

# Novel Potentiation of Interleukin 1 $\alpha$ Production in Endotoxin-Stimulated IC-21 Cells by Ambient Pressure Augmentation

Mark D. Sawyer, MD; Tom van Raaij; John Cross; Bauer E. Sumpio, MD, PhD

**Background:** We hypothesized that increased ambient pressure would increase the production of interleukin 1 $\alpha$  by endotoxin-stimulated macrophages, based on the clinical observation that patients with “pus under pressure” demonstrate systemic toxic effects (a priori hypothesis).

**Design and Setting:** In vitro experiment in the laboratory.

**Interventions:** A murine macrophage line, IC-21 cells, was seeded into 6-well plates,  $25 \times 10^4$  cells per well. Cells were incubated under atmospheric (ATM) or increased (ATM+60 mm Hg) ambient pressure (AP) in the presence or absence of endotoxin (lipopolysaccharide [LPS]). The IC-21 production of interleukin 1 $\alpha$  was determined at 2, 4, 8, and 12 hours. Four groups were examined: group 1: AP ATM, no LPS; group 2: AP ATM+60 mm Hg, no LPS; group 3: AP ATM and LPS, 500 ng/mL; and group 4: AP ATM+60 mm Hg and LPS, 500 ng/mL.

**Main Outcome Measures:** The IC-21 production of interleukin 1 $\alpha$ .

**Results:** Interleukin 1 $\alpha$  production at 2, 4, 8, and 12 hours (mean  $\pm$ SD) picograms per  $10^6$  cells) was as follows: group 1: 3.0 ( $\pm$ 5.9), 8.1 ( $\pm$ 10.3), 50.5 ( $\pm$ 51.1), and 6.1 ( $\pm$ 4.1), respectively; group 2: 228.7 ( $\pm$ 110.2), 141.0 ( $\pm$ 141.8), 112.5 ( $\pm$ 98.5), and 118.2 ( $\pm$ 79.8), respectively; group 3: 37.2 ( $\pm$ 13.3), 191.5 ( $\pm$ 86.5), 627.3 ( $\pm$ 184.3), and 600.7 ( $\pm$ 67.1), respectively; and group 4: 601.2 ( $\pm$ 49.9), 1050.9 ( $\pm$ 190.6), 2684.2 ( $\pm$ 562.2), and 3144.7 ( $\pm$ 388.4), respectively. The production of IL-1 $\alpha$  by group 3 was significantly greater ( $P < .04$ , unpaired Student *t* test) at 4, 8, and 12 hours than that by groups 1 or 2. Likewise, the production of IL-1 $\alpha$  by group 4 was significantly greater ( $P < .001$ , unpaired Student *t* test) at all time points than that by groups 1, 2, or 3.

**Conclusions:** Our data support the hypothesis that pressure may be a novel potentiator of the macrophage proinflammatory cytokine response to endotoxin. This provides a possible explanation for the phenomenon of systemic illness seen with “pus under pressure.”

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**T**HERE IS a clinical observation of long standing that “pus under pressure” produces a syndrome of systemic symptoms that includes fever, chills, leukocytosis, and malaise. Furthermore, this constellation of symptoms may be largely abrogated by release of the pressure, even though purulent material may remain incompletely drained. This scenario is common to such disorders as cholecystitis, cholangitis, and abscesses of gastrointestinal origin. Abscess pressures can reach substantially high levels and have been measured at up to 62 mm Hg.<sup>1</sup> Macrophages are thought to be the earliest and most potent instigators of the systemic inflammatory response syndrome, in particular through endotoxin-stimulated production of the proinflammatory cytokines, tumor necrosis

factor, interleukin 1 $\alpha$  (IL-1 $\alpha$ ), and interleukin 6 (IL-6).<sup>2</sup> These cytokines are responsible for many of the flu-like symptoms seen in the systemic inflammatory response syndrome. We hypothesized that the pus under pressure syndrome is caused by the increased production of IL-1 $\alpha$  by endotoxin-stimulated macrophages within the confines of a pressurized space such as an abscess. As a corollary, macrophages exposed to endotoxin in the presence of increased ambient pressure should produce increased amounts of IL-1 $\alpha$  when compared with macrophages stimulated by endotoxin under conditions of normal pressure. To test this hypothesis, a murine monocyte-macrophage cell line was exposed to endotoxin in the presence of increased ambient pressure and levels of IL-1 $\alpha$  were measured in the supernatant.

