Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide

M. CHIGNARD AND V. BALLOY
Unité de Pharmacologie Cellulaire, Unité Institut National de la Santé et de la Recherche Médicale/Pasteur 485, Institut Pasteur, 75015 Paris, France

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Chignard, M., and V. Balloy. Neutrophil recruitment and increased permeability during acute lung injury induced by lipopolysaccharide. Am J Physiol Lung Cell Mol Physiol 279: L1083–L1090, 2000.—The intranasal administration of lipopolysaccharide (LPS) to mice triggers a huge influx of polymorphonuclear neutrophils (PMNs) into the airway spaces, with a peak at 48 h. The increase in protein concentration, an index of microvascular permeability, displayed a different pattern, i.e., a first increase with a plateau between 3 and 24 h followed by a second increase peaking at 72 h. When mice were depleted of circulating PMNs, the increase in protein concentration was inhibited at 3 h but not at 24 h. The lack of PMN involvement at 24 h was confirmed by 1) an early one related to the neutrophil influx and a delayed one occurring even under neutropenic conditions.

A LUNG PATHOLOGY SUCH AS acute respiratory distress syndrome (ARDS) is accompanied by activation of the inflammatory system, characterized by the presence of polymorphonuclear neutrophils (PMNs) and proteinaceous fluid in the air spaces. The latter is due to an acute injury of the alveolocapillary barrier, resulting in increased permeability and ending in a protein-rich exudative edema (14, 17, 19, 26). There is evidence based on the awareness of their biology that PMNs could play an important role in mediating the acute injury characteristic of ARDS (16, 24). Indeed, PMNs have the potential to harm lung tissues in different ways, such as the release of serine proteinases and the generation of reactive oxygen species (6, 9, 25). Convincing proof comes from animal models of acute lung injury such as those in which drug-induced neutropenia decreased the severity of the process (4, 22). Nonetheless, in most of the studies, it is not clear whether PMNs initiate or amplify the lung injury mediated by other mechanisms, and no causal links have been firmly established (4, 22). On the contrary, several examples of lung injury have been reported in PMN-depleted animals (13). It has even been shown in humans that on its instillation in the normal lung, leukotriene B4 can recruit active PMNs into the air spaces without causing a significant change in the protein permeability of the epithelial barrier (17). More puzzling is the fact that ARDS can occur in neutropenic patients without pulmonary neutrophil infiltration (7, 18, 21).

The observation that sepsis arising as a consequence of exposure to bacterial lipopolysaccharide (LPS) is one of the main contributing factors to the development of ARDS (4) led to the establishment of experimental models based on animal challenge with LPS. With a mouse model of acute lung inflammation triggered by the nasal administration of LPS (10, 11), the aim of our study was to assess the correlation between the presence of invading PMNs and the increase in protein concentration in the air spaces as an index of lung permeability.

The present study demonstrates that the increase in the alveolocapillary permeability during lung inflammation is due to at least two distinct mechanisms, an early one related to PMN influx and a delayed one occurring even in a case of neutropenia.

METHODS

Materials. Seven-week-old male C57BL/6 mice weighing 25–30 g were provided by the Centre d’Elevage R. Janvier (Le Genest Saint-Isle, France). LPS (Escherichia coli 055:B5) was from Difco Laboratories (Detroit, MI). Hydrogen peroxide (H₂O₂), O-dianisidine dihydrochloride, hexadecyltrimethylammonium bromide (HTAB), EDTA, and N-formyl-Nle-Leu-Phe (fNLP) were from Sigma (St. Louis, MO). Diff-Quik products were from Dade Behring (Paris, France), and the Bradford reagent was from Bio-Rad (Ivy sur Seine, France).

Model of acute lung inflammation. LPS dissolved in saline was administered by an intranasal or an intraperitoneal route. In the case of intranasal administration, the animals were slightly anesthetized with diethyl ether and received 50 µl of the LPS solution directly into their nostrils. In some experiments, the animals were treated with fNLP or its
sorbance was determined as above. Ovalbumin was used as a standard. The cell-free BALF with the method of Bradford (3), with some protein concentration in the PBS, the immunoglobulin was filtered (0.22 μm) and then stored at 4°C at a final concentration of 5 mg/ml. One intraperitoneal administration of 200 μg of MAb to mice led to a complete absence of circulating neutrophils within 24 h (data not shown; similar to the data reported in Ref. 23).

