Rapid Diagnosis of Lymphocytic Meningitis by Frequency-Pulsed Electron Capture Gas-Liquid Chromatography: Differentiation of Tuberculous, Cryptococcal, and Viral Meningitis

ROBERT B. CRAVEN, JOHN B. BROOKS,* DAVID C. EDMAN,¹ JAMES D. CONVERSE,¹ JOHN GREENLEE,² DAVID SCHLOSSBERG,³ THOMAS FURLOW,² JACK M. GWALTNEY, JR.,² AND WALTER F. MINER¹

Center for Disease Control, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia 30333

Received for publication 7 March 1977

Cerebrospinal fluid specimens from patients with tuberculous (17 cases), cryptococcal (15 cases), and viral (14 cases) meningitis were analyzed by frequency-pulsed electron capture gas-liquid chromatography and mass spectrometry. Compounds that disappeared after therapy were found to be present in each of these specimens and were not detected in controls. They occurred in repetitive patterns such that these three types of meningitis could be rapidly distinguished. The compound associated with tuberculous meningitis has been tentatively identified. These findings have implications for rapid diagnosis, pathophysiological studies, and possible new therapeutic approaches.

Gas-liquid chromatography (GLC) has been used to differentiate various species of bacteria by analysis of their metabolic products in spent culture media or of their cell wall constituents (6, 8-11, 12, 19-24). More recently, the technique has been applied to the study of body fluids during infection (2, 6a, 7, 25; J. B. Brooks, R. B. Craven, D. Schlossberg, C. C. Alley, and F. M. Pitts, submitted for publication). Schlossberg et al., using GLC techniques developed in this laboratory, demonstrated the possible rapid diagnosis of cryptococcal meningitis (25). The difficulty in obtaining timely laboratory confirmation of the agent producing lymphocytic meningitis, e.g., cryptococcal, tuberculous, or viral, prompted the present investigation of GLC as a rapid etiological diagnostic method.

MATERIALS AND METHODS

CSF specimens. All specimens were the excess left after routine laboratory studies were performed. Control specimens consisted of the excess cerebrospinal fluid (CSF) from myelograms or spinal epidural anesthesia and were bacteriologically culture negative, with normal cell count, glucose, and protein.

Twelve culture-positive specimens from patients

with tuberculous meningitis were obtained from the meningitis research ward of the U.S. Naval Medical Research Unit no. 3 (NAMRU-3), Cairo, Egypt. Seven samples from cryptococcal meningitis patients were collected by us, and an additional eight samples were provided by Leo Kaufman's laboratory at the Center for Disease Control (CDC) in Atlanta, Ga. These eight samples were from specimens submitted by the University Collaborative Study of Cryptococcal Meningitis to be analyzed for cryptococcal antigen. All cryptococcal specimens were culture positive or antigen positive or both.

We collected five specimens from patients with clinically diagnosed aseptic meningitis, three from one clustered outbreak and two from another. We also obtained CSF from patients with biopsy-proven herpes simplex encephalitis and two specimens from patients with encephalitis associated with herpes zoster skin infection of the face. A number of culture-documented specimens of viral meningitis and encephalitis were provided by Richard Emmons of the California Department of Health. One specimen was from a patient with toxoplasmosis meningitis (14).

Apparatus. A Perkin-Elmer gas chromatograph, model 900, equipped with a ⁶³Ni 10-mCi frequency pulse-modulated electron capture (EC) detector, switching valves, and a 25.4-cm potentiometric recorder, was used. The instrument was operated with two coiled glass columns (0.3 cm in ID by 7.3 m in length). One column (nonpolar) was packed with Chromosorb W, 80/100 mesh (AW-DMCS H.P.), coated with 3% OV-1 (Applied Science Laboratories). The second column (polar) was packed with 3% Dexsil 410 (Analabs). The switching valves permitted a comparative analysis of the effluent from

¹ Present address: U.S. Naval Medical Research Unit no. 3, FPO, NY 09527.

² Present address: University of Virginia School of Medicine, Charlottesville, VA 22904.

