

Detergent inhibits 70–90% of responses to intravenous endotoxin in awake sheep

NORMAN C. STAUB SR.,¹ KIM E. LONGWORTH,² VLADIMIR SERIKOV,¹
E. HEIDI JEROME,¹ AND TED ELSASSER³

¹Cardiovascular Research Institute, University of California, San Francisco 94143;

²School of Veterinary Medicine, University of California, Davis, California 95616;

and ³United States Department of Agriculture, Beltsville, Maryland 20705

Received 8 August 2000; accepted in final form 21 December 2000

Staub, Norman C. Sr., Kim E. Longworth, Vladimir Serikov, E. Heidi Jerome, and Ted Elsasser. Detergent inhibits 70–90% of responses to intravenous endotoxin in awake sheep. *J Appl Physiol* 90: 1788–1797, 2001.—Sheep have reactive pulmonary intravascular macrophages, which are essential for the marked pulmonary vascular response to infusions of small quantities of endotoxin. In another species with reactive pulmonary intravascular macrophages, horses, our laboratory found that an intravenous biosafe detergent, tyloxapol, inhibited some systemic and pulmonary responses to endotoxin (Longworth KE, Smith BL, Staub NC, Steffey EP, and Serikov V. *Am J Vet Res* 57: 1063–1066, 1996). We determined whether the same detergent would inhibit endotoxin responses in awake sheep. In 10 awake, instrumented sheep with chronic lung lymph fistulas, we did a control experiment by intravenously infusing 1 $\mu\text{g}/\text{kg}$ *Escherichia coli* endotoxin. One week later, we gave 40 $\mu\text{mol}/\text{kg}$ tyloxapol intravenously 1–4 h before infusing the same dose of endotoxin. In these paired studies, we compared pulmonary hemodynamics, lung lymph dynamics, body temperature, circulating leukocyte concentrations, and circulating tumor necrosis factor for 6 h. In all 10 sheep, tyloxapol blocked 80–90% of the pulmonary responses and 70–90% of the systemic responses. Tyloxapol is safe, inexpensive, easy to use, and effective immediately. It may be a clinically useful approach to contravening many of the effects of endotoxemia, in humans as well as animals.

systemic inflammatory response syndrome; septicemia; acute lung injury; lung lymph protein clearance; sheep tumor necrosis factor; leukocyte sequestration; tyloxapol

SYSTEMIC INFLAMMATORY RESPONSE SYNDROME (SIRS) is a complex condition in which immune hyperreactivity after trauma or bacterial invasion may lead to multi-organ failure and death. Despite numerous and vigorous attempts to develop effective therapies for SIRS by means of molecular and cellular biotechnology, the fatality rate in humans remains at 50–60% (20).

Many of the features of SIRS are due to circulating bacterial endotoxin: lipopolysaccharide (LPS) (8). In several species of common laboratory animals, the administration of LPS in doses up to 1,000 $\mu\text{g}/\text{kg}$ are necessary for the development of a condition resem-

bling SIRS with a high mortality rate. LPS is recognized, bound, and internalized through various cell-surface receptors. Subsequently, it activates several cytokines pathways. Proinflammatory mediators such as tumor necrosis factor (TNF) are released primarily from LPS-stimulated cells of the mononuclear phagocyte system (mainly macrophages) (3, 43).

Among the strategies proposed for the experimental treatment of SIRS, some are directed at blockade of specific LPS receptors, some at binding and elimination of LPS, and some at blocking one or more of the proinflammatory cytokines (18). For example, the specific blockade of LPS receptors with monoclonal antibodies inhibits secretion of TNF from cultured human mononuclear cells (38). However, results of clinical trials of specific blockers of single inflammatory pathways have been disappointing (3, 18). An alternative approach to the prevention or treatment of SIRS would be to develop a broad-spectrum blockade of the hyper-reactive immune responses of macrophages, either by killing them or by reversibly inhibiting them. In addition to liver and splenic macrophages, sheep have a large population of pulmonary intravascular macrophages, which develop shortly after birth. As a result, sheep are highly sensitive to small intravenous infusions of some bacteria or endotoxin (4, 5).

Rooijen and Nieuwmegan (25–27) developed a successful method to selectively kill actively phagocytizing macrophages in rats, using liposomes containing the heavy metal chelating agent dichloromethylene bisphosphonate. Our laboratory adapted their macrophage-depletion procedure to sheep and quantified the depletion of the pulmonary intravascular macrophages (33). Depletion of the intravascular macrophages in sheep markedly inhibited both the pulmonary hemodynamic and lymph dynamic responses to intravenously infused endotoxin (34).

Our intravascular macrophage-depletion regime, although successful, is expensive and the protocol time consuming, if one wants to achieve maximum depletion. Furthermore, the depleted animals are unpro-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: N. C. Staub Sr., Box 965, Stinson Beach, CA 94970.

tected against possible bacteremia until the macrophage population regenerates. Thus we sought another approach, namely, to inhibit the macrophages without killing them. We sought a method that would be cheaper and easier to use yet give blockade of the intravascular macrophages equivalent to physical depletion.

Detergents are known to be nonspecific blockers of many receptor-mediated processes; they are also enzyme inhibitors (2). Our laboratory demonstrated that nonionic detergents block endocytosis of LPS by endothelial cells in culture (23). The release of cytokines by LPS-stimulated macrophages can be inhibited by detergents (37). We also obtained evidence that detergents block macrophage activation in intact animals. Longworth and colleagues (16) gave the detergent tyloxapol in doses of 30 $\mu\text{mol/kg}$ to awake horses without any untoward effects. The detergent inhibited most of the acute pulmonary arterial pressure rise, as well as the systemic febrile response and leukocyte sequestration that occur after small intravenous doses of *Escherichia coli* endotoxin (16).

A detergent suitable for therapeutic use ought to have a large margin of safety and be biodegradable. Some of the detergents of the Triton family satisfy these requirements. The archetype detergent Triton X-100 is a nonionic polyoxyethylene-phenol detergent (650 Da). Unfortunately, Triton is quite toxic to cells and animals, inserting into the cell membrane and, in sufficient concentrations, forming pores that cause cell lysis. The main biological use of Triton is cell disruption.

Triton WR-1339 (tyloxapol) is a mixture of linear polymers of Triton X-100 ranging between 7 and 10 Triton repeats (mean number average molecular mass is $\sim 5,000$ Da). It was developed and patented in 1948 by Rohm & Hass (Philadelphia, PA). Tyloxapol is much less toxic than Triton X-100. Although originally used as a mucolytic agent, it was soon discovered that it caused profound hyperlipemia when given parenterally because it blocks endothelial surface lipoprotein lipase. It has widely been used as a model of hyperlipidemic atherogenesis by chronic administration. Cornforth and associates (6) tested tyloxapol and several related polymers as a method to suppress acute tuberculosis infections in mice. A more thorough study of tyloxapol's possible mechanism of action was published in 1958 (21). Tyloxapol is also used in humans as part of synthetic lung surfactant (37, 42).

