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Inhibition of Pathogenic Enteric Bacteria by Hyperbaric Oxygen: Enhanced Antibacterial Activity in the Absence of Carbon Dioxide

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The antibacterial effects of 24-h exposures to high-pressure oxygen in relation to environmental CO_2 were studied at 3 atm absolute (ata) and at 1 ata. Eight gram-negative, aerobic and facultatively aerobic, pathogenic enteric bacteria (*Salmonella typhosa*, *Salmonella paratyphi*, *Salmonella schottmuelleri*, *Shigella dysenteriae*, *Shigella flexneri*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Escherichia coli*) were exposed as shallow-broth cultures and agar surface cultures. Although broths supplemented with 0.2% glucose permitted some growth of *Salmonella typhosa*, *Salmonella schottmuelleri*, *Shigella dysenteriae*, and *Shigella flexneri* during exposure to high-pressure oxygen in the presence of CO_2, the other species grew only after the exposure, indicating a bacteriostatic effect. Both bacteriostatic and bactericidal effects were demonstrated on the surface of Trypticase soy agar, where killing of *Salmonellea typhosa*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* was significantly greater after exposure to pure O_2 at 3 ata than at 1 ata. At 3 ata, significantly more killing occurred upon exposure of all species (except *Shigella dysenteriae* and *S. flexneri*) on an agar surface to 100% O_2 as compared with exposure to a mixture of 95% O_2 + 5% CO_2. Thus, deprivation of CO_2 during exposure to pure O_2 enhanced the bactericidal effect of high-pressure oxygen.

During the past decade, therapeutic application of hyperbaric oxygenation has been found to be an effective adjunct to combined surgical and antibiotic therapy in the management of some clostridial infections (18). This clinical success with clostridia has provided an impetus for studies of the antibacterial effects of high-pressure oxygen (HPO) for other bacteria, including aerobic pathogens, and for studies of the enhancement of the activities of antimicrobial drugs by HPO. Gottlieb (11), in reviewing this literature, has warned that results obtained in many in vitro studies may be influenced by the absence of CO_2 when O_2 alone is used. Thus, during exposure to HPO, bacteria may be subjected to deprivation of CO_2 along with exposure to the pressurized O_2. This phenomenon has been investigated by Brown and associates (3, 4), who found no killing of small populations of *Escherichia coli* as a consequence of exposure to O_2 in the presence of CO_2 at 1 atm absolute (ata). A bactericidal effect observed upon exposure of cells to pure O_2 was subsequently attributed to deprivation of CO_2. The nutritional state of bacteria was shown to be related to the need for CO_2. Protection against CO_2 deprivation was obtained upon a synthetic medium with either acetate or glucose as the sole source of carbon, or upon nutrient agar plus acetate (3). Although small populations (less than 10^6 bacteria dispersed per cm^2 of exposed surface) were killed, larger populations survived and grew in pure O_2. Increased numbers of cells apparently can supply greater amounts of metabolically produced CO_2 to the microenvironment.

Many bacterial species require CO_2 for growth. Removal of CO_2 from an otherwise favorable environment can inhibit growth, whereas an overabundance may also be inhibitory (6, 19). As a growth factor, CO_2 is used in biosynthetic reactions required for cellular growth and metabolism but can be replaced or "spared" by addition to simple media of hydrolyzed protein or mixtures of amino acids (7) or dicarboxylic acids (13). Thus, an exogenous source of CO_2 may not be required in a complex medium. A working hypothesis based upon the

report of Brown and Howitt (3) is that deprivation of CO_2 rather than the presence of pure O_2 may account for the observed antibacterial effects attributed to hyperoxia (100% O_2 at 1 ata). This suggests, as a corollary, that the presence of CO_2 may diminish or negate effects attributed to pure O_2. The present paper examines this corollary by in vitro exposures of eight species of aerobic and facultatively aerobic gram-negative enteric bacteria to O_2, in the presence and absence of added CO_2, at 1 ata and at the clinically useful pressure of 3 ata.

