Intravascular macrophage depletion attenuates endotoxin lung injury in anesthetized sheep

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Sone, Yasuyuki, Vladimir B. Serikov, and Norman C. Staub Sr. Intravascular macrophage depletion attenuates endotoxin lung injury in anesthetized sheep. J. Appl. Physiol. 87(4): 1354–1359, 1999.—We recently showed that we can selectively and safely deplete most (average 85%) of the pulmonary intravascular macrophages in sheep by intravenously infusing liposomes containing dichloromethylene bisphosphonate. After a 1-h stable baseline, we made a 6-h comparison after a 30-min intravenous endotoxin infusion (1 μg/kg) between six anesthetized control lambs and six anesthetized lambs in which the intravascular macrophages had been depleted 24 h previously. Three of the control lambs had been macrophage depleted and allowed to recover their intravascular macrophage population for ≥2 wk. After depletion, both the early and late pulmonary arterial pressure rises were dramatically attenuated. Our main interest, however, was in the acute lung microvascular injury response. The early and late rises in lung lymph flow and the increase in lung lymph protein dearance (lymph flow × lymph-to-plasma protein concentration ratio) were >90% attenuated. We conclude the pulmonary intravascular macrophages are responsible for most of the endotoxin-induced pulmonary hypertension and increased lung microvascular leakiness in sheep, although the unavoidable injury of other intravascular macrophages by the depletion regime may also contribute something.

Intravascular macrophages; biphosphonates; pulmonary arterial pressure; lung lymph flow; lung lymph protein clearance; acute lung microvascular injury

PULMONARY INTRAVASCULAR macrophages are a resident population of mononuclear cells in the lung capillaries of mammalian species in the orders Artiodactyla (pigs, sheep, goats) (13, 22) and Perissodactyla (horses) (8, 10, 21). These macrophages have been implicated in the acute hemodynamic and microvascular injury responses to sepsis or endotoxemia in pigs (compared with dogs) and in horses (1, 6).

Longworth et al. (11, 12) reported that newborn lambs had little or no hemodynamic response to intravenously infused foreign particles or endotoxin, but by 2 wk of age they had a large response. The development of the response paralleled the colonization and maturation of the pulmonary intravascular macrophage population, as determined by quantitative electron microscopic histology (9).

In a sheep with reactive pulmonary intravascular macrophages, a 30-min infusion of a small dose of Escherichia coli endotoxin (0.5–1.0 μg/kg) causes an early (2- to 3-h) pulmonary arterial hypertension (phase 1), followed by a prolonged increase in lung lymph flow and its protein concentration (phase 2) (2, 19). In species that do not have pulmonary intravascular macrophages, there is no apparent pulmonary microvascular response to endotoxin (21).

We were able to selectively deplete the vast majority of pulmonary intravascular macrophages in sheep (20) by using a method described by Van Rooijen and Van Nieuwemegen (24–26) in which the heavy metal chelating agent dichloromethylene bisphosphonate was incorporated into liposomes that were infused into the animals intravenously. After depletion, the animals lost their response to standardized test doses of foreign particles (microspheres, Monastral blue dye). We made extensive tests to ensure that our liposome preparation was sterile and did not contain any detectable endotoxin (20). One of the important results of those studies was that in lambs that were allowed to recover for ≥2 wk after the depletion the response to endotoxin was restored. The depletion liposomes are large particles that may be phagocytized by other macrophages that come in contact with them, such as liver or spleen intravascular macrophages (20, 25).

Our goal in this new study was to compare the responses to endotoxin infusions in control lambs with those that had their intravascular macrophages depleted. We found that depletion of the macrophages attenuated the lymph dynamic responses to endotoxin in addition to the pulmonary hemodynamic response. These results support a cause-and-effect relationship between the pulmonary intravascular macrophages and the acute lung injury caused by endotoxin in sheep.

METHODS

Liposomes. We used the recipe of Miyamoto et al. (13) combined with that of Van Rooijen (24) to make liposomes by reverse-phase evaporation, as we have described in detail (20). Briefly, we combined phosphatidyl choline, cholesterol, and phosphatidyl serine (in the molar ratios 6:4:1, respectively) to make a thin film in a rotary evaporator at 37°C. Next, we dispersed the lipid film in 10 ml PBS containing 3.0 g Clodronate (dichloromethylene bisphosphonate, a gift of Boehringer Mannheim) and filtered the liposomes through a polycarbonate filter with 0.8-μm-diameter pores (Millipore). We centrifuged the liposomes for 45 min at 100,000 g and 4°C and then removed the underlying excess Clodronate solution. We resuspended the liposomes in sterile PBS and stored them at 4°C until their use within 48 h. We also made empty liposomes that contained PBS but no chelating agent. Random cultures for aerobic and anaerobic organisms and the limulus chromotropic assay for endotoxin were negative (20).

