PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 187, 408–415 (1988)

Enhanced Production of Monokines by Canine Alveolar Macrophages in Response to Endotoxin-Induced Shock (42681)

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Abstract: The enhanced production of soluble mediators by alveolar macrophages may be responsible for promoting lung injury in canines administered endotoxin. One of the most prominent monokines, interleukin 1 (IL-1), has the potential to significantly influence the responses of host tissues. In this study we analyzed alveolar macrophages from canines that were experimentally administered endotoxin (AMEC) for their ability to produce IL-1. When concentrated AMEC supernatants from *in vitro* cultures were incubated with fresh C3H/HEJ thymocytes, a threefold greater incorporation of [^{3}H]thymidine resulted as compared to the response produced by controls. Heat treatment of the experimental preparations ablated this difference. Conversely, the activity of AMEC intracellular lysates did not significantly differ from the controls. Silver-staining the preparations separated by SDS–PAGE revealed a low-molecular-weight species (17 kD) in the AMEC supernatant lane while a similar molecular distribution was absent in all of the control preparations examined. Moreover, using the L929 cell line in a cytolytic bioassay we found that these same AMEC supernatants also contained significantly elevated levels of tumor necrosis factor. Collectively, this study suggests that during endotoxin-induced canine lung injury, the alveolar macrophages generate soluble species that can substantially regulate the hosts cellular response. This activity in the canine lung may play a critical role in the development and/or maintenance of the pathology associated with exposure to endotoxin.

In addition to their role in phagocytosis and antigen presentation, macrophages produce regulatory molecules that promote both immune and nonimmune host functions. Interleukin 1 (IL-1) production by macrophages is an important factor in the augmentation of B- and T-cell functions (1–3) and is also chemotactic for leukocytes (4). Although IL-1 has many beneficial effects, overproduction, especially if prolonged, may induce consequences that are potentially detrimental. Fibroblast proliferation (5), catabolic activity (6), and release of proteases and prostaglandins (7) are all events that may mediate tissue damage and exacerbate the clinical expression of the adult respiratory distress syndrome (ARDS). In a recently reported canine model of endotoxin-induced lung injury, similar to human ARDS, alveolar macrophages (AMEC) had altered microbicidal activity (8) and their surface receptor-mediated phagocytic response was also modified (9).

These changes may reflect important alterations in the cellular metabolism of alveolar macrophages during endotoxin-induced lung injury. Moreover, an increased release of monokines by these cells could facilitate the development/propagation of lung pathology associated with the canine model of ARDS. In the current investigation we examined the AMEC for their ability to produce IL-1 and tumor necrosis factor (TNF). Each of these monokines has the potential to induce a variety of host responses that could promote clinically apparent respiratory distress. The results demonstrated that AMEC produced IL-1 and TNF in concentrations significantly greater than those of normal controls. Moreover, the data imply that these monokines could have a valuable role in regulating events that alter the physiology of the lung in endotoxin-induced injury.

Materials and Methods. *Animals.* Stabilized adult mongrel dogs weighing 18–28 kg (average 22.2 kg) were fasted for 12 hr prior to experimental use. All animals were determined to be free from ova and parasites, including *Dirofilaria immitis.* All dogs were previously vaccinated against rabies, lepto-

0037-9727/88 $1.50

spira, hepatitis, and parvovirus by the Department of Laboratory Animal Medicine, University of Arkansas for Medical Sciences.

Endotoxin induction of canine ARDS. All animals were anesthetized with sodium pentobarbital (35 mg/kg), instrumented, ventilated, and extensively monitored exactly as previously described (8). The dogs were randomly divided into two equal groups. Experimental animals were intravenously given 2 mg/kg *Escherichia coli* 055:B5 lipopolysaccaride (Difco, Detroit, MI). This dose induces an ARDS-like, physiological response in canines (8, 10). The control dogs received an equal volume of intravenous normal saline. All animals were sacrificed 6 hr postinjection. Physiological and pathological data were used to confirm the expression of a canine model of ARDS-like lung injury in the endotoxin-treated animals (8).