MATERIALS AND METHODS

Microorganisms. The following laboratory stock strains of enteric pathogens were studied: *Salmonella typhosa* J15, *Salmonella paratyphi A* J1155, *Salmonella schottmuelleri* J1158, *Shigella dysenteriae* J1104, and *Shigella flexneri* J1106. For comparative purposes, the following opportunistic enteric pathogens were also examined: *Proteus vulgaris* J1233, *Pseudomonas aeruginosa* J694, and *Escherichia coli* ANCAR. The antibacterial effects of various gaseous atmospheres were examined by observation of the growth response of each species after exposure in shallow-broth cultures and on plates of agar medium.

Shallow-broth cultures. Each microorganism was grown in nutrient broth (Difco) for 24 h at 37 C. A portion of the culture was diluted 1:100 in nutrient broth, and 0.1 ml of this dilution was inoculated into each tube of experimental broth, which was distributed in 2.0-ml portions to cotton-plugged, optically matched tubes (125 by 15 mm) and sterilized by autoclaving. Three experimental broth media were used: nutrient broth, brain heart infusion broth (BHI; Difco), and a Trypticase broth (TNYG) containing 1.0% Trypticase (BBL), 1.0% NaCl, 0.2% yeast extract (Difco) and 0.2% glucose dissolved in 0.033 M potassium phosphate buffer (pH 7.0). The TNYG broth (final pH 6.8) was formulated for comparison of growth data obtained with BHI broth (final pH 7.4) since more dissolved CO_2 might be expected in solution under alkaline conditions. Each medium consisted of a protein hydrolysate from a different source, and, in addition, the latter two contained glucose; these constituents have been reported to spare the requirement for exogenous CO_2 (7) and to protect against toxicity due to deprivation of CO_2 (3), respectively. Inoculated tubes (in quadruplicate) were placed in a hyperbaric chamber at an angle of 130° from the horizontal to increase the surface area of the culture. Control cultures were placed in a candle jar (CO_2-rich environment). After incubation for 24 h at 37 C, growth in each tube was measured turbidimetrically at 660 nm. To facilitate these measurements, the original 2.0-ml culture volume was adjusted to 4.0 ml by addition of identical, uninoculated, sterile broth. Final absorbance values given in Table 1 thus represent a 1:2 dilution of the actual growth except for zero absorbance, which was first confirmed visually. After the determination of absorbance, 0.1 ml was transferred from tubes devoid of visible growth (before volume adjustment) to tubes containing 10 ml of an enriched broth (BHI supplemented with 0.2% yeast extract). Growth in this medium within 72 h was taken to indicate that the original conditions had been bacteriostatic (although an unknown number of cells may have been killed also). Absence of growth in this enriched broth signified that the experimental conditions had been bactericidal.

The static, 44-liter, stainless-steel hyperbaric chamber into which the freshly inoculated broth cultures were placed was evacuated of air to a pressure of 76 mm of Hg, leaving therein traces of residual N_2, calculated to be 60 mm of Hg (0.08 ata). The chamber was then filled to 1 ata (760 mm of Hg) with a mixture of 95% O_2 + 5% CO_2; 100% O_2 was then superimposed on the gases already in the chamber to achieve a final (total) pressure of 3 ata. The final, subsequent partial pressures were calculated to be 2.87 ata O_2 + 0.05 ata CO_2 + 0.08 ata N_2. Under these conditions, the tendency for metabolically produced CO_2 to escape from each culture was minimized whereas the diffusion of externally supplied CO_2 into the shallow culture was increased. Regardless of the final partial pressure, at 1 ata the chamber contained 5% CO_2, which approximated the concentration of CO_2 in the control environment (candle jar). After pressurization, the chamber and its contents were placed in an incubator and the temperature within the chamber was maintained at 37 C for 24 h. For pressure control experiments, the desired pressure was attained by adding 100% N_2 to the air (1 ata) already in the chamber, thus maintaining the partial pressures of O_2 and CO_2 equivalent to that of air at 1 ata.

