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Mechanism of Decreased *In Vitro* Murine Macrophage Cytokine Release After Exposure to Carbon Dioxide

Relevance to Laparoscopic Surgery

Michael A. West, M.D., Ph.D.,*† David J. Hackam, M.D.,‡ Jeffrey Baker, M.D.,*
Jorge L. Rodriguez, M.D.,* Janet Bellingham, B.A.,† and Ori D. Rotstein, M.D.‡

From the Department of Surgery, Hennepin County Medical Center University of Minnesota and the Laboratory for Surgical Cellular Biology and Metabolism,† Minneapolis Medical Research Foundation, Minneapolis, Minnesota; and the Department of Surgery,‡ Toronto Hospital-General Division and University of Toronto, Toronto, Ontario, Canada*

Objective
The objective of this study was to determine the effect of carbon dioxide (CO_2) on the function of peritoneal macrophages.

Summary Background Data
Laparoscopic surgery is associated with minimal pain, fever, and low levels of inflammatory cytokines. To understand the mechanisms involved, the authors investigated the effect of different gases on murine peritoneal macrophage intracellular pH and correlated these alterations with alterations in LPS-stimulated inflammatory cytokine release.

Methods
Peritoneal macrophages were incubated for 2 hours in air, helium, or CO_2, and the effect of the test gas on immediate or next day lipopolysaccharide (LPS)-stimulated tumor necrosis factor (TNF) and interleukin-1 release compared. Cytosolic pH of macrophages exposed to test gases was measured using single-cell fluorescent imaging. The *in vivo* effects of test gases were determined in anesthetized rats during abdominal insufflation.

Results
Macrophages incubated in CO_2 produced significantly less TNF and interleukin-1 in response to LPS compared to incubation in air or helium. Cytokine production returned to normal 24 hours later. Exposure to CO_2, but not air or helium, caused a marked cytosolic acidification. Pharmacologic induction of intracellular acidification to similar levels reproduced the inhibitory effect. *In vitro* studies showed that CO_2 insufflation lowered tissue pH and peritoneal macrophage LPS-stimulated TNF production.

Conclusions
The authors propose that cellular acidification induced by peritoneal CO_2 insufflation contributes to blunting of the local inflammatory response during laparoscopic surgery.

The clinical acceptance of laparoscopic abdominal surgery has been overwhelmingly positive. The largest experience has occurred with laparoscopic cholecystectomy, which has many advantages over the open operation, including a shorter length of stay, decreased postoperative pain, more rapid return to preoperative physical activities, and the absence of postoperative ileus.[1-3] The host response leading to these effects after open surgery is thought to be mediated by the local and systemic generation of inflammatory mediators. Mediators, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), prostaglandins, and reactive oxygen (O_2) intermediates, can produce hemodynamic alterations, fever, leukocytosis, and even cell injury.[4,5] Most surgeons assume that the physiologic basis for the positive outcomes observed after laparoscopic surgery is caused by the attenuation of the local and systemic inflammatory responses related to the minimal incisions required for these procedures. In keeping with this concept, Redmond et al.[6] reported reduced release of superoxide anion and TNF by inflammatory cells from patients after laparoscopic cholecystectomy compared to those having open surgery. Interestingly, the level of the stress hormone cortisol did not differ between groups.

Many different cell types produce these mediators, but cells of the monocyte–macrophage lineage are likely of particular relevance to inflammatory processes in the peritoneal cavity.[7] Under normal circumstances, resident macrophages represent the first line of host defense in the peritoneal cavity and presumably are among the first cells to respond to an inflammatory stimulus in the abdominal cavity.[8] These cells would be expected to respond to local injury, necrotic tissue, bacterial contamination, or foreign debris that might be generated in the course of abdominal surgery. In addition to the differences in the size of the incision between open and laparoscopic surgery, another major difference influencing the local inflammatory response might be the atmospheric environment to which peritoneal cells, particularly macrophages, are exposed. We hypothesized that carbon dioxide (CO_2), usually used for abdominal insufflation, might have important local effects on resident peritoneal inflammatory cells. Specifically, CO_2-mediated inhibition of inflammatory cell function might partially explain why laparoscopic surgery is so well tolerated.

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Dr. David J. Hackam is a Research Fellow of the Medical Research Council of Canada.

Address reprint requests to Michael A. West, M.D., Ph.D., Department of Surgery, Hennepin County Medical Center, 701 Park Avenue, Minneapolis, MN 55415.

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We report that murine peritoneal macrophages, after transient incubation in the presence of CO_2 *in vitro,* secrete less TNF and IL-1 in response to lipopolysaccharide (LPS) stimulation. The effect appears to be mediated by the demonstrated ability of CO_2 to cause cytosolic acidification in these cells. These findings are corroborated *in vivo* by the demonstration that peritoneal macrophages recovered after CO_2 insufflation release less TNF than those exposed to air or helium (He). Further, CO_2 insufflation reduced tissue pH in the peritoneal lining to levels that were sufficiently low to account for reduced TNF secretion by the resident peritoneal macrophages. Considered together, these studies suggest that local tissue acidification caused by peritoneal CO_2 insufflation during laparoscopy may contribute significantly to the attenuated inflammatory response reported after this type of surgery.

METHODS

Reagents

Balb/c mice and Sprague–Dawley rats were obtained from Harlan Sprague–Dawley (Madison, WI). Thioglycolate broth and 96-well polystyrene tissue culture plates were acquired from Becton Dickinson (Lincoln Park, NJ). Dulbecco's minimal essential medium, trypan blue, and Hepes buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were procured from Gibco (Grand Island, NY). Lipopolysaccharide from *Escherichia coli* 0111B4, penicillin/streptomycin, heparin, L-glutamine, dimethyl formamide, sodium dodecyl sulfate, RPMI-1640 tissue culture medium (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), actinomycin D, nigericin, and conconcanvalin A were obtained from Sigma (St. Louis, MO). Fetal calf serum (FCS) was purchased from Hyclone (Logan, UT). Modular incubator chambers were acquired from Forma Scientific, Inc (Marietta, OH). Recombinant murine interleukin-2, IL-1_β, and TNF_α were procured from R&D Systems (Minneapolis, MN). Monoclonal antimurine TNF antibody was obtained from Genzyme (Cambridge, MA). The pH-sensitive dye 2′7′ bis-(2-carboxyethyl)-5(and 6) carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR). Goat antirabbit immunoglobulin G alkaline phosphatase was obtained from Jackson Immunoresearch (Westgrove, PA). The IL-1-dependent murine T-helper cell line (D10(N4)M) graciously was provided by Stephen Hawkins (Cambridge, United Kingdom). The L929 fibroblasts (1-CCL) for the TNF bioassay were procured from ATCC (Rockville, MD).

