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Influence of Carbon Dioxide on Growth and Antibiotic Susceptibility of Coagulase-Negative Staphylococci Cultured in Human Peritoneal Dialysate

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Used peritoneal dialysis fluid was collected from patients undergoing continuous ambulatory peritoneal dialysis, and its pH and composition were assessed after incubation in either air or air with 5% CO_2. Precipitation of calcium, magnesium, phosphate, and proteins occurred in the dialysis fluid incubated in air at 37°C and was associated with a mean pH increase of 1.23 U. Incubation of dialysis fluid in air with 5% CO_2 prevented precipitation and maintained pCO_2 and pH levels at those found physiologically. Coagulase-negative staphylococcal strains isolated from patients with peritonitis tended to grow less well in dialysis fluid incubated in air than in dialysis fluid incubated in the carbon dioxide-enriched atmosphere. MICs of cefuroxime, ciprofloxacin, and vancomycin for seven strains of coagulase-negative staphylococci in dialysis fluid were markedly affected by atmosphere type (16 of 21 MICs). Of these 16 atmosphere-dependent MICs, 14 were at least fourfold higher in air than in air with 5% CO_2.

The composition of laboratory media can profoundly affect the characteristics and behavior of microorganisms in vitro, although its significance in vivo is often overlooked. However, pathogens must adapt to the hostile conditions and nutrient limitations in vivo if they are to establish an infection (2, 3, 7, 18). Culturing microorganisms in a body fluid represents a step closer to these conditions. The peritoneal cavities of patients undergoing continuous ambulatory peritoneal dialysis (CAPD) represent a unique growth environment for potential pathogens, and therefore in vitro studies of peritonitis-causing isolates have employed used peritoneal dialysis fluid (PUD) as a culture medium (8–14, 16). In addition to differences in growth kinetics, we have shown unique phenotypic changes in staphylococcal cell surface characteristics associated with growth in PUD (17, 19; S. P. Denyer, M. C. Davies, J. A. Evans, R. G. Finch, D. G. E. Smith, M. H. Wilcox, and P. Williams, *in* T. Wadstrom, I. Eliassor, I. Holder, and A. Ljungh, ed., *Pathogenesis of Wound and Biomaterial-Associated Infections*, in press). We have also shown the marked effect of a physiological concentration of carbon dioxide on coagulase-negative staphylococci grown in nutrient broth (5). This study compares the effects of two atmospheres, namely air and air with 5% carbon dioxide (5% CO_2), on both the composition of PUD and the behavior of peritonitis-causing coagulase-negative staphylococci cultured in vitro. We have examined the activities of cefuroxime, vancomycin, and ciprofloxacin in PUD, since these antibiotics may be considered useful in the treatment of CAPD patients with peritonitis.

MATERIALS AND METHODS

Bacterial isolates. A total of 13 coagulase-negative staphylococcal strains, previously isolated from the dialysis fluid of CAPD patients with peritonitis, were used. The chosen strains consisted of a core group of 10 strains which we have studied in further detail and intend to report separately. In addition, three other strains were selected, since preliminary studies had indicated that they have high growth yields in PUD. Isolates were further identified by using the API Staph-Ident system (Analytab Products, Plainview, N.Y.) as eight strains of *Staphylococcus epidermidis* (strains 8, 31, 34, 49, 59, 112, 900, and 902), two strains of *Staphylococcus warneri* (strains 75 and 79), two strains of *Staphylococcus simulans* (strains 62 and 115), and one strain of *Staphylococcus saprophyticus* (strain 907). The isolates were stored in horse serum at −70°C until required and then maintained by subculturing onto blood agar.