We investigated the effectiveness of nonspecific desensitization of the intravascular macrophages of sheep by tyloxapol as a method to prevent endotoxin-induced pulmonary and systemic responses. We present overwhelming evidence that tyloxapol, given intravenously before infusion of a standard dose of endotoxin, markedly inhibits both the pulmonary and systemic responses of unanesthetized, chronically instrumented sheep. Furthermore, we did not find any significant side effects, and the animals recovered completely.

METHODS

Overview of Studies

We conducted two sets of studies. First, we did some preliminary studies to determine the relationship between intravenous detergent dose and inhibition of pulmonary vascular reactivity (dose-inhibition experiments in 6 anesthetized sheep) and to determine the time course over days for the recovery of pulmonary vascular reactivity after intravenous detergent (recovery time course experiments in 6 awake sheep). In these studies, we used Monastral blue pigment and microspheres as test particles for pulmonary vascular reactivity. After this preliminary work, we did the main experimental studies in 10 awake sheep to determine the inhibition by detergent of the pulmonary and systemic responses to intravenous endotoxin.

Materials

Detergent. Tyloxapol (also known as Triton WR-1339; Sigma Chemical, St. Louis, MO) is viscous and slow to dissolve in water. To infuse it in the sheep, we diluted it in 500 ml of warm PBS for 1 h or more before using it.

Test particles. We used two types of particles to test the inhibition of pulmonary vascular reactivity by detergent. Our laboratory has used both of these particles extensively in previous studies to elicit an increase in pulmonary arterial pressure (14, 15, 17). Monastral blue (Sigma Chemical) pigment particles were suspended in 10 ml saline for injection. Polystyrene microspheres of 1- μm diameter (Duke Scientific, Palo Alto, CA) were suspended in 10 ml PBS for injection.

Endotoxin. We used *E. coli* endotoxin (type 055:B5; Difco, Detroit, MI) suspended in saline to test the inhibition of pulmonary and systemic responses to endotoxin by detergent.

Preliminary Studies

Dose inhibition. We used six halothane-anesthetized prone sheep (25.2 ± 6.0 kg); each was intubated and ventilated with 30% oxygen. In each animal, via an external jugular vein, we floated a triple-lumen Swan-Ganz thermodilution catheter (7F) into the pulmonary artery to measure pressure and to determine cardiac output. We also placed catheters in a carotid artery to measure systemic pressure and in the other external jugular vein for infusions. We measured systemic and pulmonary arterial pressures continuously by using pressure transducers (Medex) zeroed to the sternum and recorded on a direct-writing polygraph. We measured cardiac output in duplicate every 30 min by using 10 ml of room-temperature, 5% glucose solution injected into the proximal port of the Swan-Ganz catheter; output was calculated by a cardiac output computer (model 3500, KMA).

For each dose-inhibition curve we began with 2 $\mu\text{mol/kg}$ (10 mg/kg) of detergent and doubled the accumulated dose at each step until we achieved complete inhibition or until we had infused a total of 150 mg/kg (30 $\mu\text{mol/kg}$). After each detergent infusion, we determined the inhibition of the peak rise of pulmonary arterial pressure caused by a 1-min intravenous infusion of 5–12 mg of Monastral blue pigment particles. Using the baseline peak pressure response as 100%, we calculated the percent inhibition that occurred during each test. We allowed 1 h for recovery from tachyphylaxis between tests (19).

Recovery time course. After we were satisfied that tyloxapol was safe and caused no observable side effects, we thereafter used unanesthetized sheep. For the recovery time

course, we studied six sheep (27 ± 4 kg) that had been earlier instrumented in the manner described above. The animals were standing unrestrained in mobile metabolism cages throughout the experiment. We measured systemic and pulmonary arterial pressures and cardiac output in duplicate, as already described.

In these experiments we measured pulmonary vascular reactivity before and after infusion of detergent (tyloxapol, 30 $\mu\text{mol/kg}$ by iv drip over 1 h). We tested the pressure response to a 1-min infusion of 400 μl polystyrene microspheres twice in the baseline period, once immediately after completing the detergent infusion and then every 2–3 days thereafter until recovery was complete or 7 days had passed.

As an independent measurement of the effect of the detergent dose on the sheep, we determined plasma lipids as a measure of endothelial cell lipoprotein lipase inhibition. We took arterial samples before and after detergent on *day 1* and on every test day thereafter and measured plasma triglyceride concentration by an enzymatic, colorimetric end-point assay (Triglyceride assay kit, Roche).

In four of the six sheep, we also determined the effect of detergent on the uptake of Monastral blue particles by pulmonary intravascular macrophages. We did this by infusing Monastral blue (5 mg/kg iv over 1 h) after the first test response after the detergent infusion in the above-described experiments. To measure the plasma clearance of Monastral blue, we sampled arterial blood 1–15 min after completing the pigment particle infusion (33). When the study was completed, we killed each animal by a large dose of pentobarbital, opened the thorax, and removed, weighed, and homogenized each lung. We measured lung copper concentration by atomic absorption spectroscopy on triplicate samples of each homogenate and calculated the percentage of Monastral blue (11% copper by weight) retained in the lungs (7, 17).

Main Experimental Studies

To study the inhibition of the endotoxin response by detergent, we used 10 awake sheep (39.1 ± 2.2 kg) in which we had placed a chronic lung lymph fistula and a systemic arterial catheter for blood sampling or for pressure measurements by using our laboratory's standard procedures (28, 35). Because these experiments extended over 2–4 wk, we did not keep the pulmonary arterial catheter in place for the duration of the study. On the day before each experiment, we floated a triple-lumen 7F Swan-Ganz thermodilution catheter into the pulmonary artery via an external jugular vein. We used the proximal injection port of the catheter for slow infusions of tyloxapol or endotoxin into the right atrium.

We allowed 1 wk for recovery from the lymphatic cannulation surgery, and then we performed two or three studies in each animal over 1–4 wk. In the first study (control), after a 2-h stable baseline, we infused 1 $\mu\text{g/kg}$ *E. coli* endotoxin diluted in 30 ml saline into the right atrium over 30 min. One week later in the second study (detergent), we gave tyloxapol (40 $\mu\text{mol/kg}$; 200 mg/kg) diluted into 500 ml saline by right atrial drip over 1 h. In seven sheep we waited 4 h, and then we infused the *E. coli* endotoxin; in three we infused the endotoxin immediately after completing the tyloxapol infusion to determine whether there was any effect of the time delay on the response.

During the 8 h of each experiment, we measured systemic variables (arterial blood pressure, core temperature, hematocrit, circulating leukocyte concentration, and plasma protein concentration) and pulmonary variables [pulmonary arterial pressure, thermodilution cardiac output, lung lymph

flow, lymph-to-plasma protein concentration ratio (L/P)], and we calculated lymph protein concentration (lymph flow \times L/P). We used lymph protein clearance (LPC) as our overall measure of altered lung microvascular leakiness (34).