Cell harvest and monokine production. Alveolar macrophages (AM) were collected from bronchoalveolar lavage fluids as previously described (8, 9). Briefly a sterile number 18-French suction catheter was inserted into the left mainstem bronchus of the dog. Lavages were performed *in situ* using a total of 1 liter of cold normal saline per wash under a force of 36 cm of H_2O. The lung was completely inflated and deflated during each wash using a standardized procedure. Each animal was lavaged three times. The volume of lavage fluid recovered from individual animals was the same for all dogs.

In parallel, select lungs were instilled with 1% glutaraldehyde–4% formaldehyde in Sorenson's buffer, clamped, and held 24 hr for fixation. Subsequently lungs sliced in the anteroposterior plane for light microscopy showed focal acute lung injury manifested by a thickened interstitium, early alveolar edema, an increase in neutrophils and mononuclear cells, occlusion of multiple vessels, and margination of intravascular neutrophils and mononuclear cells (11).

All lavage preparations were washed by centrifugation at 400*g*, 10 min, and 4°C. Cell suspensions were layered over Ficoll–Hypaque 1077 (Sigma, St. Louis, MO) and centrifuged 500*g*, 30 min, and 25°C to concentrate the mononuclear cells. The cell band at the interphase was collected, washed twice, and resuspended in RPMI 1640/10% FCS (Hyclone, Logan, UT) containing 100 U/ml penicillin and 100 μg/ml streptomycin (referred to as c media) and counted on a hemocytometer. Viability was examined by trypan blue exclusion and determined to be greater than 95%. Cells were plated on 12-mm sterile glass coverslips (Bellco) contained within 24-well tissue culture plates (Costar, Cambridge, MA). After 1 hr incubation at 37°C, 5% CO_2, nonadherent cells were removed by washing the monolayers three times with warm PBS. Neutral red dye uptake and nonspecific esterase staining were used to determine the percentage of macrophages present on representative coverslips. The plating efficiency was calculated by determining the number of nonadherent AM in the preparation divided by the total number of macrophages/well, multiplied by 100. Because experimental macrophages were less adherent, it was necessary to adjust all suspensions in c media to yield a final concentration of macrophages/coverslip at the time of assay.

The crude BAL from endotoxin dogs yielded significantly more cells than those from control preparations (endotoxin, $3.8 \pm 0.8 \times 10^8$; control, $2.4 \pm 0.4 \times 10^8$; $P < 0.05$). This difference was a reflection of an influx of myeloperoxidase-positive polymorphonuclear leukocytes (PMNs) into the BAL from endotoxin treated dogs (endotoxin, $38.7 \pm 5.3\%$; control, $8.5 \pm 3.5\%$; $P < 0.05$). Although the percentage of AM in the BAL was lower for endotoxin preparations ($71.5 \pm 10.5\%$) than those for controls ($94.5 \pm 1.3\%$, $P < 0.05$), the absolute number of AM was similar (endotoxin, $2.7 \pm 1.0 \times 10^8$; control, $2.3 \pm 1.2 \times 10^8$). Also, the percentages of adherent cells that were esterase-positive following incubation were similar (endotoxin, $97.3 \pm 2.4\%$; control, $98.2 \pm 1.7\%$). Monolayer cell numbers were quantitated microscopically using stained coverslips for grid count analysis, as well as separate determinations for protein concentration, and were found to be similar for endotoxin preparations compared to controls.

In preparations used for TNF production, 10^6 macrophages/ml were reincubated in c media for 4 hr postharvest. In preparations used for IL-1 production, 10^6 macrophages/ml were incubated in RPMI 1640/10% FCS

supplemented with 25 μg/ml of LPS for 48 hr at 37°C, 5% CO_2 in a humidified chamber. All supernatants were collected, filter sterilized, and stored at −70°C until assayed. Lysates were prepared from these macrophage preparations by sonicating the cells for 10 sec at a setting of 7 (maximum) using a cell sonicator (Ultrasonics, Inc., Planeview, NY) equipped with a microprobe tip. Samples were monitored microscopically to confirm cell lysis. Preparations were cleared of all cellular debris by high-speed centrifugation at 10,000*g*, 4°C, for 30 min, and processed as above for assay.

