Possibility of Diagnosing Meningitis by Gas Chromatography: Cryptococcal Meningitis

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Cerebrospinal fluid (CSF) from eight patients with cryptococcal meningitis, from ten patients with viral meningitis, and from four control patients without meningitis were analyzed by electron-capture gas-liquid chromatography (EC-GLC). All cryptococcal specimens had similar EC-GLC profiles, and these differed from those of the controls. Viral EC-GLC patterns were different from those obtained with specimens from the patients with cryptococcal infection and from the controls. In addition, specimens from patients with various types of viral infections gave profiles that differed from each other. Two normal CSFs were inoculated with Cryptococcus neoformans; aliquots of these cultures showed an EC-GLC pattern very similar to that seen in CSF of patients with cryptococcal meningitis. The EC-GLC procedure is rapid, reproducible, and easy to perform and may hold promise as an additional aid in the diagnosis of cryptococcal infection.

Cryptococcal meningitis is frequently difficult to diagnose for the following reasons: (i) the clinical manifestations are not always distinctive; (ii) in some instances the cerebrospinal fluid (CSF) lacks a pleocytosis (7); (iii) cryptococci may not be recovered from the CSF (10); and (iv) the number of cryptococci may be too few to be detectable by India ink preparations (2, 23). Immunological techniques have proven very helpful in that cryptococcal antigen is demonstrable in infected CSF by the latex agglutination test; however, culturally proven cases do occur that are antigen negative, albeit infrequently (6, 11).

Gas-liquid chromatography (GLC) is a relatively new approach to the identification of microorganisms by analyzing for their metabolic products in spent culture media and in body fluids (4, 5, 8). GLC has been used with some success in attempts to diagnose disease (3-5). If it could be applied to infected CSF to indicate the presence of infection or of a specific infecting agent, it would be a valuable additional tool in the diagnosis of meningitis. The use of electron-capture GLC (EC-GLC) in the diagnosis of cryptococcal meningitis is described in this report.

MATERIALS AND METHODS

Specimens. Four groups of specimens were analyzed. The first group comprised eight samples of CSF from patients with cryptococcal meningitis. In all eight cases the diagnosis had been verified by a positive latex agglutination test and by culture and/or India ink preparation in seven of eight cases. Two specimens were from patients cared for by personnel in the Division of Infectious Diseases of the Department of Medicine, Emory University School of Medicine. The other six specimens had been referred to the Center for Disease Control for diagnostic confirmation via the latex agglutination test.

The second group of specimens analyzed consisted of 16 CSF samples taken from patients with proven viral meningitis. An enterovirus was identified in each of these 10 specimens. Seven different enteroviruses (ECHO 4, ECHO 5, ECHO 6, ECHO 9, coxsackie A9, coxsackie B3, and coxsackie B4) were grown.

Unfortunately, complete data on the cellular and protein content of these CSFs are not available.

The third group of four specimens used as controls comprised CSF samples taken from patients undergoing myelography. These patients were free of central nervous system infection and had normal measurements of glucose, protein, and cellular content. A fourth group of two specimens consisted of control CSF samples obtained at myelography; they were inoculated in vitro with Cryptococcus neoformans.

Preparation of derivatives. Derivatives were prepared essentially as described (3). Two milliliters of the specimen to be analyzed was acidified to about pH 2 with 0.2 ml of 50% \( \text{H}_2\text{SO}_4 \). The acidified sample was then extracted by shaking with 20 ml of diethyl ether to obtain hydroxy acids and alcohols. The sample was then made basic (about pH 10) with 8 N NaOH and re-extracted with 20 ml of chloroform. The pH 2 fraction was stored at \(-4^\circ\) C for future study. The chloroform extract was placed in 50-ml
beakers and evaporated with a gentle stream of clean, dry air to about 1 ml. Next, the samples were transferred to a test tube (12 by 75 mm) by using a disposable Pasteur pipette. Care was taken to discard any visible layer of moisture (top layer) in the pipette.