Recovery of the endotoxin response. In five sheep, we were able to maintain the lung lymph fistula long enough to repeat the control experiment with endotoxin alone. We studied one sheep each at 7 and 10 days of recovery and three sheep at 14–16 days.

TNF Measurements

We measured the concentration of TNF in plasma and lymph in a specific double-antibody precipitation RIA (12, 22), using recombinant bovine (rb) TNF (gift of Ciba-Geigy, Basel, Switzerland) as immunogen for antibody production (rabbit anti-bTNF 314/88) and the standard curve. As an assay tracer we iodinated the TNF with ^{125}I -labeled Na and iodogen (Pierce, Rockland, IL). We purified the tracer from aggregated material by chromatography through Sephadex G-57 and added it to the assay tubes under nonequilibrium conditions (24-h delayed addition). The minimal detectable quantity of TNF was 12 pg/assay tube. When we added rbTNF to plasma and lymph samples, recovery was linear and uniformly averaged 96% over the range 25–100 pg/200 μl sample (in a final tube volume of 800 μl). We assayed all samples in a single run; the coefficient of variation between duplicate samples averaged 11.4%.

Statistics

The data and statistics are presented in the eight figures as time course data; each point is the group mean \pm SE. No additional statistics were done on data from the preliminary studies (see Figs. 1 and 2) or on the time courses for individual animals in the main experimental studies (see Figs. 3 and 7). In the group summary data in the main experimental studies ($n = 10$), the baseline period and all times after infusion of endotoxin were analyzed by using a one-way analysis of variance. We used a two-way analysis of variance to compare the endotoxin alone (control) and the tyloxapol before endotoxin (detergent) series (see Figs. 4, 5, 6, and 8).

Where a statistical difference between the series occurred, we compared the individual time points by a paired *t*-test taking account of multiple comparisons (Origin, v 4.1; Microcal Software, Northhampton, MA). We accepted $P < 0.05$ as indicating statistical significance.

RESULTS

Preliminary Studies

Dose inhibition. Figure 1 shows the dose-inhibition relationship in six sheep between tyloxapol and the peak pulmonary arterial pressure rise caused by the test infusion of Monastral blue pigment particles. The pressure data are plotted as percent inhibition from the baseline response (100%). Of the six sheep, four showed 100% and two >90% inhibition at the maximum cumulative dose (30 $\mu\text{mol/kg}$).

Tyloxapol had no significant hemodynamic effects on pulmonary or systemic pressures, heart rate, breathing rate, cardiac output, or temperature. There was no detectable hemolysis as determined by visual inspection of plasma or lymph. Thus tyloxapol up to at least 30 $\mu\text{mol/kg}$ is safe to use and blocks most of the pulmonary arterial pressure responses to our test particles.

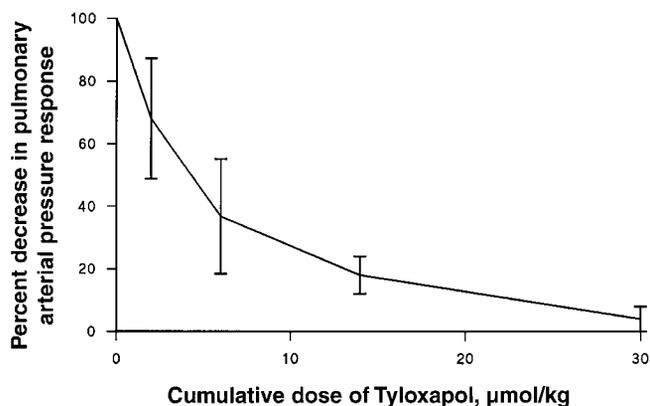


Fig. 1. Cumulative dose-inhibition relationship between tyloxapol and peak pulmonary arterial pressure after an intravenous injection of Monastral blue pigment particles. Values are means \pm SE. The pressure responses are relative (percentage of response at 0 dose). We waited 1 h between detergent doses and particle tests to avoid tachyphylaxis.

Recovery time course. Figure 2 summarizes the 7-day recovery time course of the pulmonary arterial pressure response after a single dose of tyloxapol (30 μ mol/kg) in six sheep. The response was inhibited >90% for at least 1 day ($P < 0.05$). After 3 and 7 days, the test pressure response had recovered to an average of 75%. There was considerable variability among the sheep, as indicated by the large standard error bars. We have no explanation for such interanimal variation.

We did not find any acute or chronic side effects to the tyloxapol infusion; no hemolysis was seen in plasma samples, and all sheep appeared healthy, were afebrile, and ate and drank normally until the experiment was terminated. The 30 μ mol/kg dose blocked not only most of the pressure response to the pigment particles but also the pressor response to the larger labeling dose of Monastral blue (5 mg/kg), which we gave on *day 0*, after the pressure test.

Plasma triglycerides after tyloxapol. Tyloxapol caused a visible increase in plasma opalescence during the experiment, as we expected, because of tyloxapol's known inhibition of lipoprotein lipase on the luminal surface on capillary endothelial cells. In three sheep, we measured plasma triglycerides for several days after giving tyloxapol (30 μ mol/kg). All showed a peak in the plasma lipid concentration on the second day with the plasma concentrations returning to baseline levels by 4–7 days (data not shown). More data on the early plasma lipid time course are shown in the main experimental results (see Fig. 5).

Monastral blue plasma clearance and lung retention. In four sheep, tyloxapol did not affect the clearance rate of Monastral blue from arterial blood. One minute after completion of the particle infusion, the arterial plasma showed no trace of blue pigment. At postmortem, the lungs were bright blue. The fraction of the infused Monastral blue retained in the lung averaged $78 \pm 8\%$.

Main Experimental Studies

Tyloxapol and endotoxin. We completed paired studies in 10 awake, chronically instrumented sheep with lung lymph fistulas. Thus each animal served as its own control. We include an example of data obtained over the 8-h study in two sheep (see Figs. 3 and 7). For the remainder, we show the group data because all of the animals responded in the same manner. In each graph we plotted four sets of data: 1) LPC, 2) mean pulmonary arterial pressure, 3) body temperature, and 4) circulating leukocyte concentration; two sets were pulmonary and two were systemic responses. We present these data here because they are germane to our aims in this study and because they changed significantly after endotoxin or between control and detergent experiments. Thus, whereas cardiac output was variable within $\pm 20\%$ and systemic arterial pressure was variable within $\pm 10\%$, neither showed any group trends.

Figure 3 shows two complete 8-h experiments in one sheep (L6-98). In this example, the detergent reduced the LPC increase by $\sim 95\%$, the pulmonary arterial pressure rise by 70%, the leukopenic (leukocyte sequestration) response by 90%, and the temperature response by 90%.

