INFECTION AND IMMUNITY, Mar. 1996, p. 1011–1015 Vol. 64, No. 3
0019-9567/96/$04.00+0
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Low-Dose Lipopolysaccharide (LPS) Pretreatment of Mouse Macrophages Modulates LPS-Dependent Interleukin-6 Production In Vitro

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Received 10 May 1995/Returned for modification 20 July 1995/Accepted 27 November 1995

Lipopolysaccharide (LPS) can induce mouse macrophages to produce a number of cytokines and other inflammatory mediators. Our laboratory previously reported that LPS-dependent macrophage-derived tumor necrosis factor alpha (TNF-α) production could be significantly potentiated by pretreatment with LPS at substimulatory LPS priming doses. The observed potentiation was shown to be coincident with a down-regulation of LPS-dependent nitric oxide (NO) production (X. Zhang and D. C. Morrison, J. Exp. Med. 177: 511–516, 1993). In order to determine whether these LPS reprogramming effects in mouse macrophages were selective for these two macrophage-derived mediators, we have examined the effects of LPS pretreatment on LPS-dependent interleukin 6 (IL-6) production. Thioglycolate-elicited mouse peritoneal macrophages were pretreated with various subthreshold stimulatory concentrations of LPS for 6 h, washed three times, and then stimulated with an effective stimulatory concentration of smooth LPS for 18 h. In confirmation of earlier studies, pretreatment of mouse macrophages with substimulatory doses of LPS inhibited the subsequent LPS-dependent NO production. This down-regulation was accompanied by a coordinate up-regulation of LPS-dependent IL-6 production, similar to what was shown earlier for TNF-α production. These priming effects with the substimulatory dose of smooth LPS are shown to be independent of doses of LPS used for subsequent activation and are not restricted to specific LPS stimulation. Moreover, the enhancement of the IL-6 response by LPS pretreatment is still observed in the presence of neutralizing antibody to TNF-α. These findings, therefore, provide further support for the conclusion that LPS-dependent macrophage reprogramming is likely to involve common regulatory pathways that control the secretion of both IL-6 and TNF-α.

Bacterial lipopolysaccharide (LPS) characteristically induces a variety of pathophysiologic effects on a wide variety of mammalian cells. These effects can result in endotoxic shock and, ultimately, multiple organ failure and death (16). Most endotoxic effects are known to be mediated through the activation of host immune and inflammatory cells, particularly mononuclear phagocytes, and these cells are thought to play a central role in endotoxicity through the production of numerous bioactive mediators, including tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), IL-6, and nitric oxide (NO).

To investigate the mechanisms of macrophage activation that result in the production of these cytokines and inflammatory mediators, many investigators have explored the concept of modulation of macrophage activation via various pretreatment strategies. Mathison et al. reported that rabbit macrophages stimulated in vitro and in vivo with LPS became refractory to subsequent LPS stimulation, as determined by assessing the cells for secondary TNF-α release (14). This phenomenon has been termed LPS-induced adaptation, which is also known as tolerance or desensitization. On the other hand, Pabst and Johnston reported that mouse macrophages pretreated with LPS manifested a significant increase in the generation of superoxide anion upon subsequent stimulation with phorbol myristate acetate (17). This phenomenon has been termed LPS-induced priming. These selective priming and desensitization effects of LPS on macrophage function have been well characterized in a variety of different cells, including peritoneal macrophages (6, 13, 23, 29), macrophage/monocyte-like cell lines (7, 25, 30), and human monocytes (2, 9, 15).

Our laboratory has previously reported that pretreatment of mouse macrophages with substimulatory doses of LPS significantly enhanced LPS-dependent TNF-α production and suppressed LPS-dependent NO production (27). We have concluded that these selective priming and tolerizing effects are differential manifestations of a more generalized macrophage reprogramming event. In order to extend the characterization of these pretreatment effects in mouse macrophages, we have now examined the capacity of substimulatory concentrations of LPS to modify LPS-dependent IL-6 production as well. The results of these studies support the concept that macrophage reprogramming may reflect a more generalized effect of a response to the external environment which mobilizes macrophages for selective cytokine and/or mediator production.

