Role of Alveolar Macrophages in Lipopolysaccharide-Induced Neutrophil Accumulation

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B6D2F1/TRU mice were exposed to a lipopolysaccharide (LPS) aerosol that resulted in a 1-h-postexposure lung burden of about 290 ng of LPS. This exposure caused an accumulation of neutrophils in the lung that peaked between 6 and 12 h after exposure. To determine the potential role of alveolar macrophages (AM) in the induction of neutrophil accumulation by LPS, 10^6 AM from normal or LPS-exposed mice were transferred to the lungs of groups of naive recipient mice. A third group of mice was instilled intratracheally with vehicle only. After 5 h, the lungs of the mice were lavaged and the numbers of neutrophils in the lavage fluids were determined. The instillation of AM from unexposed mice did not cause significantly more neutrophils to accumulate than did the instillation of vehicle only, whereas the instillation of AM from LPS-exposed mice caused nearly a threefold increase in the numbers of neutrophils in lavage fluids. Transfer of AM from LPS-exposed mice into cutaneous air pouches of naive mice also caused greater local neutrophil accumulation (10-fold) than did the transfer of AM from normal mice. Repeated freeze-thawing of the suspensions of AM before transfer to recipients significantly reduced the ability of the suspensions to induce neutrophil accumulation. This indicated that AM viability is necessary to cause a maximal neutrophil infiltration upon transfer of the AM. To determine the extent to which LPS-induced neutrophil accumulation depends on the presence of AM, the ability of LPS to elicit neutrophil accumulation when injected alone or together with AM into air pouches was determined. The injection of either AM or LPS alone caused few neutrophils to accumulate, whereas the injection of LPS and AM together caused a large number of neutrophils to accumulate. The results of this study indicate that LPS deposition in the lung can stimulate AM to induce neutrophil accumulation and that this may be the major mechanism by which LPS causes neutrophil accumulation.

Invasion of host tissue by gram-negative bacteria induces a vigorous inflammatory response characterized by neutrophil accumulation. This host response is thought to be important in resistance to infection by gram-negative bacteria (8, 17, 18, 21). The cell wall of gram-negative bacteria contain lipopolysaccharide (LPS), a potent producer of inflammation. Comparative studies of LPS responder and nonresponder mice suggest that the neutrophil accumulation that occurs at sites of gram-negative bacterial infection is determined by the host response to LPS (18, 21). In addition, the treatment of killed Escherichia coli with either polymyxin B or anti-LPS antibodies diminishes neutrophil infiltration and inhibits other in vivo activities associated with LPS (13). Although neutrophil accumulation induced by LPS is important in defense against infection by gram-negative bacteria, the mechanism by which LPS causes neutrophils to accumulate is not established.

LPS has been shown to have potent activity in the induction of neutrophil infiltration in vivo (6, 7). However, LPS is not chemotactic for neutrophils in vitro assays (6, 12). This suggests that LPS does not cause neutrophil accumulation by direct effects on circulating neutrophils. LPS does cause the in vitro production of interleukin-1 and tumor necrosis factor by macrophages, and these mediators are thought to cause neutrophil accumulation (1, 3). Furthermore, it has been shown that rabbit pleural exudate macrophages exposed to LPS produce a 45-kilodalton factor with neutrophil infiltration-inducing activity in vivo (14). Because of the ability of LPS to induce cytokine production by macrophages, it has been suggested that it causes neutrophil accumulation by stimulating macrophages to produce inflammatory mediators (7, 14). However, there is no direct in vivo evidence that the production of inflammation by LPS is the result of the stimulation of macrophages.

The purpose of this investigation was to determine whether exposure of mice to LPS aerosols causes neutrophil accumulation in the lung by the stimulation of alveolar macrophages (AM). The experimental approach involved the measurement of neutrophil accumulation caused by the transfer of AM from LPS-exposed donor mice to the lungs of naive recipient mice. In addition, AM were transferred to an extrapulmonary site relatively devoid of macrophages, and the ability of the AM to augment neutrophil accumulation in response to the injection of LPS at the same site was determined.