Animals

Peritoneal cells were obtained by lavage from 5- to 9-week-old Balb/c mice. Mice were killed with CO_2 before

cell harvest. For the *in vivo* studies, male Sprague–Dawley rats (range, 250–300 g) were used to measure tissue pH levels. Use of animals in these studies was approved by the Local Institutional Animal Care Committees and conformed to the Animal Care Guidelines published by the National Institutes of Health.

Peritoneal Macrophage Isolation and Culture

Peritoneal exudate cells were obtained 72 hours after injecting mice with 2-mL thioglycolate broth by lavage with 10-mL Dulbecco's minimal essential medium containing 10% FCS and heparin. Cell viability was determined by trypan blue exclusion, and the cells were enumerated with a hemocytometer. Aliquots of 1×10^5 cells in 0.1-mL Dulbecco's minimal essential medium containing 1% penicillin–streptomycin, 2-mmol L-glutamine, and 10% FCS were added to 96-well polystyrene tissue culture plates. To minimize the effects of alterations of medium pH on the results, the culture medium also contained 10-mmol pH 7.46 Hepes buffer. The cultures were incubated for 2 hours in 5% CO_2 at 37 C, and the nonadherent cells were removed by vigorous washing. The resulting adherent population consisted of more than 95% peritoneal macrophages.[9] The plates then were incubated for 48 hours before treatment to allow recovery from the isolation and adherence procedure.

Resident macrophages were harvested by peritoneal lavage with ice-cold phosphate-buffered saline. Cells then were centrifuged at 200 × g for 10 minutes, washed twice in Hanks balanced salt solution, and counted with a hemocytometer. The cells were diluted to 1×10^6/mL in RPMI-1640 containing 15-mmol Hepes and placed on ice before each experiment. The peritoneal exudate consisted of a population containing approximately 30% macrophages as assessed by light microscopy using Wright's stain. Viability exceeded 95% both before and during treatment periods as determined by trypan blue exclusion.

Apparatus for Test Gas Exposure

The apparatus used for *in vitro* exposure to the various test gases is shown schematically in Figure 1. During the interval in which macrophages were exposed to the various test gases, the tissue culture plates were placed into airtight plastic modular incubator chambers that then were flushed with the respective test gases. The total volume of the gas incubation apparatus was approximately 7 L, such that a large reservoir of atmospheric test gas was present above the cells. The volume of culture medium was adjusted so that the depth of culture medium overlying the adherent macrophages was approximately 1 mm. We have shown previously that this depth permits rapid and nearly complete equilibration between the dissolved gas in the culture medium and the overlying atmospheric gas. Four different incubation gases were investigated: 95% air/5% CO_2 (control cell culture atmospheric conditions), air (~80% nitrogen/20% O_2), 100% CO_2, and 100% He. The incubation chambers were flushed with the test gas mixtures for a minimum of 5 minutes to ensure complete test gas equilibration. We previously found that 2 minutes was sufficient to remove detectable O_2, in experiments designed to create an anoxic environment.[10]

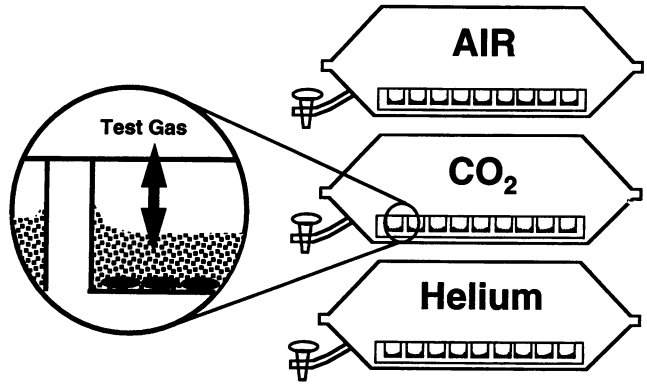

Figure 1. Schematic depiction of the experimental design used to investigate the importance of the duration of test gas incubation on alterations in lipopolysaccharide (LPS)-stimulated tumor necrosis factor and interleukin-1 secretion. Macrophages were stimulated with medium alone of medium containing 1000 ng/mL LPS and then placed immediately in the incubation chambers containing the respective test gases for 2 hours. After the 2-hour test gas incubation, the cultures were transferred to an incubator with a control atmospheric condition (95% air, 5% carbon dioxide). Macrophage supernatant for cytokine determinations was removed at the end of the 4-hour LPS stimulation interval. A parallel group of cultured macrophages was exposed to the test gases, and then LPS stimulation was delayed 24 hours.

Test Gas Incubation

To examine the effect of the test gas exposure, the gas incubation was performed as depicted in Figure 2. Cells were incubated for 2 hours in the test gas mixtures. At the completion of this period, the chambers were opened and the plates transferred to a standard tissue culture incubator with an atmosphere of 95% air/5% CO_2 for a further 2- or 24-hour incubation interval. In the 4-hour studies, cells were incubated in medium alone or medium containing 1 μg/mL LPS. For the delayed timepoint studies, cells were incubated in the presence or absence of LPS (1 μg/mL) for a further 4 hours at the end of the 24-hour recovery period. At the completion of the designated study interval, supernatant aliquots for TNF and IL-1 assays were removed and immediately frozen at −70 C to prevent cytokine deterioration.

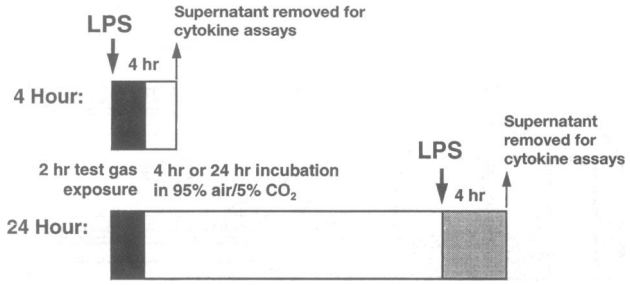

Figure 2. Schematic representation of the experimental setup used for *in vitro* test gas incubation. The macrophages, adherent to plastic tissue culture plates, were transferred to air-tight incubation chambers that were flushed for 5 minutes with humidified test gases as specified in the individual experiments. After gas flushing was complete, the chambers were sealed and transferred to incubators, and the incubation was continued at 37 C for a total of 2 hours in each gas.