Agar surface cultures. Each microorganism was grown for 24 h in 50 ml of Trypticase soy broth (BBL) in flasks incubated at 37 C on a rotary platform shaker, and was thereafter serially diluted in physiological saline solution. Samples (0.1 ml) from a previously determined dilution yielding 30 to 300 colonies were seeded upon the surface of plates of Trypticase soy agar (glucose-free; BBL) and spread with a wire loop. Plates were dried for at least 48 h at 37 C before use. Stacks of five inverted plates were placed in the hyperbaric chamber and exposed to 100% O_2 or to a mixture of 95% O_2 + 5% CO_2. Individual cylinders of compressed O_2 and CO_2 were used. The gases were mixed by passage through an anesthesia apparatus having Heidbrink variable-orifice flow meters (Ohio Chemical and Manufacturing Co., Madison, Wis.) calibrated specifically for the mixture used in this study. A temperature-regulated, flow-through hyperbaric chamber (21.6 by 21.6 by 42.5 cm) was used for these studies and has been described previously (1). Gas was passed through this 19.8-liter incubator pressure chamber (maintained at 37 C) for 24 h at the rate of 5 liters/min for exposure at 3 ata. For exposures at 1 ata, the effluent gas passed via tubing into and through a 28.3-liter, gas-tight, stainless-steel tissue culture chamber (Germfree Laboratories, Miami, Fla.). This latter chamber was maintained at 37 C by housing it in an incubator (into which the effluent gas exited) adjacent to the hyperbaric chamber. Each exposure chamber was humidified with water vapor.

Experimental design. Thirty plates of agar medium were used in each experiment with each gas and each microorganism. Each experiment was replicated on three different days and consisted of one set of 10 agar surface cultures exposed to either 100% O_2 or 95% O_2 + 5% CO_2 for 24 h in the hyperbaric chamber at 3 ata. Simultaneously, a second set of 10 plates was exposed to the respective effluent at 1 ata in the tissue culture chamber. A third set of 10 plates, kept at 37 C in an incubator in another room, served as controls exposed to air at 1 ata. Cultures were examined immediately upon removal from the chambers. Since no colonies were evident on plates exposed at 3 ata, and usually none on plates at 1 ata, these were incubated in air at 37 C for an additional 1 to 3 days until colonies appeared and were counted.

We postulated that exposure of 30 to 300 cells on an agar surface to a continuous stream of pure O_2 represented a CO_2-deprived environment and that exposure to the mixture of O_2 and CO_2 would compensate for any deprivation of CO_2. Accordingly, bactericidal activity after exposure to the mixture of O_2 and CO_2 could reasonably be attributed to the presence of O_2 rather than to deprivation of CO_2.

Statistical analysis. A preliminary analysis of variance was used to check the overall interaction (gases × pressures × replications) for each organism. Subsequently, a 2 × 2 factorial arrangement was used to test for individual comparisons involving exposure to pure O_2 or the gaseous mixture of O_2 and CO_2 at 3 ata and 1 ata (8). A quantitative measure of the bactericidal effect that accommodates experiments involving replications may be designated the index of kill, and is defined as $K = 100\ [1 - (\mu_T/\mu_c)] = 100\ [1 - \rho]$, where $\rho = (\mu_T/\mu_c)$, μ_c is the mean of the control observations (i.e., number of colonies on control plates exposed to air), and μ_T is the mean of the treatment observations (i.e., number of colonies on plates exposed in chambers). To accommodate replications of each experiment, the usual sample estimate of ρ was replaced by an unbiased ratio-type estimate developed by Hartley and Ross (5).

In this paper the index of kill is described by sample estimates of K (i.e., $k = 100\ [1 - (\bar{X}_T/\bar{X}_c)]$) and 95% confidence intervals of K. Fieller's theorem was used to calculate confidence intervals (9). It should be noted that the index of kill is merely a means of expressing the overall bactericidal effect when each replication involves a different set of control observations. No attempt was made to test the indexes of kill per se since little is known about the sampling distribution of this statistic. Differences in the numerical values of corresponding indexes of kill reflect differences in the treatment observations that were tested for statistical significance.

