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ACETAZOLAMIDE TREATMENT PREVENTS *IN VITRO* ENDOTOXIN-STIMULATED TUMOR NECROSIS FACTOR RELEASE IN MOUSE MACROPHAGES

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ABSTRACT—We previously showed that incubation in carbon dioxide (CO_2), but not air or helium (He), markedly decreased macrophage intracellular pH (pH_i) and resulted in reversible inhibition of lipopolysaccharide- (LPS) stimulated tumor necrosis factor (TNF) and interleukin-1 release. We sought to determine whether carbonic anhydrase inhibition with acetazolamide would prevent CO_2-mediated inhibition of LPS-stimulated TNF release. Murine peritoneal macrophages were treated with acetazolamide for 1 h under control atmosphere (95% air/5% CO_2) and then switched to incubator modules containing: 1) 80% CO_2/20% O_2, 2) 80% He/20% O_2, or 3) 100% air. Before transfer to experimental atmospheric conditions the macrophages were stimulated with 0 or 1 μg/mL of LPS (*Escherichia coli* 0111B4). Supernatant TNF was measured 4 h later by bioassay. In parallel experiments LPS-stimulated cytokine mRNA was estimated using reverse transcriptase polymerase chain reaction (RT-PCR) 2 h after LPS stimulation. Viability was determined using dye uptake. Incubation in CO_2 or helium had no effect on TNF production in the absence of LPS. In the absence of acetazolamide CO_2 produced marked inhibition of LPS-stimulated TNF release, but this was not blocked by the presence of acetazolamide. This CO_2-mediated inhibition of TNF was associated with normal levels of TNF mRNA. In acetazolamide-treated macrophages, LPS resulted in a dose-dependent inhibition of TNF release when the cells were incubated in air or helium. Maintenance of normal intracellular pH is required for TNF release, but not TNF mRNA induction by LPS. Factors that alter intracellular pH regulation may modulate LPS-stimulated cytokine production.

INTRODUCTION

Laparoscopic abdominal surgery is extremely well tolerated by patients but the physiologic basis of this form of surgery remains incompletely understood. In contrast to open surgery, patients undergoing laparoscopic abdominal surgery have decreased pain, few infectious complications, and lower circulating levels of inflammatory cytokines (1–5). We have been interested in the potential effect of carbon dioxide, used as the insufflation gas, in altering the response of peritoneal macrophages to inflammatory stimuli (6–8). Acidification of macrophages results in decreased immune function, including decreased cytokine secretion (9–11). We reported that carbon dioxide gas, but not air or helium, significantly inhibited endotoxin/lipopolysaccharide (LPS) stimulated cytokine production following *in vitro* exposure to the test gases (6). These effects appeared to result from a rapid, reversible acidification of the macrophages exposed to carbon dioxide. When the same cells were rechallenged 24 h after removal from the carbon dioxide, LPS-stimulated cytokine production did not differ from controls. Metabolic acidification of macrophages also resulted in a pH-dependent suppression of LPS-stimulated tumor necrosis factor (TNF) and interleukin (IL) -1 secretion (11).

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We hypothesized that one mechanism by which carbon dioxide could produce intracellular acidification may involve carbonic anhydrase-mediated generation of free hydrogen ions. Carbon dioxide is an extremely lipid soluble and rapidly diffuses through cell membranes (12, 13). Carbonic anhydrase catalyzes the dismutation of carbonic acid formed by the noncatalytic combination of water and carbon dioxide. In the presence of carbonic anhydrase, carbonic acid rapidly dissociates into bicarbonate and free hydrogen ion. In our series of experiments we sought to determine whether carbonic anhydrase inhibition, using acetazolamide, would prevent the carbon dioxide-mediated suppression of LPS-stimulated cytokine production.

