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Stimulation of Human Monocytes by Endotoxin-Associated Protein: Inhibition of Programmed Cell Death (Apoptosis) and Potential Significance in Adjuvanticity

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Mononuclear phagocytes are essential for adjuvant activity and polyclonal immunoglobulin synthesis induced by endotoxin-associated protein (EP) from *Salmonella* spp. To define the mechanisms of EP-mediated immunostimulation, we evaluated monocyte functions central to adjuvanticity following exposure to *Salmonella typhimurium* EP. In this study, we show that EP promotes the survival of monocytes by blocking programmed cell death (apoptosis), enhancing the production of the immunostimulatory cytokine interleukin-1 (IL-1) and stimulating the increased expression of HLA-DR and IL-2 receptors, which are cell membrane proteins that facilitate antigen presentation and IL-2 regulation, respectively. These results indicate that, like lipopolysaccharide, EP is a potent activator of human monocytes and suggest that EP-induced immunostimulation may be mediated, in part, by enhanced monocyte survival, cytokine release, and receptor expression.

Endotoxin-associated protein (EP) consists of a complex of four or five major proteins that range in size from 10 to 35 kDa and that are intimately associated with the lipopolysaccharide (LPS) of gram-negative bacteria (3, 10). EP can be purified from LPS by hot phenol extraction (18, 21) and contains 85% protein, 2.2% glucosamine, and ~10% certain fatty acids characteristic of lipid A (3). However, there is no evidence of 2-keto-3-deoxyoctonate and thus no LPS core in EP. Originally considered to be a superfluous carrier of LPS, EP is now recognized to have potent biological activities, some of which are unique. For example, EP is a powerful mitogen for C3H/HeJ mouse and human lymphocytes, which are hyporesponsive to LPS (4, 5, 13, 17). Additionally, EP from *Salmonella* spp. has been shown to be a strong adjuvant capable of enhancing murine antibody responses in vivo and in vitro (14–16).

Although the mechanisms involved in EP-induced adjuvanticity have not been clearly defined, EP has been shown to be a potent B cell mitogen and polyclonal activator of immunoglobulin synthesis (4, 17). However, the adjuvant effects can be dramatically reduced by depleting monocytes from the cultures (16), suggesting that EP-induced adjuvanticity is mediated, in part, by its effects on mononuclear phagocytes, cells that play an essential role in antigen presentation and release of immunoregulatory cytokines (reviewed in reference 22). In this regard, macrophages are also required for LPS-induced adjuvanticity and augmentation of B cell responses in vitro (9). Furthermore, EP, as well as LPS, has been shown to activate murine macrophages, as determined by several parameters, including increased tumoricidal activity (2), prostaglandin production (12), and release of hematopoietic growth factors (11).

In this study, we investigated the effects of purified EP on human monocyte functions that might be relevant to the adjuvant response, including cell survival, synthesis of interleukin-1 (IL-1), and HLA-DR and IL-2 receptor (IL-2R) expression. EP was purified by hot aqueous phenol extraction of *Salmonella typhimurium* LPS isolated by the trichloroacetic acid method (3). EP, the residual protein-free LPS, or *Escherichia coli* LPS (O55:B5; Difco, Detroit, Mich.) was suspended in Dulbecco's modified Eagle medium (DMEM), sonicated for ~3 s to promote dispersion, and serially diluted in DMEM supplemented with 0.1% human serum albumin (American Red Cross, Baltimore, Md.). Monocytes, obtained by leukapheresis of healthy donors, were isolated by centrifugal elutriation and cultured in suspension with or without added EP or LPS in polypropylene tubes (12 by 75 mm) as previously described (6).

Human monocytes undergo programmed cell death (PCD), or apoptosis, when cultured for >8 h in the absence of appropriate stimulation (6). However, when exposed to LPS or the cytokine IL-1 or tumor necrosis factor alpha, PCD is reduced and monocytes remain viable and functionally active. To determine whether EP, like LPS, was able to inhibit PCD, we cultured monocytes with increasing concentrations of EP for 48 h and then assessed them for viability on the basis of propidium iodide uptake and cell size (6). As shown in Fig. 1, EP and protein-free LPS from *S. typhimurium* inhibited the PCD of monocytes at concentrations of >1 to 10 pg/ml. *E. coli* LPS also prevented monocyte PCD, consistent with our previous results (6). The inhibition of monocyte PCD does not appear to be due solely to residual lipid A in the EP complex, since the concentration (weight/volume) of synthetic *Salmonella* lipid A (ICN Biochemicals, Cleveland, Ohio) or highly purified lipid A from *Salmonella minnesota* (the kind gift of Alois Nowotny, University of Pennsylvania) that rescues monocytes from PCD is 10,000-fold higher than that of EP (data not shown). Thus, like LPS,