SDS–PAGE assay. Using a 10:1 dialysate to sample ratio, all supernatant and cell lysate preparations were independently dialyzed through a 12-kD (molecular weight) cut off tubing (Sigma, St. Louis, MO) against 20 m*M* Tris–acetate buffer for 24 hr in the cold. During this period the dialysate was changed three times. Each preparation was then fractionated by using a standard 12% SDS–PAGE method as previously described (12). Subsequently all samples were visualized by using silver-staining (Bio-Rad, Richmond, CA) methods modified to maximize image intensification.

IL-1 assay. IL-1 activity was determined by the ability of macrophage supernatants or lysates to stimulate the incorporation of [^{3}H]thymidine ([^{3}H]td) into C3H/HEJ thymocytes using a modified method of Meltzer and Oppenheim (13). Briefly, all pooled preparations were concentrated approximately 100-fold by using sequential centrifugation (2500*g*, 20 min, 4°C) through Centricon concentrators (Amicon, Danvers, MA). The samples were initially separated in Centricon-10 concentrators. Filtrates from this process tested negative for IL-1 and TNF. All retentates obtained using this procedure were then run three times through Centricon-30 concentrators. These filtrates were subsequently analyzed for activity in IL-1 and TNF assays as described. Two-fold serial dilutions of the preparations were delivered into each well of a 96-well microtiter plate containing 10^6 thymocytes suspended in RPMI/FCS containing 2.5×10^{-5} *M* 2-ME, 25 m*M* Hepes, 2 m*M* L-glutamine, and 5 μg PHA. Cultures were incubated for 72 hr at 37°C, 5% CO_2 in an humidified chamber. At 18 hr before the end of the incubation period, cells were pulsed with 1 μCi of [^{3}H]td (New England Nuclear). Cells were harvested onto filter papers, mixed with a scintillation cocktail, and counted for radioactivity. Samples with molecular weights outside this range were tested and found to be consistently negative for IL-1 activity. Exogenous cell-line derived IL-1 (Genzyme, Boston, MA) was used as a standard.

TNF assay. TNF activity was determined using an adaptation of the lytic assay of Carswell *et al.* (14) as modified by Aggarwal *et al.* (15). Briefly, mouse L-929 fibroblast cells were incubated at room temperature with actinomycin D (Calbiochem) for 1½–2 hr. L-929 cells were then mixed 1:1 with serial dilutions of macrophage supernatants. Suspensions containing 30,000 L-929 cells, 0.1 μg actinomycin D, and 0.08-50 μl of sample or standard in a final volume of 0.1 ml were added to wells of plastic tissue culture trays (Corning Glass Works, Corning, NY). To determine the specificity of the assay, appropriate dilutions of selected supernatants or standard rTNF were preincubated with 5 neutralizing U of anti-human TNF monoclonal antibody (sp act 6000 neutralizing U/μg, Genentech) for 4 hr at 4°C prior to addition to actinomycin-D-treated L-929 cells. After incubation at 37°C for 18 hr, samples were decanted from the adherent L-929 cells and monolayers were washed twice with PBS. Residual cells were stained with crystal violet (0.5% in 20% methanol), washed, dried, and eluted into a 100-μl volume (eluant, 30.5 m*M* Na citrate, 1.95 *N* HCl, 47% ethanol). Optical density at 570 nm was determined in a microplate reader. Optical density of L-929 cells incubated with medium alone represented 0% lysis and cells treated with 3 *M* guanidine hydrochloride, 100% lysis. One unit TNF is defined as that amount of TNF required to produce 50% lysis. Lysis was normalized to a standard of human recombinant TNF (sp act 3.6×10^7 U/ml, Genentech).