From the Department of Surgery, Yale University School of Medicine, New Haven, Conn.

## MATERIALS AND METHODS

### CELL LINE

An immortal cell line, termed IC-21 cells (American Type Culture Collection, Rockville, Md), was created by the viral transformation of C57BL/6 murine peritoneal macrophages by the virus SV40 (simian virus 40). This cell line exhibits many of the normal features of monocytes and macrophages, including adherence (M.D.S., unpublished data, 1997), phagocytosis,<sup>3</sup> and cytokine production.<sup>4</sup> Cells were maintained in culture consisting of RPMI-1640 medium supplemented with 10% fetal bovine serum; levoglutamide; streptomycin sulfate,  $1.0 \times 10^4$  U/mL; and 1% penicillin G (Gibco BRL, Grand Island, NY). Incubators were maintained at 37°C and an atmospheric carbon dioxide content of 5%. Cells were maintained in standard culture flasks. Forty-eight hours before experimentation, cells were harvested, counted, and plated out in 6-well polystyrene plates at  $25 \times 10^4$  cells per well in 2.5 mL of medium.

### AMBIENT PRESSURE AUGMENTATION

A specially designed incubator chamber was used to subject cells to a static increase in ambient pressure while maintaining other culture conditions identical to those of our standard incubator.<sup>5</sup> Cells within the chamber were subjected to an augmentation in ambient pressure of 60 mm Hg for the duration of the experiment.

### IL-1 $\alpha$ DETERMINATION

A commercially available IL-1 $\alpha$  enzyme-linked immunosorbent assay (Genzyme Corp, Cambridge, Mass) was used to assay culture supernatant for the amount of IL-1 $\alpha$  present. The lower limit of detection of this assay is approximately 15 pg/mL, and the upper limit is 405 pg/mL. Standards of known IL-1 $\alpha$  concentration were used to generate standard absorbance curves with each 96-well assay plate, and sample absorbance was read with an enzyme-linked immunosorbent assay plate reader (Dynatech 700 microplate, Dynatech Laboratories, Chantilly, Va) at 450 nm. Macrophage supernatants

were adequately diluted with Dulbecco phosphate-buffered saline without calcium chloride or magnesium chloride to fall within the standard curve range. Assay plates were washed with a microplate washer using phosphate-buffered saline or 0.5% polysorbate (Tween 20, Sigma Chemical Co, St Louis, Mo) between assay steps. Coulter cell counts of each well were obtained at the end of each experiment; cells were collected after the application of a commercial cell dissociation solution (Sigma Chemical Co, St Louis, Mo) and gentle removal with a rubber-tipped rod. Cell numbers were used to calculate IL-1 $\alpha$  production per  $10^6$  cell basis.

### SAMPLES AND STATISTICAL ANALYSIS

Each data point represents the average of 2 separate samples. Each sample was assayed in duplicate for IL-1 $\alpha$  to minimize assay variability. Data are represented as the mean ( $\pm$ SD). Differences between samples were assessed using an unpaired Student *t* test, and *P* values of less than .05 were considered significant.