MATERIALS AND METHODS

Reagents

Balb/c mice were obtained from Harlan Sprague-Dawley, Madison, WI. Thioglycollate broth and 96-well polystyrene tissue culture plates were acquired from Becton Dickinson, Lincoln Park, NJ. Dulbecco's modified Eagle medium (DMEM), trypan blue, and HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were procured from Life Technologies, Grand Island, NY. Lipopolysaccharide (LPS) 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, and concanavalin A were obtained from Sigma Chemical Co., St. Louis, MO. Calf serum was purchased from HyClone, Logan, UT. Modular incubator chambers were acquired from Forma Scientific, Inc., Marietta, OH. Recombinant murine IL-2, IL-1β, and TNFα were procured from R&D Systems, Minneapolis, MN. The

IL-1-dependent murine T-helper cell line (D10(N4)M; was graciously provided by Stephen Hopkins, Cambridge, U.K.. L929 fibroblasts (#1-CCL) for the TNF bioassay were procured from ATCC, Manassas, VA.

Animals

Peritoneal cells were obtained by lavage from 5–9 wk old Balb/c mice. Mice were euthanized with carbon dioxide before harvesting peritoneal cells. Use of animals in these studies was approved by the 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 h after mice received injections with 2.0 mL thioglycollate broth by lavage with 10 mL of DMEM containing 10% calf serum and heparin. Cell viability was assessed using trypan blue exclusion and the cells were enumerated with a hemacytometer. Aliquots of 1×10^5 cells in .1 mL of DMEM containing 1% penicillin/streptomycin, 2 mM L-glutamine, and 10% calf serum were added to 96-well polystyrene tissue culture plates. To minimize the effects of alterations of pH on the results the culture medium also contained 10 mM HEPES buffer, pH 7.46. The cultures were incubated for 2 h in 5% CO_2 at 37°C and the nonadherent cells were removed by vigorous washing. The adherent population consisted of >95% peritoneal macrophages. Viability exceeded 95% before and during treatment periods as determined by trypan blue exclusion and MTT dye uptake. Plates were incubated for 48 h before experimental manipulations to allow recovery from the isolation procedures.

Apparatus for test gas exposure

The apparatus used for *in vitro* exposure to the various test gases has been described previously (6, 14). 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 and flushed with the experimental test gases. The volume of culture medium was chosen so that the culture medium overlying the adherent macrophages was approximately 1 mm in depth. We previously showed that this depth permitted rapid 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), carbon dioxide (CO_2: consisting of 80% CO_2 and 20% O_2), and helium (He: consisting of 80% helium and 20% O_2). We had previously shown qualitatively identical results with either 100% CO_2 or a mixture containing 20% O_2 (6, 7). In our study all experiments were performed in 20% O_2 to eliminate any potentially confounding effects of cellular hypoxia. The incubation chambers were flushed with the test gas mixtures for a minimum of 5 min to insure complete test gas equilibration. Our earlier experiments had shown that 2 min was sufficient to remove detectable O_2, in experiments designed to create an anoxic environment (14).

Test gas incubation

To examine the effect of the test gas exposure, the gas incubation was performed by incubating the cells for 2 h in the test gas mixtures (Fig. 1). 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 an additional 2 h. In all studies the cells were incubated in medium alone or medium containing 1 μg/mL of LPS. The duration of the LPS stimulation exposure was generally for 4 h. 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.

TNF assay

Bioactive TNF was determined using lysis of L929 fibroblasts using a modification of the technique of Aggarwal (15). Briefly, L929 cells were plated in 96-well plates at 2×10^4 cells/well in .1 mL and allowed to grow to near confluence overnight. Macrophages 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 and 5% CO_2. The number of viable L929 cells was determined by adding 50 μL of 2.5 mg/mL MTT dye to each well. The cells were incubated for 2 h, after which 100 μL of lysing buffer, consisting of 20% sodium dodecyl sulfate and 50% dimethyl formamide, was added to each well for an additional 2 h. MTT dye is taken up by the mitochondria of living cells, thus the color is inversely proportional to the amount of TNF present in the sample. The colored product was read at 550 nm in a SLT Model 400 ATC microplate reader (SLT Industries, Salzburg, Austria) and plotted against a standard curve of known concentrations of recombinant murine TNFα. Data are expressed as pg/mL TNF.