³ Present address: Emory University School of Medicine, Atlanta, GA 30322.

either column through a single detector. Reagents and instrument settings were as previously published (1, 3, 4). A Perkin-Elmer PEP-1 data system with Modular Software 2, Revision C, was used to refine data and to tentatively identify compounds by their retention times, as previously determined from analysis of standard mixtures (5).

Derivatization procedures. Derivatization procedures have been described in detail elsewhere (1, 3, 4). To exploit the specificity of the EC detector, we formed electron-capturing derivatives of acids, alcohols, and amines before GLC analysis as follows (15). A 2-ml specimen of CSF was acidified to approximately pH 2 by adding 0.1 ml of 50% H_2SO_4 . The acidified sample was extracted by shaking with 20 ml of 6% ethanol in chloroform to obtain acids and alcohols. The chloroform-ethanol was removed, and the sample was made basic (pH 10) and re-extracted for amines with 20 ml of chloroform. The pH 2 and pH 10 fractions were put into separate 50-ml beakers and evaporated to about 1 ml with a stream of clean dry air. The samples were transferred to 12- by 75-mm test tubes with disposable Pasteur pipettes; any visible water layer was discarded.

Samples were further dried by adding 100 mg of $MgSO_4$ to the concentrate and shaking. Next, they were centrifuged, and the solvent layer was removed to a clean 12- by 75-mm test tube. The remaining $MgSO_4$ was washed with 1 ml of chloroform and centrifuged; the chloroform layer was decanted and combined with the previously decanted solvent. The $MgSO_4$ was discarded. The samples were concentrated under a gentle stream of air to approximately 0.1 ml.

Acids and alcohols present in the pH 2 fractions were treated with trichloroethanol and heptafluorobutyric and anhydride (HFBA) to form electron-capturing derivatives as follows: 1 drop of freshly prepared trichloroethanol-chloroform (5:95) solution and 3 drops of HFBA were added to each pH 2 fraction, and these fractions were allowed to stand at room temperature for 30 min. Then, 0.1 ml of chloroform and 4 drops of 0.1 N HCl were added to each sample, which was shaken and allowed to sit for 3 min. The aqueous layer was removed and discarded, and 4 drops of 0.1 N NaOH was added to the organic layer. This was shaken and allowed to sit for 3 min. The organic layer was removed to a clean 12- by 75-mm test tube and concentrated to near dryness. Next, 0.1 ml of xylene was added, and the sample was again evaporated to near dryness in an 80 to 90°C sand bath under a stream of clean dry air. A second 0.1 ml of xylene was added as the final solvent.

Two microliters of each sample was then injected into the GLC column. The column oven temperature was increased at 4°C/min from an initial temperature of 110°C to a final temperature of 245°C, where it was held for 30 min. Under these conditions, acids varying in length from 2 to 20 carbon atoms could be detected from a single derivatization procedure and chromatographic run (4). Recently, we have found that increasing the amount of HFBA from 3 to 6 drops results in an increase in the amount of derivative formed.

Amines present in the pH 10 extraction were derivatized as follows. To the sample remaining after the $MgSO_4$ drying step was added 1 drop of a 1:3 mixture of pyridine-chloroform and 6 drops of HFBA. This mixture was evaporated by air to about 0.5 drop; the tube was then stoppered with a cork and heated for 4 min in boiling water. The tube was cooled and 0.1 ml of chloroform was added. This mixture was washed with 4 drops each of 0.1 N HCl and 0.1 N NaOH as described above, except that the sample was allowed to sit in the basic wash for 30 min. The chloroform layer from the basic wash was removed to a clean dry test tube and evaporated to near dryness under a stream of clean dry air, and 0.1 ml of ether was added as a final solvent for GLC analysis. Two microliters of each sample was injected, beginning at 90°C for 8 min, and then raised to 225°C at 4°C/min, where the temperature was held isothermally for 30 min. Total analysis of amines or acids and alcohols requires approximately 3 h.