### DESIGN

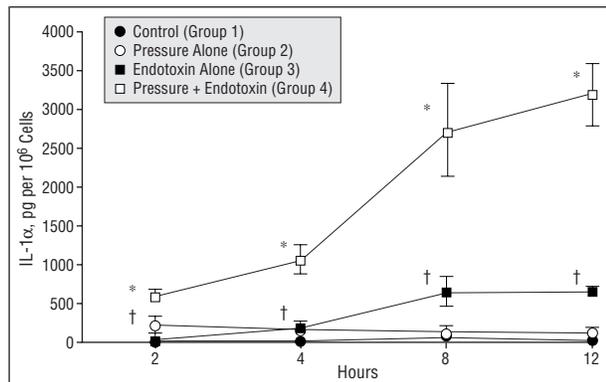
Four experimental groups were examined. Group 1 was incubated at ambient pressure with no endotoxin added to the media. Group 2 was incubated at ambient pressure plus 60 mm Hg with no endotoxin added to the media. Group 3 was incubated at ambient pressure with endotoxin (*Escherichia coli* 055:B5, Sigma Chemical Co), 500 ng/mL, added. Group 4 was incubated at ambient pressure plus 60 mm Hg with endotoxin, 500 ng/mL, added. After seeding 6-well polystyrene culture plates with  $25 \times 10^4$  cells per well, cells were allowed to adhere and adapt for 48 hours. At time 0, media were exchanged, with the media for groups 3 and 4 containing endotoxin, 500 ng/mL. Groups 1 and 3 were incubated in a standard incubator, and groups 2 and 4 were incubated in the pressure incubator for 12 hours. Supernatant was sampled at 2, 4, 8, and 12 hours and immediately frozen and stored at -80°C to prevent cytokine deterioration. Assay for IL-1 $\alpha$  was performed within 1 to 2 weeks of sample storage. Incubation conditions were identical for all groups in carbon dioxide concentration (5%) and temperature (37°C).

## RESULTS

As would be expected, groups stimulated with endotoxin (groups 3 and 4) produced significantly more IL-1 $\alpha$  than groups not receiving endotoxin (**Figure**). The combination of increased pressure and endotoxin (group 4) produced significantly higher levels of IL-1 $\alpha$  than in groups 1, 2, and 3 and demonstrated a marked synergistic effect between increased ambient pressure and endotoxin on IL-1 $\alpha$  production. The data demonstrate a difference between pressure alone and control groups that is not statistically significant. Much of these data were at or beneath the lower limit of sensitivity for the IL-1 $\alpha$  assay, and no conclusions concerning the effect of pressure alone on IL-1 $\alpha$  production can be drawn.

## COMMENT

Pressure has been noted to be an important contributing factor in the function of certain cell types, most notably cells of the vascular system.<sup>6</sup> These have been studied largely in the context of elucidating hemodynamic force effects on endothelial cellular processes. To our knowledge, this is the first study examining pressure as a potential regulatory element in macrophage cytokine production. The mechanism by which pressure is sensed by the cell and translated into an intracellular signal is unknown. Given the variety of pressure environments that exist within the body—high pulsatile pressures within arterial vessels; lower, more constant pressures within the capillaries, venous system, and interstitial spaces; slower undulations from negative to positive within the



Interleukin 1 $\alpha$  (IL-1 $\alpha$ ) production by IC-21 macrophages. A single asterisk indicates significance ( $P < .001$ ) for group 4 vs groups 1, 2, and 3; daggers, significance ( $P < .004$ ) for group 3 vs group 1, all time points.

thoracic cavity—it is possible that various pressure stimulus-response systems exist.

Interleukin 1 $\alpha$  is one of the most important mediators of the systemic inflammatory response syndrome and is released by macrophages in response to endotoxin stimulation. Its release may be by a nonclassical pathway, and a signal peptide analogous to that seen in tumor necrosis factor and IL-6 secretion has not been identified. It has been suggested that IL-1 $\alpha$  may be accumulated in the cytosol<sup>7</sup> and released during macrophage apoptosis.<sup>8</sup> In support of this hypothesis, it has been shown that *Shigella flexneri* infection of macrophages caused both apoptosis<sup>9</sup> and increased release of IL-1 $\alpha$ , but not tumor necrosis factor or IL-6.<sup>10</sup>