IL-1 bioassay

Macrophage production of bioactive IL-1β was determined using an IL-1-dependent murine T-helper cell line. A total of 50 μL of the sample was plated in triplicate on 96-well plates and serially diluted. Aliquots of 1×10^5 D10(N4)M cells/per well were added in RPMI containing 3 μg/mL conacanavalin A and 1 ng/mL of recombinant murine IL-2. These plates were incubated for 72 h, following which 50 μL of 5 mg/mL MTT dye was added to each well and incubated. After 4 h 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, were then determined on the microplate reader at 550 nm by interpolating resultant optic densities against a standard curve of known murine recombinant IL-1β concentrations. Data are expressed as pg/mL IL-1.

Semi-quantitative RT-PCR amplification of TNF and IL-1 mRNA

Total RNA was extracted from cultured macrophages by a modification of the Chomczynski single-step RNA isolation method (16) (Fig. 1). For each experimental and control group, mRNA was amplified by reverse transcription with an oligo (dT) primer (Promega, Madison, WI) as described previously (17). Specific cDNA products corresponding to mRNA for TNF, IL-1, and glyceraldehyde phosphate dehydrogenase (GAPDH) were amplified using the polymerase chain reaction (PCR) (18). Negative (no cDNA template) and positive (known TNF, IL-1 or GAPDH cDNA sequences) controls were run with their respective reaction mixture. A Model 9600 Perkin-Elmer Thermal Cycler (Perkin-Elmer, Norwalk, CT) was used for amplification with the following sequence profile: initial denaturation at 95°C for 1 min followed by 35 cycles of three temperature PCR (denaturing: 94°C for 45 s; annealing: 60°C for 45 s; extension: 72°C for 2 min) ending with a final extension at 72°C for 7 min and cooling to 4°C. Amplified PCR product was identified by electrophoresis of 5–10 μL sample aliquots on 2% agarose gel stained with .5 μg/mL ethidium bromide. The sample products were visualized by UV transillumination and the gel was photographed. Cytokine cDNA was semi-quantified by densitometric comparison with GAPDH (internal control) from the same sample after the positive image of the gel was digitized by video for computerized densitometry. Data were normalized to GAPDH and are expressed as optical density units.

Statistical analysis

Duplicate or triplicate samples were obtained by carrying parallel sets of macrophages through the entire experimental protocol. For the bioassays, each

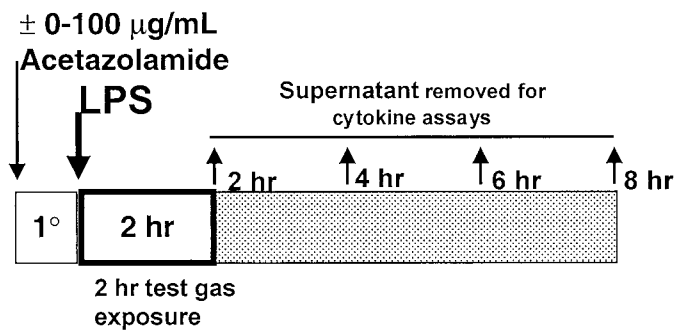

FIG. 1. **Schematic representation of the experimental design.** Acetazolamide (0–100 μg/mL) was added 1 h before activation with LPS. Macrophages were then immediately transferred to the apparatus for manipulation of the atmospheric gas and incubated in the test gases for 2 h. Following the test gas incubation cultures were switched to control atmospheric conditions and incubated for various time intervals.

sample was plated in triplicate and the results were averaged. Calculation of sample concentrations by interpolation from standard curves was performed by Deltasoft software (Biometallics Inc., Princeton, NJ). ANOVA and *t* test were used to calculate statistical difference between groups.

