Hyperbaric Oxygen Exposures for Intrahepatic Abscesses Produced in Mice by Nonsporeforming Anaerobic Bacteria

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Hyperbaric oxygen exposures were evaluated for treatment of progressive liver abscesses produced by intraperitoneal injection of combined cultures of *Fusobacterium necrophorum* plus either *Bacteroides fragilis* subsp. *fragilis* or *Fusobacterium nucleatum* in a mouse model. Infected control and hyperbaric oxygen-exposed mice were autopsied 5 or 6 weeks after inoculation of bacteria and were assigned numerical pathology scores according to the number and size of abscesses present. Seventeen daily 3-h exposures to 100% O₂ at 2 atmospheres absolute pressure begun either 1 day or 1 week after injection of bacteria significantly reduced the number and size of abscesses among mice infected with either mixture of anaerobic organisms. Abscesses due to mixed fusobacteria were often completely resolved. These data support the efficacy of hyperbaric oxygen for treatment of mixed anaerobic infection produced by *Bacteroides* and *Fusobacterium* species and suggest its further evaluation as a potential alternate or adjunct to present therapy for certain types of serious anaerobic infection.

In recent years there has been increased recognition of the role of nonsporeforming anaerobic bacteria in human infection. These organisms account for a greater proportion of clinical infections than do the clostridia, whose role as pathogens has been emphasized in the past doubtlessly because of the high mortality associated with tetanus, gas gangrene, and botulism. Recent studies utilizing rigorous specimen transport and cultural techniques capable of isolating obligately anaerobic bacteria have elucidated their high incidence in intra-abdominal, obstetric and gynecological infections, infections of the liver and biliary tract, pleuropulmonary disease, brain abscess, chronic sinusitis, and other conditions (1, 3-5, 12). Although many of these infections are mixed infections of anaerobic and facultatively anaerobic bacteria such as commonly found in abdominal abscesses, a substantial proportion are associated with an exclusively anaerobic flora.

Nonclostridial anaerobic infections are often chronic and require surgical drainage or excision in addition to prolonged antibiotic therapy. However, there are presently few antibiotics with a broad spectrum of activity against anaerobic bacteria which are also highly active against the most frequent clinical isolate, *Bacteroides fragilis* subsp. *fragilis*. This organism is resistant to usual clinical levels of many antibiotics in common use such as penicillin G, ampicillin, or the combination of cephalothin

and kanamycin (2). Hyperbaric oxygenation is a possible mode of therapy which has been incompletely investigated for nonclostridial anaerobic infections. The rationale for use of this method is the inability of most anaerobic bacteria to exist in even nominal concentrations of oxygen coupled with the technical capability of increasing oxygen tensions within tissue. Both clinical and laboratory data support the use of hyperbaric oxygen (HBO) for clostridial myonecrosis (6, 7, 10). Its use for other anaerobic infections is theoretically feasible, but almost no clinical or experimental data are available (11, 16). The present article describes an evaluation of HBO exposures for liver abscesses produced by two different combinations of nonsporeforming anaerobic bacteria in a mouse model.

MATERIALS AND METHODS

Bacterial culture. Human clinical isolates of *B. fragilis* subsp. *fragilis* NCDC no. 10903, *Fusobacterium nucleatum* NCDC no. 10206, and *Fusobacterium necrophorum* NCDC no. 9432 were obtained from the Center for Disease Control in Atlanta, Ga. Frozen samples prepared from cultures of lyophilized stocks were used to initiate growth for each experiment. Broth cultures were grown in fluid thioglycolate medium (no. 135-C, Baltimore Biological Laboratories, Cockeysville, Md.) supplemented with 0.5% yeast extract, menadione (0.5 µg/ml), hemin (5 µg/ml), and 6% rabbit serum. Blood agar plates were prepared with Trypticase

soy agar (Baltimore Biological Laboratories) supplemented with 0.5% yeast extract, 0.2% dextrose, menadione (0.5 $\mu\text{g/ml}$), hemin (5 $\mu\text{g/ml}$), and 5% sheep blood. Media were immediately placed under anaerobic conditions for storage after preparation. Inoculation, incubation (37 C), and harvesting of cultures were performed within an anaerobic glove chamber (Coy Manufacturing, Ann Arbor, Mich.) containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide. The concentration of viable bacteria was obtained by spreading duplicate 0.1-ml aliquots of 10-fold dilutions of broth cultures on the surface of blood agar plates which were incubated, and then the colonies were counted.

