Alterations in mouse fecal flora associated with hyperbaric stress

O. P. DAILY and J. D. GILLMORE

Naval Medical Research Institute, Bethesda, MD 20014

Dainty OP, Gillmore JD. Alterations in mouse fecal flora associated with hyperbaric stress. Undersea Biomed Res 1981; 8(1):23–31.—The effect of hyperbaric stress on the composition of fecal microflora was investigated in the mouse. Mice were exposed to 35 ATA with normoxic O₂ concentration in a helium atmosphere at 35°C to prevent body heat loss or to 1 ATA oxygen-helium at 25°C. Freshly discharged fecal pellets were cultured under strict anaerobic conditions, replica plated, and then cultured both aerobically and anaerobically. From experiment to experiment there was considerable variation in the total number of cultured bacteria. By replica plating, however, a consistent reduction in the relative numbers of obligate anaerobes was demonstrated, from a mean of 71% to 44%, in the pressurized mice. Bacteroides distasonis and B. multiaciidus were the obligately anaerobic species most frequently identified from mice exposed at 35 and 1 ATA. Major changes in species distribution were not encountered.

fetal flora in mice
hyperbaric stress
Bacteroides

The fecal flora of the human being and experimental animals constitute an enormous reservoir of bacteria (1–4) that under conditions of injury or stress may become important sources of infection or endotoxemia, or both (5). In an extensive study of the fecal flora of three astronauts, Holdeman et al. (2) were able to associate a change in the bacterial composition of the fecal flora with a simulated space cabin stress situation. Most experiments concerned with the influence of stress, including dietary stress, on fecal flora, however, were done in mice (6–8). Exposure of mice to increased hyperbaric pressures, mimicking those encountered by divers, were shown to alter host susceptibility to various viral and microbial agents (9–12). Recently we have shown that prolonged exposure of mice to 7.5 atmospheres absolute (ATA) of normoxic (160 mmHg) He-O₂ resulted in significant increases in Gram-negative aerobic fecal microflora (12). Gram-positive and -negative bacteria were present in livers of mice exposed to either 20 or 35 ATA of normoxic helium for 48 h. In addition, positive Limulus Amoeocyte Lysate (LAL) gelation tests (presumptive test for endotoxin) were observed in a frequency similar to the frequency of isolations of Gram-negative bacteria from the mouse livers (13). These findings, coupled with current concepts of microflora population dynamics (14–17), have led us to hypothesize that the observed bacteremia and/or endotoxemia associated with hyperbaric stress may be due, in part, to a significant shift in the proportions of
obligate and facultative anaerobic bacteria that comprise the vast majority of intestinal microflora. This shift, if it occurs, may be a key step in the invasion of host tissues by bacteria or in the release of endotoxin, or both.

The purpose of this investigation was to determine the effect of hyperbaric exposure on the populations of obligate and facultative anaerobes in mice. No attempt was made to identify all major bacterial types, but rather to examine the proportions of obligate and facultative anaerobes in the total microbial population and to identify a few of the predominant obligate anaerobes. A consistent decrease in the proportion of obligate anaerobes was demonstrated.

MATERIALS AND METHODS

Animals and diets

Female, Swiss mice, NIH/NMRI-CV, 21–23 g, were housed in Monel Metal cages with bedding (bed-o-cobs, Anderson Cob. Division, Maumec, OH) for exposure in hyperbaric chambers. The mice were fed Agway rat and mouse chow #2000 (Agway Country Foods, Long Island, NY), an antibiotic-free complete diet, and were provided water ad libitum during hyperbaric exposure.

Hyperbaric exposure

Mice were held in 150-liter hyperbaric chambers (Model 183610HP-SP., Bethlehem Corp., Bethlehem, PA) with the pressure adjusted to either 1 or 35 ATA with normoxic Po2 concentrations in a helium atmosphere. Hyperbaric exposures were at 35°C to compensate for body heat loss, and 1-ATA control mice were maintained at 25°C. Such conditions insured that colon temperatures of both groups of mice were the same (18). Chamber atmospheres were continuously vented at the rate of 1.5 liters/min. The technique of hyperbaric exposure was described previously in detail (18). Decompressions from 35 ATA following 72 h of exposure were performed according to the 6.5-h schedule described in an earlier paper (13).