Statistics. All bioassay experiments were performed in triplicate. For each preparation supernatants were pooled from cells obtained from at least eight animals per group. Comparisons between data points were analyzed by using the Student's *t* test.

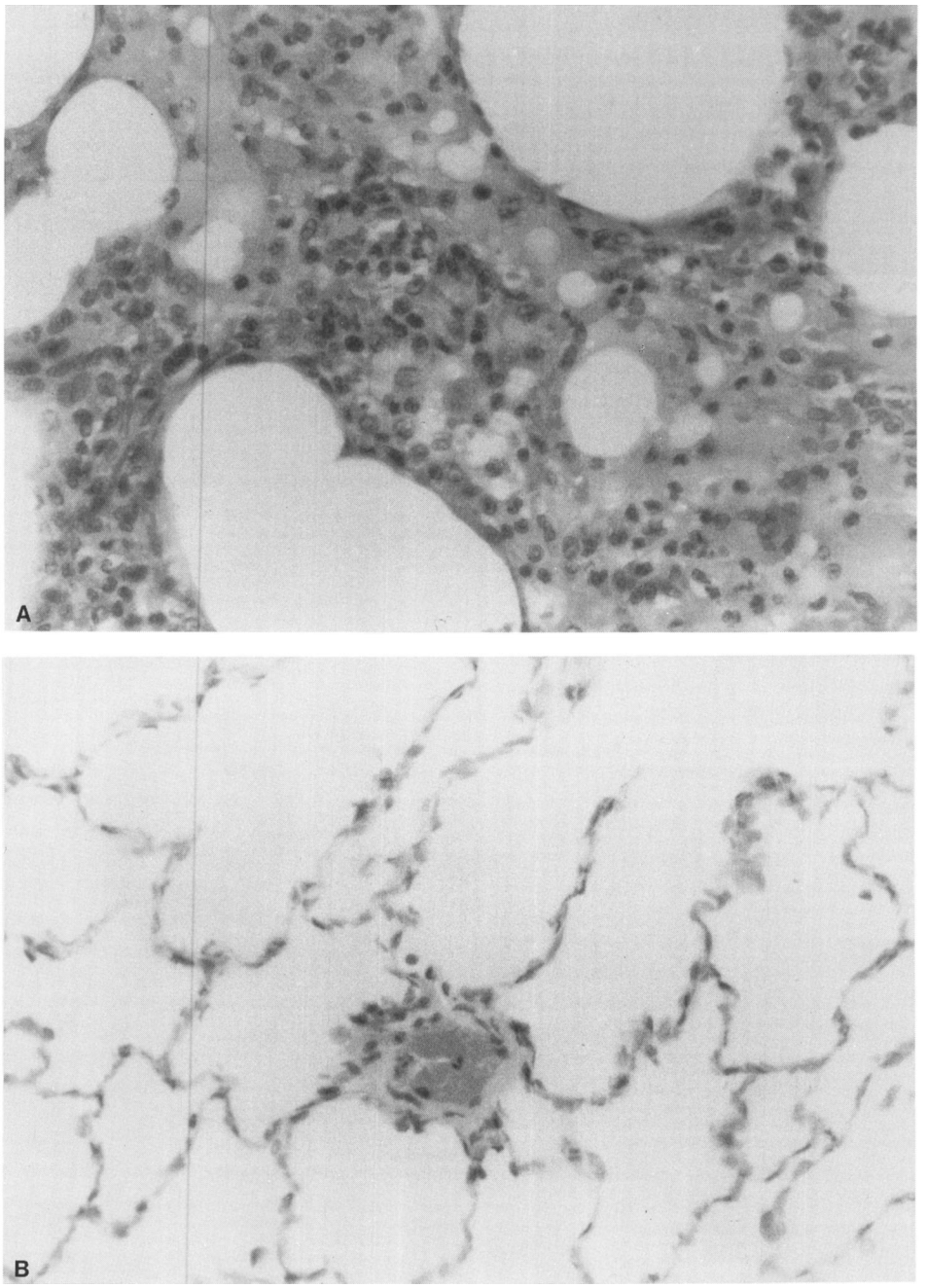

FIG. 1. Microscopic comparison of lung tissue from endotoxin-induced and control canines. Lungs were glutaraldehyde fixed and sectioned in the anteriorposterior plane. (A) Experimental and (B) control were viewed at 150× magnification.