Experimental animals. Male inbred A/J mice (The Jackson Laboratory, Bar Harbor, Me.) were used at 10 to 12 weeks of age. These mice are relatively tolerant to high oxygen tensions (8). Animals were held for 2 weeks to stabilize their health before use in experiments and were given laboratory chow and water ad libitum except during hyperbaric exposures.

Production of liver abscesses in mice. The infection model will be outlined briefly for purposes of this paper; a more complete description has been published (9). Twenty-hour broth cultures of two anaerobic species were mixed in a 1:1 ratio. An equal volume of sterile mucin (5 g/100 ml, granular mucin, type 1701-W, Wilson and Co., Inc., Chicago, Ill.) was added to the mixed bacterial culture before injecting 0.5 ml intraperitoneally into mice. Throughout these experiments the mixed inocula injected ranged between 4×10^7 to 5×10^8 bacteria per inoculation. Liver abscesses were produced by mixed inocula of *F. necrophorum* plus either *B. fragilis* subsp. *fragilis* or *F. nucleatum*.

At 24 h after injection untreated control animals appeared ill with a ruffled coat, eyes totally or partially closed, and increased respiratory rate. Animals sacrificed and autopsied at this time had a heavy growth of the two anaerobic species around the liver and particularly below the diaphragm. Blood cultures were positive for the anaerobic species in many of the animals sampled even though small inocula of blood were cultured. Often a number (infrequently to a maximum of one-third) of mice died of septicemia and peritonitis within 1 to 2 days. Animals sacrificed and autopsied after 1 week primarily had intrahepatic and perihepatic abscesses with occasional abscesses located in the thoracic cavity, abdomen, mesentery, or scrotum; these were visible macroscopically. After several weeks the majority of lesions were intrahepatic. These lesions continued to progress in size with time. If lesions were allowed to progress for 8 to 10 weeks, there were normally one to three intrahepatic abscesses present that were approximately spherical and measured about 1 cm each in diameter. The liver and particularly the spleen were enlarged. In the present experiments animals were autopsied and evaluated for lesions at 5 or 6 weeks, and control mice usually had one to three abscesses that measured between 4 and 8 mm in diameter. The injected anaerobic species were present within these abscesses in large numbers and were verified by Gram

stain smears and aerobic and anaerobic culture. Other organisms were detected in very low numbers only on rare occasion.

Exposure to hyperbaric oxygen. The method of exposure to HBO has been described previously (7). Briefly, for exposure mice were enclosed in wire mesh cages over a pan containing soda lime. The hyperbaric chamber was flushed with 100% O_2 before pressurization, and continual flow of O_2 was maintained during the exposure to avoid accumulation of expired CO_2 . The temperature inside the chamber was maintained at 21 ± 1 C. Exposure times reflect only the periods spent at maximum pressure and do not include the brief compression and decompression schedules. As controls for each experiment, two to four mice were inoculated only with sterile mucin and exposed to HBO with the infected animals. No problems with oxygen toxicity were observed with the schedules of exposure utilized.

Data analysis. In each experiment an equal-sized group (10 to 20 mice per group) of nontreated infected animals was inoculated in addition to the oxygen-exposed infected mice as a control of the pathological response. All test and control animals were autopsied together at 5 or 6 weeks after injection of bacteria. At autopsy animals were examined for lesions, and each mouse was given a numerical pathology score dependent on the number and size of abscesses present. The pathology score was obtained by measuring in millimeters the diameter of each abscess and summing the sizes for all abscesses present. The pathology scores for test and control animals were compared by using a two-sample rank test. For this reason, the median score of pathology for each group is presented rather than a mean score (Tables 1 and 2). A score of zero was assigned when no lesions were found. For certain experiments a chi-square analysis was also used to analyze for differences in the numbers of mice positive or negative for abscesses among test and control groups. Animals injected with only mucin (no bacteria) were negative for pathology on autopsy.