Lambs. Our report includes twelve 3-mo-old lambs (15.3 ± 2.1 kg) divided into two groups: depleted (n = 6) and control (n = 6). In each animal under ketamine anesthesia (30 mg/kg im), we placed polyvinyl catheters in a carotid artery to
monitor arterial pressure and draw blood samples and in an external jugular vein to infuse the liposomes. In nine lambs we depleted the intravascular macrophages by infusing liposomes four times over 2 days, as we have described in detail (20). Three of the control lambs received empty liposomes. Three of the control lambs and all six of the depleted lambs received the Clodronate liposomes. Of the latter, we maintained the three for the control group for >2 wk until their intravascular macrophages had recovered. We studied the remaining six depleted lambs 24 h after the macrophage depletion regime was completed. The three lambs that received empty liposomes and the three that were allowed to recover made up the control group.

Although the liposomes are specific for intravascular macrophages, they are not specific for pulmonary intravascular macrophages. Intravenous infusions of Clodronate liposomes cause injury or death of other intravascular macrophages in the liver and possibly the spleen, as Van Rooijen and Van Nieuwegen (25, 26) have shown. Sone et al. (20) showed that the clearance of Monostral blue pigment particles was markedly delayed after the depletion regime, which supports the conclusion that the liver macrophages were also compromised. There was no way yet to target the liposomes exclusively to the pulmonary intravascular macrophages, especially after most of the pulmonary intravascular macrophages had been incapacitated by the first two or three doses of liposomes.

Endotoxin experiment. We did the endotoxin infusion study one time only in each lamb under general anesthesia. After induction using ketamine (15 mg/kg iv), we intubated the lamb and maintained ventilation and anesthesia with 1–2% halothane in 30% oxygen at 15 ml/kg tidal volume by using a positive-pressure pump. Through a left thoracotomy via the fourth intercostal space, we placed catheters in the pulmonary artery and the left atrium to measure pressures, and we placed a 3.5-F thermodilutor catheter in the pulmonary artery to measure cardiac output by thermodilution. Through a right thoracotomy in the sixth intercostal space, we cannulated the main efferent duct of the caudal mediastinal lymph node with heparin-containing (TDMAC, Polysciences, Warrington, PA) polyvinyl tubing (0.8 mm ID) and then ligated and resected the tail of the node and cauterized across the diaphragm to eliminate possible abdominal contributions to the lymph (17, 23).

We placed the animals in the prone position for the 7-h-long experiments and collected lung lymph in heparinized, weighed test tubes. After a stable 1-h baseline, we infused 1 µg/kg E. coli endotoxin (055:B5, Difco Laboratories, Detroit, MI). We had to modify the endotoxin regime in the control group because three lambs died before the 30-min endotoxin infusion was completed. Their pulmonary arterial pressures increased until right heart failure developed; cardiac output was too low to measure. Those three animals are not included in our results because we did not obtain any useful data. To prevent further losses in the control group, we gave indomethacin (5 mg/kg) to partially block thromboxane production (19) 1 h before the endotoxin infusion (3 lambs) or we reduced the total dose of endotoxin (3 lambs). All six control lambs survived the entire experiment. The dose of endotoxin we gave the control lambs varied between 0.50 and 1.0 µg/kg (mean 0.9 µg/kg). The macrophage-depleted lambs, on the other hand, had minimal hemodynamic responses to the endotoxin and so easily tolerated 1 µg/kg of endotoxin; they did not receive any indomethacin.

Measurements. During each 7-h study, we measured pulmonary arterial and left atrial pressures by connecting the respective catheters to lightweight pressure transducers (Novatrans MX860, Medex) and a direct-writing polygraph (model 7, Grass Instruments). We measured cardiac output every 30 min by thermodilution (5 ml iced saline) by using a cardiac output computer (model 3500, KMA) and calculated pulmonary vascular resistance. We weighed the lymph at 15-min intervals and collected it every 30 min for measurement of its total protein. We drew heparinized blood samples hourly for total protein. We calculated lung lymph protein clearance [LPC; product of lymph flow and lymph-to-plasma protein concentration (L/P) ratio] as an index of lung microvascular barrier leakiness. On systemic arterial blood samples, we counted the circulating leukocyte concentration (model Z, Coulter) and, using differential smears, calculated the concentration of neutrophils.