MATERIALS AND METHODS

Mice. B6D2F1/TRU mice were obtained from the animal breeding facilities, Trudeau Institute. Male mice maintained under standard laboratory conditions were used between the ages of 8 and 12 weeks. The B6D2F1 mice used were sensitive to LPS, as determined by the ability of LPS to cause proliferation of B6D2 spleen cells and induce cytokine production by B6D2 macrophages (results not shown).

LPS aerosol exposures. Mice were exposed in a Plexiglas chamber to a LPS aerosol generated by a disposable nebulizer (Hudson Oxygen Therapy Co., Temecula, Calif.). The nebulizer reservoir was loaded with 200 μg of LPS from E. coli serotype O128:B12 (Sigma Chemical Co., St. Louis, Mo.) in 5 ml of water. The LPS solution was nebulized at an airflow rate of 5 liters/min until it was gone; this typically took about 16 min.
In some experiments, fluorescein isothiocyanate (FITC)-labeled LPS from E. coli serotype O128:B12 (Sigma) was used to determine the amount of LPS deposited in the lungs of exposed mice. Mice were exposed to the FITC-LPS as described above. One hour after exposure, the mice were killed and their lungs were lavaged as described below. The lungs were then homogenized in 3 ml of phosphate-buffered saline. The FITC-LPS content of the lung homogenate, lung lavage fluid, and cells in the lung lavage fluid was determined by a fluorometric technique. The fluorescent intensity of the samples was determined by using a fluorimeter at an excitation wavelength of 483 nm and an emission wavelength of 517 nm (15). The content of FITC-LPS in the samples was calculated from standard curves generated by the addition of known amounts of FITC-LPS to samples of the respective lung fractions obtained from normal mice.

Lung lavage. To obtain AM for cell transfer studies, the lungs of mice exposed to room air or LPS aerosols were lavaged. The procedure used was similar to that described for rat lung lavage (5). The mice were anesthetized with halothane gas and exsanguinated. The trachea was exposed and intubated, and the lungs were lavaged with ten 1-ml volumes of Hanks balanced salt solution (HBSS) without Ca²⁺ and Mg²⁺ (GIBCO Laboratories, Grand Island, N.Y.) but containing 3 mM EDTA (HBSS-EDTA). The cells were washed three times in HBSS, counted, and suspended to 10⁶ cells/ml of HBSS. The HBSS used in these experiments contained less than 0.02 ng of LPS per ml, as determined with the E-Toxate kit (Sigma). The cell populations recovered from both control and LPS-exposed mice (1 h after exposure) typically contained at least 95% AM, with the remaining cells composed of neutrophils, lymphocytes, and epithelial cells. Differential cell counts were done by light microscopy examination of cell smears produced with a cytocentrifuge (Shandon, Pittsburgh, Pa.) and stained with Diff-Quik (American Scientific Products, McGaw Park, Ill.).

Lung lavage was also used to assess neutrophil accumulation in the lungs. The procedure was essentially as described above except that five 1-ml portions of HBSS-EDTA solution were used for lavage.

Transfer of AM to lungs of recipient mice. AM were instilled into the lungs of mice by a technique previously described for rats (2). The mice were anesthetized with halothane gas and placed in a vertical position. A 20-gauge, blunted needle was passed through the oral pharynx into the trachea. Placement of the needle in the trachea was confirmed by the feel of the cartilaginous rings as the needle end was moved up and down. A 0.1-ml solution containing 10⁶ AM was injected into the lung through the needle.

Injection of air pouches. Air pouches were produced on the backs of the mice by the subcutaneous injection of 0.4 ml of air (4, 9, 20). Suspensions of 10⁴ AM, 10⁵ AM plus LPS, or LPS only were immediately injected into the air pouches. In some experiments, suspensions containing 10⁶ normal AM or LPS-exposed AM were repeatedly frozen and thawed before injection. These AM suspensions contained fewer than 5% viable AM, as determined by trypan blue dye exclusion. After 5 h, the mice were killed by CO₂ inhalation and a 20-gauge needle was inserted into the lumen of the air pouch. Placement of the needle end in the lumen of the air pouch was confirmed by the aspiration of air from the air pouch. The air pouch was lavaged by injection of 2 ml of HBSS-EDTA, followed by aspiration of the fluid. Total cells in the lavage fluids were determined by use of a hemacytometer. Differential cell counts were done from cell smears stained with Diff-Quik.