PUD. Approximately 2-liter volumes of PUD were collected from 10 CAPD patients and, within 10 min, samples (10 ml) were transferred aseptically to glass universal containers with loose-fitting tops. These were incubated for 24 h at 37°C in (i) air, (ii) air with 5% CO_2, and (iii) air at room temperature (18 to 20°C). The samples were then analyzed for pH and pCO_2 by using an automated blood gas analyzer (model IL1312; Allied Instrumentation Laboratory, Milan, Italy). For comparison, five PUD samples from the patients were analyzed for pH and gas content immediately after collection.

A working pool of approximately 55 liters of PUD (pooled PUD) was collected from 25 CAPD patients. This was stored in samples at −20°C until use and then thawed in air with 5% CO_2 at 37°C. A minor amount of precipitation did occur following each freeze-thaw cycle, and for this reason such manipulation of PUD was limited to two occasions. The chemical composition of pooled PUD was determined, and the pH and gas content were measured after incubation as described above. Sodium and potassium were measured by flame photometry (model 343; I. L. UK Ltd., Cheshire, England); calcium, phosphate, urea, and creatinine were measured by a discrete chemistry analyzer (Olympus 5000; British Drug Houses, Dorset, England); and magnesium, chloride, and bicarbonate also were measured by a discrete

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chemistry analyzer (Roche Mira; Roche, Herts, England). Protein content was assessed by using a sulfosalicylic acid precipitation assay. The in-house variability of these assays is less than 2%.

All collected PUD was confirmed to be sterile by aerobic and anaerobic incubation of 10-ml samples in blood culture bottles for 72 h and then subcultured onto blood agar. Any samples demonstrating antibacterial activity (zone of inhibition around wells in a blood agar plate seeded with the Oxford *Staphylococcus aureus* NCTC 6571) were excluded; PUD from 3 of 28 patients demonstrated such activity.

PAGE of proteins. The protein profiles of the following pooled PUD samples were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (19): freshly thawed pooled PUD; pooled PUD after 24 h of incubation at 37°C in air with 5% CO_2; supernatant and precipitate (after centrifugation at 3,000 × *g* for 10 min) from pooled PUD after incubation for 24 h at 37°C in air; and supernatant and protein precipitate of pooled PUD following the addition of 10% (vol/vol) trichloroacetic acid and a double wash with acetone.

The variously treated pooled PUD samples were electrophoresed on SDS–12.5% PAGE gels (approximately 10 μg of protein per lane) and then fixed and stained in acetic acid-methanol with 1% Coomassie blue (Sigma Chemical Co. Ltd., Dorset, England).

Bacterial growth. The growths of the 13 coagulase-negative staphylococcal isolates were compared in 5-ml samples of pooled PUD incubated at 37°C for 18, 24, and 48 h in air and in air with 5% CO_2. An initial inoculum of approximately 10^6 CFU/ml taken from a culture in pooled PUD was used. Glass bijoux with loose-fitting tops were used for culture, and samples were vortex-mixed vigorously for 15 s prior to viable counting. Counting was performed in triplicate by a modified method of Miles and Misra on blood agar, and the mean counts were calculated. The pH of each culture was measured after 24 h of incubation by using a pH meter (Kent EIL3055; Analytical Supplies Ltd., Surrey, England).

MICs. Seven strains of coagulase-negative staphylococci were selected to compare the MICs of three antimicrobial agents (cefuroxime from Glaxo, Middlesex, England; ciprofloxacin from Bayer, Berkshire, England; and vancomycin from Eli Lilly, Hampshire, England) in pooled PUD incubated at 37°C for 24 h under both atmospheric conditions. A microtiter tray system with an initial inoculum of 10^6 CFU/ml (see above) was used and challenged with doubling dilutions of antibiotics (final well volume, 100 μl). MICs were determined in triplicate and were read both visually and by viable counting, as described above. MICs determined by viable counting were defined as the lowest concentration of antibiotic that prevented an increase in the cell number of the initial inoculum.

