Rapid diagnosis of anaerobic infections by gas-liquid chromatography

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SUMMARY It was postulated that the short chain fatty acids (SCFA) produced by anaerobic bacteria might serve as microbial markers in purulent material. Eighteen pus specimens from various sources were analysed by gas-liquid chromatography (GLC), and the SCFA detected were compared with the microorganisms isolated by conventional methods. It was found that the detection of propionic, isobutyric, butyric, or isovaleric acids by direct GLC of pus specimens is strong evidence for anaerobic infection but not specific for Bacteroides fragilis. It was also shown that the presence of succinic acid in pus specimens does not necessarily indicate infection by anaerobes. It can be concluded that direct GLC of purulent material provides a rapid and reliable presumptive method for the differentiation between anaerobic and aerobic infections.

Since certain anaerobic infections may be fulminating, the need for early diagnosis is mandatory. In these cases, effective treatment depends on the recognition of the aetiological agent in the bacteriology laboratory. This is time-consuming, even in the best bacteriological hands, because clinical specimens treated by conventional methods require about one week for complete identification of the isolated strains (Gorbach and Bartlett, 1974).

The production of short-chain fatty acids (SCFA) is a known metabolic activity of anaerobic bacteria, and it is used in vitro for taxonomic purposes (Moore, 1970; Holdeman et al., 1977). Such end products can be detected in clinical specimens by gas-liquid chromatography (GLC) and can be used as a presumptive test for the diagnosis of anaerobic infections (Gorbach et al., 1974; Gorbach et al., 1976; Phillips et al., 1976; Nord, 1977). In addition, Gorbach et al. (1974, 1976) and Nord (1977) support the view that the detection of significant amounts of either isobutyric, butyric, or succinic acids by GLC in purulent material is strong evidence of anaerobic infection, notably by Bacteroides fragilis. On the other hand, Phillips et al. (1976) believe that specific identification of anaerobes present in purulent material is not possible by GLC.

In view of this controversy, we decided to analyse by GLC pus specimens from various sources. The detected SCFA from each specimen were compared with the microorganisms isolated by conventional bacteriological methods.

Material and methods

SPECIMENS

Eighteen samples of pus from patients suffering from a variety of superficial and deep infections were included in the study: namely, peritonitis (6), subphrenic abscess (1), buttock gangrene (1), perirectal abscess (1), pilonidal sinus abscess (1), compound comminuted fractures of tibia with abscess formation (1), brain abscess (1), buttock abscess caused by intramuscular injections (3), and abdominal fistula (3).

Each specimen was taken with an air-sealed syringe and subsequently divided into four samples. The first one (a few drops) was used for Gram staining. The second (0·5 ml) was inoculated into a 45-ml culture bottle containing Peptone Broth II (Becton-Dickinson, Rutherford, New Jersey 07070, USA), which was immediately placed in an incubator set at 37°C. The third sample (0·2 ml) was transported to the bacteriology laboratory in a Port-A-Cul vial (BBL, Cockeysville, Maryland 21030, USA) within a few hours of collection and was used for anaerobic and aerobic cultures. The remaining pus (3 ml) was...

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put into an ice-cold tube and was used for direct GLC.

BACTERIOLOGICAL STUDIES
Each pus specimen was cultured on the following media: (1) Three freshly prepared Columbia human blood agar plates for incubation in aerobic, aerobic with 10% carbon dioxide, and anaerobic with 10% carbon dioxide atmosphere (Gas Pak anaerobic system, BBL); (2) Columbia human blood agar plate containing 0.1 mg/ml neomycin sulphate for anaerobic incubation (Willis, 1977); (3) McConkey’s agar as enteric differential medium (Finegold et al., 1977); (4) Chapman’s agar; and (5) Sabouraud’s agar. All plates were incubated at 37°C and were examined at 24 and 48 hours. The incubation of primary anaerobic plates was continued for 96 hours.