Animals. Female C3HeB/FeJ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and used at 6 to 20 weeks of age.

Thioglycolate-elicited peritoneal macrophages. Mice were injected intraperitoneally with 1.5 ml of 4% (wt/vol) Brewer thioglycolate broth (Difco Laboratories, Inc., Detroit, Mich.). Five days later, cells were harvested by peritoneal lavage with RPMI 1640 (JRH Biosciences, Lenexa, Kans.) containing 100 U of streptomycin (JRH Biosciences) per ml, 100 μg of penicillin (JRH Biosciences) per ml, and 4.0 mM glutamine (JRH Biosciences). The cells were washed twice, and 10^6 peritoneal cells per ml in the same medium containing 10% heat-inacti-

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vated fetal bovine serum (endotoxin concentration, <0.0125 ng/ml; Atlanta Biologicals, Norcross, Ga.) were then added to each well of 24-well cluster plates (Costar Corp., Cambridge, Mass.). The cells were incubated overnight at 37°C in a humidified 5% CO_2 incubator to allow macrophages to adhere to the plates. The plates were then washed twice with 0.5 ml of warm Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (JRH Biosciences) to remove nonadherent cells.

Stimulation of mouse peritoneal macrophages. *Escherichia coli* O111:B4 smooth LPS (S-LPS) was purchased from List Biological Laboratories (Campbell, Calif.). Recombinant gamma interferon (rIFN-γ) was purchased from Schering-Plough through the American Cancer Society (Atlanta, Ga.). rTNF-α was purchased from Pharmingen (San Diego, Calif.). Stock solutions of LPS were prepared in sterile double-distilled water at 2.5 mg/ml, sonicated for 3 min, and then diluted into working solutions with cell culture medium. The cells were then incubated at 37°C in a humidified 5% CO_2 incubator for 18 h. Supernatants from three identically treated wells were pooled and assayed for the presence of IL-6, TNF-α, and/or NO. In priming experiments, macrophages were pretreated with various subthreshold stimulatory concentrations of LPS for 6 h, washed three times with 0.5 ml of HBSS, and then stimulated with an effective stimulatory concentration of LPS and, in some instances, a combination of rIFN-γ and rTNF-α for 18 h. For some experiments, subsequent LPS-dependent stimulation of mouse macrophages was carried out in the presence of either rabbit neutralizing anti-TNF-α polyclonal antibodies (a gift from R. E. McCallum, Texas A&M University, College Station) or control rabbit immunoglobulin G and immunoglobulin M (purified by our laboratory). The amount of IL-6 in each well was measured by proliferation of the IL-6-dependent 7TD1 mouse hybridoma cell line (American Type Culture Collection, Rockville, Md.), as previously described (24) with slight modification (3), and the amount of TNF-α was quantified by the L929 bioassay (20). The amount of NO was determined by measuring the amount of nitrite, a metabolic product of NO (22), in culture supernatants. In brief, 100 μl of Griess reagent (1:1, [vol/vol] 0.1% *N*-[1-naphthyl]ethylenediamine dihydrochloride [Sigma Chemical Co.] in H_2O and 1.0% sulfanilamide [Sigma Chemical Co.] in 5% H_2PO_4) was added to 100 μl of culture supernatant in each well of 96-well culture plates. The A_{570} was measured with a microplate reader. The amount of nitrite in culture supernatants was calculated from an $NaNO_2$ standard curve.

Preliminary studies have established that approximately 0.5 to 1.0 ng of S-LPS per ml is the minimal threshold dose required to induce IL-6 production in thioglycolate-elicited mouse macrophages. These concentrations of S-LPS are also the approximate concentrations below which NO responses in thioglycolate-elicited mouse macrophages cannot be detected (27). It is on concentrations of LPS below 1.0 ng/ml, therefore, that we have focused in order to assess LPS-dependent reprogramming of the macrophage response to subsequent activating doses of LPS.