Sampling procedure

The experimental mice, usually two groups of five animals each, for both hyperbaric and control exposures were placed individually in clean 1/4-pint (0.118-liter) perforated ice cream cartons immediately following removal from hyperbaric chambers. The cartons were locked through the vacuum air lock of an anaerobic glove box asphyxiating the mice under an oxygen-free environment. One fresh fecal pellet from each of 5 mice was collected within the anaerobic glove box; the pellets were pooled and used to prepare a primary dilution (approximately 1:10) accomplished by grinding the pellets in a sterile mortar and pestle in a reduced anaerobic salts solution (19). Pools of 5 pellets from mice of the same age and weight have been shown by repeated weighings to average 0.104 g wet wt ± 0.015 Standard Deviation (SD). Killing of the mice and the collection and preparation of serial dilutions of the pooled fecal samples in the anaerobic atmosphere eliminates the disadvantage of weighing and homogenizing in an oxygen atmosphere before instituting strict anaerobic conditions for incubation. Serial 10-fold dilutions were prepared in the reduced anaerobic salts solution, and duplicate 0.1-ml samples were plated on Brain Heart Infusion Agar (BHIA) containing resazurin as a redox indicator (19) and spread within the glove box for enumeration of the viable
FECAL FLORA CHANGES IN HYPERBARIC STRESS

Microflora representative of those microorganisms found in the mouse large colon but not attached to the gut wall.

All initial isolations and replica-plating experiments were performed in an anaerobic glove box (Germfree Laboratories, Inc., Miami, FL). Glove box atmosphere was maintained in an oxygen-free state by use of 85% nitrogen, 10% CO₂, and 5% hydrogen mixture circulated constantly through HEPA filters over palladium catalysts. Resazurin solutions (19), normally pink but changing to a colorless form under anaerobic conditions (−150 to −200 mV) were used to indicate anaerobiosis in the glove box (20). All experimental procedures and plating were performed only when the glove box environment was anaerobic as judged by the color change in these solutions. The glove box, with a vacuum air lock, contained an internal incubator at 35°C. Aerobic incubation (for facultative anaerobes) took place at 35°C in 10% CO₂ in air.

The primary plating medium utilized in all experiments was the Virginia Polytechnic Institute and State University (VPI) formulation (19) for BHIA supplemented with cysteine, vitamin K, heme, and resazurin. All plates were prepared fresh and reduced for 24 h in the anaerobic glove box atmosphere before use. Anaerobic salts solution (19) was freshly prepared and similarly reduced and used for dilution blanks. All plates and dilution blanks were carefully inspected for complete reduction of the resazurin indicator before use, to insure pre-reduced conditions necessary for the cultivation of anaerobes highly sensitive to oxygen.

Replica-plating procedure

To determine the relative percentage of obligate and facultative anaerobes present in a given sample, duplicate initial isolation plates, having a good distribution of countable colonies (approximately 50 to 80 colonies), were replica plated to fresh BHIA plates using the velveteen pad technique (21, 22). These replica plates from each fecal pool were prepared in quadruplicate, with two plates incubated under strict anaerobic conditions and the other two incubated aerobically. After 48 h, a determination of the total microflora was made by counting the colonies on the strict anaerobic plates. These counts represent both obligate and facultative anaerobic microflora. The counts on the aerobically incubated plates represent the facultative anaerobic microflora. We used the following formulae to determine the percentage of the total microflora composed of either obligate or facultative microorganisms:

\[
\% \text{ obligate anaerobes} = \frac{\text{counts on strict anaerobic plates} - \text{counts on aerobic plates}}{\text{counts on strict anaerobic plates}} \times 100, \quad (1)
\]

\[
\% \text{ facultative anaerobes} = 100 - \% \text{ obligate anaerobes}. \quad (2)
\]

By examining the percentage of obligate and facultative anaerobes by replica plating, we eliminated fluctuations from experiment to experiment due to sample size and other variables.

Characterization of anaerobic isolates

Sterile plastic transfer needles were used to pick colonies from each of the representative replicate plates. Depending upon the number of colonies available, 16 to 32 colonies were subcultured from each of the anaerobic hyperbaric and control fecal pool plates and were
streaked in duplicate on freshly reduced BHIA plates. One-half of the subcultures remained within the glove box for anaerobic incubation, and the duplicates were incubated aerobically. Only those isolates exhibiting growth after 48 h of incubation under the oxygen-free environment were selected for identification as possible obligate anaerobes. Following Gram staining for purity and morphological and tinctorial appearance, peptone yeast glucose (PYG) and peptone yeast (PY) broth were inoculated for analysis of metabolic end products and determination of biochemical activities.