In this experiment, we have demonstrated a synergistic effect of increased ambient pressure and endotoxin on the elaboration of IL-1 $\alpha$  by a macrophage cell line. This is supportive of the hypothesis that the systemic toxic effects anecdotally associated with purulent collections under increased pressure may be caused, at least in part, by increased levels of IL-1 $\alpha$ . However intriguing, these data are not sufficient to presume cause and effect in vivo. This mechanism may not be physiologically important or perhaps represents only one of many stimuli that account for the clinical phenomenon. As an example, alternative explanations for the pus under pressure phenomenon include the extrusion of bacteria and proinflammatory cytokines into the systemic circulation by the pressure gradient and increased production of other cytokines or molecular mediators. Although the ideal experiment to fulfill Koch's postulates would entail the clinical measurement of abscess pressure, as well as abscess and serum IL-1 $\alpha$  levels before and after decompression, correlated with signs and symptoms, this experiment nonetheless provides important information regarding the behavior of macrophages under abnormal pressure conditions. These data suggest that macrophages are able to sense increases in static ambient pressure and somehow translate this information into an intracellular signal affecting the production of IL-1 $\alpha$ .

The model used in these studies was designed with the goal of an easily reproducible one in which to

### Statement of Clinical Relevance

Current in vitro models of macrophage function are performed at atmospheric pressure, a condition that is the exception rather than the rule in the body. This experiment is a limited characterization of the effects of ambient pressure on the macrophage response to endotoxin. Our goal was to simulate more closely the conditions affecting macrophages within an in vivo abscess with regard to ambient pressure while retaining the reproducibility and control possible with an in vitro experimental system. Refinements and adaptations of this model will hopefully allow us to explore the physiology of abscesses and macrophages with a greater degree of clinical relevance.

assess pressure effects on many cellular proteins and functions. The results obtained raise some questions, and other models or clinical studies may address elements of these more appropriately. Although the IC-21 cells have many of the characteristics of normal monocytes and macrophages, they are nonetheless an immortal, nonhuman cell line. To ascertain the applicability of this effect to human illness, this effect will need to be assessed in normal human monocytes. Furthermore, the use of an enzyme-linked immunosorbent assay to determine cytokines gives no information concerning the biological importance of these effects, and an in vivo or clinical investigation would be necessary for these determinations.

The current model serves as a basis for ongoing investigations in our laboratory. A small, consistent, but statistically nonsignificant effect of pressure alone on IL-1 $\alpha$  production was noted in our studies, most probably due to the fact that IL-1 $\alpha$  levels in the pressure-only group were near the lower limit of detection of the IL-1 $\alpha$  enzyme-linked immunosorbent assay. Further refinement of assay and experimental conditions may allow us to demonstrate a pressure effect on IL-1 $\alpha$  production independent of endotoxin stimulation with statistical significance. Ongoing studies in our laboratory include the elucidation of pressure effects on other cytokines and inflammatory surface proteins, confirmation of these data using normal human peripheral blood monocytes, clinical correlation in patients with abscesses, and investigation of the pathways by which increased pressure affects intracellular mechanisms. We conclude that ambient pressure may be an important regulator of IL-1 $\alpha$  expression in endotoxin-stimulated macrophages.

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Corresponding author: Mark D. Sawyer, MD, Department of Surgery, Yale University School of Medicine, 333 Cedar St, LH 118, New Haven, CT 06510.