RESULTS

Carbon dioxide inhibits LPS-stimulated TNF and IL-1 production

Fig. 2 shows that LPS-stimulated TNF production was profoundly inhibited by a 2 h exposure to carbon dioxide. Incubating macrophages in either air or helium for 2 h after LPS stimulation produced no alterations compared with macrophages incubated in the control atmosphere. Carbon dioxide caused no alteration in TNF production in the absence of LPS. There were no alterations in macrophage viability detectable by trypan blue exclusion or MTT dye uptake (data not shown).

RT-PCR of macrophages incubated in various test gases ± LPS-stimulation

To gain some insight into the mechanism responsible for the CO_2-mediated inhibition we used RT-PCR to estimate the amount of cytoplasmic cytokine mRNA with or without LPS stimulation. Fig. 3 shows that in the presence of carbon dioxide there were abundant levels of cytokine mRNA following stimulation with 1000 ng/mL of LPS. Note that these same cells had significantly decreased LPS-stimulated TNF and IL-1 production. The qualitative pattern of mRNA production seen after LPS stimulation was similar in control atmospheric conditions, air, CO_2, or helium. In the absence of LPS Fig. 3 shows that there was no detectable TNF or IL-1 mRNA.

Effect of various concentrations of acetazolamide on LPS-stimulated cytokine secretion

We hypothesized that carbonic anhydrase blockade with acetazolamide would prevent CO_2-mediated inhibition of macrophage cytokine secretion by preventing dismutation of CO_2 that diffused through cell membranes. To test this hypothesis we pretreated macrophages with a range of acetazolamide concentrations for 1 h before stimulation with 1,000 ng/mL of LPS. Immediately after the addition of medium containing LPS, the macrophage cultures were transferred to the incubation chambers containing air, helium, or CO_2. Fig. 4 shows that acetazolamide had no effect on CO_2-mediated inhibition at any concentration tested. Instead, increasing concentrations of acetazolamide inhibited LPS-stimulated cytokine production under control or helium atmospheric conditions. Qualitatively similar results were seen with both TNF (Fig. 4*A*) and IL-1 (Fig. 4*B*). In the absence of LPS stimulation, cytokine production was at the limits of detection irrespective of the acetazolamide concentration (data not shown). Fig. 4*C* shows that acetazolamide had no effect on cell viability, measured using MTT dye uptake.

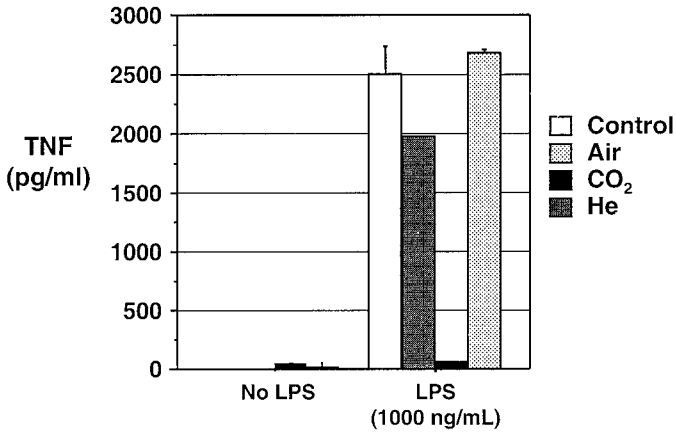

FIG. 2. **Effect of 2 h incubation in various test gases on cytokine production by macrophages stimulated with 1,000 ng/mL of LPS.** Medium alone or medium containing 1,000 ng/mL of LPS was added to macrophages immediately before transferring the cells to incubation chambers that were then flushed with control, air, carbon dioxide, or helium test gases. Incubation in the test gases was continued for 2 h, following which the atmosphere was replaced with control atmosphere (95% air, 5% CO_2) for an additional 2 h. After a total incubation in intervals of 4 h the supernatants were harvested for cytokine determinations. Results are expressed as mean ± SEM of cytokine protein in pg/mL.