Histology. Lung tissues were fixed, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. The tissues were examined to determine whether the responses of cells in lung lavage fluids were representative of the cellular responses occurring within the tissues. Differences between means were analyzed for statistical significance by the Student t test. Probabilities less than or equal to 0.05 were considered significant.

RESULTS

Exposure of mice to an LPS aerosol. Exposure of mice to an aerosol of LPS generated from 3 ml of H₂O containing 200 μg of FITC-LPS resulted in a total lung content of about 290 ± 87 ng of LPS (x ± standard deviation, n = 3) 1 h after exposure. Of this, about 210 ± 56 ng was in the lung lavage fluids, 6 ± 3 ng was associated with the cells in the lung lavage fluids, and about 75 ± 31 ng was in the homogenates of the tissues of the lavaged lungs.

Neutrophil accumulation that occurred in the lung over time, as determined by analysis of cells in the lung lavage fluids of groups of exposed mice, is shown in Fig. 1. Neutrophils first appeared in the lavage fluids at 3 h, reached a peak between 6 and 12 h, and decreased in number by 24 h. The number of AM in the lung lavage fluids did not change significantly during the 24-h period after LPS exposure.

Histological examination of the lung tissue revealed an interstitial accumulation of neutrophils at 3 h and the presence of neutrophils at 6 h both in the lumina of the alveoli and in the lung interstitium. By 12 h, neutrophils were still present in the alveoli but fewer were observed in the interstitium. At 24 h, few neutrophils were present and only in the alveolar lumina and airways.

Transfer of normal and LPS-exposed AM to the lungs of naive recipients. AM were obtained by lung lavage from normal mice and from mice exposed 1 h previously to LPS. Groups of mice were instilled intratracheally with 10⁶ AM from either unexposed or LPS-exposed mice. A third group of mice was instilled intratracheally with HBSS (vehicle) only as a control. AM were obtained from donor mice 1 h after LPS exposure since the previous experiment indicated that at this time the lung lavage cell population did not yet

![FIG. 1. Numbers of neutrophils and AM in lung lavage fluids at different times after LPS aerosol exposure. Bars represent means ± 1 standard error (n = 4). Note that the x axis does not represent a linear progression of time.](http://iai.asm.org/Downloadedfrom)
lavage fluids were determined. The results of one of three experiments are shown in Fig. 2. Similar results were obtained in all three experiments. The instillation of normal AM into the lungs of naive mice did not cause significantly more neutrophils to accumulate than did the instillation of vehicle only. On the other hand, the instillation of AM from LPS-exposed mice caused significantly more neutrophils to accumulate than did the instillation of either normal AM or vehicle.

**Instillation of normal AM, LPS, or AM plus LPS into skin air pouches.** Investigators in this laboratory and others have observed that newly formed cutaneous air pouches contain relatively few macrophages (9, 20). Thus, the air pouch provides an in vivo location to examine the effects of the presence of transferred AM, with minimal effects due to resident macrophage populations.

Air pouches were formed on the backs of the necks of mice by the subcutaneous injection of 0.4 ml of air. The mice were separated into eight groups. The air pouches were then injected with HBSS (vehicle), 10⁶ AM from normal mice, different concentrations of LPS alone, or 10⁶ AM together with different concentrations of LPS. The results are shown in Fig. 3. The injection of either vehicle or AM alone caused little neutrophil accumulation. The injection of LPS alone caused moderate neutrophil accumulation in a dose-dependent manner. The addition of AM to the LPS significantly increased neutrophil accumulation at all three doses of LPS injected. The injection of AM together with 5,000 ng of LPS caused about a sixfold increase in neutrophils compared with the additive effects of the injections of AM only and 5,000 ng of LPS only.