Experiments were thus carried out to determine the effects of LPS-dependent reprogramming in mouse macrophages following pretreatment with different substimulatory doses of S-LPS. For these studies, macrophages were pretreated with various substimulatory doses of LPS and then challenged with stimulatory doses of 100 ng of S-LPS per ml; the effects of LPS pretreatment on secretion of IL-6 and NO were then determined and compared. The results of one such experiment are shown in Fig. 1, and they demonstrate that, as shown previously, pretreatment of mouse macrophages with substimulatory doses of LPS significantly inhibits subsequent LPS-dependent NO production, with reductions in NO production of up to 85% compared with that of non-LPS-pretreated control macrophages. In contrast to these results, equivalent pretreatment conditions with LPS significantly enhanced LPS-dependent IL-6 production. Thus, potentiating effects similar to those demonstrated earlier for TNF-α production occurred for IL-6 secretion. In studies whose results are not presented here, we have established that these findings are not dependent upon either the concentration of LPS used in the secondary stimulatory phase of macrophage activation or the chemotype of LPS used (data not shown).

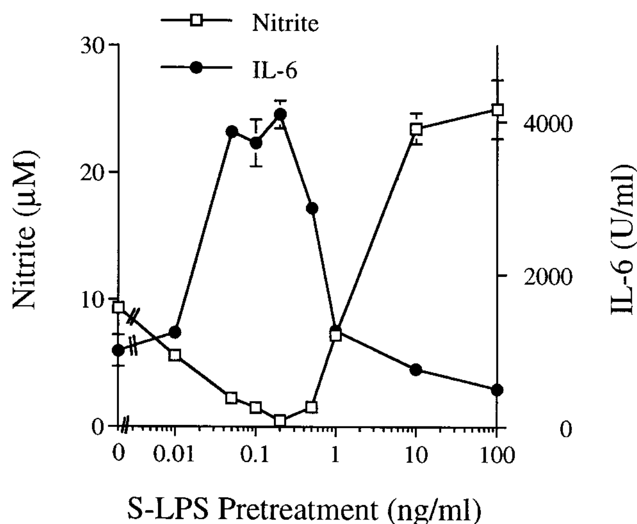

FIG. 1. Low-dose-LPS-induced reprogramming of macrophages enhances IL-6 and inhibits NO production. Various concentrations of S-LPS were added to the macrophage cultures as indicated. After 6 h, LPS-reprogrammed macrophage cultures were washed three times with HBSS and then stimulated with 100 ng of S-LPS per ml for 18 h. The amounts of both IL-6 and nitrite in the supernatants were assessed as described in the text. Each point represents the mean of triplicate values ± the standard error of the mean.

Our previous data have shown that pretreatment of mouse macrophages with substimulatory doses of LPS up-regulates TNF-α production in a manner coordinate with the suppression of NO production. Those data are similar to the results shown here for IL-6 production. TNF-α has been reported to potentiate IL-6 production in macrophages (8, 21); however, NO cannot be induced by TNF-α alone (4). In order to determine whether the effects of enhanced IL-6 production by LPS-dependent reprogramming were due solely to the effect of LPS pretreatment on enhanced subsequent LPS-dependent TNF-α production, secondary activation of mouse macrophages was carried out in the presence of rabbit neutralizing anti-TNF-α polyclonal antibodies. As shown in Fig. 2, this anti-TNF-α polyclonal antibody completely neutralized the TNF-α activity present in culture supernatants. In contrast, control rabbit antibody did not neutralize the activity of secreted TNF-α (data not shown). Importantly, however, the data shown in Fig. 2 also demonstrate that the presence of neutralizing anti-TNF-α antibody has only modest effects on the observed enhancement of IL-6 responses caused by LPS-dependent reprogramming of macrophages. Furthermore, anti-TNF-α antibody has no detectable effects on the observed suppression of NO responses initiated by LPS-dependent reprogramming. These data, therefore, indicate that the observed enhancement of the IL-6 response by LPS-dependent reprogramming is not strictly the result of enhanced TNF-α production.