RESULTS

Shallow-broth cultures. The effect of exposure to HPO on bacterial growth in liquid media was studied in a CO_2-enriched milieu. HPO was bacteriostatic for the entire collection of eight different enteric bacteria in nutrient, BHI, and TNYG broths (Table 1). However, *Salmonella typhosa*, *Salmonella schottmuelleri*, and *Shigella dysenteriae* were protected from the toxicity of O_2 in each of the two glucose-containing broths (BHI and TNYG) to a greater extent than in nutrient broth as evidenced by growth of these three organisms. Some protection was also afforded to *Shigella flexneri* which, in the presence of HPO, grew only in the TNYG broth. The normobaric, nutrient broth control cultures generally showed poorer growth than the normobaric BHI and TNYG broth controls.

With respect to any one species, similar levels of growth in the three broth media were obtained when cultures were incubated for 24 h either in the candle jar normobarically or in the hyperbaric chamber pressurized to 3 ata with N_2 but with the O_2 tension equivalent to that of normobaric air. Thus, as noted in Table 1, the

TABLE 1. *Growth[a] of enteric bacteria during hyperbaric exposure to O_2 enriched with CO_2[b] at 3 ata*

Organism	Nutrient broth		BHI broth		TNYG broth	
	HPO	Controls	HPO	Controls	HPO	Controls
Salmonella typhosa	0.000[c]	0.056 ± 0.002	0.173 ± 0.049	0.298 ± 0.013	0.013 ± 0.001	0.111 ± 0.004
S. paratyphi	0.000	0.033 ± 0.006	0.000	0.134 ± 0.003	0.000	0.116 ± 0.003
S. schottmuelleri	0.000	0.095 ± 0.006	0.250 ± 0.012	0.348 ± 0.009	0.116 ± 0.003	0.483 ± 0.022
Shigella dysenteriae	0.000	0.030 ± 0.000	0.025 ± 0.005	0.153 ± 0.002	0.010 ± 0.003	0.126 ± 0.002
S. flexneri	0.000	0.003 ± 0.001	0.000	0.183 ± 0.025	0.008 ± 0.002	0.095 ± 0.003
Proteus vulgaris	0.000	0.026 ± 0.002	0.000	0.240 ± 0.012	0.000	0.262 ± 0.021
Pseudomonas aeruginosa	0.000	0.105 ± 0.014	0.000	0.175 ± 0.014	0.000	0.372 ± 0.026
Escherichia coli	0.000	0.213 ± 0.009	0.000	0.107 ± 0.010	0.000	0.311 ± 0.021

[a] Growth is expressed in absorbance units at 660 nm. Each figure represents a numerical average of four separate cultures ± the maximum deviation of any reading from the average figure.

[b] All hyperbaric exposures were at 37 C for 24 h in the presence of 2.87 ata of O_2 plus 0.05 ata of CO_2 plus 0.08 ata of N_2. Controls were incubated in a candle jar at 1 ata at 37 C for 24 h.

[c] 0.000 indicates that growth was inhibited by bacteriostatic conditions. Growth occurred in all subcultures.

effects of exposure to HPO on growth were related to increased O_2 tension at 3 ata and not to elevated pressure per se.

Agar surface cultures. The antibacterial effect of exposure to HPO was examined, using organisms directly exposed on an agar surface rather than in a shallow broth. Such an examination allowed for a more precise statistical analysis of any bactericidal effects. Further, since the agar medium used was formulated without added glucose, it could not be considered protective in view of the data shown in Table 1. Plates were observed after removal from hyperbaric and normobaric chambers for colonial growth but were not counted until they were subsequently incubated in air for at least 24 h. In most experiments, minute colonies of *Salmonella schottmuelleri*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *E. coli* were noted after a 24-h exposure at 1 ata to pure O_2 and to the mixture of O_2 and CO_2. The mixture at 1 ata also supported the growth of minute colonies of *Salmonella typhosa* and *Shigella flexneri*. No colonies were observed after exposure at 3 ata to either O_2 or the mixture.