Statistics. We report all data as means ± SE for each group. We made our statistical analysis by using the SuperANOVA statistics program (Abacus Concepts, CA). Between the two groups we used a repeated-measures, two-way (time and group) analysis of variance for the entire time course. Then we compared the individual time points by using the Bonferroni-Dunn test for multiple comparisons. We accepted P < 0.05 as indicating statistical significance.

RESULTS

The control lambs were more sensitive to endotoxin than we had anticipated, probably because they were young (2 mo old) and because they were anesthetized and ventilated with the chest open, thereby lacking some of the compensatory venous return mechanisms that older or intact sheep possess. As we planned our experiments to study the phase 2 lung microvascular injury response, we modified the experimental protocol, as described in METHODS, to attenuate phase 1. This reduced the hypertensive response without significantly affecting the lymph dynamic responses. The group data are summarized in Figs. 1–5. We used absolute data in our graphs and statistics.

In Fig. 1 we compare the time course of the pulmonary arterial pressure between groups. There were no differences in the baseline period. Overall, the depleted sheep are significantly different from the control group (P < 0.0001). At the completion of the endotoxin infusion, pulmonary arterial pressure in the control group had peaked, whereas the depleted sheep showed little or no change from baseline. During the time period between 1.0 and 2.5 h, the differences between groups at each time point are significant (P < 0.05). After that, although pressure remained elevated in the control group, the differences were not significant.

In Fig. 2 we compare the time course of pulmonary vascular resistance between groups. There were no differences in the baseline period. Overall, resistance was increased in the control group compared with the depleted group (P < 0.05). However, because of the large SE bars for the control group at the individual time points, there were no statistically significant differences.

In Fig. 3 we compare the time course of lung lymph flow between groups. There were no differences during the baseline period. After endotoxin in the control lambs, there was the expected surge during the pulmonary hypertension (phase 1), followed by a sustained rise during the final 3 h (phase 2). In the depleted lambs...
there was only a small rise in lymph flow. Overall, the groups are significantly different ($P < 0.0001$). Compared with the control group, the lymph flow from 1.0 to 6.0 h in the depleted lambs was markedly and statistically less ($P < 0.05$).

In Fig. 4 we compare the time course of the L/P. There were no differences during the baseline period. After endotoxin there was the expected large decrease in the control group during the high-pressure phase 1. The nadir occurred at 1.5 h, and then L/P rose steadily as phase 2 developed. During the final 2 h the average control group L/P was above that of the depleted group. Overall, there is a significant difference between the groups ($P < 0.02$). When the individual time points are compared, those between 1.0 and 2.0 h are significantly different ($P < 0.01$). Again, the most important feature is that the L/P of the depleted lambs did not change much at any time after endotoxin and was not significantly different from the baseline mean L/P.

In Fig. 5 we compare the LPC, which is lymph flow $\times$ L/P. In the control group there was a steady rise after endotoxin ($\sim$4-fold above baseline by the final hour), whereas the depleted lambs showed only a modest gain ($\sim$1.5-fold maximum). Overall, the LPC was significantly different ($P < 0.0001$) between the groups. From 1.0 to 6.0 h the differences between the individual time points were significant ($P < 0.05$). Furthermore, the LPC of the control group had not yet reached a plateau when we terminated the study.

**DISCUSSION**

Acute lung injury in sheep caused by bacteremia (4) or *E. coli* endotoxin (3) has been extensively studied.

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**Fig. 1.** Comparison of 7-h time course of pulmonary arterial pressure between 6 control (dashed line) and 6 depleted lambs (solid line) before and after 30-min intravenous infusion of *Escherichia coli* endotoxin (1 µg/kg) (hatched bar at time 0). Values are means ± SE. *Groups are significantly different between 0.5 and 2.5 h (phase 1), $P < 0.05$. Later (phase 2), pressure remained $\sim$5–7 cmH$_2$O higher in control lambs than in depleted lambs, but difference was not statistically significant because of large variance in control group.

**Fig. 2.** Comparison of 7-h time course of pulmonary vascular resistance between 6 control (dashed line) and 6 depleted lambs (solid line) before and after a 30-min intravenous infusion of *E. coli* endotoxin (1 µg/kg) (hatched bar time 0). Values are means ± SE. None of the individual time point differences between groups are significantly different ($P > 0.05$), even though mean resistance in control group was always higher; the reason is that cardiac output of control lambs varied widely after endotoxin. This did not happen in depleted group, in which variance was small.
our experiments all components of the pulmonary reaction to intravenous endotoxin in sheep were attenuated by intravascular macrophage depletion. We expected, on the basis of our recent report on the depletion regime itself (20), that the hemodynamic response would be inhibited. The data in Figs. 1 and 2 show that, after depletion of the intravascular macrophages, endotoxin caused only a slight rise in the pulmonary arterial pressure and pulmonary vascular resistance scarcely changed in marked contrast to the control lambs. Because it is known that the pulmonary hemodynamic effects are due to the release of thromboxane by the intravascular macrophages (7, 11, 13) and that the hemodynamic effects of endotoxin in the sheep lung can be attenuated by cyclooxygenase inhibitors (15, 19), the data we present here are confirmatory.