The effects of LPS pretreatment on a combination of

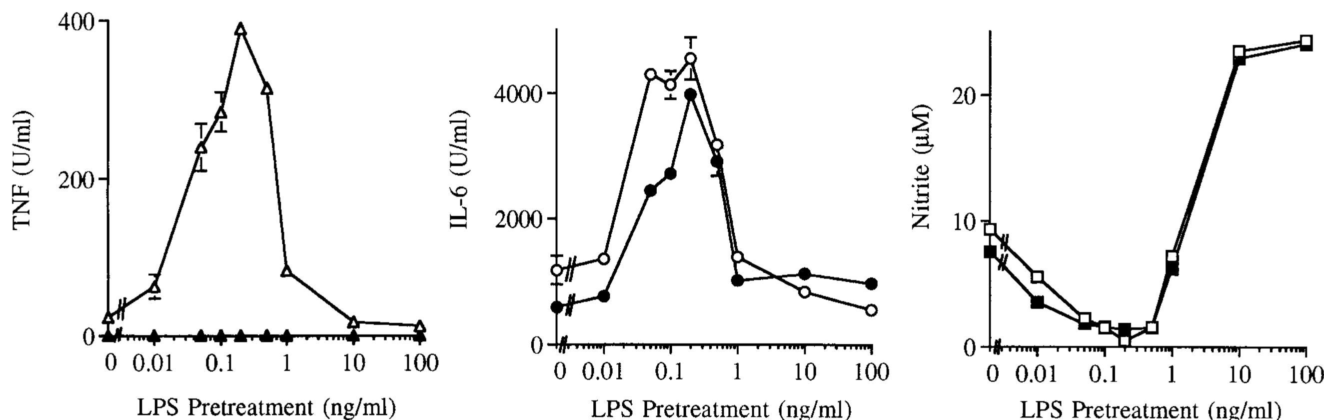

FIG. 2. Role of TNF-α in selective LPS reprogramming of macrophages. Various concentrations of S-LPS were added to the macrophage cultures as indicated. After 6 h, LPS-reprogrammed macrophage cultures were washed three times with HBSS and then stimulated with 100 ng of S-LPS per ml in the presence (closed symbols) or absence (open symbols) of anti-TNF-α polyclonal antibodies (150 U of neutralizing activity per ml) for 18 h. The amounts of IL-6, TNF-α, and nitrite in the culture supernatants were assessed as described in the text. Each point represents the mean of triplicate values ± the standard error of the mean.

rIFN-γ- and rTNF-α-mediated stimulation of macrophages were also assessed. In the absence of pretreatment of macrophages with LPS, very little IL-6 (20 ± 3 U/ml) was induced by the combination of rIFN-γ (100 U/ml) and rTNF-α (60 U/ml) (Fig. 3). These concentrations of cytokines were, however, sufficient to induce high levels of NO production (7.5 ± 0.3 μM) in mouse macrophages. It is of interest that pretreatment of macrophages with subthreshold stimulatory concentrations of LPS initiated reprogramming of the cells such that, as was shown earlier for LPS stimulation, subsequent macrophage responses for NO production were significantly reduced. The ability of LPS-pretreated macrophages to secrete IL-6 in response to TNF-α and IFN-γ is less clear. In this respect, while higher pretreatment concentrations of S-LPS do result in detectable IL-6 responses, these responses are at the threshold of direct S-LPS-mediated activation concentrations (Fig. 1). It is, in any case, clear that LPS-dependent reprogramming of macrophages for enhanced cytokine-mediated IL-6 secretion is significantly less efficient than that observed with LPS stimulation.