Figure 4 summarizes the data for the both experiments in all 10 sheep. There are no differences in the baseline period between control and tyloxapol experiments. After tyloxapol, all of the sheep responded similarly to endotoxin, as indicated by the small error bars. Overall, tyloxapol reduced the expected LPC increase by $\sim 90\%$, the pulmonary arterial pressure rise by 75%, the leukopenic (sequestration) response by 80%, and the temperature response 70%. After the baseline, the data are significantly different between the control and detergent studies, except for one temperature, two leukocyte, and two pressure points. Al-

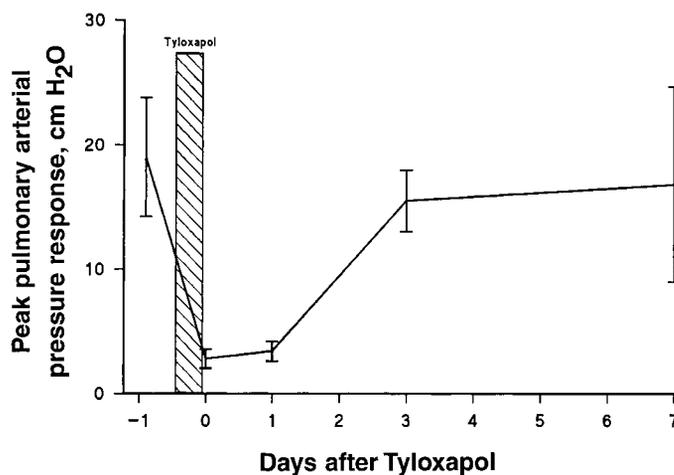


Fig. 2. Mean time course of recovery in 6 sheep of the pulmonary arterial pressure response after blockade by the detergent tyloxapol (30 μ mol/kg iv). Values are means \pm SE. The response was >90% suppressed for at least 24 h. Recovery was $\sim 75\%$ after 1 wk, although the large SE bars on *day 7* indicate considerable individual variation.

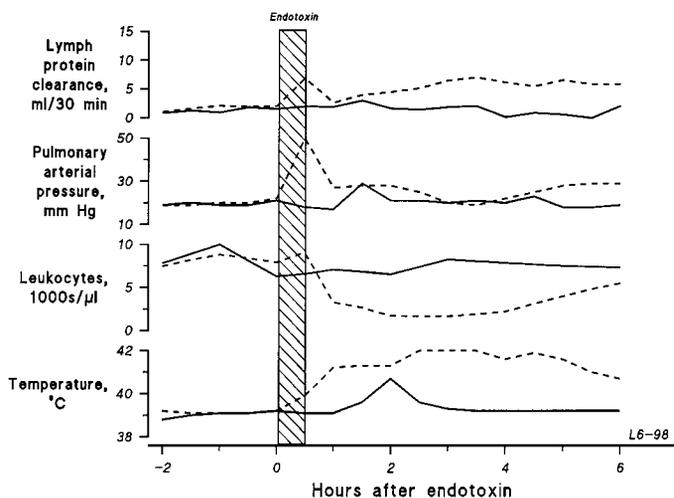


Fig. 3. Time course of 4 variables during 2 experiments in 1 awake sheep (L6-98) performed 1 wk apart. From top to bottom, the y-axes show lymph protein clearance, mean pulmonary arterial pressures, circulating leukocyte concentration, and body temperature. Compared with the control study (dashed lines) all variables are markedly attenuated after tyloxapol (40 μ mol/kg iv; solid lines) was infused before endotoxin.

though $P < 0.05$ was the statistical significance level chosen, the large majority of data pairs have much smaller P values ($P < 0.01$ or < 0.001).

Effect of tyloxapol on plasma triglyceride levels. Figure 5 shows that tyloxapol caused a linear rise in plasma lipids in every animal. Regardless of whether the detergent was given 4 h before endotoxin or immediately before endotoxin, the rate of rise of the circulating triglycerides was the same (slopes not significantly different). Endotoxin alone did not affect the

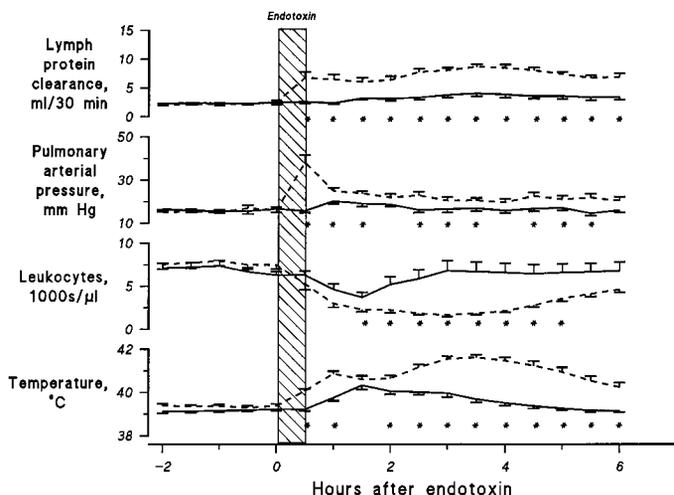


Fig. 4. Summary of time course of 4 variables during 2 experiments in each of 10 awake sheep studied 1 wk apart. From top to bottom, the y-axes show lymph protein clearance, mean pulmonary arterial pressures, circulating leukocyte concentration, and body temperature. Values are means \pm SE. Compared with the control study (dashed lines), the effect of endotoxin on all variables is markedly attenuated after tyloxapol (solid lines). *Points of statistical significance ($P < 0.05$) between the data pairs; the majority of P values were < 0.001 .

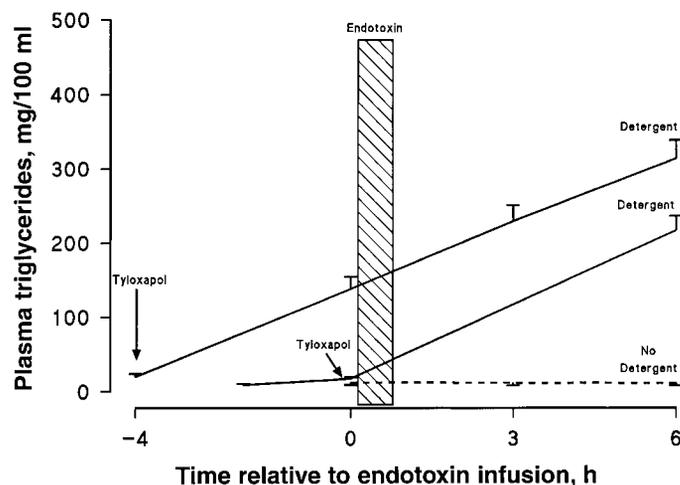


Fig. 5. Effect of endotoxin alone and tyloxapol followed by endotoxin on the rise in plasma lipids in 10 awake sheep. Values are means \pm SE. The detergent was given 4 h before endotoxin in 7 animals (top solid line) and immediately before endotoxin in 3 animals (bottom solid line). The rise in plasma triglycerides indicates blockade of the endothelial luminal surface enzyme lipoprotein lipase. Regardless of when tyloxapol was given, plasma lipids increased in a linear fashion afterward. Endotoxin alone did not affect the circulating lipid concentration.

circulating lipid concentration, nor did it affect the rate of rise of the lipids after the detergent.