Results. In the canine model of ARDS, we previously observed very rapid physiological responses (within 2 hr), especially pyrogenic activity, postendotoxin administration (8). In the current study we initially examined representative areas of the canine lungs microscopically (Figs 1A and 1B). The results confirmed that there were definite morpho-

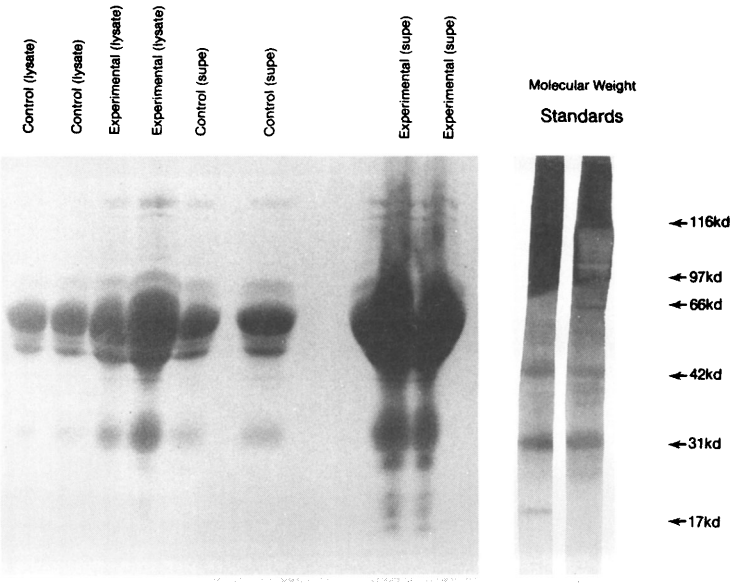

FIG. 2. SDS-PAGE analysis of monokines produced by alveolar macrophages from endotoxin-induced canines. Alveolar macrophage supernatants (Experimental, supes) and lysates (Experimental, lysate) from endotoxin-induced animals were separated using 12% SDS-PAGE. Similarly, supernatants (Control, supe) and lysates (Control, lysate) from control alveolar machrophages were analyzed on this gel. Both low (17–42 kD) and high (42–116 kD) molecular-weight standards were run in parallel for comparison. Results represent data obtained from two independent experiments. All preparations were visualized by silver-staining the gel preparation.

logic changes in the experimental group as compared to the controls. Focal acute lung injury was readily apparent in the experimental tissue as evidenced by a thickened interstitum, alveolar edema, and an increase in mononuclear cells (Fig. 1A). This sharply contrasted the fine, lacey morphology demonstrated in the sections taken from the normal control lung (Fig. 1B). Because of the distinct differences in tissue morphology as well as mononuclear cell prominence, we hypothesize that these manifestations were associated with the presence of soluble mediators. Thus, the generation of acute modulatory monokines could perhaps serve to exacerbate the development of ARDS in the pulmonary compartment.

In subsequent work we examined both the unfractionated supernatant (supe) and the cell-associated preparations (lysate) from AMEC by SDS-PAGE to determine if there were any differences in protein content from normal controls (Fig. 2). The experimental supes prominently displayed a band at 17 kD while this same protein was absent in all other soluble control preparations. When only the fractionated preparations from these samples were tested, the data reproduced exactly. Since the two major reactive monokine species under investigation lie within this molecular weight range, we hypothesized that IL-1 and TNF were contained within the band. This suggested that the AMEC were producing enhanced quantities of either IL-1, TNF, or both. Therefore, all supes and lysates were functionally analyzed for their IL-1 content by using the thymocyte proliferation assay (Fig. 3). The AMEC supes generated a cumulative response that was significantly greater than that of normal control macrophage supes. Moreover, the IL-1 activity detected in the experimental samples substantially exceeded that produced by the matched control supes. These differences were readily apparent through a dilution of 1 to 8. Conversely, the same analysis performed on lysates from all samples showed no significant differences at