Tumor Necrosis Factor Assay

Tumor necrosis factor was measured by two techniques. The TNF bioactivity was determined using lysis of L929 fibroblasts as described previously.[11] Briefly, L929 cells were plated in 96-well plates at 2×10^4 cells/well in 0.1 mL and allowed to grow to near confluence overnight. Peritoneal macrophage supernatants were added to the L929 cells in triplicate and serially diluted. The cells and supernatants were incubated in medium containing 1 μg/mL actinomycin D overnight at 37 C in 95% air, 5% CO_2. To determine the number of viable L929 cells, 50 μL of 2.5 mg/mL MTT dye was added to each well and incubated for 2 hours, after which 100 μL of lysing buffer, consisting of 20% sodium dodecyl sulfate and 50% dimethyl formamide, was added to each well for 2 hours. Because MTT is a dye taken up by the mitochondria of living cells and TNF results in cellular killing, the color released by the lysing buffer is inversely proportional to the amount of TNF present in the sample. The resulting colored product was read at 550 nm in an SLT Model 400 ATC microplate reader (SLT Industries, Salzburg, Austria) and plotted against a standard curve of known concentrations of recombinant murine TNF_α. In some studies, supernatant TNF was measured by enzyme-linked immunosorbent assay (ELISA). The antibody sandwich was detected by fluorescence as described previously.[12] Microtiter plates were coated with a monoclonal antimurine TNF antibody, incubated with samples, washed, and then exposed to the polyclonal rabbit antimurine TNF antibody. The enzyme sandwich then was incubated with goat antirabbit immunoglobulin G alkaline phosphatase for 1 hour at room temperature before the addition of the substrate solution, 5-fluorosalicyl phosphate, and the developing reagent terbium-ethylene diaminetetraacetic acid. The fluorescence was measured with a time-resolved fluorometer, and the calibration curve and the data reduction were performed by automatic immunoanalyzer (Cyber Fluor 615). Data are expressed as picograms per milliliter TNF for both assays.

Interleukin-1 Bioassay

Macrophage production of bioactive IL-1 beta was determined using an IL-1-dependent murine T-helper cell line (D10(N4)M. Fifty microliters per well of sample was plated in triplicate on 96-well plates and serially diluted. Aliquots of 1×10^5 D10(N4)M cells/well were added in RPMI containing 3 μg/mL conconcanvalin A and 1 ng/mL of recombinant murine interleukin-2. These plates were incubated for 72 hours, after which 50 μL of 5 mg/mL MTT dye was added to each well and incubated. After 4 hours' incubation, 50 μL of lysing buffer was added to each well, plates were incubated overnight, and the resulting color, proportional to the number of viable cells, was read at 550 nm. Sample IL-1 concentrations, proportional to the number of cells that took up MTT, then were determined on the microplate reader at 550 nm by interpolating resultant optic densities against a standard curve of known murine recombinant IL-1 beta concentrations. Data are expressed as picograms per milliliter IL-1.

Extracellular pH Measurement

The pH of the extracellular medium was measured to quantitate the magnitude of the acidification that occurred after transient exposure to the test gases and how quickly perturbations of pH returned to normal after return of the cultures to control atmospheric conditions. The pH of the culture medium of a parallel group of cultured macrophages was measured immediately after removal from the test gas exposure using a calibrated pH probe (Dow Corning, Corning, NY). The cell cultures were returned to control (95% air/5% CO_2) cell culture atmosphere, and the pH was measured again 2 hours later, corresponding to the time immediately before aspiration of supernatant for cytokine determinations.

Intracellular pH Measurement

Measurements were performed using video microscopy and fluorescence ratio imaging. For each experiment, 1 mL of suspended cells was placed on a glass coverslip for 30 minutes at 37 C and washed three times in phosphate-buffered saline. The resulting cell population is enriched for cells of monocyte–macrophage lineage. Coverslips then were inserted into a thermostated perfusion chamber (Open Perfusion Micro-Incubator; Medical Systems Corp, Greenvale, NY) and placed on the stage of an inverted microscope equipped with epifluorescence optics

(Nikon Diaphot TMD, Nikon Canada, Toronto). Cells then were perfused with 2 mmol of the acetoxymethylester form of the pH-sensitive dye BCECF for 10 minutes. Cleavage of the ester linkages of this hydrophobic compound by cytoplasmic esterases renders it hydrophilic, thereby trapping it within the intracellular compartment.[13]

Previous measurements of intracellular pH have been performed using cell population spectrofluorimetry. Significant methodologic problems of this approach include a decrease in fluorescence associated with nonselective dye leakage from cells, heterogeneous emission from nonviable cells, as well as failure to precisely identify the cell population being studied in mixed populations. Hence, we performed two wavelength ratio imagings while simultaneously observing macrophage cell morphology. Alternate excitation of BCECF at 490 and 440 nm was provided by a computer-controlled shutter and filter wheel assembly (Metaltek, Empix Imaging, Toronto, ON), while continuous 620-nm illumination (allowing for visualization of cell morphology) was achieved by filtering the transmitted incandescent source. The red light was directed to a video camera, allowing continuous visualization of the cells, while the fluorescent light was directed onto a 535- ± 25-nm filter and imaged with a slow-scan cooled (−45 C) (charge coupled device) CCD camera (Star-1, Photometrics, Tucson, AZ). The large separation between the fluorescence and bright field signals (535 and 620 nm) resulted in no measurable "cross-talk" between the two light paths. Control of image acquisition and of excitation filter selection was achieved using the Metafluor software package (Universal Imaging Corp, Sterling Hts, MI), running on a Pentium-100 Dell computer (Dell, Inc, Mississauga, Canada), interfaced to the Photometrics camera by way of a 12-bit GPIB II/IIA board (National Instruments, Austin, TX).

A pixel-by-pixel ratio of the 490-nm and 440-nm fluorescence images was obtained after background noise was subtracted. The resulting ratio images were displayed online, and regions of interest were defined encompassing the cells. The averaged ratio values of the regions of interest were calculated and plotted to follow the kinetics of the pH_i changes throughout the experiment.

Cytosolic pH Measurements During Insufflation of Test Gases

The cytoplasmic pH (pHi) of cells in response to test gases was determined as follows. Adherent BCECF-loaded cells were perfused in RPMI-1640 containing 24-mmol sodium bicarbonate. The test gas of interest was bubbled into the perfusion chamber at a rate of approximately 0.25 L/minute, and continuous fluorescent ratio images were recorded. At the end of each experiment, a calibration curve of cellular fluorescence *versus* pH was obtained *in situ* by sequentially perfusing the cells with potassium chloride (KCl)-rich medium containing 5-mmol nigericin and buffered at four different pH values ranging from 6 to 7.5.

Effect of Intracellular Acidification on Tumor Necrosis Factor Production

Having defined that CO_2 perfusion caused a marked reduction in the intracellular pH of peritoneal macrophages, studies were performed to determine whether changes in cytosolic pH had an effect on LPS-induced TNF production. To accomplish this, a pharmacologic approach was used to "clamp" intracellular pH at a defined level before LPS stimulation.[14] Briefly, 2 mL of cells (1×10^6/mL) was resuspended in KCl at varying extracellular pH with 5-mmol nigericin for 1 hour at room temperature. Cells then were pelleted, resuspended in RPMI plus 10% FCS, and incubated with LPS for 4 hours at physiologic pH. The concentration of TNF in the supernatant was determined by ELISA. Cell viability was confirmed throughout by way of trypan blue exclusion.