Recovery of endotoxin response after tyloxapol. We studied the recovery from the detergent in five sheep by doing another control experiment 1–3 wk after giving tyloxapol. In two sheep (7 and 10 days after giving tyloxapol), recovery was incomplete (data not shown). This was unexpected because the plasma lipids returned to control levels within 1 wk, showing that the blockade of lipoprotein lipase had disappeared. However, the time course of recovery of the pulmonary hemodynamic response (Fig. 2) did suggest a delayed final recovery.

In three sheep, after 2 wk or more recovery, all responses had returned essentially to the initial control level, as summarized in Fig. 6. The before and after tyloxapol control studies are essentially identical, indicating complete recovery from the effects of the detergent after 2 wk. There was no indication of any development of tolerance, even though these animals had received three doses of endotoxin over a period of 4 wk.

Effect of tyloxapol on circulating cytokine concentration. Figure 7 shows the time course of both plasma and lymph TNF concentration in one sheep (L8-97), together with two functional attributes; LPC (pulmonary) and body temperature (systemic). In the control experiment, TNF began to increase by the end of the endotoxin infusion, peaking in plasma at the 1-h sample and in lymph at the 2-h sample. Furthermore, the plasma peak concentration was nearly twice the peak lymph concentration. Body temperature peaked at 42°C \sim 1 h after the peak of plasma TNF occurred. Similarly, LPC peaked after both plasma and lymph TNF had peaked. After tyloxapol treatment, the rises

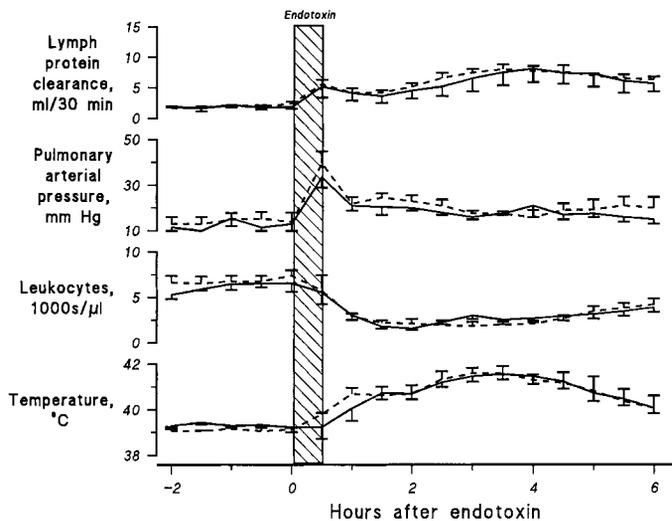


Fig. 6. Summary of the time course of responses after endotoxin infusions (control), before tyloxapol, and again 2–3 wk after recovery from tyloxapol treatment in 3 sheep. Values are means \pm SE. The data are essentially identical. Dashed lines, initial control data; solid lines, recovery control data.

in plasma and lymph TNF were markedly inhibited (>90%), as were the prolonged rises in temperature and LPC.

Figure 8 summarizes the time course of mean plasma and lymph TNF in the 10 sheep for the control and tyloxapol experiments. There were no significant differences in either plasma or lymph concentrations between the two groups of experiments during the baseline period. The group data confirm what occurred in the single sheep. After endotoxin alone (control), the plasma TNF concentration peaked at 1 h. The plasma level peaked at almost twice the maximum lymph concentration. After tyloxapol treatment, the rises in plasma and lymph TNF were not only markedly atten-

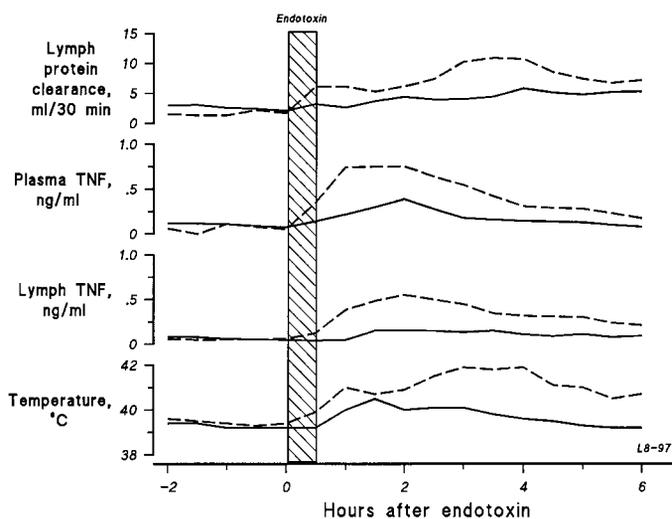


Fig. 7. Time course of lymph protein clearance, plasma and lymph tumor necrosis factor (TNF) concentrations, and body temperature in 1 sheep (L8-97). Values are means \pm SE. Dashed lines, endotoxin alone; solid lines, tyloxapol given before endotoxin. All 4 variables are markedly attenuated by the detergent.

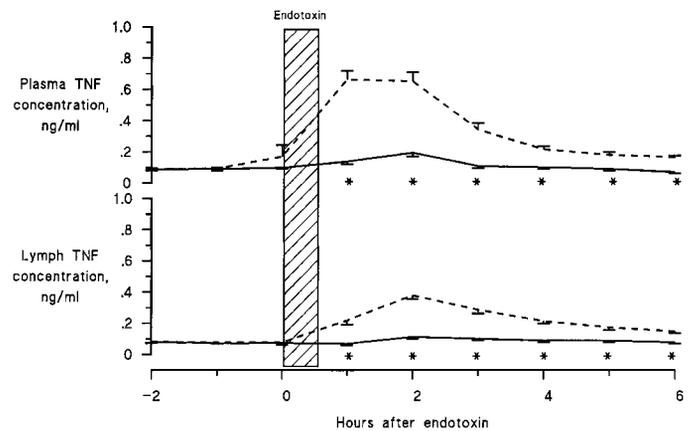


Fig. 8. Summary of time course of plasma and lymph TNF concentrations in 10 awake sheep for 2 experiments each. Dashed lines, endotoxin alone; solid lines, endotoxin after tyloxapol treatment. Tyloxapol inhibited the circulating concentration of TNF by >90%. *Differences between groups are significant ($P < 0.001$) at all times after endotoxin.

uated but also there was almost no differences among sheep as the tiny error bars indicate. The inhibition of the rise in plasma TNF was >90% and was significant at all times after the endotoxin infusion, even at 6 h ($P < 0.001$).

DISCUSSION

Our purpose in performing these experiments was to determine whether the detergent inhibited either the pulmonary or systemic responses to acute endotoxemia. That tyloxapol did so is self-evident from the data for individual sheep (Figs. 3 and 7) and for the whole group of 10 sheep (Figs. 4 and 8). The increase in pulmonary vascular pressure and microvascular leakiness (LPC), the febrile response, and the inflammatory response (leukopenia and increased plasma TNF concentration) caused by endotoxin were markedly attenuated by tyloxapol. Furthermore, the detergent effects were completely reversible over 2 wk (Fig. 6). We believe our results using tyloxapol are unique among experimental studies of endotoxemia in whole animals (sheep and horses).