RESULTS

Mixed *F. necrophorum*-*F. nucleatum* infection. HBO exposures were evaluated for liver abscesses produced by injection of a mixture of *F. necrophorum* plus *F. nucleatum*. Since this infection was chronic and progressive, a prolonged schedule of HBO exposures was tested using 2 atmospheres absolute pressure (ATA) to allow longer exposure sessions. HBO exposures were initiated either 1 day or 1 week after injection of anaerobic bacteria. Single 3-h exposures to 100% O_2 at 2 ATA were continued daily for 17 days. This schedule of exposures significantly reduced pathology in this mixed anaerobic infection (Table 1). A significant difference was observed by comparing the number of animals in the infected control and oxygen-exposed groups that were

TABLE 1. *Effect of HBO exposures on liver abscess infection with Fusobacterium necrophorum plus Fusobacterium nucleatum in mice*

Period between injection of bacteria and initiation of HBO ^a (days)	Lesions at autopsy ^b				
	Mice positive for lesions in group (%)		Median pathology score of lesions in group		Probability ^c of lesion scores
	Infection control	Oxygen-exposed	Infection control	Oxygen-exposed	
Expt 1 (1)	71	38	8	0	<0.005
Expt 2 (1)	79	11	8	0	<0.01
(7)		32		0	<0.005
Expt 3 (1)	100	46	13	0	<0.0001
(7)		38		0	<0.0001
Expt 4 (7)	100	22	7	0	<0.001

^a HBO, 17 3-h daily exposures to 100% O₂ at 2 ATA.

^b Autopsy of surviving control and oxygen-exposed mice at 5 weeks after injection of bacteria. Each group initially consisted of 10 to 20 mice. The numerical pathology score of lesions for each mouse = summation of diameters (in millimeters) of all abscesses present.

^c Probability value by a two-sample rank test.

TABLE 2. *Effect of HBO exposures on liver abscess infection with Bacteroides fragilis subsp. fragilis plus Fusobacterium necrophorum in mice*

Period between injection of bacteria and initiation of HBO ^a (days)	Lesions at autopsy ^b				
	Mice positive for lesions in group (%)		Median pathology score of lesions in group		Probability ^c of lesion scores
	Infection control	Oxygen-exposed	Infection control	Oxygen-exposed	
Expt 1 (1)	100	73	14.5	10	<0.25
Expt 2 (1)	100	86	17	8	<0.005
Expt 3 (1)	90	69	9.5	7	<0.1
(7)		65		5	<0.01

^a HBO, 17 3-h daily exposures to 100% O₂ at 2 ATA.

^b Autopsy of surviving control and oxygen-exposed mice at 6 weeks after injection of bacteria. Each group initially consisted of 10 to 20 mice. The numerical pathology score of lesions for each mouse = summation of diameters (in millimeters) of all abscesses present.

^c Probability value by a two-sample rank test.

positive for lesions (chi-square analysis). Also, the numerical pathology scores of the test and control groups were significantly different using a two-sample rank test. Thus, there were fewer animals positive for lesions in the oxygen-exposed group, and those positive animals had smaller and fewer abscesses present than in the nontreated control group.

One week after injection of bacteria perihepatic and intrahepatic abscesses were well developed and visible in infected control mice (Fig. 1A), yet initiation of HBO exposures after 1 week was as effective as initiation of therapy after 1 day. Oxygen exposures usually arrested further progression of abscesses and, in most cases, reduced the size of the abscesses or completely resolved the lesion. An example of the differences in livers and spleens excised from infected control and oxygen-exposed mice (ex-

posures begun after 1 week) at autopsy 5 weeks after inoculation of bacteria is presented in Fig. 1B and C. Examples of multiloculated abscesses that are most frequently produced by *F. nucleatum* in combination with *F. necrophorum* are obvious in certain of these livers (Fig. 1B). The enlargement of infected livers and particularly the associated spleens is also demonstrated. Although a portion of animals died from septicemia or peritonitis in each of these experiments, there were no significant differences in the number of deaths among HBO-exposed and control mice on this schedule of exposures.

An initial study to test whether the HBO exposure schedule could be reduced from 17 daily exposures and still be effective was encouraging. Nine daily 3-h exposures to 100% O₂ at 2 ATA begun 1 week after injection

FIG. 1. Excised livers and spleens from mice injected with *F. necrophorum* plus *F. nucleatum*. (A) From untreated infected control mice 1 week after injection; (B) from untreated infected control mice 5 weeks after injection; (C) from HBO-exposed infected mice 5 weeks after injection. Daily 3-h exposures to 100% O_2 at 2 ATA for 17 days, initiated 1 week after injection of mice.

of bacteria also significantly decreased the number and size of intrahepatic abscesses (two-sample rank test, $P < 0.01$).