In Vivo Measurement of Tissue pH and Macrophage Tumor Necrosis Factor Production During Abdominal Insufflation of Test Gases

The effect of test gases insufflated into the peritoneal cavity on peritoneal membrane pH was determined as follows. Rats were anesthetized using halothane, nitrous oxide inhalation, and after a small midline laparotomy, a 14-gauge angiocatheter was inserted into the left lower quadrant of the peritoneal cavity under direct vision. The test gas of interest was insufflated via this catheter to an abdominal pressure of 12 to 15 mmHg. Insufflation pressures were monitored by way of a pressure transducer connected to the angiocatheter through a three-way adapter. Through a separate midline incision, a plane between the peritoneal lining and its underlying preperitoneal fascia was identified and dissected. An 18-gauge needle microelectrode (Microelectrodes Inc, Lebanon, NH) was placed into this plane and connected to a calibrated Orion 611 pH meter (Boston, MA).

Having assessed the ability of test gases to reduce the pH of the peritoneal cavity, we next determined the effect of abdominal insufflation with such gases on macrophage TNF production. After 1 hour of insufflation with the test gas, the peritoneal cavity was lavaged with 50 mL of ice-cold phosphate-buffered saline. The effluent then was centrifuged at 200 × g for 10 minutes, cells were washed twice in Hanks balanced salt solution, and counted with a hemocytometer. Cells were resuspended in RPMI-1640 containing 10% FCS and incubated with or without LPS (100 ng/mL) for 2 hours

at 37 C. Cells were pelleted and the concentration of TNF in the supernatant determined using ELISA.

Statistical Analysis

Duplicate or triplicate samples were obtained by carrying parallel sets of macrophages through the entire experimental protocol. For the bioassays, each sample was plated in triplicate and the results were averaged. For the ELISA assay, samples were done in duplicate. Calculation of sample concentrations by interpolation from standard curves was performed by Deltasoft software (Biometallics Inc, Princeton, NJ). One-way analysis of variance and Student's t test were used to calculate statistical difference between groups.

RESULTS

Lipopolysaccharide-Stimulated Cytokine Production After Exposure to Test Gas

Figure 3 shows the effect of transient exposure of macrophages to each test gas mixture on LPS-induced cytokine release. Both TNF and IL-1 production in the absence of LPS stimulation were low (Figs. 3A and 3B, respectively). As expected, LPS stimulation of macrophages incubated in 95% air:5% CO_2 resulted in a significant enhancement of both TNF and IL-1. By contrast, incubation for 2 hours in 100% CO_2 caused a significant reduction of both TNF and IL-1 release. Incubation in 100% air or 100% He did not significantly alter LPS-stimulated TNF or IL-1 release by LPS. Furthermore, in the absence of LPS stimulation, transient exposure to the test gases was insufficient to produce any alterations in basal cytokine production. To determine whether the effect of CO_2 on cytokine bioactivity was caused by a reduction in bioactive protein secretion, LPS-stimulated TNF levels in the supernatant after exposure to test gases were evaluated by ELISA. As listed in Table 1, incubation in CO_2 also reduced TNF protein release, whereas He had no effect compared to air-exposed cells.

To evaluate the reversibility of the inhibitory effect of CO_2 exposure, LPS-stimulated cytokine release was studied 24 hours after 2-hour test gas exposure. In these experiments, exposure of macrophages to the respective test gases was performed identically to that described above. After a 2-hour test gas exposure, the macrophages were incubated for a further 24 hours in control atmospheric conditions (95% air/5% CO_2). The medium then was discarded and replaced with medium containing 0 or 1 μg/mL of LPS for a further 4 hours. The supernatant was aspirated and cytokines measured. Using this protocol, LPS enhanced TNF and IL-1 release after exposure to air or transient exposure to 100% He, and there was no late stimulation of cytokine production by any of the test gases in the absence of LPS stimulation (Fig. 4).

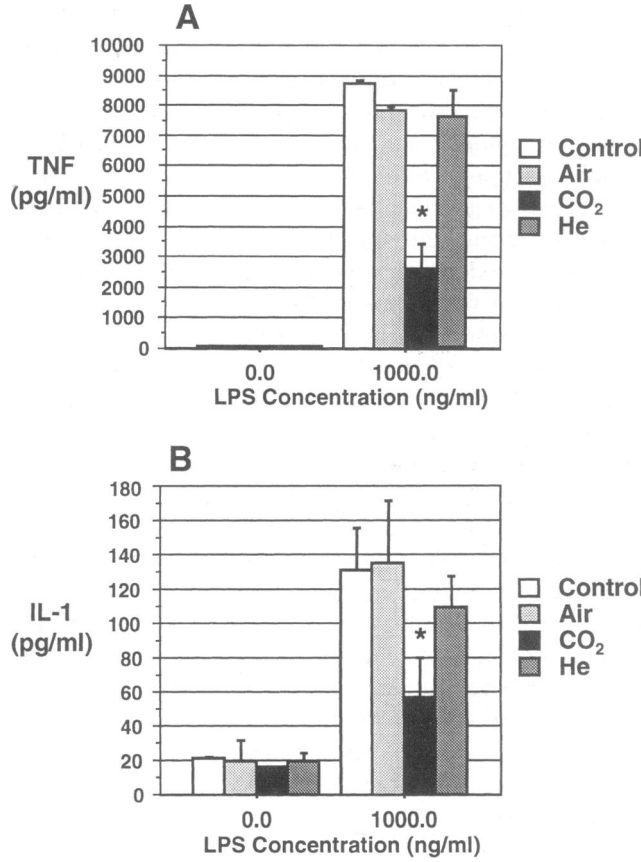

Figure 3. Effect of 2-hour exposure to test gases on lipopolysaccharide (LPS)-stimulated. (A) Tumor necrosis factor (TNF) and (B) interleukin-1 secretion in murine peritoneal macrophages. Exposure to 100% carbon dioxide (CO_2) produced significant inhibition of both LPS-stimulated TNF and interleukin-1 release, whereas no inhibition was seen with either air or 100% helium. The total duration of LPS stimulation was 4 hours. Cultures were exposed to 95% air, 5% CO_2 control (white bars), air (stippled bars), CO_2 (black bars), or helium (shaded bars) for 2 hours in the presence of medium alone or 1000 ng/mL LPS. Representative cytokine bioassay results, expressed as picogram per milliliter ± standard error of the mean, from one of six experiments is shown. *$p < 0.05$ *vs.* control incubation.