Comparison between tyloxapol and macrophage depletion. When we compare these new results with our laboratory's previous macrophage depletion studies using liposomes containing the heavy metal chelating compound, dichloromethylene bisphosphonate (Clodronate, Boehringer Ingelheim) (33, 34), we conclude that the use of a detergent is simpler, less expensive, and quicker. Inhibition of the effect of experimental endotoxemia by tyloxapol is equally, possibly more, effective than depletion. The full effect of the detergent appears to be immediate, as we showed for the three sheep in which we gave endotoxin immediately after infusing tyloxapol (main experimental studies). Even on a worst-case basis, tyloxapol was fully effective in all sheep within 4 h.

Other important advantages of tyloxapol over macrophage depletion are that it is more effective in block-

ing some of the systemic responses and it is safer. After macrophage depletion, our laboratory had two episodes of uncontrollable bacteremia (33); no such problem occurred in the present study. The explanation is suggested by our Monastral blue clearance data; even when endotoxin responses are inhibited by tyloxapol, phagocytosis by the intravascular macrophages still occurs. This is an important consideration for clinical applications of macrophage inhibition.

Benefit of Detergent Nonspecificity

The data indicate that the detergent binds nonspecifically to various intravascular cells (macrophages, endothelium, leukocytes), whereas the toxic liposomes we used for depletion are almost exclusively phagocytized by intravascular macrophages (25, 33). Thus, although the inhibition of lung lymph dynamic responses are similar for the two blockade procedures, the systemic responses to endotoxin are inhibited much more by tyloxapol. The only direct comparison we have is the leukopenia response, which is ~70% inhibited over 6 h after detergent, whereas after macrophage depletion, there was only a small effect (34).

Dose of Tyloxapol

The dose of tyloxapol we used (40 $\mu\text{mol/kg}$; 200 mg/kg) was completely safe, according to all of our evidence; no sheep became ill or died after receiving tyloxapol. We probably could have safely increased the dose to 60 $\mu\text{mol/kg}$ (300 mg/kg) because Patnode et al. (21) gave this larger dose subcutaneously three times in 1 wk to guinea pigs to provoke intense lipemia. On the other hand, tyloxapol may be effective in smaller doses with the potential of more rapid recovery. The 40 $\mu\text{mol/kg}$ dose not only inhibited the intravascular macrophage responses to endotoxin but also blocked endothelial surface lipoprotein lipase enzyme, causing a prolonged increase in the total circulating plasma lipids. The lipoprotein lipase blockade is independent of pulmonary intravascular macrophage inhibition because it occurs in mammals that do not have them (6). Different doses of tyloxapol and alternate routes of administration remain to be explored.

Sensitivity of Intravascular Macrophages

As far as various populations of intravascular macrophages are concerned, the detergent is as nonspecific as are toxic liposomes (34). We do not know of any reliable method to selectively inhibit the pulmonary intravascular macrophages and avoid the liver intravascular macrophages. One advantage of the slow intravenous infusion we used is that the pulmonary intravascular macrophages have the first opportunity to interact with tyloxapol. The pulmonary intravascular macrophages are exposed to 100% of cardiac output, whereas the liver macrophages only receive ~25–30% of cardiac output; thus they interact with only about one-fourth of whatever tyloxapol, endotoxin, or particles pass through the lungs on the first circulation. We found that the pulmonary intravascular macrophages

retained ~80% of infused Monastral blue particles. In our laboratory's macrophage-depletion study, the control sheep retained ~85% of the infused Monastral blue (33). Warner and co-workers (41) reported high pulmonary retention of colloidal iron particles and of labeled endotoxin in sheep on the first pass through the lungs.

Effect of Tyloxapol on Circulating TNF

The dramatic effects of tyloxapol in reducing the endotoxin-induced rise in circulating TNF are similar to the effects of tyloxapol in reducing the other physiological changes after endotoxin. Our data are highly reproducible among the 10 sheep, as indicated by the small standard error bars, so that overall the differences between the groups, after the endotoxin infusion, are highly significant ($P < 0.01$); even at 6 h, when the experiment ended, the paired differences are still significant ($P < 0.05$).

As Fig. 8 shows, the source of TNF is most likely intravascular because the concentration peaked earlier and reached higher concentrations in plasma than in lymph in every animal. In reports by others, the principal source of plasma TNF in several mammalian species (none of which had pulmonary intravascular macrophages) appears to be the liver intravascular macrophages (13, 39). However, there is one unexplained failure to block the rise of plasma TNF in the rat after partial liver intravascular macrophage inhibition using gadolinium trichloride (10).

Now that assays for TNF are available for pig (11), and cattle and sheep (12), the source of the endotoxin-induced rise in plasma TNF needs to be reevaluated. The pulmonary intravascular macrophages make up a huge population, comparable to the mass of liver intravascular macrophages. We believe more attention needs to be given to assays that quantify circulating cytokines in large animals, in which critically important physiological data can be easily obtained.

Tyloxapol's Mechanism of Action

At suitable doses, detergents stabilize biological membranes and modulate membrane-related processes in cells (2). Surfactants may affect the conformation of membrane receptors for LPS (CD14) or adhesion receptors (such as CD11/CD18), thus acting as antagonists. The addition of detergent (sodium desoxycholate or Tween 80) to cell culture media reduced the phagocytosis of *Staphylococcus epidermidis* by human neutrophils fourfold (40). Antibiotics of the polymyxin group (polycationic peptide detergents) penetrate bacterial membranes, causing the appearance of numerous protrusions or blebs. Polymyxin also decreases the release of TNF from stimulated alveolar macrophages (36). Pluronic F68 inhibits the adherence and migration of isolated granulocytes and also decreases the appearance of leukocytes in alveolar lavage fluid in bleomycin-instilled rat lungs (44). Human pulmonary surfactant reduces the ability of monocytes and alveolar macrophages to phagocytize bacteria (9). The syn-

thetic surfactant Exosurf, which contains 6% tyloxapol, inhibits activation of alveolar macrophages by LPS and blocks the release of TNF and other cytokines. The mechanism of detergent action as a blocker of receptor-ligand interaction appears to be a hydrophobic association with receptor nonpolar groups.

Cornforth et al. (6) thought that tyloxapol suppressed the growth of tubercle bacilli because of the plasma hyperlipemia. In that way, they explained the fact that tyloxapol suppressed the bacilli in whole animals but not in cell culture. However, Rees (24) showed that the lipemic effect and the therapeutic effect of tyloxapol on tuberculosis infections in mice could be separated, leading Patnode et al. (21) to conclude that "... cellular surfaces are somehow altered by Triton."

