> Significantly decreased ( $P < 0.05$ ) compared with LPS-treated cells without PmB.

TABLE 3. Effect of PmB on basal and LPS-induced GM-CSF and IL-6 production<sup>a</sup>

LPS concn (ng/ml)	PmB concn (μg/ml)	GM-CSF concn (pg/ml) on day 5	IL-6 concn (pg/ml) on day 5
		0	0
0	1	<1.5 (<1.5–<1.5; 4)	5.6 (2.9–6.2; 4)
0	5	<1.5 (<1.5–<1.5; 3)	9.8 (5.2–11.3; 3)
0	20	<1.5 (<1.5–<1.5; 3)	54.1 (7.8–87.3; 3)
0	50	163.2 (<1.5–531.9; 4)	9,504.0 (114.0–15,620.0; 4)
1	0	<1.5 (<1.5–1.9; 4)	231.4 (12.3–626.1; 4)
1	1	<1.5 (<1.5–<1.5; 4)	3.3 (0.8–8.2; 4)
1	50	73.4 (<1.5–562.3; 4)	5,507.5 (317.1–20,960.0; 4)
100	0	755.0 (40.6–1,349.5; 4)	24,410.0 (12,635.0–37,640.0; 4)
100	1	<1.5 (<1.5–2.3; 4)	65.1 (6.5–1,733.0; 4)
100	5	<1.5 (<1.5–<1.5; 3)	147.1 (30.2–264.1; 3)
100	20	<1.5 (<1.5–4.3; 3)	159.5 (3.8–930.4; 3)
100	50	189.7 (<1.5–884.2; 4)	12,501.0 (1,090.0–21,340.0; 4)

<sup>a</sup> Monocytes were cultured with PmB or LPS alone or in combination from the initiation of cell culture. Supernatants were harvested after 5 days and analyzed for their GM-CSF and IL-6 contents. Data are median (range; number of experiments).

15). In a serum-free monocyte culture, 1 ng of LPS per ml is a sufficient dose to induce IL-6 production but not GM-CSF production by the cells (Table 3). At 100 ng/ml, LPS stimulated the production and release of considerable amounts of both IL-6 and GM-CSF in the monocytes from all donors examined (Table 3). It is well known that LPS-induced cytokine production may be efficiently inhibited by PmB (4, 14). As shown in Table 3, 1 to 20 μg of PmB per ml suppressed LPS-induced IL-6 and GM-CSF release by more than 95%. However, a higher PmB dose (50 μg/ml) added together with LPS again stimulated the release of IL-6 and GM-CSF by monocytes (Table 3).

The effect of PmB on LPS-induced complement production was even more complex. LPS is a potent stimulator of monocyte C3 (Table 1) and factor B (Table 2) production. When low doses of PmB were added together with 1 ng of LPS per ml, a significant inhibition of both LPS-induced C3 and factor B biosynthesis was found (Tables 1 and 2). When a higher dose of PmB (50 μg/ml) was used, the C3 and factor B concentrations obtained in the monocyte supernatants were again high (Tables 1 and 2), similar to the observations for cytokine production (Table 3). A possible explanation for these findings is that the intrinsic, dose-related stimulatory capacity of PmB is responsible for the augmented cytokine and complement factor release compared with that from the control, even when the stimulatory effect of LPS is neutralized.

In contrast to this, the increased level of C3 production found after treatment with a high dose of LPS (100 ng/ml) was not suppressed by any dose of PmB (Table 1). The reason for this result may be that PmB is unable to suppress the effects of this relatively high dose of LPS on C3 production. However, the cytokine and factor B release induced by 100 ng of LPS per ml was efficiently inhibited by PmB (Tables 2 and 3), and we therefore speculated whether other mechanisms could also be involved. We have previously shown that GM-CSF suppresses basal and LPS-induced C3 production (12). Since PmB inhibited the LPS-induced release of GM-CSF, the decreased levels of this cytokine combined with the intrinsic stimulatory effect of PmB could have contributed to the persisting high C3 concentrations compared with that from the control.