Metabolic end products were determined after 48 h of anaerobic incubation in PYG broth by the methods described by Holdeman et al. (19). Both fractions were analyzed with a Dohrman gas-liquid chromatograph (GLC) using CAPCO columns (Clinical Analysis Products Co., Sunnydale, CA) packed with “15% CE 2225 on Chromasorb W/AW, 45/60 mesh.” Helium was used as a carrier gas with a flow rate of 120 ml/min for volatile fatty acids and 60 ml/min for the nonvolatile methylated fatty acids. Columnar and thermal conductivity temperature was maintained at 115°C to 118°C, whereas injection port temperature was maintained at 145°C.

Biochemical activities were determined by use of the Minitek (BBL, Division of Becton Dickinson, Cockeysville, MD) anaerobic differentiation system (23). The Minitek plates with differential disks were inoculated within the glove box with 0.05 ml of a 48-h pure PY broth culture of the anaerobic strain, and incubated anaerobically for 48 h before determination of biochemical activities. Determination of indole production from tryptophan was determined on a 0.15-ml inoculum. While utilization of standard GLC and Minitek procedures does not permit identification of all species within the various anaerobic genera, a combination of both analyses, with reference to the VPI manual, does provide a reproducible tentative identification within each genus that fills the need of this investigation.

RESULTS

Enumeration of mouse fecal flora by replica plating

By utilization of replica-plating technique, identical copies of the master plate were subjected to both anaerobic and aerobic-CO₂ regimes. Since obligate aerobic organisms constitute a very small percentage of the normal large colon microflora (24), we could reasonably assume that strict anaerobic incubation resulted in a very good approximation of the total members of viable microorganisms present in our mouse fecal samples.

Table 1 lists the obligate and facultative anaerobic bacterial counts from pooled fecal samples from mice exposed to either 1- or 35-ATA normoxic helium for 72 h. There is reasonable agreement with previous studies (4, 7, 14, 25), both in total counts normally reported in the 10⁹/g to 10¹⁰/g range, and in variation (as much as fivefold among mice in the same experiment). In our study, variation among individual mice was not measured, since the fecal pellets of 5 mice were pooled, but variation between two identically treated groups in the same experiments was less than threefold. For unknown reasons, however, counts in Experiment 2 were approximately five times greater than those in Experiments 1 and 3. In only one case (Experiment 2, Group 2) the counts in a 35-ATA group were substantially higher than those at its 1-ATA counterpart; otherwise the data presented in Table 1 provide no evidence that the total number of bacteria was greater in the pressurized mice.

In contrast to the variation encountered between experiments, the examination of replica plates divided from the same “master plate” yielded highly reproducible results. In every
FECAL FLORA CHANGES IN HYPERBARIC STRESS

TABLE 1
ENUMERATION OF OBLIGATE AND FACULTATIVE ANAEROBIC BACTERIA IN POOLED FECAL SAMPLES FROM 5 MICE FOLLOWING EXPOSURES TO EITHER 1 ATA OR 35 ATA NORMOXIC HELIUM FOR 72 H

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Pressure (ATA)</th>
<th>Group (5 mice)</th>
<th>Total Microflora</th>
<th>Facultative Anaerobic Microflora*</th>
<th>Obligate Anaerobic Microflora**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>1</td>
<td>10.2†</td>
<td>4.5† (44)</td>
<td>5.7† (56)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2</td>
<td>5.1</td>
<td>2.9 (56)</td>
<td>2.2 (44)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>5.7</td>
<td>2.1 (36)</td>
<td>3.6 (64)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>7.9</td>
<td>2.0 (25)</td>
<td>5.9 (75)</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>1</td>
<td>29.5</td>
<td>12.5 (42)</td>
<td>17.0 (58)</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>2</td>
<td>82.5</td>
<td>46.0 (56)</td>
<td>36.5 (44)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>25.0</td>
<td>4.5 (18)</td>
<td>20.5 (82)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>21.0</td>
<td>1.3 (6)</td>
<td>19.7 (94)</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>1</td>
<td>8.5</td>
<td>7.2 (84)</td>
<td>1.3 (16)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>7.8</td>
<td>4.7 (60)</td>
<td>3.1 (40)</td>
</tr>
</tbody>
</table>

*Percentages in parentheses.  **Obligate anaerobes numbers are calculated by subtracting facultative counts from the total microflora.  †Value = x 10^5 for 1.0 g of fecal matter.