We wondered whether the rise in plasma lipids contributed to tyloxapol's action in endotoxemia. Whether the endotoxin was given immediately after or 4 h after tyloxapol, the responses were the same, even though the concentrations of plasma lipids were different (Fig. 5). We conclude that the effect of tyloxapol in blocking responses to endotoxemia is due to the detergent itself and not because it raises plasma lipid concentration.

The uptake and internalization of endotoxin by macrophages in mammals is mainly, if not entirely, facilitated by receptor-mediated endocytosis (39) and thus is a likely target for the detergent's action. The general process of endotoxin internalization involves the formation and invagination of clathrin-coated pits containing the receptors. However, the mechanism of initialization of coated pit formation is unknown (1). Serikov et al. (31) hypothesized that changes in cell membrane-surface free energy may be the trigger. They made a theoretical analysis of surface free-energy nonuniformity as the possible triggering mechanism for endocytosis. In a computer model, their results adequately explained vesicle formation (30).

To obtain experimental evidence, Serikov and Staub (32) blood-perfused six *in situ* sheep lungs at constant blood flow and found that Triton X-100 delayed the removal from the perfusate of detoxified FITC-labeled lipopolysaccharide. The results suggested that receptor-mediated endocytosis was inhibited by the detergent. In a follow-up temperature-sensitivity study, Powers et al. (23) found that Triton X-100 completely blocked the internalization of labeled LPS at 40°C but did not block surface binding of the LPS at 8°C. Later, Serikov and colleagues (29) did *in vitro* studies and found that tyloxapol blocked several human neutrophil receptors: CD4, CD8, CD14. However, neutrophil migration and phagocytosis of *S. aureus* at 50 μ M of tyloxapol remained intact (29). The latter effect is the same as what we found in the chronic sheep experiments of the present study.

Random Order of Experiments

One potential problem with our experiments was that they were not done in random order. We always

did the endotoxin control first to avoid a prolonged recovery period before we could do the detergent experiment. To circumvent the problem of randomness, we planned to repeat the endotoxin control after recovery from tyloxapol (2 wk), if we could keep the lung lymph fistula patent. Despite our best efforts, lymph flow stopped in half of the sheep. Among the five recovery control experiments we did complete, one each at 7 and 10 days showed incomplete recovery, although both showed that recovery was in progress. This incomplete recovery is consistent with the delayed recovery time course we found in our preliminary studies (Fig. 2). Although our comparative data on endotoxin before and after recovery from tyloxapol are limited to three sheep, these are sufficient because the data are essentially identical for all measured variables (Fig. 6). We conclude that the detergent effect is completely reversible and that our repeated small doses of endotoxin did not provoke tachyphylaxis or tolerance.

Our experiments were designed to study the effect of tyloxapol on endotoxin-induced pulmonary and systemic responses in intact animals. Thus our results cannot solve the basic cellular mechanism by which tyloxapol inhibits endotoxin. However, the results are consistent with the view of Patnode et al. (21) that the principal action of tyloxapol is at membrane surfaces. The results are also consistent with the hypothesis of Serikov et al. (31) that variation in surface free energy is involved in triggering endocytosis.

Clinical Implications

There are a number of possibilities for the therapeutic use of detergents in disease. Here we are interested mainly in endotoxemia, which is a substantial problem in veterinary medicine. Our laboratory has shown in horses and now in sheep that tyloxapol safely inhibits the systemic as well as the pulmonary responses to intravenous endotoxin (16). Thus we believe it will be safe and effective in a variety of animals, those with and without pulmonary intravascular macrophages. We recommend that clinical trials of tyloxapol be undertaken for the prevention or treatment of acute endotoxemia in valuable livestock.

Many humans die annually from SIRS, as a complication of trauma or sepsis. Thus far, biotechnology has not had much positive impact in human sepsis; mortality remains high. Available experimental animal studies and the new data we have presented suggest that tyloxapol offers promise for the prevention or treatment of acute human endotoxemia. We believe this despite the hyperlipidemia, because it did not appear to cause any problems in our sheep. Naturally, studies of side effects, safety, and dose must be done. We are confident that tyloxapol (or some other detergent) will be found to be safe and effective in humans.

We could never have completed this study without the generous and continuing support of Dr. Robert Gunther, Director, Health

Sciences Research Surgery Laboratory, School of Medicine, University of California, Davis, and his staff. Special thanks to Jessica Davis and Linda Talken, who made the sheep surgical preparations, looked after the animals on a daily basis, and helped us with other technical aspects of the experimental studies. The preliminary experiments were done in the Sheep Facility at the University of California San Francisco (UCSF).

Dr. K. E. Longworth, Assistant Research Physiologist, Department of Surgical and Radiological Sciences, School of Veterinary Medicine, University of California, Davis, coordinated and collaborated in all of the experiments.

Dr. E. H. Jerome, Associate Professor of Anesthesia, Department of Pediatric Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, New York, collaborated on the preliminary experiments at the UCSF sheep facility.

This work was supported by an American Heart Association grant to N. C. Staub.

Present address of V. Serikov: Institute of Molecular Pharmacology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0828.