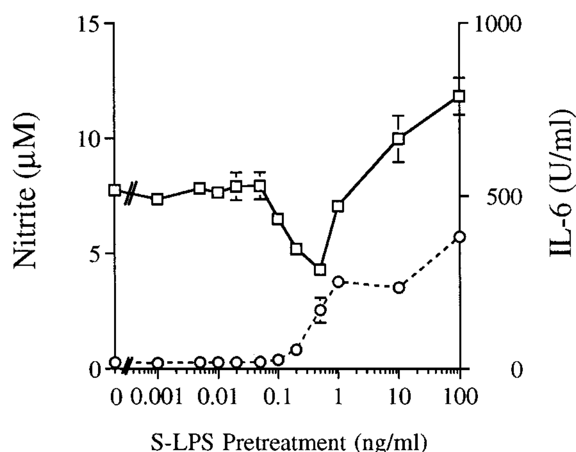

FIG. 3. LPS-induced selective priming effects on macrophage responses are not restricted to specific LPS stimulation. Various concentrations of S-LPS were added to the macrophage cultures as indicated. After 6 h, LPS-reprogrammed macrophage cultures were washed three times with HBSS and then stimulated with a combination of 100 U of rIFN-γ and 600 U of TNF-α per ml for 18 h. The amounts of nitrite (squares) and IL-6 (circles) in the culture supernatants were assessed as described in the text. Each point represents the mean of triplicate values ± the standard error of the mean.

Discussion. In the present study, we confirmed and extended our previous findings that have demonstrated that substimulatory doses of LPS can effectively reprogram macrophages for altered responses to subsequent activation by LPS and other microbial stimuli. While the earlier studies focused on decreased NO production and enhanced TNF-α production, we now show that an equivalent enhancement is observed with IL-6 production following LPS-dependent reprogramming. The observed enhancement of the IL-6 response by LPS-dependent reprogramming is not strictly dependent upon enhanced macrophage responsiveness for TNF-α production, since neutralizing antibody to mouse TNF-α only partially reduces the IL-6 response. Collectively, these data support the conclusion that the LPS-dependent reprogramming event involved a more generalized intracellular regulatory event that controls both IL-6 and TNF-α in a coordinate manner.

Pretreatment of macrophages/monocytes with LPS has been reported to initiate tolerance (desensitization) to subsequent LPS-dependent responses (2, 6, 7, 9, 14, 23, 25, 30). In the present study, we have shown that pretreatment of macrophages with relatively low doses of LPS (~0.2 ng/ml) causes potentiation of the subsequent LPS-dependent IL-6 response whereas higher pretreatment doses (1.0 to 10 ng/ml) of LPS reverse that enhancement. However, it is of interest that, unlike in several earlier studies, although a reduction in the IL-6 response is consistently observed, we have not been able to totally abrogate the subsequent IL-6 response even with the very high (100-ng/ml) LPS concentrations used for pretreatment of macrophages. In many of the tolerance (desensitization) studies previously reported, however, relatively longer times (18 h to 3 days) were used as pretreatment periods for macrophages/monocytes, and it may well be that the 6-h pretreatment period may not be sufficient to induce IL-6-specific tolerance. It is also possible that the reduced responsiveness may be the result of the majority of the IL-6 response occurring in the first 6 h.

Several investigators have described the effects of pretreatment of macrophages/monocytes with LPS on subsequent LPS-dependent IL-6 production (6, 9, 12, 15, 24). Most of these studies have suggested that pretreatment of macrophages/monocytes with LPS would inhibit subsequent LPS-induced TNF-α and IL-6 production in a similar fashion (6, 9,