Anaerobic bacteria were identified by the use of the API-20 anaerobe system (La Balme Les Grottes, 38390 Moltalieu Vercieu, France) and by SCFA analysis by GLC according to Holdeman et al. (1977).

Strains belonging to Enterobacteriaceae were identified biochemically by the API-20 Enterobacteriaceae system (La Balme Les Grottes, 38390 Moltalieu Vercieu, France).

The Phadebact Streptococcus Test (Pharmacia Diagnostics, Uppsala, Sweden) and the Lancefield precipitation method (Lancefield, 1933) were used for the grouping of beta-haemolytic streptococci.

GAS-LIQUID CHROMATOGRAPHY
Hewlett Packard model 5750 G research chromatograph equipped with a thermal conductivity detector was used for the SCFA analysis. The operating conditions were: injection port temperature 140°C, column temperature 120°C, detector block temperature 120°C, bridge current 150 milliamps, attenuation 1, and helium carrier gas of chromatographic grade purity at a flow rate of 120 ml/min. The stainless steel chromatographic columns (6 feet long × ¼ inch diameter) contained 5% Carbowax 20 M on Chromosorb G-AWDMCS, 60-80 mesh (Hewlett Packard). Chromatographic deflections were visualised on a Hewlett Packard strip chart recorder, model 7127A, with an operating speed of 0.25 in/min.

Titrated aqueous solutions of SCFA were diluted in human sterile ascitic fluid, to prepare the control solution, which contained 0.05 mEq/ml of each of the following acids: acetic, propionic, isobutyric, butyric, isovaleric, valeric, caproic, lactic, and succinic. Samples from this solution were extracted and chromatographed to obtain the standard values. The minimum detectable quantities of each one of the above-mentioned acids were: 4.2, 4.2, 0.7, 3.5, 0.3, 0.9, 1.9, 1.2, and 0.8 μmol/ml respectively.

Pus specimens were prepared for GLC as described by Nord (1977). In cases where the chromatogram showed the presence of acetic acid only, the ether extract was concentrated by gentle evaporation in a water bath set at 40°C. The residual volume of the ether extract was usually 0.2-0.1 ml and was subsequently rechromatographed, but after that manoeuvre we were not able to perform quantitative measurements, since, by warming the ether extract, some of the diluted SCFA was lost. In this way the sensitivity of the method was increased up to 20-fold (Fig. 1: Compare chromatograms (a) and (c)). If there were still no detectable SCFA the incubated Peptone Broth II culture was used for successive chromatograms every 24 hours.

Results
Analysis of purulent material by GLC showed the presence of a number of SCFA. As can be seen from the Table, specimens from cases 1 to 12 which yielded anaerobic bacteria showed a series of volatile acids. Thus, butyric acid was present in all the 12 specimens, isobutyric in 10, propionic and