Statistical analysis. Preliminary analysis of variance revealed a statistically significant three-way interaction effect (gas × pressure × replication) for six of the eight microorganisms. These six were tested for simple effects, and only *Salmonella schottmuelleri* and *Shigella flexneri* were tested for main effects. There was a significant ($P < 0.01$) main effect due to gases for *Salmonella schottmuelleri*, but the main effect due to pressures was not significant. For *Shigella flexneri*, main effects due to both gases and pressures were not significant. The bactericidal effect of pure O_2, as examined by the 2×2 factorial analysis, is shown in the left half of Table 2. The mean number of colonies on plates exposed to HPO was fewer for six of the eight microorganisms, but this difference was significantly less only for *Salmonella typhosa*, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. Conversely, the mean number of colonies of *Salmonella paratyphi* and *Shigella flexneri* was greater after exposure at 3 ata; this difference was significant for the former, but not for the latter. The right half of Table 2 presents the index of kill, which relates the mean number of colonies on exposed plates to that on unexposed control plates. Exposure to HPO produced a greater index of kill for six microorganisms. The exceptions again were *Salmonella paratyphi* and *Shigella flexneri*.

The role of CO_2 in diminishing the bactericidal effect of exposure to pure O_2 at 3 ata was evident after exposure to the mixture (Table 3). The mean number of colonies on plates exposed to the mixture (as compared with those exposed to 100% O_2) was significantly greater for six of the eight miroorganisms. The exceptions were *Shigella flexneri*, for which the increase was not statistically significant, and *S. dysenteriae*, for which the CO_2-enriched mixture was significantly more toxic. The index of kill after exposure to the mixture was less for the same six species but greater for *S. flexneri* and *S. dysenteriae*. Data for the response of *E. coli* to exposure at 1 ata are included in Table 3. In addition to being similar to those for exposure at 3 ata, they confirm the report of Brown and Howitt (3) that bactericidal effects of O_2 at 1 ata on *E. coli* are negated by addition of CO_2 to the gaseous environment.

DISCUSSION

The growth response of eight enteric bacteria

TABLE 2. *Normobaric and hyperbaric exposure of enteric bacteria to 100% O_2*

Organism	Mean no. of colonies on 30 plates[a]			Index of kill (95% confidence interval)	
	Exposed at 1 ata	Exposed at 3 ata	Significance (P)	Exposure at 1 ata	Exposure at 3 ata
Salmonella typhosa	127.38	1.70	<0.01	37.18 (35.29–39.03)	99.15 (98.15–100.15)
S. paratyphi	0.80	29.93	<0.05	99.44 (95.72–103.16)	76.85 (72.92–80.73)
S. schottmuelleri	62.87	38.67	NS[b]	−4.43 (−9.01–0.02)[c]	35.86 (31.91–39.71)
Shigella dysenteriae	173.00	156.17	NS	24.16 (21.91–26.37)	30.72 (28.65–32.76)
S. flexneri	122.80	156.97	NS	−41.90 (−60.68 to −25.07)	−99.25 (−129.26 to −73.52)
Proteus vulgaris	131.50	58.30	<0.01	22.57 (20.67–24.48)	65.93 (64.28–67.55)
Pseudomonas aeruginosa	149.27	1.97	<0.01	11.45 (9.90–12.99)	98.87 (98.15–99.60)
Escherichia coli	133.03	129.17	NS	24.44 (20.84–27.95)	26.63 (23.09–30.08)

[a] Ten plates in each experiment were replicated on 3 different days.
[b] NS, Not significant; denotes $P < 0.05$.
[c] Negative indexes designate stimulation of growth rather than killing relative to unexposed controls.

TABLE 3. *Hyperbaric exposure (3 ata) of enteric bacteria to pure O_2 and to O_2 enriched with CO_2*

Organism	Mean no. of colonies on 30 plates[a]			Index of kill (95% confidence interval)	
	Exposed to 100% O_2	Exposed to 95% O_2 + 5% CO_2	Significance (P)	Exposure to 100% O_2	Exposure to 95% O_2 + 5% CO_2
Salmonella typhosa	1.70	151.43	<0.01	99.15 (98.15–100.15)	36.50 (34.20–38.75)
S. paratyphi	29.93	67.60	<0.01	76.85 (72.92–80.73)	40.62 (33.71–47.27)
S. schottmuelleri	38.67	123.17	<0.01	35.86 (31.91–39.71)	4.34 (−0.02–8.77)
Shigella dysenteriae	156.17	41.17	<0.01	30.72 (28.65–32.76)	70.92 (68.46–73.35)
S. flexneri	156.97	205.53	NS[b]	−99.25 (−129.26 to −73.53)[c]	13.21 (5.74–20.33)
Proteus vulgaris	58.30	122.73	<0.01	65.93 (64.28–67.55)	34.66 (32.76–36.54)
Pseudomonas aeruginosa	1.97	53.13	<0.01	98.87 (98.15–99.60)	68.64 (66.82–70.44)
Escherichia coli	129.17	183.50	<0.01	26.63 (23.09–30.08)	−10.54 (−13.36 to −7.78)
E. coli at 1 ata	133.03	189.60	<0.01	24.44 (20.84–27.95)	−13.82 (−16.31 to −11.36)