30). Those findings would be consistent with our results reported here showing parallel potentiation and suppression of IL-6 and TNF-α production induced by pretreatment of macrophages with low and high doses of LPS, respectively. On the other hand, Mengozzi et al. (15) recently reported that human monocytes preexposed to LPS did not secrete TNF-α following LPS restimulation but retained the ability to produce IL-1β, IL-6, and IL-8. Furthermore, Li et al. (12) reported that pretreatment of mouse macrophages with LPS suppressed subsequent LPS-induced TNF-α secretion but augmented the IL-1, IL-6, and prostaglandin E_2 responses. It is, however, difficult to directly compare these earlier findings with those of the present study because the concentration of LPS and time of exposure to LPS for pretreatment of macrophages/monocytes were not consistent among the earlier experiments. It is of potential interest that LaRue and McCall have recently reported that the cellular expression of IκB-α correlates with the down-regulation of IL-1β gene expression during the induction of endotoxin tolerance (11); however, the detailed mechanisms for the regulation of this and other cytokine genes are not clear.

While the data showing modification of macrophage IL-6 responses are, in one sense, reflective of previously published results obtained by this laboratory with TNF-α, it should be pointed out that there exists no a priori reason to predict that the effects of LPS-dependent reprogramming should be parallel. In regard to this, there is evidence from inhibitor studies that LPS-dependent activation of macrophage TNF-α responses can be virtually completely suppressed without inhibition of IL-6 responses (8, 18), suggesting, at least, that the molecular mechanisms for the induction of these two cytokines are not identical. The parallel profiles of IL-6 and TNF-α responses following LPS reprogramming, therefore, support the conclusion that the mechanism involved affects some component common to both pathways.

The cellular mechanism(s) of the enhancement (priming) induced by pretreatment of macrophages with low (substimulatory) doses of LPS is unclear. Although prostaglandin E_2 has been reported to suppress LPS-induced macrophage activation (10), Renz et al. (19) have reported that low concentrations of prostaglandin E_2 can enhance TNF-α production in mouse macrophages. Furthermore, both IFN-γ or IFN-β are well recognized for their abilities to enhance LPS-induced macrophage activation (5, 26). Recently, Calandra et al. (1) reported that low concentrations of LPS that are not sufficient to induce TNF-α would still induce macrophage migration inhibitory factor. It is of interest that recombinant migration inhibitory factor has been shown to induce the RAW 264.7 mouse macrophage cell line to produce TNF-α. Thus, the interplay of one or more of these macrophage-derived mediators with the cell line during the pretreatment period may be of relevance to the observed reprogramming event.

Alternatively, our laboratory has previously reported that pretreatment of mouse peritoneal macrophages with pertussis toxin (PT) enhanced LPS-induced TNF-α production (28). However, LPS-pretreatment-induced enhancement of TNF-α production was refractory to regulation by PT (29). Moreover, pretreatment of mouse peritoneal macrophages with PT has no effect on subsequent LPS-induced IL-6 production (7a). A relationship between PT-dependent alterations in macrophage responsiveness and LPS-dependent reprogramming is, therefore, less likely.

Overall, we have shown that the phenomenon of macrophage tolerance (desensitization) by pretreatment with low doses of LPS cannot be applied uniformly to all macrophage responses. These and other data from our laboratory have established that low-dose-LPS pretreatment induces reciprocal and biphasic dose-dependent enhancement and inhibition of TNF-α, IL-6, and NO. The establishment of the concept of reprogramming of LPS-induced macrophage activation may allow a better understanding and potential control of the macrophage response to non-self-inflammatory stimuli in order to maximize its host defense potential for killing tumor cells and/or bacteria. Our current research efforts are directed toward the elucidation of the cellular and biochemical mechanisms responsible for the observed modulation of macrophage activation responses by these LPS-dependent reprogramming events.

We express our appreciation to R. E. McCallum for providing rabbit neutralizing anti-TNF-α polyclonal antibodies for use in these studies.

This research was supported by NIH grants R37-AI23447 and PO1-CA54474. Nobuyuki Hirohashi is a scholar of the Kansas Health Foundation Cancer Research and Training Program.

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Editor: R. E. McCallum