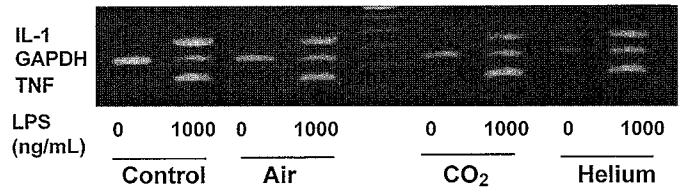

FIG. 3. **Effect of 2 h incubation in various test gases on cytokine mRNA, measured with RT-PCR, from macrophages stimulated with 1,000 ng/mL of LPS.** Medium alone or medium containing LPS was added to macrophages immediately before transferring the cells to incubation chambers that were then flushed with control, air, carbon dioxide, or helium test gases. Incubation in the test gases was continued for 30 min, following which the plates were removed and the cells immediately lysed. Cytoplasmic mRNA was extracted as described under "Materials and Methods" and RT-PCR was performed. Results are expressed as mean ± SEM of cytokine protein in pg/mL. (Representative result from experiment repeated five times.)

Kinetics of LPS-stimulated TNF secretion ± acetazolamide

Macrophages possess energy-dependent mechanisms by which the intracellular pH can be normalized in the presence of an acid load (19–21). For example, Swallow et al. (20, 21) described a proton-ATPase in peritoneal macrophages (19). We reasoned that such compensatory mechanisms might permit resumption of cytokine production at longer duration after LPS stimulation. Fig. 5 shows the kinetics of TNF secretion following LPS stimulation with 1,000 ng/mL. Under controlled atmospheric conditions we saw that acetazolamide again resulted in significant inhibition of TNF for up to 4 h after LPS stimulation; however, by 8 h the cells secreted TNF. In contrast, macrophages incubated under controlled atmospheric conditions showed progressive accumulation of TNF throughout the entire 8 h study interval. No TNF production was seen in the absence of LPS stimulation (data not shown).

DISCUSSION

The results showed that incubation of LPS-stimulated macrophages in a CO_2-rich atmosphere depressed release of TNF and IL-1 compared with similar cells incubated under normal atmospheric conditions. Cytokine inhibition from CO_2 incubation did not alter TNF or IL-1 mRNA levels, suggesting that the CO_2-mediated alterations did not occur via a transcriptional