Further, in contrast to the CO_2-induced inhibition of both cytokine seen immediately after test gas exposure, there was complete recovery of LPS-stimulated IL-1 release and partial recovery of TNF release when cells were allowed to recover in control atmosphere for 24 hours. There was no difference in the viability of the cells as a function of the test gas exposure (data not shown).

Lipopolysaccharide-Stimulated Cytokine Release 4 Hours After Exposure to Test Gases Containing 20% Oxygen

Although the studies using 100% He suggested that the inhibitory effect of CO_2 was not caused by an effect of

Table 1. THE EFFECT OF DIFFERENT GAS EXPOSURE ON SECRETION OF TNF BY MURINE PERITONEAL MACROPHAGES MEASURED BY ELISA

	Treatment*		
	Air	Helium	CO_2
TNF† (pg/mL)	592 ± 50	490 ± 45	<1‡
pH_i	7.3 ± 0.1	7.3 ± 0.1	6.1 ± 0.1

Data are mean ± SEM of three independent studies.
TNF = tumor necrosis factor; ELISA = enzyme-linked immunosorbent assay.
* Cells were incubated in the designated test gas for 1 hour and then stimulated for 4 hours at 37 C with LPS.
† TNF measured by ELISA.
‡ $p < 0.05$ *vs.* air and helium.

anoxia, the possibility that trace amounts of molecular O_2 in the 100% He gas might have prevented an anoxia-mediated inhibition of cytokine release could not be ruled out. To confirm that lack of O_2 was not responsible for the observed results, we obtained (Minneapolis Oxygen, Minneapolis, MN) mixtures of the respective test gases that contained normal atmospheric concentrations of O_2 (20%). Figure 5 shows that qualitatively identical results were obtained when the cells were incubated in 80% CO_2 and 20% O_2 compared to those shown earlier with 100% CO_2. Again, there was a profound inhibition of LPS-stimulated TNF and IL-1 release after 2-hour exposure to an atmosphere consisting of 80% CO_2 and 20% O_2. As shown earlier, no inhibition was seen with air or He, and there also was no CO_2-mediated alteration in cytokine production in the absence of LPS. As shown in Figure 6, macrophages incubated for 24 hours after transient exposure (2-hour) to the CO_2 containing 20% O_2 exhibited complete recovery of TNF release as well as an augmentation of IL-1 release compared to both air and the He/O_2 mixture.

Effect of Test Gas Exposure on pH_i and pH_o of Peritoneal Macrophages

Acute exposure of cells to CO_2 is known to cause a reduction in pH_i.[15] Because cytosolic acidification has been shown to cause cellular dysfunction in a variety of cell types including macrophages, it was hypothesized that the inhibitory effect of CO_2 on cytokine release might be mediated by its ability to reduce pH_i. To test this possibility, pH_i was examined in cells exposed to each of the test gases. Figure 7 shows representative tracings of the changes in pH_i in peritoneal macrophages exposed to air, He, and CO_2. Although neither air nor He altered pH_i during the period of exposure, CO_2 induced a rapid and profound cytosolic acidification to a pH_i of approximately 6.1. The data summarizing three independent studies are listed in Table 1. Replacement of CO_2 gas with air resulted in rapid recovery of pH_i back to control levels (data not shown).

In a similar fashion, CO_2 selectively reduced the extracellular pH of the buffered medium in which the cells were incubating. Although the cells were inaccessible to measurement during the time of test gas exposure in the incubation chambers, the pH of the incubation medium immediately after removal of the cells from the chamber is listed in Table 2. The pH of the extracellular medium was measured at approximately 7.2 after incubation in control atmosphere, air, or 100% He. In contrast, cultures that were incubated in 100% CO_2 had a significant depression in the pH of the extracellular medium of 6.1 ± 0.3.

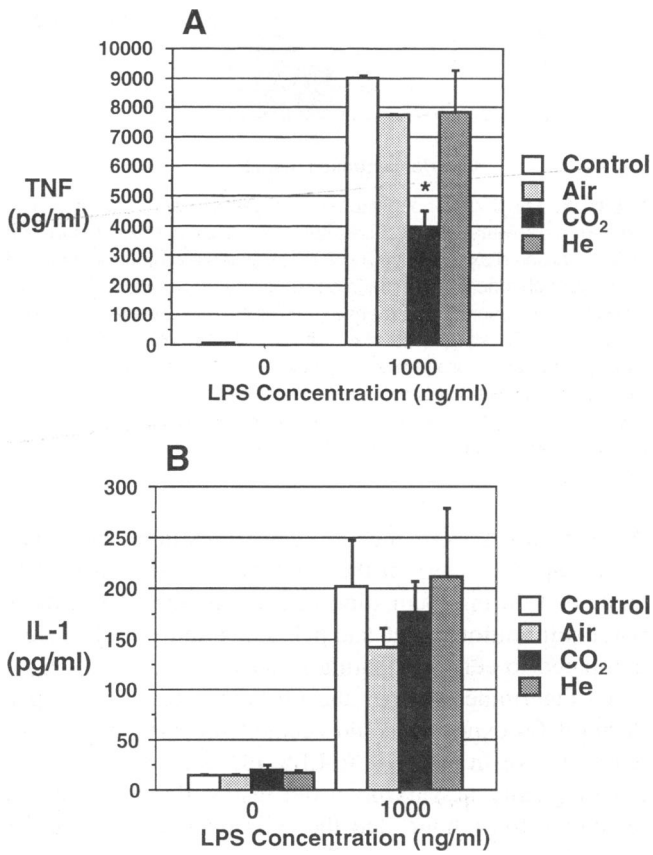

Figure 4. Effect of test gas exposure on murine peritoneal macrophage lipopolysaccharide-stimulated tumor necrosis factor (A) and interleukin-1 (B) secretion 24 hours after removal from the test gas. Reversal of carbon dioxide (CO_2)-mediated inhibition of interleukin-1 was observed 24 hours after incubation in CO_2, along with some return of tumor necrosis factor release. Test gas incubation groups are as follows: 95% air, 5% CO_2 control (white bars), air (stippled bars), CO_2 (black bars), or helium (shaded bars). Representative cytokine bioassay results, expressed as picogram per milliliter ± standard error of the mean, from one of three experiments is displayed. *$p < 0.05$ *vs.* control incubation.