After the unexpected deaths of three control lambs, we gave indomethacin or reduced the total dose of endotoxin to blunt the pulmonary vasoconstrictor response. This reinforces our depletion data because, even though the hemodynamic response in the control lambs was attenuated, there were large and statistically significant differences between the groups. It is likely that the differences would have been greater had we not modified the endotoxin regime in the control group.

Many variables may affect the individual animal's response: age, type, dose and method of endotoxin infusion, experimental conditions (awake or anesthetized), and open or closed thorax. However, after the colonization of the lung capillaries by monocytes and their maturation into macrophages, all lambs we have...
studied respond in the manner shown here for the control group (11). The sudden death due to low cardiac output in three lambs may be a peculiarity of the acute study conditions. Longworth et al. (12) also had problems getting anesthetized 2-wk-old lambs (with their chests open for the lung lymphatic cannulation procedure) through phase 1 in acute experiments with endotoxin. They did not use indomethacin or reduce the endotoxin dose.

The effects of endotoxin on lung liquid and protein exchange in macrophage-depleted sheep was not known before we did these experiments. What was known was that blocking the hemodynamic effects using cyclooxygenase inhibitors does not block the increased vascular leakiness that develops over several hours (phase 2) (15, 19). Thus our new contribution is the dramatic reduction of the lymph flow response (Fig. 3) and the stable L/P in the depleted group (Fig. 4). Multiplied together, these variables make up the LPC (Fig. 5), which, as our index of vascular leakiness, was also markedly attenuated after macrophage depletion.

The increase in LPC reached fourfold by the end of the control experiments (6 h after endotoxin). Increases in LPC up to three times baseline have been reported in exercising sheep in which there is no reason for supposing that microvascular leakiness has increased (5, 14). We have long used a rise on LPC of three times baseline as the demarcation between hemodynamic and increased microvascular leakiness. This is the conservative approach. It does not mean that lesser changes are not due to increased microvascular leakiness, only that hemodynamic effects could account for the increase in LPC. No matter whether one uses a conservative or liberal basis, intravascular macrophage depletion blocked nearly all of the increase in LPC after intravenous endotoxin infusion.

Our main conclusion to this study is that the effects of intravenous endotoxin in sheep, as the model for other mammals with reactive pulmonary intravascular macrophages, are mainly caused by the responses of the pulmonary intravascular macrophages to the circulating endotoxin. As a corollary, our study supports most of the published evidence, namely, that there is no apparent pulmonary vasoconstriction in response to endotoxin in animals that do not have pulmonary intravascular macrophages (7, 11, 18).

Although we did not make a detailed study of the systemic responses in these lambs, we did take blood samples for total leukocyte concentration and percent neutrophils. The reason we did so is that the leukocyte sequestration reaction (leukopenia) after endotoxin occurs in all mammals and is maximal at low circulating concentrations of endotoxin, such as we used (maximum circulating concentration in our lambs < 20 ng/ml plasma). Because the leukopenia occurs in all species, it ought not to be related to the presence or functional state of the pulmonary intravascular macrophages. What we observed was exactly that. The maximal leukocyte concentration decrease occurred between 0.5 and 2 h and was 74 and 61% in the control and depleted groups, respectively. By 6 h, circulating concentrations were rising in both groups. The maximum neutrophil concentration decrease occurred between 0.5 and 2 h and was 94 and 86% in the control and depleted groups, respectively. By 6 h, circulating concentrations were rising.

On the basis of the lung copper concentrations terminally and also corroborative electron-microscopic analysis, the liposome-mediated macrophage depletions averaged 85% (20). Although that is excellent depletion, it was not complete, which could account for the small changes in LPC that occurred after endotoxin in the depleted group. We wondered why some intravascular macrophages are resistant to depletion. We considered whether some lung units had no blood flow during the depletion procedure. However, that requires no blood flow on four occasions over 2 days, which seems unlikely (20). Another possibility is that during our studies, some macrophages were not actively phagocytizing particles and thus escaped "suicide" (24).
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