Fig. 1 Chromatographic recordings of (a) SCFA and (b) methyl esters analysis from pus specimen from case 7 (Table); (c) SCFA analysis from the same pus specimen after condensation of the original ether extract.
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<table>
<thead>
<tr>
<th>Case</th>
<th>Source</th>
<th>Short chain fatty acids</th>
<th>Culture</th>
<th>Anaerobes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>Peritonitis</td>
<td>A, P, iB, B, L</td>
<td>E. coli</td>
<td>B. fragilis spp fragilis</td>
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<tr>
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<td>A, P, iB, B, iV, L, S</td>
<td>E. coli</td>
<td>B. fragilis spp vulgatus</td>
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<td>3</td>
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<td>K. pneumoniae</td>
<td>F. nucleatum</td>
</tr>
<tr>
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<td>E. coli</td>
<td>B. fragilis spp fragilis</td>
</tr>
<tr>
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<td>E. coli</td>
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<td>Str. group D</td>
<td>Peptostreptococcus species</td>
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<td>8</td>
<td>Buttock gangrene</td>
<td>A, P, B, iV, L, S</td>
<td>E. coli</td>
<td>B. fragilis spp vulgatus</td>
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<tr>
<td>9</td>
<td>Perirectal abscess</td>
<td>A, iB, B, iV, L, S</td>
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<td>F. nucleatum</td>
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<td>10</td>
<td>Pilonidal sinus abscess</td>
<td>A, B, V, L, S</td>
<td>Str. group G</td>
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<td>11</td>
<td>Gas gangrene after compound fracture of tibia</td>
<td>A, iB, B, iV, L, S</td>
<td>Str. group F</td>
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<td>Brain abscess</td>
<td>A, P, iB, B, L, S</td>
<td>Candida species</td>
<td>B. melaninogenicus spp asaccharolyticus</td>
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<td>Buttock abscess</td>
<td>A, L, S</td>
<td>Staph. aureus</td>
<td>Pc. magnus</td>
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<td>Buttock abscess</td>
<td>A, L, S</td>
<td>Str. group D</td>
<td>B. melaninogenicus spp asaccharolyticus</td>
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<tr>
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<td>A, L, S</td>
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<td>Peptostreptococcus species</td>
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<td>Cl. sporogenes</td>
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<tr>
<td>17</td>
<td>Abdominal fistula</td>
<td>L</td>
<td>K. pneumoniae</td>
<td>Gram-positive anaerobic coccus</td>
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<td>18</td>
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<td>A, L, S</td>
<td>E. coli</td>
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<td></td>
<td></td>
<td>Pr. mirabilis</td>
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<td>K. pneumoniae</td>
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<td></td>
<td></td>
<td></td>
<td>K. pneumoniae</td>
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</table>

A, acetic; P, propionic; iB, isobutyric; B, butyric; iV, isovaleric; V, valeric; iC, isocaproic; L, lactic; S, succinic.

isovaleric in nine, while valeric acid was detected in one case and isocaproic in two. On the other hand, specimens from cases 13 to 18, which did not yield anaerobic bacteria but only aerobic, did not show any one of the above-mentioned SCFA. However, all the 18 specimens showed lactic acid, 16 showed

Fig. 2 Chromatographic recordings of SCFA analysis (a) before and (c) after condensation of the ether extract, as well as (b) methyl esters analysis from pus specimen from case 13 (Table).
acetic, and 15 succinic acid. It seems, therefore, that
the presence of either propionic, isobutyric, butyric,
or isovaleric acid suggests the existence of anaerobes
in a pus specimen. This becomes evident when
comparing the chromatographic patterns in Figures
1 and 2. Figure 1 shows chromatographic patterns
of (a) SCFA and (b) methyl esters analysis from the
pus specimen from case 7 (Table). Chromatographic
pattern (c) is an analysis of SCFA from the same pus
specimen after condensation of the original ether
extract. Pattern (a) shows the presence of propionic
acid, while pattern (c) shows propionic, isobutyric,
butyric, isovaleric, and isocaproic acids. Culture of
this pus specimen yielded Bacteroides melaninogenicus,
Eubacterium lentum, and Peptostreptococcus anaero-
bius. On the other hand, in Fig. 2, SCFA analysis
before (a) and after (c) concentration, as well as
methyl esters analysis (b) shows acetic, lactic, and
succinic acids. Cultures from this pus specimen (case
13) did not yield anaerobes. The only isolated bacteri-
un was Staphylococcus aureus.

Examination of Gram-stained slides of the
18 purulent materials revealed Gram-negative
bacilli in 14 cases, Gram-positive cocci in 11,
Gram-positive bacilli in three cases, and Gram-
negative diplococci and oval yeast-like forms in one
case respectively.

Cultures of the purulent materials from cases 1
to 12 yielded anaerobic and aerobic bacteria in each
specimen, while cultures of the pus specimens from
cases 13 to 18 yielded only aerobes (Table). Thus
B. fragilis subspecies were found in seven specimens,
B. melaninogenicus subspecies in three specimens,
and Fusobacterium species in three cases, while
clostridium was found in only one specimen and
Eubacterium lentum in two specimens. All the above
anaerobes coexisted with anaerobic cocci such as
Peptococcus asaccharolyticus, Peptococcus magnus,
and Peptostreptococcus anaerobius.