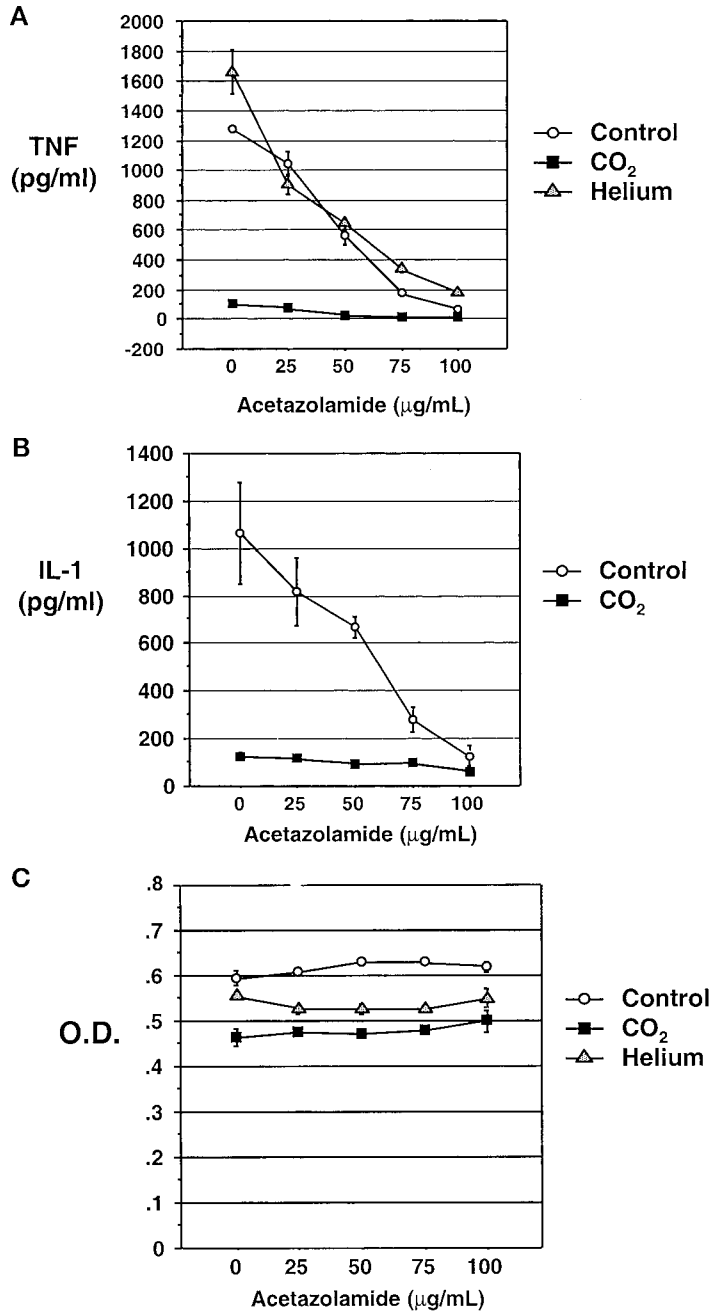

FIG. 4. **Effect of acetazolamide on LPS-stimulated TNF (Fig. 3*A*) and IL-1 (Fig. 3*B*) secretion, or macrophage viability (Fig. 3*C*).** Cells were pretreated with medium alone or 25, 50, 75, or 100 μg/mL of acetazolamide for 1 h, stimulated with 1,000 ng/mL of LPS, then transferred to incubation chambers containing air, helium, or CO_2. Cytokines were measured using specific bioassays and the results are expressed as mean ± SEM of cytokine protein in pg/mL. Viability was measured using MTT dye uptake and the results are expressed as optical density units ± SEM. (Representative result from experiment repeated three times.)

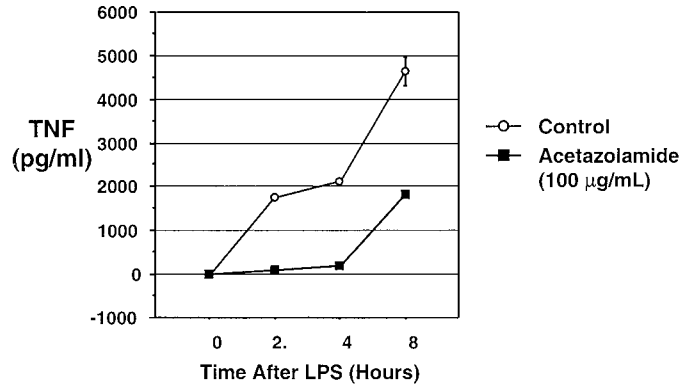

FIG. 5. **Kinetics of LPS-stimulated TNF secretion in the presence of acetazolamide.** Macrophage cultures were incubated with medium alone (open circles) or medium containing 100 μg/mL acetazolamide (black squares) for 1 h before stimulation with 1,000 ng/mL of LPS. Supernatant levels of TNF were measured using parallel cultures of macrophages at the following timepoints: 0, 2, 4, or 8 h after LPS stimulation using a bioassay. Results are expressed as mean ± SEM of cytokine protein in pg/mL. (Representative result from experiment repeated three times.)

control mechanism. Exposure to the CO_2 atmosphere, in the absence of LPS stimulation, had no significant effect on cytokine production. We found that acetazolamide did not block carbon dioxide-mediated inhibition of cytokine production, but rather resulted in a dose-dependent inhibition of LPS-stimulated TNF and IL-1 production under control atmospheric conditions. Acetazolamide treatment before LPS-stimulation did not alter CO_2-mediated inhibition of cytokine synthesis. These results suggest that alterations in intracellular pH may be an additional mechanism regulating cytokine production in inflammatory cells.