**Transfer of AM from normal and LPS-exposed mice into air pouches of naive recipient mice.** The transfer of AM from normal mice into air pouches increased the number of neutrophils that accumulated in response to the local injection of LPS. The transfer of AM from LPS-exposed mice into the lungs of naive recipient mice also caused more neutrophils to accumulate than did the transfer of AM from

![Graph](imageURL)

**FIG. 2.** Numbers of neutrophils in lung lavage fluids of mice 5 h after intratracheal instillation of HBSS (vehicle), 10⁶ AM from normal mice (normal AM), or 10⁶ AM from mice exposed 1 h previously to an LPS aerosol (LPS-AM). Bars represent means ± 1 standard error (n = 5). The value for LPS-AM is significantly different from that for normal AM (P < 0.02).

![Graph](imageURL)

**FIG. 3.** Numbers of neutrophils in lavage fluids from cutaneous air pouches 6 h after injection of different doses of LPS, 10⁶ AM, or different doses of LPS together with 10⁶ AM. Bars represent means ± 1 standard error (n = 4). For each dose of LPS, the addition of AM to the air pouches significantly increased the number of neutrophils in the lavage fluids (P < 0.01).
control mice (Fig. 2). To determine whether AM transferred into air pouches function similarly to AM transferred into the lungs, $10^6$ AM from either control or LPS-exposed mice were injected into the air pouches of recipient mice. A third group of mice was injected with vehicle (HBSS) only. After 5 h, the air pouches of the mice were lavaged and the numbers of neutrophils in the lavage fluids were determined. The numbers of neutrophils that accumulated in the air pouches after the transfer of LPS-exposed AM varied between individual experiments. However, in all experiments the relative numbers of neutrophils that accumulated in response to the injection of control and LPS-exposed AM were similar. Representative results from one of three experiments are shown in Fig. 4. The injection of vehicle only or normal AM caused few neutrophils to accumulate. However, the injection of AM from LPS-exposed mice caused significantly more (10-fold) neutrophils to accumulate than did the injection of normal AM.

To determine whether the viability of AM transferred into air pouches affected the ability of AM to cause neutrophil accumulation, the following experiment was done. Suspensions of AM from control or LPS-exposed mice were repeatedly frozen and thawed to lyse the AM. The resulting suspensions of lyzed AM and suspensions of intact AM from control and LPS-exposed mice were then injected into the air pouches of groups of mice. After 5 h, the air pouches were lavaged and neutrophil numbers in the lavage fluids were determined (Fig. 5). As in previous experiments, injection of AM from LPS-exposed mice caused significantly greater neutrophil accumulation than did the injection of normal AM. Lysing of the AM before injection decreased the ability of the AM suspensions to cause neutrophils to accumulate. However, the difference was significant only between the lyzed and intact AM from the LPS-exposed mice. The injection of a suspension of lyzed AM from LPS-exposed mice, however, still caused significantly more neutrophils to accumulate than did the injection of normal AM. There was no significant difference between neutrophil accumulation caused by the injection of suspensions of lyzed LPS-exposed AM and intact normal AM.

**DISCUSSION**

The transfer of AM from LPS-exposed mice into the lungs of naive mice caused significantly more neutrophils to accumulate than did the transfer of normal AM. This suggests that LPS aerosols can induce neutrophil accumulation in the lung, at least in part, through the stimulation of AM. Similar results were also found when AM from normal and LPS-exposed mice were transferred into cutaneous air pouches of mice.

It is possible that the increased neutrophil response induced by the transfer of AM from LPS-exposed mice, compared with control mice, was the result of the transfer of LPS. However, experiments with FITC-labeled LPS indicated that AM obtained from LPS-exposed mice contained only about 6 ng of LPS. Instillation of this amount of LPS into the lung did not cause significantly more neutrophils to accumulate than did the instillation of vehicle only (results not shown). It was also found that 5 ng of LPS injected into air pouches of mice did not induce a significant neutrophil response (Fig. 3). Furthermore, injection of suspensions of AM from LPS-exposed mice, lyzed by repeated freeze-thawing, caused significantly fewer neutrophils to accumu-
late than did the injection of intact AM from LPS-exposed mice. Since the same amount of LPS was in both the intact-AM and lysed-Am suspensions, it is probable that neutrophil accumulation induced by intact AM from LPS-exposed mice was not solely the result of the transfer of LPS. However, the possibility that the LPS that is bound to the macrophages is the component of LPS that is the most inflammatory cannot be ruled out.