RESULTS

The pH and gas composition of both individual and pooled PUDs are shown in Table 1. PUDs from individual patients showed results very similar both to each other and to pooled PUD. Incubating PUD in an atmosphere containing 5% CO_2 maintained both the carbon dioxide and pH levels close to those found in dialysate analyzed immediately after collection from patients. However, if PUD was either left at room temperature in air or incubated at 37°C in air for 24 h, the carbon dioxide content fell with a corresponding rise in pH (mean increase in pH, 1.23 in air at 37°C) and the formation of a visible precipitate. By 2 and 8 h, 25 and 70%, respectively, of the total pH change (at 24 h) had occurred; 50% of the total precipitation occurred by 8 h. Changes in pH and precipitate yield in air were greater in PUD incubated at 37°C than in PUD left at room temperature. Measurements of atmosphere-induced pH and pCO_2 changes in glass vessels and microtiter tray wells were identical.

TABLE 1. pH and gas composition of patient PUDs (and pooled PUD) following different incubation conditions

PUD[a]	pH[b]	pCO_2 (kPa)[b]
Immediately after collection (5)	7.27 ± 0.03	5.24 ± 0.06
After incubation at 37°C in air with 5% CO_2 (10)	7.37 ± 0.03 (7.36 ± 0.03)	4.76 ± 0.16 (4.90 ± 0.38)
After incubation at 37°C in air (10)	8.60 ± 0.03 (8.61 ± 0.08)	0.59 ± 0.02 (0.68 ± 0.01)
After incubation at room temp in air (10)	8.10 ± 0.05 (8.07 ± 0.01)	1.01 ± 0.06 (1.18 ± 0.14)

[a] Number in parentheses refers to number of individual patient PUDs tested.

[b] Mean ± standard error of the mean. Figures in parentheses are means ± standard error of the mean for pooled PUD (number of sampling repeats = 4). Samples were incubated for 24 h.

The biochemical profiles of pooled PUD supernatant left in each of the three atmospheric conditions are shown in Table 2. Marked reductions in the concentrations of dissolved calcium (24%), magnesium (30%), phosphate (19%), and total protein (12%) were observed in pooled PUD incubated at 37°C in air, compared with pooled PUD incubated in air with 5% CO_2. Comparatively small changes were seen in pooled PUD left in air at room temperature.

The results of SDS-PAGE of pooled PUD proteins are shown in Fig. 1. The protein profiles of pooled PUD when freshly thawed and when incubated in air with 5% CO_2 were almost identical. The precipitate formed in air (Fig. 1, lane 4) contained most of the proteins present in pooled PUD, but there appeared to be some selective precipitation (arrow).

The majority of coagulase-negative staphylococcal strains (9 of 13) grew significantly better (the growth was more than the mean colony count plus 2 standard deviations) in air with 5% CO_2, with viable counts at least 10-fold in excess of those achieved in air alone (Fig. 2). Of the remaining four strains, growth in air did not achieve a better-than-fivefold increase in yield over that in air with 5% CO_2. Irrespective of the strain used, the pH of pooled PUD following 24 h of growth was 1 or more pH units higher in the 100% air atmosphere

TABLE 2. Chemical composition of pooled PUD incubated under three different conditions for 24 h

Substance	Composition[a] in air:		
	With 5% CO_2 (37°C)	At 37°C	At room temp
Na	133	133	133
K	4.3	4.3	4.3
Ca	1.55	1.18	1.50
Mg	1.0	0.7	1.1
Cl	108	108	107
HCO_3	16	16	19
PO_4	1.39	1.13	1.39
Urea	24.5	24.5	22.3
Creatinine	864	869	873
Protein	840	740	800
Glucose	25.7	26.5	25.9

[a] Units are in millimoles per liter, except for creatinine (micromoles per liter) and protein (milligrams per liter).