**Collection of BALF.** At different time intervals as indicated in Figs. 1–8, the mice were euthanized by intraperitoneal administration of pentobarbital sodium (12 mg/mouse). The tracheae were cannulated, and the lungs were washed eight times with 0.5 ml of PBS to provide 4 ml of BALF. Total cell counts were measured in the BALF with a Coulter Counter (Coulter Electronics, Margency, France), and cell differential counts were determined after cytoospin centrifugation and staining with Diff-Quik products. Myeloperoxidase (MPO) activity and protein concentration were measured in the cell-free BALF obtained after centrifugation (300 g for 10 min).

**Determination of MPO activity.** MPO activity was determined in the lung tissues and in the cell-free BALF following a previously described method (12), with minor modifications. After BALF collection, the lung vessels were flushed via a perfusion of saline into the right ventricle to discard circulating blood, and the lungs were removed from the thorax, blotted with gauze, and frozen at −20°C until further use. The collected frozen lungs were homogenized for 30 s (Potter-Elvehjem glass homogenizer, Thomas, Philadelphia, PA) at 4°C in 1 ml of PBS. The extracts were centrifuged (10,000 g for 10 min at 4°C), and the supernatants were discarded. The pellets were resuspended in 1 ml of PBS supplemented with HTAB (0.5% wt/vol) and EDTA (5 mM) and homogenized again. After centrifugation, 100 μl of supernatant was placed in a test tube with 200 μl of PBS-HTAB-EDTA, 2 ml of Hank's balanced salt solution, 100 μl of O-dianisidine dihydrochloride (1.25 mg/ml PBS), and 100 μl of H₂O₂ (0.05% = 0.4 mM). After 15 min of incubation with shaking at 37°C, the reaction was stopped by the addition of 100 μl of NaNO₂ (1% wt/vol). MPO activity is expressed as a change in absorbance at 450 nm. For the determination of MPO activity in the BALF, aliquots of 50 μl of cell-free BALF were mixed in microtiter plates with 200 μl of O-dianisidine dihydrochloride (1.25 mg/ml PBS) supplemented with BSA (0.1% wt/vol) and containing H₂O₂ (0.05% = 0.4 mM). Absorbance was determined as above.

**Protein concentration in the BALF.** Lung permeability was determined by measuring the total protein concentration in the cell-free BALF with the method of Bradford (3), with ovalbumin as a standard.

**Preparation of the anti-granulocyte antibody.** RB6–8C5 (anti-Ly-6G) is a rat IgG2b monoclonal antibody (MAb) (5) that binds selectively to and depletes mouse neutrophils and eosinophils but not lymphocytes or macrophages (23). The anti-granulocyte MAb was purified from ascitic fluids (cloned hybridomas kindly provided by Dr. G. Millon, Institut Pasteur, Paris, France) through precipitation with 45% (vol/vol) saturated ammonium sulfate. After dialysis at 4°C against PBS, the immunoglobulin was filtered (0.22 μm) and then stored at 4°C at a final concentration of 5 mg/ml. One intraperitoneal administration of 200 μg of MAb to mice led to a significant decrease in the number of mononuclear cells compared with the control mice.

**Results**

**Cell counts in the BALF after intranasal administration of LPS.** LPS was given at different concentrations (3.3, 10, 33, 100, and 330 μg/kg), and cell counts in the BALF were evaluated at 24 h (Fig. 1). The number of PMNs was significant on administration of 3.3 μg/kg of LPS, reached a maximum with 100 μg/kg, and then plateaued up to 330 μg/kg. In contrast, no significant change in the number of mononuclear cells was observed.