We previously showed that carbon dioxide mediated inhibition of TNF and IL-1 production was accompanied by intracellular acidification (11, 22). In those experiments intracellular pH decreased to a level of 6.1 within 3 to 5 min (11). The intracellular pH did not decrease further than 6.1, consistent with the pK_a of buffering capacity for carbonic acid. That cytokine production should be so responsive to a 1 pH unit change in intracellular pH suggests that such mechanisms could be an additional potential mechanism regulating LPS-stimulated cytokine production. Because acetazolamide inhibits carbonic anhydrase the observed inhibitory effects on cytokine production might also be secondary to intracellular acidification. In our previous experiments intracellular acidification was associated with a lesser, but statistically significant, acidification of the extracellular culture medium. In contrast to our previous results with incubation in the presence of CO_2, we saw no acidification of the culture medium with high doses of acetazolamide under the control atmosphere. We did not measure intracellular pH in our experiments, therefore it remains unknown whether the acetazolamide effects seen herein are secondary to cytoplasmic acidification.

Carbonic anhydrase is an ubiquitous enzyme that is present in a wide variety of cell types. Other investigators have suggested that high doses of carbonic anhydrase inhibitors may sometimes produce effects via inhibition of other enzyme systems. At acetazolamide concentrations of more than 10^{-3} molar such effects may come in to play (12). In our experiment the maximal acetazolamide concentration used was approximately 5×10^{-4} molar. Although we did not measure carbonic anhydrase activity directly we saw statistically significant inhibition at acetazolamide concentrations of one-tenth of this amount, suggesting that the observed effects should result from carbonic anhydrase inhibition. The dose of LPS used in these

studies, 1,000 ng/mL, is much higher than the concentrations of LPS present in sepsis. Nonetheless, the observation that CO_2 and acetazolamide inhibited cytokine secretion in response to these pharmacologic doses of LPS underscore the regulatory potential of these agents.

We had hypothesized that CO_2-mediated inhibition of macrophage cytokine production occurred because CO_2 traversed the cell membrane and then dismutated, via carbonic anhydrase, to form free intracellular H^+ ion. Our results do not support this hypothesis. An alternative interpretation of our data is that carbonic anhydrase normally participates in elimination of metabolic acids from activated macrophages. Acetazolamide did not alter cytokine production in the absence of LPS stimulation, whereas it produced a dose-dependent inhibition in cytokine production in LPS-stimulated macrophages. The inhibitory effects of acetazolamide cannot be ascribed to a direct toxic effect because the viability, assessed by MTT dye uptake and trypan blue exclusion, was not altered by the highest concentrations used.

Although alterations in pH are known to effect a wide variety of cellular biochemical pathways, carbon dioxide is not considered to be a second messenger molecule. Recently, several lines of evidence have suggested that other short-lived gases, such as nitric oxide (23) and carbon monoxide (24), may serve as intracellular second messengers. Isotopic exchange experiments show that there is complete equilibration of radiolabeled carbon dioxide across the cell membrane almost instantaneously (13). Thus, the cell membrane represents no diffusion barrier to carbon dioxide. The fact that carbonic anhydrase is present in most cell types and that carbon dioxide rapidly equilibrates across cell membranes suggests that such a system could be operative in many cells.