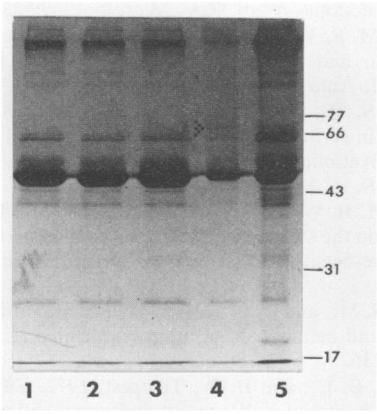

FIG. 1. SDS-PAGE of pooled PUD proteins. Pooled PUD immediately after thawing (lane 1) and after incubation at 37°C for 24 h in air with 5% CO_2 (lane 2), supernatant after incubation at 37°C for 24 h in air (lane 3), precipitate after incubation at 37°C for 24 h in air (lane 4), and precipitate after the addition of trichloroacetic acid (lane 5). Arrow indicates selective protein precipitation (compared with lanes 1 to 3). Numbers at right indicate molecular mass (kilodaltons).

than in air containing 5% CO_2 (mean pH in air, 8.46; mean pH in air with 5% CO_2, 7.18). Growth yield in PUD in which a precipitate had been allowed to form (by leaving PUD to stand at room temperature for 24 h in air) was identical to that in controls, providing that culture was performed in air with 5% CO_2.

MICs of each antibiotic in pooled PUD varied considerably between the two atmospheric conditions (Table 3). A total of 76% of MICs differed by a factor of 4 or more. No

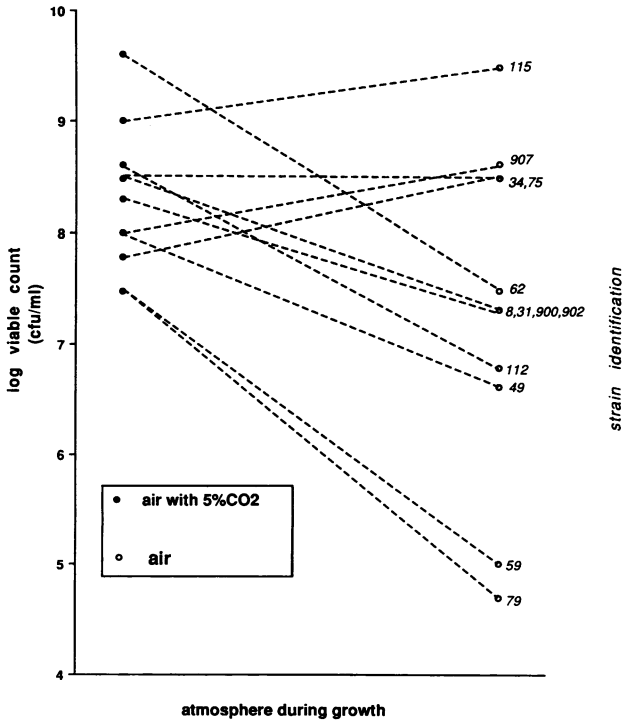

FIG. 2. Growth of 13 coagulase-negative staphylococcal strains in pooled PUD at 24 h after incubation in air or in air with 5% CO_2.

TABLE 3. Effect of atmosphere on MICs of cefuroxime, ciprofloxacin, and vancomycin for coagulase-negative staphylococci

Strain	MIC in air with 5% CO_2 (MIC in air) (mg/liter)		
	Cefuroxime	Ciprofloxacin	Vancomycin
8	4 (32)	0.5 (8)	2 (8)
49	0.5 (8)	0.25 (1)	4 (16)
59	0.25 (0.25)	0.125 (1)	2 (8)
62	0.5 (0.5)	0.125 (4)	1 (4)
75	>64 (16)	8 (1)	1 (8)
79	1 (0.5)	0.5 (1)	1 (4)
112	16 (16)	0.25 (4)	4 (16)

obvious atmosphere-dependent pattern of altered antibiotic susceptibility was noted, except in the case of vancomycin, in which case growth in air produced higher MICs for all four strains. Of the 16 MICs which differed by a factor of 4 or more, 14 were higher following growth in air.