Results of the experiments in which AM from LPS-exposed mice were transferred to the lungs or air pouches of naive mice indicate that LPS can cause neutrophils to accumulate through the stimulation of AM. However, these experiments did not establish the relative contribution of this mechanism to the overall neutrophil accumulation in the lung caused by LPS aerosols. The most direct approach to answer this question would be to determine the effects of the specific depletion of AM on LPS-induced neutrophil accumulation in the lung. However, there is currently no means of specifically depleting AM in vivo. Therefore, an alternative approach was pursued, that of using an extrapulmonary site at which the effects of the presence of AM could be determined. The newly formed cutaneous air pouch appears to be a suitable alternate site since it contains relatively few resident macrophages and since LPS-exposed AM injected into the airpouch have effects on neutrophil accumulation similar to those of LPS-exposed AM transferred back into the lung. The results of the present experiments in which the cutaneous air pouch was used indicate that the ability of doses of LPS between 5 and 5,000 ng to cause neutrophil accumulation is greatly enhanced by the presence of AM (Fig. 3). This indicates that the presence of AM at the site of LPS deposition can significantly enhance the neutrophil-eliciting ability of LPS in vivo. The slight neutrophil accumulation that occurred after the injection of LPS only could have been the result of the stimulation of the few resident macrophages in the interstitium of the air pouch. Thus, the production of inflammation by LPS is probably highly dependent on its ability to stimulate macrophages at the site of deposition.

LPS has numerous effects in vivo, and several mechanisms by which LPS can induce inflammation have been suggested. Haslett et al. (11) found that LPS can have direct effects on neutrophils, causing them to marginate in the lung vasculature. It has also been suggested that LPS induces neutrophil accumulation by directly affecting the pulmonary endothelium (16) or through activation of complement (19). The B6D2 strain of mice used in the present experiments has been reported to have approximately one-half the amount of serum C5 present in complement-sufficient mice (10). This finding has been confirmed in this laboratory in the B6D2 mice bred at the Trudeau Institute. The sera of the two parental strains, DBA2 and C57BL/6, had hemolytic titters of 0 and 64, respectively, while that of the B6D2F1 strain had a hemolytic titer of 32 (results not shown). Taken together, the results of the present investigation do not rule out possible involvement of the complement system in LPS-induced neutrophil accumulation. However, few neutrophils accumulated in air pouches injected with LPS if AM were not also injected. It is difficult to predict how extensively the slightly reduced level of C5 found in the sera of B6D2 mice affects the biological role of complement in these mice. It is also possible that under the influence of LPS, the AM released factors that then interacted with complement components to generate chemotactic factors. Other investigators have suggested that LPS induces neutrophil accumulation through stimulation of resident macrophages. Isseku et al. (14) found that macrophages challenged in vitro or in vivo with LPS produced a 45-kilodalton factor which caused neutrophil accumulation in vivo. Cybulska et al. (7) observed cross-tachyphylaxis between interleukin-1 and LPS and suggest that interleukin-1 mediates LPS-induced neutrophil emigration. Results of AM transfer studies in the present investigation support the premise that LPS can induce neutrophil accumulation through the stimulation of macrophages. In addition, the presence of AM in cutaneous air pouches strongly enhanced LPS-induced neutrophil accumulation, suggesting that macrophage-dependent mechanisms are a major means by which LPS causes neutrophil accumulation.

The mechanism by which the LPS-exposed AM induced neutrophil accumulation was not determined, although it seems likely that the LPS caused the AM to produce inflammatory mediators. Identification of mediators of inflammation, their source, and their targets is of much interest. However, the complexity of the response in vivo has made this task a difficult one. The results of the present investigation for transfer of AM suggest that AM can be a source of inflammatory mediators in vivo. Macrophage transfer offers a potential means of dissecting the complex inflammatory response and may be a useful approach for the investigation of the role of mediators in inflammatory responses.

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LITERATURE CITED