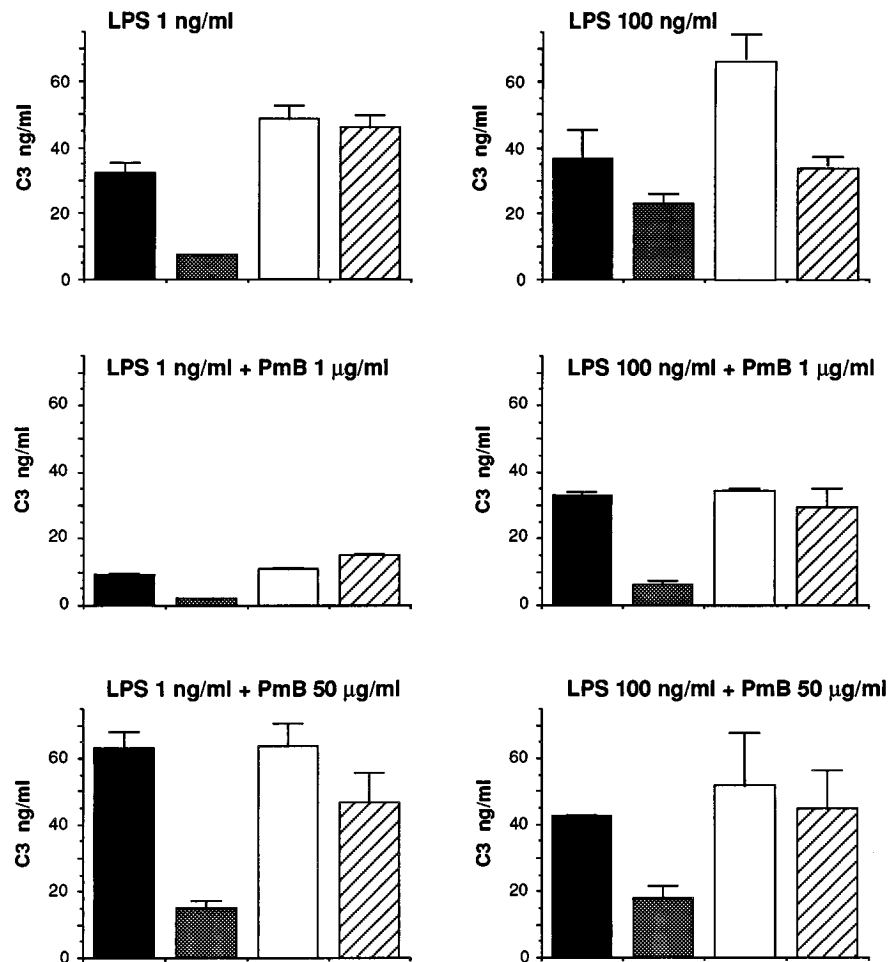


FIG. 1. LPS (1 or 100 ng/ml), PmB (1 or 50 µg/ml), recombinant human GM-CSF (10 ng/ml), anti-GM-CSF (20 µg/ml), and control IgG (20 µg/ml) were added at the initiation of monocyte culture, and supernatants were harvested after 5 days for determination of their C3 contents as described in Materials and Methods. Data from one of four representative experiments are shown and are expressed as means  $\pm$  standard deviations of duplicate monocyte cultures. All cultures contained LPS with or without PmB as indicated. ■, no further additives; ▨, GM-CSF; □, anti-GM-CSF; ▤, control IgG.

To support this hypothesis, we added either recombinant human GM-CSF (10 ng/ml), an anti-GM-CSF antibody (20 µg/ml), or a normal goat IgG (20 µg/ml) to monocyte cultures treated with LPS in the presence or absence of PmB. This anti-GM-CSF antibody neutralized the inhibitory effect of 10 ng of GM-CSF per ml on monocyte C3 production, while the control IgG had no effect (data not shown). As described earlier (12) and as shown in Fig. 1, recombinant GM-CSF is more effective in suppressing LPS-induced C3 production obtained at low doses of LPS (1 ng/ml) than that obtained at high doses (100 ng/ml), probably because endogenous GM-CSF has nearly saturated the inhibitory effects of this cytokine in the latter case. However, GM-CSF efficiently suppressed the C3 production induced by the combination of 100 ng of LPS per ml and 1 µg of PmB per ml (Fig. 1), which is a culture condition under which no endogenous GM-CSF was released, according to the data presented in Table 3.

The addition of an anti-GM-CSF antibody together with LPS at 100 ng/ml resulted in increased monocyte C3 production, probably because of the neutralization of LPS-induced endogenous GM-CSF (Fig. 1). Since there was no endogenous GM-CSF present in monocyte cultures treated with the combination of LPS and low doses of PmB, the addition of anti-GM-CSF to such cultures had no effect (Fig. 1). However,

there seemed to be a small but consistent stimulatory effect of anti-GM-CSF added together with LPS at 100 ng/ml with PmB at 50 µg/ml, indicating neutralization of endogenous GM-CSF, which is in agreement with the results presented in Table 3.

In contrast to what was observed for C3, the LPS-induced (100 ng/ml) factor B production was suppressed by both low and high doses of PmB (Table 2). This indicates that although recombinant GM-CSF suppresses both LPS-induced C3 and factor B production (12), this cytokine may be less important in the autocrine regulation of monocyte factor B biosynthesis than in the regulation of C3 production.

Taken together, we have demonstrated that PmB has a dose-dependent stimulatory effect on monocyte C3, factor B, IL-6, and GM-CSF production. This is in agreement with a previous report describing a stimulatory effect of PmB on monocyte IL-1 production at doses higher than 10 µg/ml (3). Moreover, we have shown that PmB inhibits well-known effects of LPS but that PmB may be more effective in suppressing cytokine and factor B production than C3 production. The resulting low level of GM-CSF, an inhibitor of monocyte C3 production, may have contributed to the increased C3 concentrations in the supernatants of monocytes treated with the combination of a high dose of LPS and PmB compared with those in the supernatants of monocytes treated with LPS alone or untreated

control monocytes. The results of these experiments support the view that monocyte-produced GM-CSF regulates C3 biosynthesis through an autocrine negative feedback loop.

As already pointed out by others (3, 7), the direct and indirect effects of PmB on various cellular functions should be taken into consideration both when this agent is used to neutralize possible LPS contamination in a given test system and when it is used in the treatment of experimental septic shock.

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