Stimulation of macrophages with LPS results in activation with increases in the metabolic activity of the cells and increased hydrogen ion production (25). Carbonic anhydrase could facilitate conversion of hydrogen ion into carbonic acid that dismutates into CO_2. The CO_2 released into the cytoplasm by metabolically active cells would diffuse through the cell membrane and thus be effectively removed from the cell. Carbonic anhydrase inhibition could result in H^+ accumulation within the cytoplasm or in key organelles. A schematic depiction of this postulated role for carbonic anhydrase in regulating free intracellular hydrogen ion concentration is shown in Fig. 6. This scheme could also explain why acetazolamide did not alter the function of quiescent cells, i.e., they were producing little CO_2. Inhibition of cytokine production seen when LPS stimulation took place in a high CO_2 atmosphere could occur because high levels of dissolved carbon dioxide in the extracellular medium would diffuse across the cell membrane and impede the enzymatic conversion of hydrogen ion into carbonic acid by mass action. Our previous data showed that alterations in intracellular pH accompanied CO_2 exposure and suggested that alterations in pH_i may regulate LPS-stimulated cytokine release. However, because pH_i was not directly measured in these experiments it is conceivable that the acetazolamide effects were due to an entirely different, pH-independent, mechanism.

It is unclear whether these data have direct applicability to clinical laparoscopic surgery. In most instances insufflation is performed with carbon dioxide. We previously suggested that CO_2-mediated intracellular acidification could inhibit or prevent secretion of inflammatory cytokines after laparoscopic surgery (6). There are anecdotal reports showing that circulating cytokine levels were higher if laparoscopic surgery was performed with pneumoperitoneum, rather than CO_2 insufflation (26). Our results would suggest the possibility that pharmacologic agents might also have the potential to ameliorate inflammatory cytokine secretion by resident peritoneal macrophages.

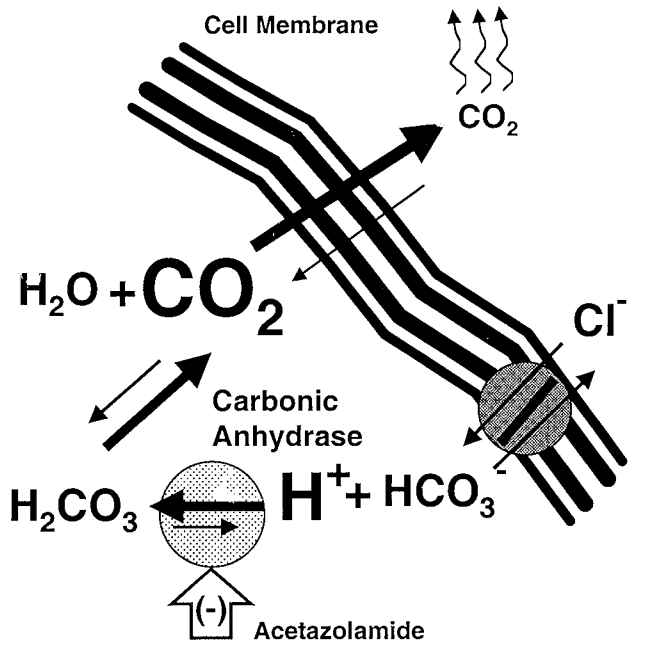

FIG. 6. **Schematic depiction illustrating our current hypothesis of how acetazolamide inhibition of carbonic anhydrase could produce acute accumulation of intracellular hydrogen ion in metabolically active cells.** Under normal conditions H^+ could combine with HCO_3^- and subsequently dismutate into CO_2 and H_2O. Excess CO_2 (acid equivalent) could then diffuse through the cell membrane and dissipate into the atmosphere. With high levels of atmospheric CO_2 there could be net cellular CO_2 uptake that could acidify the cytoplasm by the reverse reaction, or upset the equilibrium for dismutation of carbonic acid.

In conclusion, these findings provide preliminary evidence suggesting that factors that modify intracellular pH may regulate LPS-stimulated TNF and IL-1 production. Whether such effects are physiologically important remains unclear. Macrophages may encounter acidic conditions within the body frequently during sepsis and therefore it is important to understand how pH control mechanisms can modulate the inflammatory response. Further exploration regarding the role of intracellular pH in the control of the inflammatory response may reveal novel regulatory points that could be exploited therapeutically.

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