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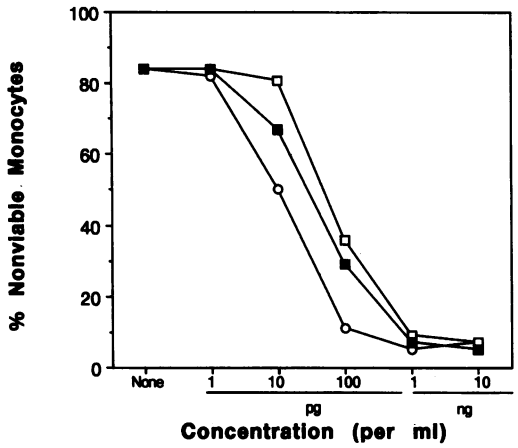

FIG. 1. Inhibition by EP of monocyte PCD. Human monocytes (2×10^6/0.2 ml of DMEM) were cultured with the indicated concentrations of *S. typhimurium* EP (□) or LPS (○) or *E. coli* LPS (■) for 48 h. The percentage of nonviable cells was assessed on the basis of decreased cell size and enhanced propidium iodide uptake (6). The results are the means of duplicate tests and are representative of five similar experiments. Standard deviations were less than 10% of the means.

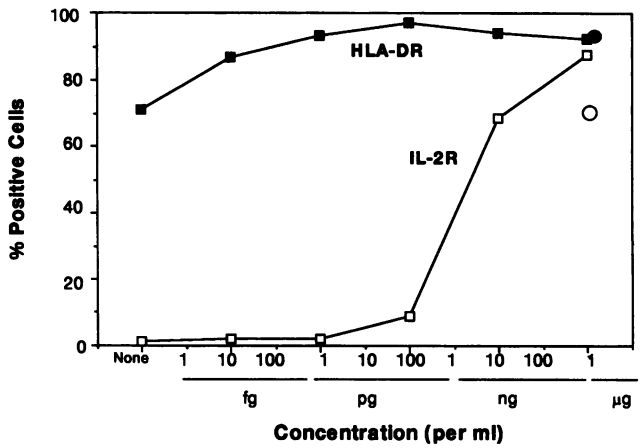

FIG. 3. Up-regulation by EP of the expression of HLA-DR and IL-2Rs. Human monocytes (2×10^6/0.2 ml of DMEM) were cultured with the indicated concentrations of *S. typhimurium* EP (■ and □) or, for comparison, with 1 µg of *S. typhimurium* LPS per ml (● and ○). Cells were stained with monoclonal antibodies specific for HLA-DR (■ and ●) or IL-2Rs (□ and ○) (Becton Dickinson, Mountain View, Calif.). Cells with fluorescence distinctly more intense than that of control cells stained with subclass-matched irrelevant monoclonal antibodies were considered positive (20). Results are representative of three similar experiments.