RESULTS

The 12 samples from patients with proven tuberculous meningitis in the early stage were remarkably similar in both the amines detected in the HFBA derivative from the pH 10 extract and in the acids and alcohols detected in the trichloroethanol derivative from the pH 2.0 extract. The same pattern was found in CSF from five clinically suspected cases. Figure 1 shows chromatograms obtained from the CSF of four different patients with culture-proven tuberculous meningitis by using the pH 10 HFBA derivative. Figure 2 shows a fifth patient with tuberculous meningitis whose CSF demonstrates the differences noted between specimens obtained during the acute phase of illness (Fig. 2A) and after successful treatment (Fig. 2B). The presence of several compounds in CSF obtained early in the course of tuberculous meningitis contrasts sharply with their virtual absence both in successfully treated patients (Fig. 2B) and in the uninfected controls.

Peaks with the same numbers in Fig. 1A through D have the same retention times and may be used to study similarities in the profiles of patients. Peaks numbered 1 through 7 were common to all tracings; however, peaks 6 and 7, which were present in small amounts in the original chromatogram, were not visible in the reduced tracing (Fig. 1B) and hence are not labeled in that tracing. The most important peak in these tracings is labeled KHI. It has been identified by mass spectrometry as 3-(2-ketohexyl)indoline. Data supporting this identification will be reported elsewhere (6a).

KHI has been found in every case of acute tuberculous meningitis but not in the other types of meningitis studied. In serial CSF speci-

FIG. 1. HFBA derivatives of the pH 10 extractions of CSF from four patients with meningitis due to *Mycobacterium tuberculosis*. The peak labeled KHI has been identified by mass spectrometry as 3-(2-ketohexyl)indoline and is characteristic of tuberculous meningitis.

mens of five patients, KHI disappeared slowly over the first 2 months of therapy; however, when steroid therapy was used, it tended to disappear more rapidly. Fig. 1D represents a partially treated case, and the reduced amount of KHI present is evident. Figure 1C was obtained from a 1.5-ml sample of CSF. The effect of reduced sample size on the profile in general, as well as on KHI, may be seen. The value of this compound in distinguishing tuberculous from cryptococcal and viral infections may be seen by comparing Fig. 3A to the other tracings in the figure. By noting the presence of KHI as part of the typical pattern shown in Fig. 1, we have been able to identify correctly two cases of tuberculous meningitis before the culture results were available from NAMRU-3.

Two patterns of cryptococcal meningitis are encountered in the pH 10 extraction (Fig. 3B and C). The occurrence of the B and I or J peaks, however, distinguished this disorder from viral meningitis in the 15 samples studied. The type II pattern (Fig. 3B) is the same as that previously found by Schlossberg et al. (25) in this laboratory and was present in 7 of the 15

specimens studied. The type I pattern contains two different compounds, labeled A and I. We suspect that the occurrence of the type II pattern may be due to lesser severity of disease or to the specimen being obtained early in the treatment course. However, in specimens from two acute culture-positive untreated cases, a type II pattern was obtained. Figure 2C and D show the effects of treatment with amphotericin B on the CSF of a patient with cryptococcal meningitis. Figure 2C was from a CSF obtained early in the treatment course, and it is a type II pattern. Figure 2D shows a normal pattern in this patient after 6 weeks of therapy.

A further possible cause for the two patterns may be real metabolic differences among the three serogroups of *Cryptococcus neoformans*. To study this, we inoculated serogroups A, B, and C into neisseria defined medium and analyzed the spent culture medium for metabolic products, using the procedures described earlier (19). Serogroup A did not produce an amine that was produced by B and C. Serotype C did not produce five acids that were produced by both A and B. Thus, metabolic differences do exist between serogroups.

Figure 3D and E demonstrate the major pat-

FIG. 2. Effect of treatment on acute tuberculous and cryptococcal meningitis. HFBA derivatives of amines in CSF of a patient with acute (A) and treated (B) tuberculous meningitis and of a patient with acute (C) and treated (D) cryptococcal meningitis. B and D are like normal CSF.

FIG. 3. Comparison of amine patterns associated with tuberculous, cryptococcal, viral, and toxoplasma meningitis. The distinctive compound associated with tuberculous meningitis, 3-(2'-ketoethyl)-indoline, may be seen in tracing A (peak labeled KHI).

terns associated with viral central nervous system (CNS) infections. Chromatographic peaks of importance for distinguishing viral from *C. neoformans* infection are under brackets in Fig. 3B through E. Further, the viral patterns (Fig. 3D and E) do not have peaks B, I, or J, which were present in cryptococcal meningitis, but they do have peaks 1 or 1 and 2 plus the FGH complex.