[a] Ten plates in each experiment were replicated on 3 different days.
[b] NS, Not significant; denotes $P < 0.05$.
[c] Negative indexes designate stimulation of growth rather than killing relative to unexposed controls.

to HPO indicated that the pressurized gas was bacteriostatic for broth cultures and both bacteriostatic and bactericidal for agar surface cultures. Bacteriostasis occurred in shallow-broth cultures in the presence of gaseous O_2 and CO_2 plus an alleged sparing substance (protein hydrolysate). However, four of the enteric species (two salmonellae and two shigellae) grew during such exposures when glucose was incorporated in the medium and were thus protected from O_2 toxicity to a greater extent in media containing added glucose than in nutrient broth. Both bacteriostatic and bactericidal effects could be demonstrated more clearly on agar surface cultures. With such cultures, killing was greater after exposure to pure O_2 at 3 ata than at 1 ata. In addition, after exposure to O_2 enriched with CO_2, less killing occurred. Thus, deprivation of CO_2 enhanced the bactericidal effect of pure O_2.

The antibacterial activity of HPO is a function of species and strain differences as well as differences in the duration and intensity of exposure. Species differences seem to account for the exceptions observed here. Gottlieb and Pakman (12) have previously reported that *Salmonella* and *Shigella* species were generally more resistant to HPO than were other enteric bacteria. In the present study, the bactericidal effect of HPO on agar surface cultures, as contrasted with bacteriostasis in shallow-broth cultures, can be explained by the different means used to measure the antibacterial effects of HPO. Differences can also be explained by the numbers of cells exposed. Fewer than 300 bacteria were seeded onto the agar surface, whereas from 1,000 to 10,000 were inoculated into broth. Differences in response to HPO between surface and broth cultures would also be expected to result from limitations of diffusion of O_2 in culture media (17). Diffusion of O_2 from the external environment has been a consideration in the use of HPO to combat infections but is not completely analogous to our in vitro systems. In vivo, only the O_2 carried by the circulatory system during exposure to HPO is readily diffusible to all parts of the body, and it has even been measured within the colon (2).

Varying degrees of nutritional protection obtained by the use of glucose-containing media have been shown for enteric bacteria exposed in broth to the antibacterial activity of HPO. This increased ability of organisms to survive and even grow in the presence of HPO may be attributed to the utilization of an appropriate carbon source by respiratory metabolism during the exposure to the gaseous environment under elevated pressure. Gottlieb (10) suggested that O_2 toxicity represented a disturbance of metabolism. There have been several reports indicating that HPO can decrease cellular adenosine triphosphate concentration (14, 15) as well as inhibit aspects of pyruvate (14) and folic acid (16) metabolism. Accordingly, nutritional reversal of O_2 toxicity might thus act by supplying substances that are readily oxidizable to provide energy, that can bypass blocked anabolic reactions, or that stimulate endogenous metabolism. Under these conditions, the O_2 ten-

sion in the growth medium would be expected to become locally reduced, with subsequent survival or growth of cells more likely to occur.

This study demonstrates that agar surface cultures may be used to provide a simple, experimental method for quantitative evaluation of nutritional protection from HPO. A statistical treatment for analysis of bactericidal effects of HPO has been described and used, and may find application in other studies where each replication of an experiment involves a different control population of bacteria. Combined with initial screening of bacteria in shallow-broth cultures, the quantitative agar surface method can be used to study the relationship between gaseous CO_2 and chemical compounds in protecting against antibacterial effects of HPO.

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