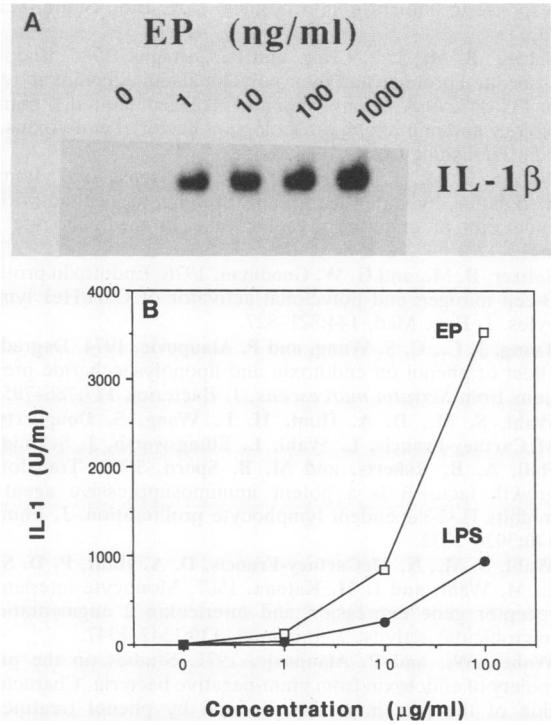

FIG. 2. Induction by EP of IL-1 release. (A) Monocytes (15×10^6) were cultured with the indicated concentrations of *S. typhimurium* EP for 24 h. Total RNA was extracted, processed for Northern (RNA) blot analysis, and probed for IL-1β as described previously (8). Equal amounts of RNA (5 µg) were loaded per lane and confirmed by the intensity of 18S or 28S rRNA bands (data not shown). (B) Supernatants from monocytes (10^6/ml) cultured with the indicated concentrations of *S. typhimurium* EP (□) or LPS (●) were assayed for IL-1 activity (19). Results are from pooled duplicate samples and are representative of two similar experiments.

EP is an extremely potent inhibitor of PCD in human monocytes. The capacity of EP to prevent monocyte PCD could increase the number of monocytes or macrophages in vivo through enhanced accumulation and subsequent differentiation of viable monocytes.

LPS-stimulated monocytes produce IL-1 (7), a cytokine that prevents monocyte PCD (6) and up-regulates numerous components of the host immune response (1). Thus, the capacity of EP to stimulate monocytes to release this cytokine might be important in the adjuvant effects of EP. To investigate this possibility, we examined the production of IL-1 by monocytes cultured with EP. Monocytes demonstrated enhanced levels of IL-1 mRNA when stimulated with EP at concentrations of ≥1 ng/ml (Fig. 2A). In addition, elevated amounts of biologically active IL-1 protein were detected in the supernatants of cultures stimulated with EP at concentrations of >1 µg/ml (Fig. 2B). At concentrations of ≥10 µg/ml, EP was consistently three- to fourfold more active than LPS in promoting the synthesis and release of active IL-1. These data indicate that EP is a potent inducer of IL-1 production by monocytes.

In addition to IL-1 production, augmented antigen presentation might also contribute to EP-induced adjuvanticity. Therefore, we next evaluated HLA-DR expression on EP-stimulated monocytes. HLA-DR, a cell surface protein involved in antigen presentation to helper T lymphocytes, is constitutively expressed on the majority of resting monocytes but is up-regulated following stimulation with LPS (20). Monocytes cultured with EP for 24 h were stained with monoclonal antibodies specific for HLA-DR and then analyzed by flow microfluorimetry (20). As shown in Fig. 3, the percentage of total monocytes expressing high levels of HLA-DR (~72%) increased in a concentration-dependent manner after stimulation with EP, and ≥90% of the monocytes were positive for this antigen when cultured with 1 µg of either EP or LPS per ml. Although the expression of HLA-DR on resting monocytes ranged from 70 to 90%

depending on the donor, EP stimulation consistently increased both the percentage of HLA-DR-positive cells and the fluorescence intensity on the cells (data not shown), indicating the up-regulation of this molecule.

The levels of monocyte IL-2Rs, which are not expressed on resting monocytes but whose levels are increased dramatically after stimulation with LPS (20), also increased after EP stimulation (Fig. 3). At 1 μg/ml, EP was slightly more effective than LPS in inducing IL-2R expression. The interaction of IL-2 with IL-2Rs enhances cytokine (e.g., IL-1) release by, oxygen radical production by, and microbicidal activity of monocytes (20). These results indicate that EP can enhance the expression of surface proteins important in antigen presentation and increased monocyte functions.

Our results indicate that *S. typhimurium* EP stimulates human peripheral blood monocytes and promotes cell survival, IL-1 synthesis, and expression of HLA-DR and IL-2Rs. These responses parallel the effects of LPS on monocytes and may be involved in the adjuvanticity induced by both EP and LPS. Although LPS is recognized as an effective activator of monocytes, EP appeared to be superior to LPS in activating certain monocyte responses, such as the expression of IL-2Rs and the release of IL-1. Whether each of the proteins associated with the EP complex activates monocytes in a qualitatively or quantitatively similar manner remains to be determined. Because mononuclear phagocytes are a crucial cellular component of the host inflammatory and immune responses, the activation of monocytes by EP may play a significant role in the pathogenesis associated with gram-negative infections and contribute to the adjuvant effects ascribed to EP.

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