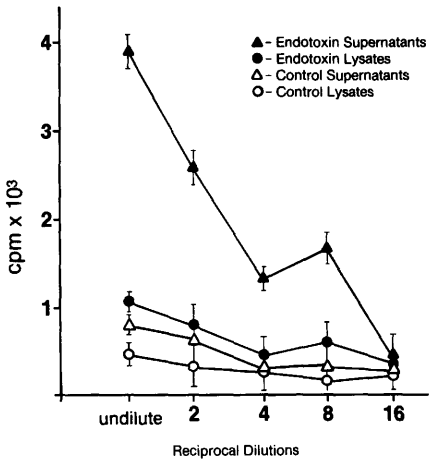

FIG. 3. Production of interleukin 1 by alveolar macrophages from endotoxin-induced canines. Preparations from alveolar macrophage supernatants and lysates (control △, ○, and endotoxin-induced ▲, ●) were diluted twofold and analyzed for their ability to stimulate the proliferation of C3H/HEJ mouse thymocytes. The results are reported as mean counts per minute ± SE of experiments performed in triplicate. Endotoxin supernatants were significantly different from controls up to a 1/16 dilution ($P < 0.01$).

TABLE I. COMPARISON OF IL-1 ACTIVITY IN PREPARATIONS DERIVED FROM ALVEOLAR MACROPHAGES[a]

	Untreated	Heat-treated[b]
Unfractionated		
Exptl[c] Supe	360 ± 64	10 ± 8
Lysate	100 ± 40	10 ± 5
Control Supe	50 ± 18	10 ± 8
Lysate	20 ± 12	10 ± 8
Fraction 1[d]		
Exptl Supe	490 ± 53	10 ± 6
Lysate	150 ± 27	10 ± 6
Control Supe	110 ± 12	10 ± 7
Lysate	70 ± 19	10 ± 5
Fraction 2[e]		
Exptl Supe	20 ± 12	10 ± 9
Lysate	10 ± 7	10 ± 6
Control Supe	10 ± 8	10 ± 4
Lysate	10 ± 7	10 ± 7

[a] Normalized for IL-1 standard, reported as units per milliliter ± SE.
[b] Samples were heated 56°C, 60 min.
[c] Endotoxin-induced animals.
[d] Molecular weight range, 10–30 kD.
[e] Molecular weight range, >30 kD.

any level of the dilutions examined. In parallel experiments when supes and lysates were independently heat-treated, the ability of each preparation to induce thymocyte proliferation was ablated (Table I). Because both IL-1 and TNF are frequently involved in generating parallel host responses (16) which may be associated with tissue damage, we examined AMEC supes for their TNF content as well. The results were markedly positive when compared to those of the control samples (Table II).

Discussion. Alveolar macrophages (AM) may represent a potent contributor to lung injury that develops postendotoxin administration. Recent studies performed using the canine model, which mimics human ARDS, show distinct alterations in select defense functions of the AM (8, 10). These results corroborate the work by Davis *et al.* (17) that demonstrated similar changes in human AM exposed to endotoxin *in vitro*. Therefore, functional modifications of a prominent lung cell type may readily dirsupt the normal physiological activity of this organ.

Moreover, alterations in the regulatory role of AM may significantly contribute to prolonged inflammatory reactions and subsequent tissue damage. Although AM IL-1 can serve the host in a regulatory capacity (18), such activity requires only very modest concentrations of the monokine. This was established by Rupp *et al.* who showed that slightly elevated levels can promote extensive effector cell activity (9).