**PMN count, MPO release, and protein concentration in the BALF after intranasal administration of LPS.** The optimal concentration of 330 μg/kg of LPS was used to evaluate PMN migration and protein concentration as a function of time at 0, 3, 15, and 24 h and each 24 h up to 168 h (Fig. 2). A bell-shaped curve was observed for PMNs. A significant increase was detected as soon as 3 h after LPS challenge, with a peak at 48 h followed by a decrease to the basal level by 120 h. Mononuclear cell counts also varied (data not shown) but with different kinetics (peak at 96 h) and within a lower range (2.03 ± 0.27 × 10⁶ cells/BALF at 96 h; n = 4 mice).

MPO activity was evaluated in the cell-free supernatant of the BALF. The kinetics were strictly superimposable on the kinetics of the PMN count (Figs. 2, inset, and 3), indicating that PMNs present in the air spaces were activated.

In contrast, the kinetics of the increase in protein concentration were different as shown in Fig. 2. A plateau was rapidly reached beyond 3 h and lasted for 24 h. This was followed by a second increase, with a peak at 72 h, before a return to the basal value at 168 h (7 days).

**Effect of PMN depletion on PMN recruitment in the BALF after intranasal administration of LPS.** Mice were treated intraperitoneally with the anti-granulocyte MAb RB6–8C5 at a concentration of 200 μg/mouse. Circulating PMNs, which represented 20.1 ± 1.5% (n = 4 mice) of blood cells before treatment, were totally absent within 24 h and up to 96 h after treatment (data not shown). We also looked at the marginalized pool of PMNs in the lung vasculature by measuring MPO activity in whole lungs, an index that correlates with the number of PMNs (12). For control mice, the value (optical density at 460 nm) was 0.029 ± 0.003 (n = 5), corresponding to ~4.4 × 10⁴ adherent PMNs. This figure, although quite small, was further lowered 24 and 72 h after treatment with the anti-granulocyte MAb, with MPO values of 0.015 ± 0.001 (n = 5 mice) and 0.012 ± 0.002 (n = 5 mice), respectively. On intranasal administration of LPS 24 h after
RB6–8C5 pretreatment, PMNs were not present in the BALF 3 h later but started to be detected in small significant numbers at 24 h (Fig. 3A). At 48 and 72 h, PMNs were present in higher amounts, although in far lower amounts than those detected in control LPS-challenged animals. It was observed that PMNs present in the BALF after LPS administration to RB6–8C5-treated mice were of two kinds, i.e., either with ring-shaped or with polylobular nuclei. It is of note that ring-shaped nuclei cells cannot be totally referred to as PMNs because it was demonstrated that they additionally encompass different types of mononuclear-like ring cells (2). It is thus assumed that the number of authentic PMNs present in the air spaces was overestimated when cells with ring-shaped nuclei were included. MPO activity in the cell-free BALF increased up to 48 h and declined thereafter with LPS administration in control mice (Fig. 3B). In contrast, when mice were pretreated with the anti-granulocyte MAb, LPS challenge did not trigger a significant increase in MPO activity. These data indirectly support the fact that a small number of mature PMNs are recruited in the air spaces under this condition.

Effect of PMN depletion on protein concentration in the BALF after intranasal administration of LPS. Protein concentration analyzed 48 and 72 h after LPS administration was not significantly different whether or not the mice were neutropenic. As an example, the concentrations at 72 h were 1.5 ± 0.2 and 1.6 ± 0.2 mg/BALF for control and RB6–8C5-treated mice, respectively (n = 11/group). To reduce the potential contribution of the relatively large presence of PMNs in the BALF at 48 and 72 h, protein concentration was analyzed after PMN depletion at two earlier time points, namely 3 and 24 h.