For this series of experiments, mice had all been autopsied after 5 weeks-18 days or 12 days, respectively, after termination of the 17 HBO exposures begun either 1 day or 1 week after challenge with bacteria. With only nine HBO exposures as just discussed an additional 8-day delay was added between the termination of HBO exposures and measurement of lesions at autopsy. Although this delay after discontinuance of HBO purposefully provided information on more long-term effects of the exposures, it was uncertain whether the lesions observed at 5 weeks were waning or actively enlarging once again. To obtain information on this question, a single large experiment compared groups of HBO-exposed animals autopsied immediately after completion of HBO exposures with other HBO-exposed animals autopsied after a delay of 8 days after the completion of an HBO schedule. Schedules of 9 and 17 HBO exposures were used in this test. Equal numbers of nontreated infected animals were autopsied as controls for pathology for each HBO-exposed group. When examined at autopsy, the pathology scores were somewhat less in the HBO-exposed mice autopsied 8 days after completion of either of the exposure schedules than in those autopsied immediately, indicating that lesions did not resume growth shortly after termination of the HBO exposures.

Mixed *B. fragilis*-*F. necrophorum* infection. The effect of exposures to HBO using the same 17-day schedule at 2 ATA as described above was also tested on a mixed infection initiated with *B. fragilis* plus *F. necrophorum*. This mixed infection was more resistant to HBO than the abscesses produced by mixed *Fusobacterium* species. Significant differences in the pathology scores for infected control and oxygen-exposed mice were demonstrated in some but not all of the tests (Table 2). However, a statistical test (14) utilizing the combined probabilities for the entire series on *B. fragilis* plus *F. necrophorum* infection demonstrated a significant difference in pathology scores for the series overall. In this mixed infection the intrahepatic abscesses were normally fairly large, entire, and spherical; numerous smaller (multiloculated) abscesses were not seen as frequently as with the *F. necrophorum* plus *F. nucleatum* infection.

Additional trials of HBO. An immediate and more contracted schedule of HBO exposures was tested on liver abscesses produced by *F. necrophorum* singly or in combination with *B. fragilis* or *F. nucleatum*. This schedule consisted of five 90-min exposures to 100% O_2 at 3 ATA within 48 h (at 2, 8, 20, 26, and 32 h after inoculation of bacteria). With this HBO schedule the lesions were not significantly different among infected control and HBO-exposed groups autopsied after 5 or 6 weeks; the number of animals positive for lesions and the pathology scores were similar. Thus, exposures at a

higher pressure of oxygen initiated 2 h after injection of bacteria and continued intermittently for 48 h were ineffective in changing the progression of these infections.

DISCUSSION

Therapy currently available for infections involving nonsporeforming anaerobic bacteria is somewhat limited. Surgery, drainage, or excision remains a mainstay for most of the closed space anaerobic or mixed infections which are amenable to surgery. Some multiloculated lesions, of course, are not. Also, many infections continue to smolder even after surgical intervention and appropriate antibiotic therapy. These chronic anaerobic infections are difficult to cure, giving a prolonged clinical course which requires continued therapy for weeks and even months. Until very recently the lack of suitable model infections in animals has prevented testing, under controlled experimental conditions, of the antibiotics in current use or other potential therapeutic agents for anaerobic infection. The liver abscess model used for the present studies mimics the common characteristics of human infection with nonsporeforming anaerobic bacteria, including a chronic and progressive course. Approximately one-half or more of intrahepatic abscesses in humans are associated with anaerobic bacteria (15). Thus, this model of a deep-seated infection was appropriate for *in vivo* testing of HBO as a potential therapeutic agent.

Although certain applications of HBO including the treatment of clostridial myonecrosis have been successful (10), other early and tentative uses have been discontinued. Evaluations of its therapeutic efficacy for other potential applications are currently under investigation. A potential use in a variety of infections caused by anaerobic bacteria is theoretically valid and is supported by the data presented herein. Hyperbaric chambers are available (Vickers, Ltd., England) that are portable and can be located on a ward with a single technician in attendance. These small chambers provide for HBO treatments at quite reasonable cost and have been used successfully, as have the large fixed chambers, in treatment of patients seriously ill with clostridial myonecrosis.