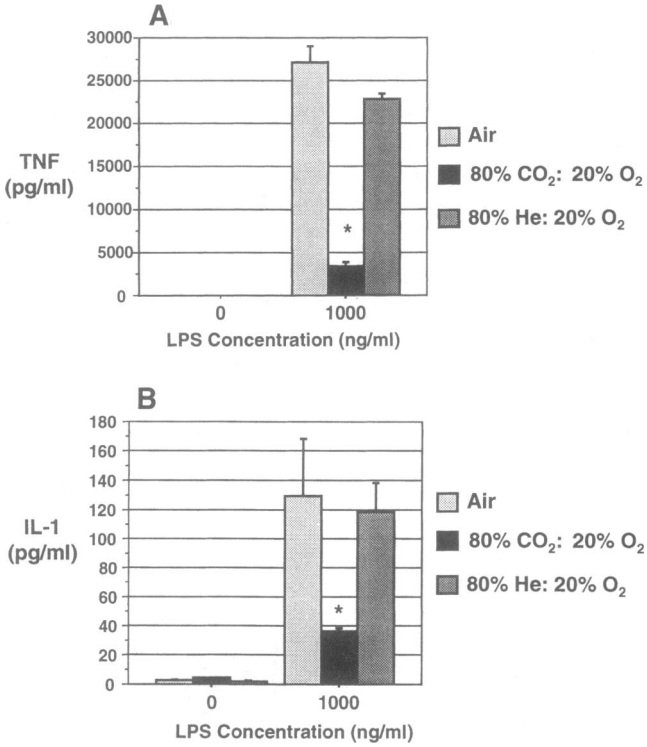

Figure 5. Effect of 2-hour exposure to test gases containing 20% oxygen to eliminate the potential effects of cellular hypoxia. Incubation in 80% carbon dioxide containing 20% oxygen still profoundly inhibited lipopolysaccharide (LPS)-stimulated tumor necrosis factor (A) and interleukin-1 (B) secretion in murine peritoneal macrophages. The total duration of LPS stimulation was 4 hours. Cultures were exposed to air (stippled bars), carbon dioxide (black bars), or helium (shaded bars) for 2 hours in the presence of medium alone or 1000 ng/mL LPS. The figure shows representative cytokine bioassay results, expressed as picogram per milliliter ± standard error of the mean, from one of three experiments. *$p < 0.05$ *vs.* incubation in air.

After removal from the test gas incubation, there was rather rapid recovery of the extracellular pH_o. These data show that during incubation of cells in 100% CO_2, there was equilibration of pH_i and pH_o at markedly acidic levels compared to cells incubating in air or He.

To determine whether the observed reduction in pH_i during CO_2 exposure could account for the impaired release of TNF in response to LPS, pH_i was clamped pharmacologically at varying levels using the K^+/nigericin technique for 1 hour, and the cells then were tested for their ability to release TNF during the ensuing 4 hours at physiologic pH. As listed in Table 3, clamping of pH_i at levels of 6.5 or less markedly reduced LPS-stimulated TNF release when compared to pH_i levels of 7 or greater. Using this technique, reduction in pH_i is accomplished by lowering pH_o under conditions in which ionic gradients are dissipated. Thus, TNF release is measured under various clamp conditions. It is not possible to discern whether the inhibitory effect is a result of a reduction in pH_i or pH_o. To differentiate these, cells were incubated for 1 hour in KCl medium at various pH_o levels (range, 7.4–6) in the absence of nigericin. Under these circumstances, pH_i is nominally affected because of the normal pH homeostatic mechanisms (Table 3). The LPS-stimulated TNF release during the following 4 hours in physiologic medium did not differ between groups. Thus, when considered in conjunction with data in Table 1, the findings suggest that the CO_2-induced cytosolic acidification was sufficient to account for the impaired TNF release.

In Vivo Measurement of Peritoneal Tissue pH Levels During Test Gas Insufflation and Effect on Lipopolysaccharine-Stimulated Tumor Necrosis Factor Release by Resident Peritoneal Macrophages

Resident peritoneal macrophages reside on the peritoneal lining and respond to local trauma or inflammatory

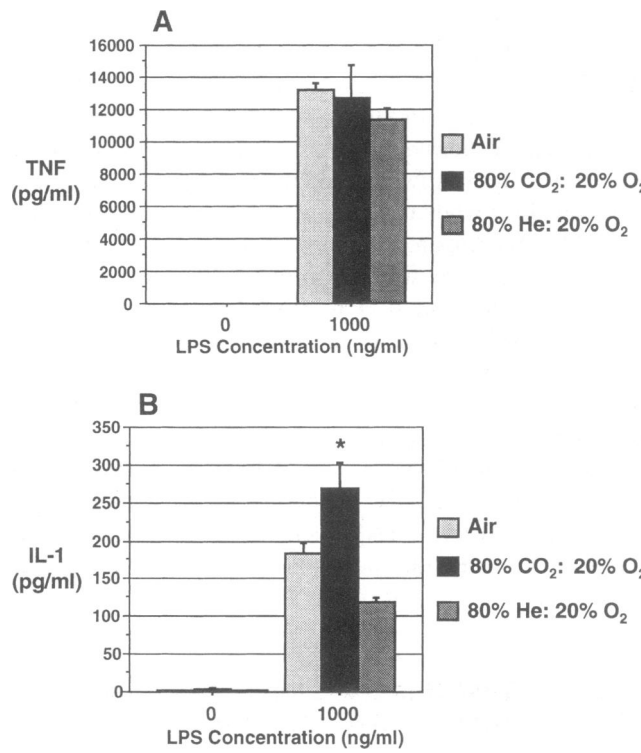

Figure 6. Recovery of murine macrophage lipopolysaccharide (LPS)-stimulated tumor necrosis factor (A) and interleukin-1 (B) secretion 24 hours after a 2-hour exposure to test gases containing 20% oxygen. There was complete recovery of the tumor necrosis factor inhibition observed previously after incubation in 80% carbon dioxide containing 20% oxygen and augmentation of LPS-stimulated interleukin-1 secretion. The total duration of LPS stimulation was 4 hours. Cultures were exposed to air (stippled bars), carbon dioxide (black bars), or helium (shaded bars) for 2 hours in the presence of medium alone or 1000 ng/mL LPS. The figure shows representative cytokine bioassay results, expressed as picogram per milliliter ± standard error of the mean, from one of three experiments. *$p < 0.05$ *vs.* incubation in air.