Although the distinctive FGH complex is consistent in the 14 viral specimens studied to date, different patterns do seem to be associated with different viruses. This may be seen in Figure 3D, obtained from a patient with herpes simplex encephalitis. Peak L and the unlabeled peak preceding K are not present in the sample from an ECHO-4 meningitis patient (Fig. 3E). Figure 3F shows the amine pattern associated with a case of *Toxoplasma gondii* meningitis.

This pattern recurred with the exacerbations of this patient's illness, which has been described in detail elsewhere (14). The MNO complex of peaks distinguishes this type of meningitis from the other types studied.

A further demonstration of differences in viral cases may be seen in Fig. 4, which compares the pH 2 derivatized extract of samples obtained from patients in two separate clustered outbreaks of clinically diagnosed aseptic meningitis, one patient with biopsy-proven herpes simplex encephalitis, and two patients with herpes zoster-associated encephalitis. The patterns are easily distinguishable by visual inspection of the chromatograms.

DISCUSSION

The theoretical and practical considerations involved in applying GLC to the analysis of infected body fluids were discussed by Cherry and Moss in 1969 (13). Recently, Brooks has reviewed advances in methodology and equipment which have established the feasibility of this approach to diagnosis (J.B. Brooks, *Microbiology—1975*, p. 45–54, American Society for Microbiology, Washington, D.C., 1975). Previously, Mitruka et al. suggested that the interaction of the host and infecting agent could produce compounds whose detection by GLC might lead to a rapid diagnostic technique (17). The implications and desirability of this approach have been discussed recently by Schloss-

FIG. 4. GLC profiles of various viral CNS infections (pH 2 extraction). Trichloroethanol derivatives of acids and alcohols detected in viral meningitis and encephalitis.

berg et al. (25).

That distinctive compounds are present in the course of infection and not in the same patient after treatment or in normal controls has been demonstrated in this study, as well as in studies of cryptococcal meningitis (25), tuberculous meningitis in primates (J. B. Brooks, L. Thacker, R. Broderson, and E. R. Beam, unpublished data), and septic arthritis (7). Using a different GLC technique, Amundson et al. have also shown changes occurring in response to cryptococcal and other meningitidies (2). The presence of a large number of compounds during infection and the ability to detect them by ultrasensitive GLC techniques offer possibilities for future investigations.

The origin of these compounds detected by GLC, whether from infected host cells, inflammatory response, or metabolic products of the organism itself, is unknown. Schlossberg et al. showed that *C. neoformans* grown in vitro in normal human CSF produced a chromatographic pattern similar to that detected in naturally infected CSF (25). In the case of viral infection, however, it is reasonable to assume that the compounds are from the infected target tissue or associated inflammatory cells. Whatever the origin of these compounds, the possibility of their being involved in the pathophysiology of meningitis is an important question for further study. If they are shown to be important in the disease process, then a new neuropharmacological approach to therapy might be possible.

The compound KHI found in tuberculous meningitis is of interest because of its structural similarities to indolic-type compounds of neurochemical importance, such as melatonin. The amine derivatization procedure described above does not derivatize hydroxy-substituted indolic compounds, such as serotonin and 5-hydroxyindole acetic acid. This may be a result of degradation of these compounds at the high pH used during extraction (pH 10). Thus, we may assume that amines of this type are not producing the peaks noted in the chromatograms of Fig. 3. Ultimate determination of the structures of these compounds by mass spectrometry will require obtaining them in greater concentration, since the EC detector is much more sensitive than the mass spectrometer for these types of derivatives. This difficulty may be overcome by trapping the compounds as they pass through the EC detector or by extracting much larger samples for CSF for subsequent analysis by mass spectrometry.

In agreement with the findings of Schlossberg et al. (25), our data show that different viruses have different chromatographic pat-

terns. However, these findings must still be interpreted with care, since we have not collected a large enough series of samples of each virus to be assured of pattern reproducibility. Further, we need to study specimens from patients with CNS neoplasia and demyelinating disorders. The patterns associated with the clustered aseptic meningitis and the herpes zoster-related encephalitis, however, suggest that these patterns may indeed be specific for certain viruses. If further analyses of well-documented specimens continue to differentiate systematically between CNS infections produced by different viruses, then GLC will be a very useful clinical and epidemiological tool, especially when contrasted with the poor yield from culture attempts and the time and effort required to obtain seroconversion data.