In experiment, the percentage of obligate anaerobes was observed to decrease in response to the hyperbaric exposure (Table 1). The obligate anaerobic population in the experimental group declined from a mean of 71% at 1 ATA to a mean of 44% at 35 ATA. This difference, due to hyperbaric exposure, was found to be highly significant when tested with a two-way analysis of variance (P<0.01).

In addition to the replica-plating experiments, random colonies were selected from the initial isolation plates and screened for their ability to grow as either obligate or facultative anaerobes. Table 2 lists the distribution of obligate and facultative anaerobes obtained from randomly picked colonies. This experiment as well as the previous one shows an increase in the facultative anaerobic population with respect to the obligate anaerobic population in mice.

TABLE 2
DISTRIBUTION OF OBLIGATE ANAEROBES AMONG RANDOMLY PICKED COLONIES FROM THE INITIAL ISOLATION PLATES

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Colonies Picked (1 ATA + 35 ATA)</th>
<th>Incidence of Obligate Anaerobes (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 ATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35 ATA</td>
</tr>
<tr>
<td>1</td>
<td>16 + 16</td>
<td>10/16 (62.5)</td>
</tr>
<tr>
<td>2</td>
<td>16 + 16</td>
<td>13/16 (81.3)</td>
</tr>
<tr>
<td>3</td>
<td>32 + 32</td>
<td>14/32 (43.8)</td>
</tr>
<tr>
<td>Average for 3 experiments</td>
<td>37/64 (63)</td>
<td>23/64 (36)</td>
</tr>
</tbody>
</table>

*Percentages in parentheses.
subjected to hyperbaric exposure. The average of obligate anaerobes for the three experiments, i.e., 63% for 1 ATA and 36% for 35 ATA, agree quite well with those from the replica-plate experiments (probability of random distribution by chi-square analysis = 0.03).

**Identification of isolated anaerobes**

Colonies of obligate anaerobes selected at random from the replica plates were subcultured and tentatively identified on the basis of Gram stain reaction, biochemical differentiation, and gas-liquid chromatographic analysis of metabolic end products (fatty acids). Table 3 lists the predominant obligate anaerobes present in the 1-ATA and 35-ATA mice. These results are in agreement with mouse obligate anaerobic microflora listed in previous studies (3, 4, 26). In addition to *Bacteroides distasonis*, *B. multiacidus*, *B. vulgatus*, and *Fusobacterium mortiferum* we identified several isolates as bacteria of the genus *Bacteroides* but were unable to determine species under the limited identification protocols we used. Pronounced changes in species distribution were not encountered, with the possible exception of *B. multiacidus*, which was isolated less frequently in mice held at 35 ATA. No attempts were made to identify the facultative anaerobes, as the predominant types had been described previously (12).

**DISCUSSION**

Our results indicate that exposure of NIH/NMRI-CV mice to 35 ATA of normoxic helium (i.e., hyperbaric stress) results in a significant decrease in the proportion of obligate anaerobes in the total fecal microflora freshly discharged from the colon. This appeared to be in part due to an increase in the total number of facultative anaerobes, although not necessarily in the total number of viable bacteria. These results extend our earlier laboratory studies, which noted that Gram-negative facultative anaerobes, primarily *E. coli*, *Klebsiella*, and *Enterobacter* species, increased in absolute number in the mouse fecal flora as a result of long periods of hyperbaric exposure (12). In our earlier work no attempt was made, however, to compare

**TABLE 3**

**Tentative Identification of Randomly Picked Colonies from Initial Isolation Plates**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total Isolates Identified</th>
<th>Bacterial Identification</th>
<th>Number of Isolates from Mice at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 ATA</td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td><em>B. distasonis</em></td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td><em>B. distasonis</em></td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td><em>B. multiacidus</em></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. species</em></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. multiacidus</em></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. species</em></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. distasonis</em></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. multiacidus</em></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. vulgatus</em></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. species</em></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>F. mortiferum</em></td>
<td>2</td>
</tr>
</tbody>
</table>

*B = Bacteroides; F = Fusobacterium.
FECAL FLORA CHANGES IN HYPERBARIC STRESS

relative proportions of facultative and strict anaerobes in the stressed animals, since the
techniques for the isolation and enumeration of the obligate anaerobes were not as satisfactory
as those used in the present study.