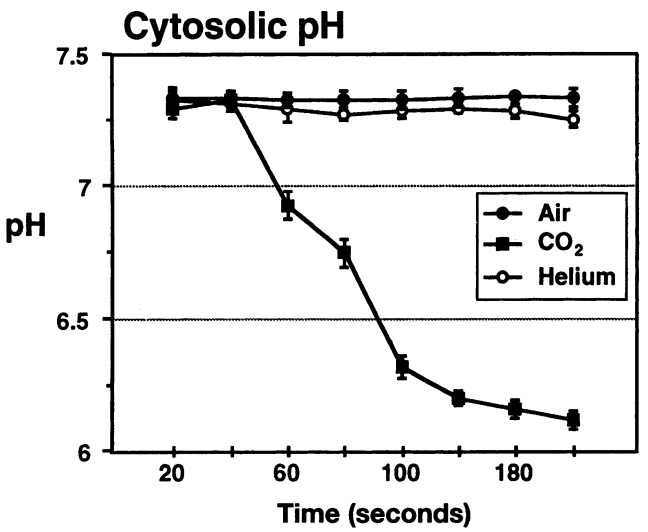

Figure 7. Cytosolic pH of murine peritoneal macrophages during insufflation of peritoneal carbon dioxide. Peritoneal macrophages were adhered to glass coverslips and loaded with the pH sensitive dye 2′7′ bis-(2-carboxyethyl)-5(and 6) carboxyfluorescein (BCECF). Cytosolic pH was determined using fluorescent imaging as described in the Methods section during insufflation of the indicated gases. As can be seen, a rapid and profound cytosolic acidification to a pH_i of approximately 6.1 occurred during carbon dioxide insufflation. In contrast, there was little change in pH_i during insufflation with air or helium. A representative tracing that displays the mean cytosolic pH ± standard error of the mean of n = 12 cells during insufflation is shown.

stimuli. To determine whether CO_2 insufflation induced local acidification, extracellular tissue pH was assessed by inserting an electrode just beneath the peritoneal lining. Rats were used in these experiments because mice were too small to permit accurate localization of the pH microelectrode. Figure 8 shows a representative tracing of tissue pH levels measured during peritoneal insufflation

Table 2. ALTERATIONS IN EXTRACELLULAR pH IMMEDIATELY AFTER REMOVAL FROM TEST GAS INCUBATION OR AT THE COMPLETION OF THE LPS STIMULATION INTERVAL

	Test Gas			
	Control	Air	CO_2	Helium
End of test gas*	7.1 ± 0.2	7.2 ± 0.3	6.1 ± 0.3‡	7.2 ± 0.3
2 hr after test gas†	7.0 ± 0.3	7.3 ± 0.3	7.2 ± 0.4	7.4 ± 0.3

Data are mean ± SEM of two independent studies.
LPS = lipopolysaccharide.
* Cells were incubated in the designated test gas for 2 hours.
† Cells were incubated in test gas for 2 hours, then incubated in control atmosphere for 2 hours before pH measurement.
‡ $p < 0.05$ *vs.* control, air, or helium.

Table 3. THE EFFECT OF CYTOSOLIC pH MANIPULATION ON LPS-INDUCED TNF RELEASE

Extracellular pH	KCl/Nigericin		KCl	
	pH_i*	TNF†	pH_i‡	TNF†
7.4	7.4	400 ± 45	7.3 ± 0.02	440 ± 50
7.0	7.0	330 ± 35	7.2 ± 0.01	390 ± 60
6.5	6.5	20 ± 5‡§	6.9 ± 0.03	350 ± 50
6.0	6.0	20 ± 5‡§	6.8 ± 0.05	330 ± 60

Data are mean ± SEM of three independent studies.
LPS = lipopolysaccharide; TNF = tumor necrosis factor.
* By using nigericin, the pH_i is pharmacologically adjusted to equal the extracellular pH.
† Cells were incubated in the stated conditions for 1 hour at 37°C and then returned to RPMI containing 10% fetal calf serum and 100 ng/mL LPS for 4 hours.
‡ The data represent the mean ± SEM of three independent studies. Triplicate measurements of pH_i were done in 10 cells per study.
§ $p < 0.01$ *vs.* extracellular pH 7.4.

with the various test gases. The CO_2 insufflation but not air or He insufflation caused a marked and rapid reduction in local tissue pH. The pH of blood recovered from these animals did not differ between groups (data not shown). Further, cells lavaged from the peritoneal cavities of each group after a 1-hour exposure to the test gas were evaluated for their ability to release TNF in response to LPS. As illustrated in Figure 9, peritoneal cells derived from animals exposed to intraperitoneal CO_2 released significantly less TNF than did cells from animals exposed to either air or He.

DISCUSSION

Patients undergoing abdominal laparoscopic surgery have been shown to have blunted local and systemic inflammatory responses. The current studies suggest that cytokines from resident peritoneal macrophages contribute a previously underappreciated source of inflammatory mediators after elective abdominal surgery. The data showed that both *in vitro* and *in vivo,* transient exposure to CO_2 caused a marked impairment in LPS-induced cytokine release. The *in vitro* inhibition was reversible, was not related to cellular anoxia, and was shown in both resident and elicited macrophages. Several lines of evidence suggest that the effect was mediated by the ability of CO_2 to induce cytosolic acidification. First, exposure to CO_2, but not air or He, caused a marked cytosolic acidification. Second, pharmacologic induction of intracellular acidification to levels caused by CO_2 exposure recapitulated the inhibitory effect. In the *in vitro* studies, peritoneal CO_2 insufflation was associated with a tissue

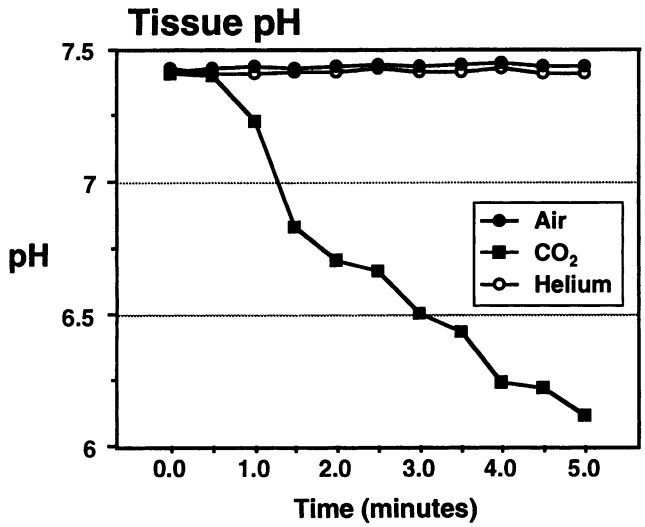

Figure 8. The *in vivo* effects of test gas insufflation on peritoneal pH. Sprague–Dawley rats underwent insufflation with the indicated gas to a pressure of 12 to 15 mmHg through an angiocatheter placed into the peritoneal cavity. A microelectrode was surgically placed into the plane located between the peritoneal lining and its underlying preperitoneal fascia as described in the Methods section and attached to a pH meter. A representative tracing of the changes in tissue pH during insufflation is displayed. There was a marked and rapid acidification of this tissue to pH approximately 6.1 during insufflation with carbon dioxide but not with helium or air.

pH level sufficiently low to account for the observed impairment of cytokine release. Third and finally, LPS stimulation of TNF secretion in resident peritoneal macrophages from animals that underwent *in vivo* CO_2 insufflation was markedly diminished compared to air or He insufflation. Considered together, these data support the concept that resident peritoneal macrophage cellular acidification induced by peritoneal CO_2 insufflation contributes to blunting of the local inflammatory response during laparoscopic surgery.