The problems of conventional diagnostic techniques, combined with geographically scattered incidence of viral CNS infections and the inadequacy of sample size for analysis, have been the major factors preventing conclusions on the validity of the GLC technique for differentiating between viral etiologies of CNS infections. We hope the data presented will encourage other investigators to provide documented 2-ml specimens for further study of this technique.

Aside from the importance of adequate sample size, other equipment, reagent, and methodological details associated with this study should be noted. The extraction and derivatization procedures are not complex, but they do require practice to achieve reproducibility. The quality of reagents used in the analytical procedure is critical because of the extreme sensitivity of the EC detector, and substitutions are not recommended. A temperature-programmable gas chromatograph equipped with 24-foot (ca. 7.32-m) columns must be used to obtain the necessary resolution of these complex mixtures. Finally, in our experience, neither the thermal conductivity nor the flame ionization detector is selective or sensitive enough to use in this application.

The data presented suggest that EC GLC can be used to differentiate between tuberculous, cryptococcal, viral, and parasitic infections of the CNS. These differences are a result of the presence of compounds that disappear as patients recover and are not present in normal CSF. By combining GLC with mass spectrometry, it may be possible to identify some of these compounds and thus gain further insights into the pathophysiology of CNS infections. If these compounds are toxic, new therapeutic approaches to treating patients with meningitis may also be possible.

ACKNOWLEDGMENTS

We thank John Bennett of the National Institutes of Health for the assistance of the University Collaborative Study of Cryptococcal Meningitis in securing specimens and Amy Stine and Leo Kaufman of the CDC for verifying these specimens. We also thank Sharon Blumer of CDC for providing the serogrouped specimens of *C. neoformans* and Michael Hattwick and colleagues for securing a wide variety of well-documented viral specimens.

This research was supported by Naval Medical Research and Development Work Unit MR000.01.01.3121.