REFERENCES

1. **Albert B.** (Editor). *Molecular Biology of the Cell* (3rd ed.). New York: Garland, 1994, p. 636.
2. **Attwood D and Florence TP.** *Surfactant Systems*. London: Chapman & Hall, 1983.
3. **Bone RC.** Toward a theory regarding the pathogenesis of the systemic inflammatory response syndrome: what we do and do not know about cytokine regulation. *Crit Care Med* 24: 163–172, 1996.
4. **Brigham KL, Bowers RE, and Haynes J.** Increased sheep lung vascular permeability caused by *Escherichia coli* endotoxin. *Circ Res* 45: 292–297, 1979.
5. **Brigham KL and Meyrick B.** Endotoxin and lung injury. *Am Rev Respir Dis* 133: 913–927, 1986.
6. **Cornforth JW, Hart PD, Rees RJW, and Stock JA.** Antituberculous effect of certain surface-active polyoxyethylene ethers in mice. *Nature* 168: 150–153, 1951.
7. **Evenson MA.** Measurement of copper in biological samples by flame or electrothermal atomic absorption spectrophotometry. *Methods Enzymol* 158: 351–357, 1988.
8. **Galanos C and Freudenberg MA.** Mechanisms of endotoxin shock and endotoxin hypersensitivity. *Immunobiology* 187: 346–356, 1993.
9. **Geertsman MF and Broos HR.** Ingestion of pulmonary surfactant by human monocytes inhibits their antibacterial functions. In: *Mononuclear Phagocytes*, edited by van Furth R. Dordrecht, The Netherlands: Kluwer, 1992, p. 308–314.
10. **Iimuro Y, Yamamoto M, Kohno H, Itakura J, Fujii H, and Matsumoto Y.** Blockade of liver macrophages by gadolinium chloride reduces lethality in endotoxemic rats—analysis of mechanisms of lethality in endotoxemia. *J Leukocyte Biol* 55: 723–729, 1994.
11. **Jesmok G, Lindsey C, Duerr M, Fournel M, and Emerson T Jr.** Efficacy of monoclonal antibody against human recombinant tumor necrosis factor in *E. coli*-challenged swine. *Am J Pathol* 141: 1197–1207, 1992.
12. **Kenison DL, Elsasser TH, and Fayer R.** Radioimmunoassay for bovine tumor necrosis factor: concentration and circulating molecular forms in bovine plasma. *J Immunoassay* 11: 177–198, 1990.
13. **Kluger MJ.** Fever: role of pyrogens and cryogens. *Physiol Rev* 71: 93–127, 1991.
14. **Longworth KE, Albertine KH, and Staub NC.** Ultrastructural quantification of pulmonary intravascular macrophages in newborn and 2-week-old lambs. *Anat Rec* 246: 238–244, 1996.
15. **Longworth KE, Jarvis KA, Tyler WS, Steffey EP, and Staub NC.** Pulmonary intravascular macrophages in horses and ponies. *Am J Vet Res* 55: 382–388, 1994.
16. **Longworth KE, Smith BL, Staub NC, Steffey EP, and Serikov VB.** Use of detergent to prevent initial responses to endotoxin in horses. *Am J Vet Res* 57: 1063–1066, 1996.
17. **Longworth KE, Westgate AM, Grady MK, Westcott JY, and Staub NC.** Development of pulmonary intravascular macrophages in lambs: hemodynamics and uptake of particles. *J Appl Physiol* 73: 2608–2615, 1992.
18. **Lynn WA.** Anti-endotoxin therapeutic options for the treatment of sepsis. *J Antimicrobial Chemotherapy* 41, Suppl A: 71–80, 1998.
19. **Miyamoto K, Schultz K, Heath T, Mitchell MD, Albertine K, and Staub NC.** Pulmonary intravascular macrophages and hemodynamic effects of liposomes in sheep. *J Appl Physiol* 64: 1143–1152, 1988.
20. **Nyström PO.** The systemic inflammatory response syndrome: definition and aetiology. *J Antimicrobial Chemotherapy* 4, Suppl A: 1–7, 1998.
21. **Patnode RA, Hudgins PC, and Janicki BW.** Studies on the effect of Triton (WR-1339) on guinea pig tissues. *J Exp Med* 107: 33–61, 1958.
22. **Perkowski SZ, Sloane PJ, Spath JA, Elsasser TH, Fisher JK, and Gee MH.** TNF and the pathophysiology of endotoxin-induced acute respiratory failure in sheep. *J Appl Physiol* 80: 564–573, 1996.
23. **Powers M, Serikov V, and Staub NC.** Triton X-100 reduces fluid-phase endocytosis and receptor internalization but not ligand binding in cultured endothelial cells (Abstract). *FASEB J* 7: A236, 1993.
24. **Rees RJW.** Antituberculosis activity of certain non-ionic detergents. *Proc R Soc Med* 46: 581–583, 1953.
25. **Rooijen NV.** The liposome-mediated macrophage “suicide” technique. *J Immunol Methods* 124: 1–6, 1989.
26. **Rooijen NV.** Liposome elimination of macrophages. *Res Immunol* 143: 215–219, 1992.
27. **Rooijen NV and Nieuwmegan RV.** Elimination of phagocytic cells in the spleen after intravascular injection of liposome-encapsulated dichloromethylene-diphosphonate: an enzyme histochemical study. *Cell Tissue Res* 283: 355–360, 1984.
28. **Roos PJ, Wiener-Kronish JP, Albertine KH, and Staub NC.** Removal of abdominal sources of caudal mediastinal node lymph in anesthetized sheep. *J Appl Physiol* 55: 996–1001, 1983.
29. **Serikov VB, Glazanova TV, and Boobnova T.** Tyloxapol blocks human lymphocyte surface receptors (Abstract). *FASEB J* 10: A4427, 1996.
30. **Serikov VB, Malinin YA, Cherkaev AV, Gibyansky LA, and Serikova NV.** Configuration of the membrane surface forms during local change of surface energy: application to the endocytosis processes. *Biol Membrany* 7: 326–334, 1990.
31. **Serikov VB, Serikova NV, and Belyakov A.** Theoretical approach to the description of microvesicular transport in endothelium. *Arch Anat Histol Embryol* 95: 5–11, 1988.
32. **Serikov V and Staub NC.** Low concentration of detergents blocks endocytosis in perfused sheep lung (Abstract). *Am Rev Respir Dis* 147: A428, 1993.
33. **Sone Y, Nicolaysen A, and Staub NC Sr.** Effect of particles on sheep lung hemodynamics parallels depletion and recovery of intravascular macrophages. *J Appl Physiol* 83: 1499–1507, 1997.
34. **Sone Y, Serikov V, and Staub NC.** Intravascular macrophage depletion attenuates lung injury in anesthetized sheep. *J Appl Physiol* 87: 1354–1359, 1999.
35. **Staub NC, Bland RD, Brigham KL, Demling RH, and Erdmann AJ III.** Preparation of chronic lung lymph fistulas in sheep. *J Surg Res* 19: 315–320, 1975.
36. **Stokes DC, Shenep JL, Fishman M, Hildner WK, Bysani GK, and Rufus K.** Polymyxin B prevents lipopolysaccharide-induced release of tumor necrosis factor-alpha from alveolar macrophages. *J Infect Dis* 160: 52–57, 1989.
37. **Thomassen MJ, Meeker DP, Antal JM, Connors MJ, and Wiedemann HP.** Synthetic surfactant (Exosurf) inhibits endotoxin-stimulated cytokine secretion by human alveolar macrophages. *Am J Respir Cell Mol Biol* 7: 252–260, 1992.
38. **Tobias PT and Ulevitch RJ.** Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. *Immunobiology* 187: 227–232, 1993.
39. **Ulevitch RJ.** Recognition of bacterial endotoxin by receptor-dependent mechanisms. *J Adv Immunol* 53: 267–289, 1993.

40. **Van Oss C, Gillman CF, and Neumann AW.** *Phagocytotic Engulfment and Cell Adhesiveness*. New York: Dekker, 1975, p. 69-72.
41. **Warner AE, DeCamp MM, Molina RM, and Brain JD.** Pulmonary removal of circulating endotoxin results in acute lung injury in sheep. *Lab Invest* 59: 219-230, 1988.
42. **Weg GJ, Bulk RA, Tharratt RS, Jenkinson SJ, Jagdip JB, Zaccardelli D, Horton J, and Pattishall EN.** Safety and potential efficacy of an aerosolized surfactant in human sepsis-induced adult respiratory distress syndrome. *JAMA* 272: 1433-1438, 1994.
43. **Welbourn CR and Young Y.** Endotoxin, septic shock and acute lung injury: neutrophils, macrophages and inflammatory mediators. *Br J Surg* 79: 998-1003, 1992.
44. **Williams JH, Chen M, Drew J, Panigan E, and Hosseini S.** Modulation of rat granulocyte traffic by a surface active agent in vitro and in bleomycin injury. *Proc Soc Exp Biol Med* 188: 461-470, 1988.