The samples were then dried as follows. First, about 100 mg of MgSO₄ was added to the concentrate. The contents of the test tube were shaken and briefly centrifuged, and the ether layer was decanted into another 12- by 75-mm test tube. A 1-ml amount of ether or chloroform was added to the sedimented MgSO₄, the sample was shaken and centrifuged, and the solvent layer was decanted and combined with the previously decanted layer. The MgSO₄ was discarded. The samples were further concentrated by air to about 0.1 ml. Next, 0.2 ml of chloroform was added to the ether extracts. The contents of the test tube were shaken, and the sample was again evaporated by air to about 0.1 ml; then the contents of both tubes were treated with 1 drop of pyridine, which had been diluted with 3 parts of chloroform, and 6 drops of heptfluorobutyric anhydride. The chloroform was evaporated to about 0.5 drop, and the tube was stoppered with a cork, tapered, and heated in a boiling-water bath for 4 min. Then samples were treated with 2 drops of 0.1 N HCl. The contents of the test tube were shaken to extract into the aqueous layer substances that interfere with GLC analysis. Next, the contents were drawn up into a disposable pipette, and the aqueous layer was discarded. The washing procedure was repeated by using 0.1 N NaOH. The sample was permitted to sit for 30 min. The chloroform layer was deposited into a clean, dry test tube, and the aqueous layer was discarded. Finally, the chloroform was evaporated to near dryness in the test tube with a gentle stream of dry air, and 0.1 ml of ether was added as a final solvent for GLC analysis.

Cultural procedures for in vitro studies. Two control spinal fluids were checked for contaminants by EC-GLC and for bacteriological sterility. Two milliliters of the sterile fluid was adjusted to pH 6 with 0.1 N HCl and inoculated with a clinical isolate of C. neoformans. The samples were incubated for 1 week at 35 C. Heavy growth was obtained, as judged by the turbidity of the fluid after incubation. The samples were acidified and processed as described above.

The heptfluorobutyric anhydride derivatives were analyzed on two different gas chromatographs: (i) a Perkin-Elmer model 900 instrument (ca. 5% of the samples) equipped with frequency pulse modulation (3, 16) and UN detector operated in the frequency pulse mode and (ii) a Barber-Colman instrument (ca. 95% of the samples) equipped with a H⁺ detector operated in the DC mode. Both chromatographs were equipped with dual glass columns (0.3-cm ID for the Perkin-Elmer and 0.6-mm ID for the Barber-Colman by 7.3 m in length). One column (nonpolar) was packed with Chromosorb W 80/100 mesh (dimethylchlorosilane high performance) coated with 3% OV-1 (Applied Science Laboratories), and the second column (polar) was packed with TSP (Regis Chemical Co.). The operating conditions for the Barber-Colman (4) and the Perkin-Elmer chromatographs (3) are described. The carrier gas for the Perkin-Elmer was 95% argon-5% methane, and for the Barber-Colman oxygen-free nitrogen (Matheson, Coleman & Bell flush gas was used as described for the Perkin-Elmer (3). The Barber-Colman had a flow of 40 ml/min, with a combined flow of carrier gas and flush gas (as measured from the detector) equal to 50 ml/min. Two microliters of the sample was injected for each analysis. Cleansing of the injector syringe and injection technique are important (4).

RESULTS

Similar EC-GLC patterns were obtained for specimens of CSF from all eight patients with cryptococcal meningitis. Examples of patterns obtained from CSF of two of these patients on the Barber-Colman are shown in Fig. 1 (chromatograms B and C). Analysis of the peaks showed not only a consistency in their presence, but also a remarkable similarity in size, shape, and retention time. As shown (Fig. 1, chromatogram A) the EC-GLC profile of the CSF from myelography controls was devoid of peaks and was different from the EC-GLC profiles obtained from the cryptococcal meningitis patients (chromatograms B and C).

Analysis of the chromatograms from all eight patients with cryptococcal meningitis is shown in Table 1. All samples have seven or more of the peaks present. Peaks 1 through 6 were present in every sample.

The strain of C. neoformans grew well in the control spinal fluid and produced basic extractable compounds that were reactive with heptfluorobutyric anhydride and detectable by EC-GLC (Fig. 2, chromatogram B). In addition, many of the compounds produced in vitro by the organism in CSF had retention times similar to those detected by EC-GLC analysis of CSF samples taken from patients with cryptococcal meningitis. Compare peaks 2, 3, 4, 5, 6, 7, and 8 (Fig. 2, chromatograms B and C). The EC-GLC patterns of CSF from patients with viral meningitis were different from patterns of CSF obtained from both the cryptococcus patients and noninfected controls (Fig. 3, chromatograms A–D). Furthermore, the profiles obtained from the CSFs of viral meningitis patients were different from each other. Compare chromatograms B and C (Fig. 3). The peaks found in the cryptococcal meningitis CSF are numbered to correspond to those observed in viral meningitis CSF. Peaks found only in viral meningitis cases are lettered. Peaks that show differences between the cryptococcal meningitis patients and the viral meningitis patients are darkened.