DISCUSSION

In the in vitro study of microbial pathogenicity, increasing efforts are being made to simulate in vivo conditions more closely, particularly by using pooled body fluids. However, little attention has been given to the significance of gas tension for the composition of body fluids and its subsequent effect on microbial growth and pathogenic behavior (1, 6). We have recently shown that altering the carbon dioxide tension, to approximate that found physiologically, produces marked changes in bacterial cell surface structure, physicochemistry, and adherence (5). In the presence of CAPD with peritonitis caused by coagulase-negative staphylococci, we have also explored the phenotypic changes in bacteria induced by growth in PUD (Denyer et al., in press).

It is well known that isolates from CAPD patients with peritonitis often grow poorly or not at all in the fresh acidic dialysis fluid that is instilled into the peritoneal cavity (11, 14). Furthermore, the composition of this fluid changes rapidly during dialysis, and so many workers have instead used PUD as a culture medium (8–14, 16). The pH of PUD quoted in these references ranged from 7.0 to 8.6. In addition, some workers have centrifuged, filtered, or acidified the fluid prior to use (9–12, 14). In no case was it reported that increased levels of carbon dioxide were used during culture.

The work presented in this study shows the marked changes that occur in PUD composition and the behavior of coagulase-negative staphylococci when no attempt is made to buffer the culture system with CO_2. When sterile PUD, either from individual patients or pooled and then freeze-thawed (in air with 5% CO_2), was incubated at 37°C or room temperature in air, a marked precipitate formed. A corresponding pH increase of more than 1 U was also observed. This precipitate contained significant quantities of the PUD-derived calcium, magnesium, phosphate, and protein. SDS-PAGE of PUD proteins seemed to indicate that some degree of selective protein precipitation was also occurring. By using an atmosphere of air with 5% CO_2 combined with PUD bicarbonate as a physiological buffer, the pH increase and precipitate formation were prevented.

We have attempted to demonstrate the significance of these changes for in vitro studies with PUD by using simple microbiological assays. The pH increases seen in cultures of coagulase-negative staphylococci in PUD incubated in air were often accompanied by a pronounced reduction in

bacterial growth (9 of 13 strains) of greater than or equal to 1 log unit. Determination of the MICs of three antibiotics for seven strains of coagulase-negative staphylococci showed that three-quarters of these varied by fourfold or more, according to atmosphere type. Culture of strains in air most often produced a higher MIC than that in air with 5% CO_2. Other workers have shown that MICs in PUD are often significantly higher than those in routine susceptibility testing media (8, 12, 14, 16). Our findings indicate that these results may be exaggerated if culture were performed in air.

The relatively large variations in MIC observed are likely to be due to a combination of factors such as altered bacterial growth yield and hydrogen ion availability and consequent changes in cation concentrations and protein binding. Cation concentrations and pH level are known to affect aminoglycoside activity (4, 15). Therefore, we would expect that in PUD cultured in various carbon dioxide tensions, significant differences in behavior would also be seen with these antimicrobial agents. It should be recognized that visual determination of the MIC endpoint cannot be achieved with certainty by using PUD in air because of the precipitation simulating growth which occurs. Likewise, optical density measurements of growth in PUD in air are likely to overestimate bacterial yield.

Significantly different conditions have been employed in the study of microbial growth and antibiotic activity in PUD (8–14, 16). It is clear that a comparison of studies using such different approaches cannot be easily made. Therefore, we argue strongly for standardizing the methods by which this fluid is handled. In particular, we recommend that studies with PUD employ an atmosphere of air with 5% CO_2 to maintain the pH, carbon dioxide, and biochemical levels found in vivo. PUD stored at −20°C should be thawed out in air with 5% CO_2 to prevent precipitation from occurring, and procedures such as filtration, centrifugation, or the addition of acids should be avoided.

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