As a result, on LPS administration, the increase in protein concentration in the BALF was significantly reduced at 3 h in the RB6–8C5-treated group compared with that in the nontreated group, with recovered amounts not significantly different from the values measured in control mice receiving intranasal saline in place of LPS (Fig. 4B). In contrast, the increase in protein concentration observed 24 h after LPS challenge was not significantly different whether or not animals were pretreated with the RB6–8C5
At this time point, the number of PMNs in the BALF of neutropenic mice was significantly higher than the number recovered from control animals receiving intranasal saline (Fig. 5A), a situation not observed 3 h after LPS administration (Fig. 4A). It could be deduced from these data that the increase in permeability is dependent on PMN recruitment, even if the latter is small. Nonetheless, 24 h after LPS administration, the presence of PMNs in the air spaces was dramatically reduced in neutropenic animals compared with that in control animals (Fig. 5A), whereas the protein contents were equivalent, thus implying that PMNs did not account for the increase in permeability.

**Effect of in situ activation of exudated PMNs on protein concentration in the BALF.** To delineate their possible participation in the increase of lung permeability at 24 h, PMNs present in the air spaces at this time point after intranasal administration to control mice were activated in situ by an intranasal administration of fNLP (2 mg/kg). The effective in situ activation of PMNs was evidenced 1 h later by a significant increase in MPO activity in the BALF (Fig. 6). Nonetheless, under these conditions, it was never possible to note an increase in protein concentration whether measured at 1 or 6 h (Fig. 6).

**PMN count and protein concentration in the BALF after LPS administration through another route.** In another set of experiments, mice were given LPS intraperitoneally at 1 mg/kg. In confirmation of previous MAb (Fig. 5B)
work (12, 15), PMNs did not migrate from the blood to the air spaces. Indeed, a nonsignificant number (compared with that in untreated mice) were recovered in the BALF collected 6 and 27 h later (Figs. 7A and 8A, respectively). Nonetheless, it was demonstrated that under these experimental conditions, PMNs are sequestered in the lung vessels and are ready to cross the alveolocapillary barrier (15). To trigger their migration, fNLP (2 mg/kg) was given intranasally 3 h after LPS challenge. As expected, PMNs were recovered in the BALF 3 h after fNLP administration and in an even larger number after 24 h (Figs. 7A and 8A, respectively). Protein concentration was measured in the BALF under these different conditions. Interestingly, at 3 h, the concentration only increased when PMNs crossed, i.e., with the LPS (intraperitoneal) plus fNLP (intranasal) combination (Fig. 7B), whereas at 24 h, it increased even in the absence of PMN migration, i.e., with LPS alone (Fig. 8B). It is of note that fNLP by itself, i.e., when given to naive mice, had no effect in that PMN count and protein concentration did not increase. To confirm this difference between 3 and 24 h, the mice were rendered neutropenic on pretreatment with the anti-granulocyte MAb RB6–8C5. It was observed that the increase in protein concentration induced by the combination of LPS (intraperitoneal) plus fNLP (intranasal) was suppressed at 3 h but was left unchanged at 24 h (data not shown), i.e., exactly as the data presented in Figs. 4 and 5.

**DISCUSSION**

Intranasal administration of LPS to mice triggers an acute lung inflammation. The main feature is a huge...
influx of PMNs into the air spaces. It is thought that PMNs can create injury to lung tissue, leading to the flooding of alveoli by plasma liquid and proteins. The increase in protein permeability across the endothelial and epithelial barriers of the lung is an early characteristic of lung injury.

As mentioned in the introduction, there are controversies concerning whether PMNs play a role in protein permeability. Our present experimental data support both possibilities. First, the kinetics of protein accumulation in the BALF is clearly biphasic, with an early increase within the first 3 h and a further increase starting at around 24 h. The peak of the latter is observed at 72 h, thus 24 h after the peak of PMNs. In fact, the whole kinetics of the second wave of proteins appears delayed by roughly 24 h with the entire kinetics of the PMN count and, more importantly, of MPO activity. Such a shift fits with a potential involvement of PMNs in the increase in lung permeability. This is reminiscent of the data reported by Fowler et al. (8), who observed that alveolar PMN influx in patients at high risk for ARDS occurs before development of the syndrome. The explanation would be that PMNs, while migrating and settling into the air spaces, would release harmful molecules (reactive oxygen species and/or proteinases) that would injure tissues with time (within 24 h), leading to increased permeability.