Discussion

In recent years, anaerobic bacteria have been
implicated in a wide range of infective diseases such
as intra-abdominal and pelvic sepsis, aspiration
pneumonia and pneumonia secondary to obstructive
process, lung, brain, liver, subphrenic, and perirectal
abscesses as well as endocarditis and septicemia
(Gorbach and Bartlett, 1974). Because most of these
infections are fulminating and often fatal without
early and proper antibiotic treatment, rapid recog-
nition of the infective agent is imperative. However,
anaerobes are not only slow-growing organisms but
they almost always coexist with aerobes, especially
in purulent material. In addition, many anaerobes
require specialised anaerobic techniques such as
glove box and roll tube. Thus, the isolation and
identification of an anaerobic bacterium is laborious
and takes at least one week. Therefore, the detection
of SCFA from pus specimens by the use of GLC is
a very helpful screening technique for the pre-
sumptive diagnosis of anaerobic infections because
of its rapidity and simplicity.

Using this method we were able to inform the
clinician of the nature of the infection 1 hour after
the sample had reached the laboratory. In addition,
like Phillips et al. (1976) and Gorbach et al. (1976),
we had no false-positive results. However, we
occasionally obtained false-negative chromatograms,
but this we attributed to our less sensitive thermal
conductivity detector. By warming the ether extracts
in a waterbath we were able to obtain concentrated
solutions up to 20-fold. Samples from these extracts
did not give false-negative results, apart from case 12.
That was a brain abscess which had been treated
intensively with antibiotics for 20 days before
incision. The Gram-stained slides from the original
pus specimen examined by direct microscopy showed
scanty Gram-positive cocci which were grown in
pure culture but they were not identified. SCFA
analysis of an original pus sample, as well as a
concentrated ether extract solution, did not visualise
any fatty acid on the chart. However, SCFA analysis
of a sample from the incubated Peptone broth II at
48 hours showed the existence of propionic, iso-
butyric, and butyric acids. It seems certain that the
bacterium was an anaerobic Gram-positive coccus
whose multiplication had been greatly reduced by
the antibiotic treatment.

A comparison of the bacteriological studies with
the SCFA analysis reveals that acetic and lactic
acids are not specific for the diagnosis of anaerobic
infection. Gorbach et al. (1974, 1976) and Nord
(1977) have claimed that the detection of succinic
acid is strong evidence for the existence of anaerobes,
notably B. fragilis, in pus specimens. Nevertheless,
in specimens from cases 13, 14, 15, and 18 we
detected succinic acid (2-1, 3, 1-4, and 1-7 μmol/ml
respectively) but we failed to isolate anaerobes. It
seems certain, therefore, that succinic acid is not
specific for the diagnosis of anaerobic infections.
Besides, it is known (Nord, 1977) that the end
products of glucose metabolism from Escherichia
coli, Klebsiella pneumoniae, and Proteus mirabilis
include succinic acid. In our experience, Staphylococcus
aureus also produces succinic acid in vivo (Table:
cases 13, 14, 15) (Fig. 2b) and in vitro. However,
propionic, isobutyric, butyric, and isovaleric acids
were recorded in almost every chromatogram from
pus samples which yielded different species of
anaerobes.

In conclusion, the data presented here are in
agreement with those reported by Phillips et al. (1976) and indicate that the detection of propionic, isobutyric, butyric, or isovaleric acids by direct GLC of purulent material is strong evidence for anaerobic infection but not specific for B. fragilis. In addition, we obtained evidence that the detection of succinic acid by GLC does not necessarily indicate infection by anaerobes.

Since an early report from the bacteriology laboratory is of much greater clinical value than a definite taxonomic report one week later, it must be stressed that direct GLC of pus specimens is very useful because it can almost always set the diagnosis of anaerobic infection.

References


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