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## DISCUSSION

**Kenneth Burchard, MD, Lebanon, NH:** Dr Sawyer and his colleagues have provided us with an interesting perturbation on the theme of cytokine production, prompted by the clinical recognition that the draining infected material, be it in such locations as an abscess or in the common bile duct, characteristically results in significant resolution of systemic inflammation. Common wisdom, however, argues that the release of pressure decreases the passage of organisms or inflammatory mediators from the infected site into surrounding tissue, and I would certainly think that 60 mm Hg in an abscess would promote that effect, as

this has been well studied in models of common duct obstruction where pressures of 20 mm Hg are considered high and will allow the passage of either bacteria themselves or breakdown products into liver parenchyma. So, since pressures of 20 mm Hg would be considered high, why was 60 mm Hg chosen? It was not clear in the paper, but should we have a dose-response curve about this effect? Should we see what 5, 10, 15, 20, 25, etc, mm Hg does to the IL-1 production? And then, why would high pressure cause the macrophage to produce more IL-1? I would not expect 60 mm Hg to increase the PO<sub>2</sub> in the cell significantly, but I usually consider abscesses to be a relatively anaerobic environment. What would happen if the FIO<sub>2</sub> [fraction of inspired oxygen] was reduced, and what happens when you increase the FIO<sub>2</sub> in the area of a macrophage under normobaric conditions?

I think these data are intriguing and look forward to experimental manipulations that more closely simulate clinical circumstances.

**Dr Sawyer:** First, regarding the pressure gradient, our preliminary experiments did look at various pressure gradients from 20 to 80 mm Hg. We chose 60 mm Hg. We seemed to get an optimal response from the cells at that level, and in addition, we did have the supporting paper from the literature as to why that particular level was chosen. So we have done dosage-response curves at least in an informal way with that. Similarly, we chose 500 ng/mL of endotoxin as that seemed to give us the optimal response from the IC-21 cells.

Why more IL-1? We're not sure. It does seem to be an isolated effect. We have some other data with tumor necrosis factor and IL-6 that we did not complete in time for this presentation that suggest it may be due to IL-1's rather unique method of release, which may involve cellular apoptosis.

In terms of the FIO<sub>2</sub> in the experiment, there is a paper by Michael West at Hennepin County in Minnesota who has looked at increased oxygen tensions up to 100% and has noted an increase under these conditions, too. Because of the apoptotic release mechanisms of IL-1, it may be that elements which cause increased cell turnover or cause toxic effects in the cell may be contributing to the increase seen. As to a decrease in the FIO<sub>2</sub>, we have not examined this effect, but it would certainly be interesting.

## IN OTHER AMA JOURNALS

### ARCHIVES OF INTERNAL MEDICINE

#### Is Obesity Related to Microvascular and Macrovascular Complications in Diabetes? The Wisconsin Epidemiologic Study of Diabetic Retinopathy

Ronald Klein, MD, MPH; Barbara E. K. Klein, MD, MPH; Scot E. Moss, MA

**Background:** There are few epidemiological data describing the relationship of obesity to the incidence of microvascular and macrovascular complications in patients with diabetes.

**Methods:** In a population-based study performed in southern Wisconsin, 1370 persons diagnosed as having diabetes when they were 30 years of age or older (mean [±SD] age, 66.6 ± 11.3 years) participated in a baseline examination, 987 in a 4-year follow-up examination, and 533 in a 10-year follow-up examination. Height and weight were measured using standard protocols. Individuals were characterized as underweight, normal weight, overweight, and obese by body mass index (expressed as weight in kilograms divided by the square of the height in meters) status. Outcomes included incidence and progression of retinopathy, incidence of gross proteinuria, incidence of lower-extremity amputation, and death due to ischemic heart disease, death due to stroke, and all-cause mortality.

**Results:** The prevalence of being obese was 25.2%. After other risk factors were controlled for, being underweight was associated with higher incidence of diabetic retinopathy, death from stroke, and all-cause mortality. Body mass was not associated with progression of retinopathy, incidence of gross proteinuria, amputation of a lower extremity, or death due to ischemic heart disease.

**Conclusions:** These data suggest that obesity in persons with older-onset diabetes is not related to the long-term incidence of microvascular and macrovascular complications. *Arch Intern Med.* 1997;157:650-656

Reprints: Ronald Klein, MD, MPH, Department of Ophthalmology and Visual Sciences, University of Wisconsin Medical School, 610 N Walnut St, 460 WARF, Madison, WI 53705-2397.