A summary of the cryptococcal peaks present or absent in all the viral chromatograms is provided in Table 2. Nine of the 10 viral patterns had only three peaks or less. Peak 2 was not
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FIG. 1. Cryptococcal meningitis compared to control. EC-GLC chromatograms of basic chloroform extracts of CSF treated with heptafluorobutyric anhydride-pyridine-ethanol to form electron-capturing derivatives. The analyses were made on a 3% OV-1 glass column, 0.3-cm ID by 7.3 m in length. A tritium electron-capture detector was used.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Peaks present</th>
<th>No. of peaks absent</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
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<td>10</td>
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<td>39</td>
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<td>+</td>
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<tr>
<td>Total (%)</td>
<td>100</td>
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*+, Peak present.
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Cryptococcal meningitis

Patient No. 15

Control CSF inoculated with Cryptococcus neoformans

Control CSF

TIME (MIN)

90°

220°

Centigrade

FIG. 2. Cryptococcal meningitis compared to C. neoformans in vitro. EC-GLC chromatograms of basic chloroform extracts of CSF treated with heptafluorobutyric anhydride-pyridine-ethanol to form electron-capturing derivatives. The analyses were made on a 3% OV-1 glass column, 0.3-cm ID by 7.3 m in length. A 3H electron-capture detector was used.

gated as a possible tool in the rapid diagnosis of cryptococcal infection.

GLC is not presently being used in the diagnosis of meningitis. Its most routine bacteriological use is in the identification of anaerobes (13). There are several reports on the differentiation of bacteria at the species level by GLC analysis of their metabolic profiles (4, 5, 12, 13, 17-19, 21). Further, GLC has been used to study metabolites of toxigenic fungi (22) and the sugars present in controls and patients infected with cryptococci (1). In vivo studies have focused mainly on the analysis of body fluids; infected serum (20), urine (4), and synovial fluids (5) have been studied.

In this study the spinal fluids from eight patients with cryptococcal meningitis were analyzed by EC-GLC. All eight specimens showed a similar pattern, and this pattern was repro-
produced in vitro by incubating C. neoformans in CSF. Four noninfected control CSF specimens produced no peaks, whereas specimens from 10 patients with viral meningitis produced patterns that differed from those of the cryptococcal and control specimens. However, until the procedure is proved to be of diagnostic value, serological tests for antibody as well as antigen continue to play an important role in diagnosis and should be performed in every suspected case of cryptococcosis.

The identity of the compounds that are the source of the peaks in CSF from cryptococcal infection is not known. For the present they represent only a fingerprint. Some of these peaks may represent metabolites elaborated by the organism. This view is supported by the fact that the organism can produce similar patterns in vitro. Alternatively, the peaks may represent products manufactured by the host's neural tissue or by leukocytes.

It is not known whether the GLC pattern observed in cryptococcosis is specific for that fungus or whether it represents a less specific response to fungal infection or, perhaps, a more general response to infection of the CSF. This last possibility is unlikely in view of the preliminary results obtained in the study of viral in-
fection. Although only seven different enteroviruses were analyzed, they are relatively common causes of viral meningoitis, and the dissimilarity of their patterns from the cryptococcal pattern suggests that this type of analysis may be useful in distinguishing viral from cryptococcal central nervous system infection, a not uncommon differential diagnosis. The few peaks common to cryptococcal and viral infection may indicate a nonspecific host response. The possibility of obtaining specific EC-GLC profiles for viral meningoitis caused by various agents holds exciting possibilities for both a new approach to diagnosing viral meningoitis and identification of some of the compounds produced as a result of viral infection. Work in this area is currently under way.

Because the differential diagnosis of "lymphoctic" meningoitis commonly includes consideration of tuberculous and partially treated bacterial meningoitis, as well as fungal and viral infection, representative specimens from patients with these disorders must also be studied. Preliminary work (R. B. Craven and J. B. Brooks, personal communication) has shown that CSF specimens from 10 patients with pyogenic (pneumococcal, meningococcal, and Haemophilus influenzae) meningoitis and from two patients with tuberculous meningoitis subjected to the same GLC analysis described in this paper have produced patterns different from the cryptococcal patterns we obtained.

The process of extraction and derivitization preparatory to analysis by GLC takes about 3 h (5), and several samples can be prepared simultaneously. The chromatographic run then takes about 40 min. If, on the basis of metabolites produced by an infecting organism or by the infected host, GLC could be used (i) to detect the presence of infection, (ii) to differentiate between groups of infectious agents, such as bacteria, fungi, and viruses, or (iii) to identify a specific agent in infected CSF, the technique would be an invaluable tool in the diagnosis of meningoitis. In those instances in which an etiologic agent grows slowly or not at all (tuberculosis, cryptococcosis, and partially treated bacterial meningoitis) or definitive studies delay diagnosis appreciably (viral meningoitis), a diagnostic procedure that takes only a few hours could prove extremely valuable.

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LITERATURE CITED

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