Previous experience with HBO and *Clostridium perfringens* indicated that the increased oxygen tensions attainable through HBO produced direct inhibitory and lethal effects on this organism and probably raised the oxidation-reduction potential in tissues surrounding the infection, which also acted to halt anaerobic growth (6, 7). The alpha-toxin produced by *C.*

perfringens is not inactivated by exposure to HBO (13). The high oxygen tension appears to reduce the production of toxin through a direct inhibitory effect on these anaerobic bacteria (17). Most of the anaerobic bacteria except *B. fragilis* commonly associated with clinical infections are less tolerant to oxygen than is *C. perfringens* and exhibit greater inactivation by HBO. This is known from cultural characteristics and from HBO inactivation studies of five nonsporeforming anaerobic species which have been tested in this laboratory. Since HBO is active against *C. perfringens* infections, it is reasonable to expect a good response in infections involving anaerobes with similar or greater sensitivity to oxygen. Thus, high oxygen tension (or HBO) has the unique advantage as a potential therapeutic agent of being toxic to all species of anaerobic bacteria even though some relative differences in susceptibility may exist among species.

The present experimental data support the potential usefulness of this mode of therapy in the treatment of anaerobic infections. Exposures to HBO reduced the pathology in two models of mixed infection caused by *Bacteroides* and *Fusobacterium* species. Although the therapeutic effect was greater in the mixed fusobacteria infection, HBO exposures were still effective for mixed infection with *B. fragilis*. The decreased response to HBO demonstrated by the infection which involved *B. fragilis* probably relates to the greater tolerance to oxygen of this organism. The therapeutic effect observed may reflect the lesser response of *B. fragilis* to the oxygen exposures and/or a disruption of its synergistic combination with *F. necrophorum*, which is more oxygen sensitive. In a survey of the literature on anaerobic bacteria in liver abscess in humans (15), *F. necrophorum* (older terminology *Sphaerophorus necrophorus*) was the most common isolate; fusobacteria (including *Sphaerophorus*), bacteroides, particularly *B. fragilis*, and species of gram-positive cocci, respectively, were the three groups of anaerobes most frequently isolated.

The contrasting efficacy of the different HBO schedules tested again emphasizes the lesson learned from experience with model clostridial gas gangrene, that the schedule of HBO exposures must be matched to the natural history of the infection. An intensive schedule of 90-min exposures at 3 ATA completed within 48 h after challenge reduced mortality and morbidity among mice (7) with model clostridial gas gangrene, a fulminating infection; yet a similar 48-h schedule (initiated 2 h after challenge) tested against these intrahepatic abscesses in

mice did not prevent or reduce pathology. However, by lengthening each exposure and continuing exposures for a longer interval to match the chronic and progressive nature of the liver abscess infection, pathology was significantly reduced among HBO-exposed mice. This effective HBO schedule (daily 3-h exposures at 2 ATA) was not simply prophylactic but was also therapeutic. A similar good response was noted when this schedule was initiated 1 day after injection of bacteria or after first allowing the liver abscesses to develop for 1 week. The prolonged 17-day schedule was used initially to test for an effect. Data from the mixed fusobacteria infection tested with nine daily HBO exposures indicate that the 17-day schedule can be reduced and still be effective.

In conclusion, the present studies with model intrahepatic abscesses support the efficacy of HBO exposures in treatment of anaerobic infections produced by mixtures of *B. fragilis* and *Fusobacterium* species. The significant reduction in the pathology of closed abscesses within the viscera using HBO alone without antibiotic therapy or surgical intervention is encouraging. A more complete evaluation of HBO for anaerobic infections is suggested. Additional tests on mixtures of exclusively anaerobic species and mixtures with facultative and aerobic bacteria, on optimal exposure schedules, and on additional models of infections such as bacteremia or pleuropulmonary disease would better define the range of potential usefulness of HBO. One potential application would include the addition of HBO to antibiotic therapy in patients with anaerobic infections who are considered to be high surgical risks. Also, its addition to present therapy may have the potential of making certain infections more amenable to surgery and antibiotic therapy, thus reducing the hospitalization and extensive treatment time presently required for many chronic anaerobic infections.

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