The disruption of the normal intermicrobial antagonisms, resulting in the relative propor-
tions of various bacterial species that are reported here, may contribute to the previously
reported bacterial invasion of host tissues caused by hyperbaric stress (13). A stress-induced
shift, from the predominant obligate anaerobic microflora normally present to a more faculta-
tive anaerobic microflora, may permit colonization and penetration of the intestinal mucosal
barrier by facultative anaerobes. This sublethal invasion of the host tissue by facultative
anaerobic microorganisms could compromise host resistance to subsequent infections. In-
deed, we have previously observed increased host susceptibility to endotoxin in animals
challenged immediately following hyperbaric exposure (13).

Several types of stress, through mechanisms that are as yet obscure, are known to induce
alterations in the normal intermicrobial antagonisms that occur in the host’s microflora (2–7).
Microbial population levels in the intestine are maintained by a combination of host and
microbial factors that include the concentration of volatile fatty acids, sugars, and other
nutrients, peristalsis, pH, Eh, and local immunity (15–17). Normally, the obligate anaerobes
form a more stable intestinal population, whereas the less numerous facultative organisms,
such as Gram-negative coliforms, vary more dramatically in response to stress (5). The
mechanism is not known by which hyperbaric stress provides a selective advantage to one group of
organisms at the expense of another. Our experiments were not sufficiently detailed to show
an effect on a particular species, as was the case in the experiments of Holdeman et al. (2) on
human fecal flora. One possibility is that during hyperbaric stress the facultative anaerobes
transport sugars and other nutrients more efficiently than the obligate anaerobes. This
hypothesis is consistent with our previous observation that E. coli transports β-galactosides
more efficiently at hyperbaric than at normobaric pressures (27, 28).

In conclusion, hyperbaric stress induces significant shifts in the proportions of obligate and
facultative anaerobes in the fecal flora freshly excreted from the colon of the mouse. The
facultative population appears to increase at the expense of the obligate anaerobic population.

This study was supported by the Naval Medical Research and Development Command, Navy Department, Research
Task No. MF51.524.014.6021 and M0099NNo02.5061. The opinions and statements contained herein are the private ones
of the authors and are not to be construed as official or reflecting the views of the Navy Department or the naval service
at large. The animals used in this study were handled in accordance with the provisions of Public Law 89-44 as amended
by Public Law 91-579, the "Animal Welfare Act of 1970," and the principles outlined in the "Guide for Care and Use of
Laboratory Animals." U.S. Department of Health, Education and Welfare Publication No. (NIH) 73-23. The authors
wish to acknowledge the discussion of protocols and the invaluable editorial assistance of Dr. E. Weiss, the assistance of
Dr. R. C. Bailey in the statistical calculation of the two-way analysis of variance, and the excellent technical support of
Mr. James E. Perry. —Manuscript received for publication August 1980; revision received November 1980.

Daily OP, Gillmore JD. Altérations dans la flore fécale de la souris associées à la tension hyperbare.
microflore fécale ont été observés chez la souris. Les souris ont été exposées à 35 ATa avec une
concentration normoxique de PoO dans une atmosphère de hélium à 35°C pour empêcher la perte de
chaleur corporelle ou à une atmosphère d’oxygène-hélium de 1 ATa à 25°C. Une culture de matière
fécale fraîche a été faite sous des conditions strictes anaérobiques; ces cultures ont été reproduites
et une nouvelle culture aérobie et anaérobie a été faite. On a noté une variation considérable
dans le nombre total des bactéries soumis à la culture, d’expérience en expérience. Néanmoins,
la reproduction des cultures a provoqué une réduction consistente dans le nombre relatif des
anaérobies fixes, d’une quantité de 71 % à 44%, dans les souris soumis à la pression. Les Bactéroïdes
distosus at les Bactérioides multiformes ont été les espèces anaérobies fixées les plus fréquemment identifiées dans les souris exposées à 35 à 1 ATA. Des changements majeurs dans la distribution des espèces n’ont pas été notés.

flore fécale de la souris
tension hyperbare
Bactérioides

REFERENCES

FECAL FLORA CHANGES IN HYPERBARIC STRESS