LITERATURE CITED

- Alley, C. C., J. B. Brooks, and G. Choudhary. 1976. Electron capture gas-liquid chromatography of short chain acids as their 2,2,2-trichloroethyl esters. *Anal. Chem.* 48:387-390.
- Amundson, S., A. I. Braude, and C. E. Davis. 1974. Rapid diagnosis of infection by gas-liquid chromatography: analysis of sugars in normal and infected cerebrospinal fluid. *Appl. Microbiol.* 28:298-302.
- Brooks, J. B., C. C. Alley, and R. Jones. 1972. Reaction of nitrosamines with fluorinated anhydrides and pyridine to form electron capturing derivatives. *Anal. Chem.* 44:1881-1884.
- Brooks, J. B., C. C. Alley, and J. A. Liddle. 1974. Simultaneous esterification of carboxyl and hydroxyl groups with alcohols and heptafluorobutyric anhydride for analysis by gas chromatography. *Anal. Chem.* 46:1930-1934.
- Brooks, J. B., C. C. Alley, J. W. Weaver, V. E. Green, and A. M. Harkness. 1973. Practical methods for derivatizing and analysing bacterial metabolites with a modified automatic injector and gas chromatograph. *Anal. Chem.* 45:2083-2087.
- Brooks, J. B., W. B. Cherry, L. Thacker, and C. C. Alley. 1972. Analysis by gas chromatography of amines and nitrosamines produced in vivo and in vitro by *Proteus mirabilis*. *J. Infect. Dis.* 126:143-153.
- Brooks, J. B., G. Choudhary, R. B. Craven, D. Edman, C. C. Alley, and J. A. Liddle. 1977. Electron capture gas chromatography detection and mass spectrum identification of 3-(2'-ketoethyl)indoline in spinal fluids of patients with tuberculous meningitis. *J. Clin. Microbiol.* 5:625-628.
- Brooks, J. B., D. S. Kellogg, C. C. Alley, H. B. Short, H. H. Handsfield, and B. Huff. 1974. Gas chromatography as a potential means of diagnosing arthritis. I. Differentiation between staphylococcal, streptococcal, gonococcal, and traumatic arthritis. *J. Infect. Dis.* 129:660-668.
- Brooks, J. B., D. S. Kellogg, L. Thacker, and E. M. Turner. 1971. Analysis by gas chromatography of fatty acids found in whole cultural extracts of *Neisseria* species. *Can. J. Microbiol.* 17:531-543.
- Brooks, J. B., D. S. Kellogg, L. Thacker, and E. M. Turner. 1972. Analysis by gas chromatography of hydroxy acids produced by several species of *Neisseria*. *Can. J. Microbiol.* 18:157-168.
- Brooks, J. B., and W. E. C. Moore. 1969. Gas chromatographic analysis of amines and other compounds produced by several species of *Clostridium*. *Can. J. Microbiol.* 15:1433-1447.
- Brooks, J. B., C. W. Moss, and V. R. Dowell. 1969. Differentiations between *Clostridium sordellii* and *Clostridium bifermentans* by gas chromatography. *J. Bacteriol.* 100:528-530.
- Brooks, J. B., R. E. Weaver, H. W. Tatum, and S. A. Billingsley. 1972. Differentiation between *Pseudomonas testosteroni* and *P. acidovorans* by gas chromatography. *Can. J. Microbiol.* 18:1477-1482.
- Cherry, W. B., and C. W. Moss. 1969. The role of gas chromatography in the clinical microbiology laboratory. (Editorial) *J. Infect. Dis.* 119:658-662.
- Greenlee, J. E., W. D. Johnson, Jr., J. F. Campa, L. S. Adelman, and M. A. Sande. 1975. Adult toxoplasmosis presenting as polymyositis and cerebellar ataxia. *Ann. Intern. Med.* 82:367-371.
- Maggs, R. J., P. L. Jones, A. J. Davies, and J. E. Lovelock. 1971. The electron capture detector—a new mode of operation. *Anal. Chem.* 43:1966-1971.
- Mitruka, B. M., and M. Alexander. 1967. Ultrasensitive detection of certain microbial metabolites by gas chromatography. *Anal. Biochem.* 20:548-550.
- Mitruka, B. M., L. E. Carmichael, and M. Alexander. 1969. Gas chromatographic detection of an vitro and in vivo activities of certain canine viruses. *J. Infect. Dis.* 119:625-634.
- Morse, C. D., J. B. Brooks, and D. S. Kellogg. 1976. Electron capture gas chromatographic detection of acetyl methylcarbinol produced by *Neisseria gonorrhoea*. *J. Clin. Microbiol.* 3:34-41.
- Moss, C. W., and W. B. Cherry. 1968. Characterization of the C15 branched-chain fatty acids of *Corynebacterium acnes* by gas chromatography. *J. Bacteriol.* 95:241-242.
- Moss, C. W., and C. M. Kaltenbach. 1974. Productions of glutaric acid: a useful criterion for differentiating *Pseudomonas diminuta* from *Pseudomonas vesicularis*. *Appl. Microbiol.* 27:437-439.
- Moss, C. W., D. S. Kellogg, D. C. Farshy, M. A. Lambert, and J. D. Thayer. 1970. Cellular fatty acids of pathogenic *Neisseria*. *J. Bacteriol.* 104:63-68.
- Moss, C. W., M. A. Lambert, and W. H. Merwin. 1974. Comparison of rapid methods for analysis of bacterial fatty acids. *Appl. Microbiol.* 28:80-85.
- Moss, C. W., S. B. Samuels, J. Liddle, and R. M. McKinney. 1973. Occurrence of branched-chain hydroxy fatty acids in *Pseudomonas maltophilia*. *J. Bacteriol.* 114:1018-1024.
- Moss, C. W., S. B. Samuels, and R. E. Weaver. 1972. Cellular fatty acid composition of selected *Pseudomonas* species. *Appl. Microbiol.* 24:596-598.
- Schlossberg, D., J. B. Brooks, and J. A. Schulman. 1976. The possibility of diagnosing meningitis by gas chromatography. I. Cryptococcal meningitis. *J. Clin. Microbiol.* 3:239-245.