Previous reports have documented that CO_2 laparoscopy is associated with impaired TNF release by LPS-stimulated macrophages–monocytes. Redmond et al.[6] showed that circulating monocytes obtained from patients after laparoscopic cholecystectomy exhibited reduced TNF release compared to those from patients who had open cholecystectomy. Similarly, these investigators reported that peritoneal macrophages derived from animals undergoing CO_2 laparoscopy released less TNF in response to LPS than those undergoing air laparoscopy.[16] These authors suggested that endotoxin contamination in the air primed cells for this response. Our data point to the alternate possibility that the response in cells derived from CO_2-treated animals might have been decreased as an explanation for the observed difference. More important, the current studies are the first to define the mechanism whereby peritoneal CO_2 insufflation might attenuate the local inflammatory response. Other investigators have shown profound inhibition of human peritoneal macrophage cytokine production when these cells are incubated in an acidic extracellular environment. For example, incubation of macrophages in commercial peritoneal dialysate solution lowers intracellular pH and causes impaired cytokine release.[14] Similarly, Carozzi et al.[17] showed decreased spontaneous release of IL-1, IL-6, IL-8, and TNF_a when incubations were performed in pH 5.5 medium compared to much higher cytokine levels from cells incubated in medium with a pH of 7.4. Further, in the absence of short-chain fatty acids, we and others have shown that cytosolic acidification caused by pharmacologic inhibition of the normal pH_i homeostatic mechanisms can alter macrophage function. The mechanisms whereby transient intracellular acidification after exposure to CO_2 impairs LPS-stimulated TNF and IL-1 release require further investigation. However, some insight may be gained from studies investigating the ability of commercial dialysate solutions to inhibit peritoneal macrophage TNF release. The presence of lactate in these low pH solutions (pH 5.2) serves as a shuttle to move protons into the intracellular space, resulting in profound cytosolic acidification. Cells subsequently stimulated with LPS exhibit reduced levels of TNF mRNA. Because TNF mRNA stability was unaffected and DNA binding activity of the transcription factor NF-kB was reduced, the authors concluded that the

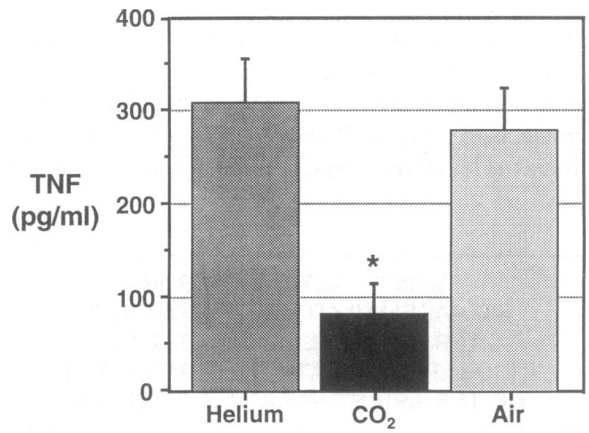

Figure 9. The effect of *in vivo* carbon dioxide insufflation on tumor necrosis factor (TNF) production by rat peritoneal macrophages. Animals were insufflated with the test gas for 1 hour as shown in Figure 8, and the peritoneal cavity was lavaged with 50 mL of ice-cold phosphate-buffered saline. Cells were washed and resuspended in RPMI-1640 containing 10% fetal calf serum and incubated with lipopolysaccharide (100 ng/mL) for 2 hours at 37 C. Cells were pelleted, and the concentration of TNF in the supernatant was determined by way of enzyme-linked immunosorbent assay. There was a marked reduction in TNF production after insufflation with carbon dioxide as compared to air or helium. Supernatant TNF, expressed as mean picogram per milliliter ± standard error of the mean, of n = 3 separate experiments is displayed. *$p < 0.05$ *vs.* air.

acid-induced inhibition of TNF release was at the level of transcription or earlier. Presumably, a comparable mechanism is operative in CO_2-exposed cells, which are acidified to levels comparable to those reported by Douvdevani et al.[18] Studies presently are underway to further define the mechanisms underlying the observed acid-induced alterations in macrophage activation.

We have not examined whether other macrophage functions are likewise inhibited by CO_2. Specifically, there is no information on whether CO_2 incubation impairs phagocytosis or O_2 radical production. Collet et al.[19] reported that peritoneal bacterial clearance was greater in animals undergoing laparoscopic operations compared to open procedures, and they observed no differences in stimulated oxidative burst between the two groups. Conversely, Swallow et al.[20] showed a 36% decrease in superoxide production by murine peritoneal macrophages when the intracellular pH was decreased pharmacologically from 6.80 to 6.60. Macrophages incubated in CO_2 probably would not be capable of a normal oxidative burst response because of the absence of molecular O_2. *In vivo,* it is conceivable that sufficient O_2 might be available to peritoneal macrophages so that they might retain some degree of antimicrobial oxidative activity. If peritoneal macrophage of patients undergoing laparoscopic procedures were significantly impaired in their ability to deal with bacterial contamination, one might expect that such patients would be at increased risk for infectious complications. No increase in infectious complications has been observed in large series of laparoscopic cholecystectomies, appendectomies, or herniorrhaphies.[1,21–23] However, these surgical procedures are associated with a relatively low peritoneal bacterial load, a feature that probably precludes evaluation of infectious risk after laparoscopy.

In conclusion, the data presented show that short-lived exposure of peritoneal macrophages to atmospheric conditions containing a high percentage of CO_2 results in profound, but reversible, alterations in their ability to release cytokines in response to bacterial lipopolysaccharide. Extrapolating to the human clinical situation, these data may provide a partial scientific explanation for the observation that patients have scant inflammatory reaction to laparoscopic abdominal surgery. The mechanisms by which local CO_2 gas exposure produces alterations of macrophage function currently are under investigation. The data also raise the intriguing question as to the role of CO_2 laparoscopy in cancer surgery. Presumably, local cytokine (including TNF) release contributes to the host–tumor balance in the peritoneal cavity. The CO_2 insufflation may negatively affect this balance, thus promoting tumor spread. In this regard, the neoplastic L929 fibroblast cell line used in the bioassay for TNF activity exhibited improved viability after CO_2 exposure. Understanding how local factors within the peritoneal cavity alter the function of resident peritoneal inflammatory cells may therefore have important implications for open as well as laparoscopic abdominal operations.

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