The above-mentioned assumption proved to be false in light of the experiments conducted with PMN-depleted mice. Indeed, at 48 and 72 h, permeability was not modified on PMN depletion. Nonetheless, it is of note that a significant number of PMNs were recovered from the BALF. This was surprising because at the same time, PMNs were undetectable in the blood and, more importantly, were largely removed from the vascular lung compartment. In fact, such a discrepancy has been previously observed (14, 20). It is assumed that this influx of PMNs in RB6–8C5-treated mice occurring in the context of lung vascular neutropenia represents a maximal deployment of the limited pool of newly born PMNs (20). To avoid the 48- and 72-h time

![Fig. 7. PMN count (A) and total protein concentration (B) in BALF 3 h after a combination of an intraperitoneal administration of LPS (1 mg/kg) and an intranasal administration of fNLP (2 mg/kg) 3 h later. BALF was collected 3 h after fNLP (i.e., 6 h after LPS) for PMN count and protein concentration determinations as described in Fig. 2. Results are means ± SE; n = 4–6 mice. *P < 0.05.](http://ajplung.physiology.org/)

![Fig. 8. PMN count (A) and total protein concentration (B) in BALF 24 h after a combination of an intraperitoneal administration of LPS (1 mg/kg) and an intranasal administration of fNLP (2 mg/kg) 3 h later. BALF was collected 24 h after fNLP (i.e., 27 h after LPS) for PMN count and protein concentration determinations as described in Fig. 2. Results are means ± SE; n = 4–6 mice. *P < 0.05.](http://ajplung.physiology.org/)
periods susceptible to giving inconclusive data, experiments were performed at 3 and 24 h. It was observed that 3 h after LPS challenge, the increase in permeability was totally suppressed in neutropenic mice, indicating the involvement of a PMN-dependent mechanism. In contrast, 24 h after LPS administration, increases in protein concentration were comparable whether or not the mice were neutropenic. As an explanation, a PMN-independent mechanism can be put forward because the presence of these cells in the BALF was very highly reduced (> 80%) when the mice were pretreated with the anti-granulocyte MAb. Nonetheless, the number of PMNs present in the BALF under the latter condition was not negligible and could have accounted for the increase in permeability. Such a possible mechanism at 24 h was, however, doubtful because in LPS-challenged neutropenic mice, the increase in protein concentration over the basal level was ~0.70 mg/BALF (see the difference of the means in Fig. 5B), with an influx of 0.50 \( \times 10^6 \) ± 0.09 \( \times 10^6 \) PMNs (Fig. 5A), whereas in the case of the PMN-dependent increase in permeability, i.e., with LPS-challenged control mice at 3 h, the increase in protein concentration was only ~0.30 mg/BALF (see the difference of the means in Fig. 4B), with an influx as high as 0.77 \( \times 10^6 \) ± 0.12 \( \times 10^6 \) PMNs. Because there was apparently no relationship between the number of PMNs and the increase in permeability at 24 h, it was assumed that this increase was PMN independent. This was supported by experiments performed with fNLP. Indeed, intranasal administration of this agonist to control mice 24 h after LPS challenge induced in situ activation of PMNs as checked by the increase in MPO activity. In such a case, there was no increase in permeability. It is inferred that beyond 3 h and before 24 h, other process(es) would overwhelm the participation of PMNs and would totally account for injury to the alveolocapillary barrier. This was exemplified by another experimental approach with intraperitoneal administrations of LPS, mimicking not a more local lung inflammation but rather a systemic endotoxemia. In that case, PMNs accumulate in lung vessels (12, 15), and intranasal administration of fNLP allows them to migrate into the air spaces (15). Clearly, 3 h after fNLP, the increase in permeability was related to PMN migration, whereas at 24 h, the increase was totally independent of this migration.

In conclusion, the present data support both of the two opposing arguments of the controversy concerning the role of PMNs. It is thus possible that in human acute lung injury, PMNs participate in the early phase of the disease but that their participation vanishes for longer periods of time, being supplanted by other mechanism(s) that have to be delineated.

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