Therefore, our initial experiments were performed to determine if there were any qualitative differences in the types of proteins produced by AM from canine ARDS as compared to those derived from normal control AM. We chose to study AM as a corollary to the work that we have previously performed on these cells using the canine model of ARDS (8, 10, 11). Since our interest was focused upon lung injury, we selected the AM as the target cell that best repre-

TABLE II. ANALYSIS OF ALVEOLAR MACROPHAGE PREPARATIONS FOR TNF[a]

	Control	Experimental[b]
Supernatant preparations	56 ± 36	316 ± 114

[a] Normalized for TNF, reported as units per milliliter.
[b] From endotoxin-induced animals.

sented the local environment. Finding distinct bands in the 17-kD region from AMEC supes that were absent in control supes suggested that IL-1 production was significantly increased in the canine ARDS model. This assumption is supported by studies that show vigorous AM IL-1 production in sarcoidosis (20), AIDS, and idiopathic pulmonary fibrosis (21). An examination of the fractionated samples demonstrated that the 10- to 30-kD preparation contained enhanced IL-1 activity while all the other preparations expressed a response that was equal to the control level. However, these functional studies did not exclude the possibility that IL-1 precursor molecules were present in the larger fractions (i.e., > 30 kD). In fact, Bayne *et al.* (22) have shown that lysates routinely contain the 33-kD precursor IL-1 protein. This suggests that the probability of finding a 17-kD species in the cytosol is very low. Nevertheless, our results demonstrated that the active molecule was more readily available in AMEC supes than in any other preparation. The absence of a 17-kD band in AMEC lysates coupled with negligible thymocyte proliferation indicated that in the canine model, an active IL-1 was produced and rapidly released. Whether IL-1 *de novo* synthesis or a conversion from a 33- to a 17-kD form was accelerated in these AM remains to be determined. Moreover, the rate of staging this protein for release via incorporating it into the plasma membrane may similarly represent an important event that influences the quantities of active IL-1 present. Although the membrane fraction was not analyzed in our study, Bakouche has recently provided evidence that membrane-bound IL-1 is an active protein (23). The enhanced AMEC release of IL-1 is significant since spontaneous IL-1 production in quiescent AM is routinely very low (24). Thus, alterations in the concentration of this mediator may be one mechanism that can sustain an inflammatory response and subsequently promote tissue damage.

Although the macrophage activation mechanisms subsequent to LPS administration are not clearly defined, Pace *et al.* have shown that these cells usually require at least a preliminary priming signal (25). Similarly, Eden and Turino have shown that human AM when exposed to interferon-γ and LPS produce markedly enhanced levels of IL-1 (26). Because of the short incubation period used in our study we suspected that a rapidly formed signal may be responsible for both priming macrophages and directly inducing localized alterations associated with the observed pulmonary damage. Since TNF can meet both these criteria, it could provide the initial stimulus necessary to activate macrophages or neutrophils in the pulmonary microcirculation or lung parenchyma. It was reasonable to consider TNF involvement since this mediator can promote IL-1 production (27) and is recognized as an immunopotentiating agent capable of sustaining inflammatory reactions (28). More recently, IL-1 and TNF have been shown to produce synergistic immunologic activity (29) that may prove to be important in lung injury as well. Such concordant activity has the potential to play a significant role in the development of tissue damage associated with ARDS.

This study demonstrated that both IL-1 and TNF were present in increased concentrations in AMEC during the development of endotoxin-induced lung injury in the canine. Such acute production of monokines may facilitate the progression of lung injury by substantially altering the integrity of the pulmonary compartment. Therefore this response could exacerbate the injury by sustaining enhanced levels of either mediator.

Collectively these data imply that both monokines (i.e., IL-1, TNF) may significantly contribute to the disease progression and subsequent physiological dysfunction associated with lung injury.

We thank Rai Smith for carefully typing this manuscript and C. Lary and C. Connaughton for their excellent technical assistance throughout these experiments. This investigation was supported by NIH Grant HL 32081.

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Received July 10, 1987, P.S.E.B.M. 1988, Vol. 187.
Accepted December 1, 1987.