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STUDIES OF METABOLIC CHANGES IN CELL CULTURES INFECTED WITH FOUR SEROTYPES OF DENGUE FEVER VIRUSES BY FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

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SUMMARY

Monkey kidney cell cultures were infected with four serotypes of dengue viruses, and the supernatant fluids of the cell cultures were extracted for amines, alcohols, carboxylic acids,

and hydroxy acids. The derivatized extracts were then analyzed by frequency-pulsed electron-capture gas–liquid chromatography (FPEC–GLC). FPEC–GLC profiles of the hydroxy acids showed peaks that were different for different serotypes and the FPEC–GLC carboxylic acid profiles differed from the control medium. These differences were reproducible when the same lot of medium was used. There were differences in profiles between lots of control media due apparently to different fetal bovine sera used in the growth medium. Therefore, the same lot of medium was necessary to reproduce profiles. The data obtained from the study indicate that FPEC–GLC can be used to detect changes in cellular metabolism caused by viral infection, and that these metabolic changes might be useful for detection of genetic differences in viruses as reflected by detectable changes in the metabolism of the infected cell.

INTRODUCTION

Gas–liquid chromatography has been used to study chemical changes in various chemical components present in body fluids and tissue culture as a results of bacterial and viral infections [1–8], and frequency-pulsed electron-capture gas–liquid chromatography (FPEC–GLC) has been used to detect differences in chemical components in sera and cerebrospinal fluids of persons infected with different types of viral diseases [5, 9].

Diagnosis of viral infections, such as those produced by various serotypes of dengue, through direct serum analysis by FPEC–GLC would be desirable. The major advantage of the new technique would be in the amount of time that would be saved if the virus did not have to be cultured. However, before analyzing more complex human specimens with FPEC–GLC, it seemed important to evaluate the method in controlled conditions such as tissue culture. If metabolic differences detected in infections caused by dengue serotypes, which produce epidemics throughout the world [10–12], could be detected in tissue culture, then metabolic differences produced in the host cell may also be detected in disease cases involving humans. In addition these studies may provide clues to metabolic changes that take place in virus infected cells.

MATERIALS AND METHODS*

Tissue culture and virus infection

Rhesus monkey kidney cells, LLC-MK_2, were seeded in 25 cm^2 Falcon tissue culture flasks in aliquots of 2 million cells per 8 ml growth medium per flask. The flasks were incubated at 35°C for three days. The growth medium used was medium-199 with Hanks' salts containing 10% new born calf serum. The pH of the medium was adjusted to 7.4 with sodium bicarbonate. The histories of the virus strains used are described in Table I. All viruses were diluted in virus diluent [20% fetal bovine serum (FBS); pH 7.3]. The viruses were inoculated into replicate cell culture flasks from which growth medium had been removed. After virus adsorption at 35°C for 1 h, each flask was rinsed twice with phosphate buffered saline (PBS) and incubated at 35°C with 5 ml of maintenance medium which contained 2% FBS in medium-199. Flasks

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

HISTORY OF DENGUE VIRUS STRAINS USED IN THE STUDY

CDC accession number	Serotype	Virus designation and passage history	Year isolated	Place
CA1816	Dengue 1	Hawaiian prototype One monkey, one mosquito and seven tissue culture passages	1944	Hawaii
CA1817	Dengue 1	H-13806a Four tissue culture passages	1977	Jamaica
CA1818	Dengue 1	H-23333a Four tissue culture passages	1977	Puerto Rico
CA1819	Dengue 1	H-45509a Two tissue culture passages	1980	Mexico
CA1821	Dengue 2	H-14241a Three tissue culture passages	1977	Puerto Rico
CA1822	Dengue 2	H-20919a Two tissue culture passages	1977	Puerto Rico
CA1820	Dengue 2	New Guinea 'C'; prototype Twenty four suckling mouse and six tissue culture passages	1944	New Guinea
CA1823	Dengue 3	H-87; prototype Passages (one monkey and nineteen tissue cultures)	1956	Philippines
CA1824	Dengue 3	PR-6 Thirteen suckling mouse passages	1963	Puerto Rico
CA1825	Dengue 3	H-21326 Two tissue culture passages	1977	Puerto Rico
CA1826	Dengue 4	H-241; prototype Seven suckling mouse and six tissue culture passages	1956	Philippines
CA2039	Dengue 4	H-54101 Two tissue culture passages	1981	Dominica
CA2040	Dengue 4	H-54157 Two tissue culture passages	1981	Saint Barthelemy

inoculated with virus diluent alone and uninfected tissue culture in growth medium served as controls. For the time study, supernatant fluid from each flask was removed daily for seven days and on the tenth day after inoculation, centrifuged at 200 *g* for 10 min, and stored frozen at −70° C until analysis. For other studies, the procedure was the same except that the supernatant fluids were removed on the seventh day after inoculation. All tests except the time study were repeated. Virus titers were determined by means of plaque assay, according to the method of Eckels et al. [13].

Extraction and derivatization procedures

A 2-ml aliquot of each tissue culture supernatant fluid was placed in a 50-ml round-bottomed centrifuge tube with a PTFE-lined screw cap; then heptanoic acid (11.43 nmol in 0.3 ml of distilled water, made basic with sodium hydroxide to obtain solubility), 2-hydroxyisovaleric acid (2.68 μmol

in 0.1 ml of distilled water), and di-*n*-butylamine (1.19 μmol in 0.4 ml of distilled water made acidic to increase solubility) were added to each sample as internal standards. Next, the samples were acidified to about pH 2 with 0.1 ml of 50% (v/v) sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell Wrist Action Shaker at a setting of 10. The residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *N* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction, to obtain the amines. Then, the residual basic aqueous phase was reacidified to about pH 2 with sulfuric acid and extracted with 20 ml of diethyl ether (Fisher reagent grade stabilized with butylated hydroxytoluene) to obtain the hydroxy acids. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carbxylic acids and HFBA esters of alcohols as described [14, 15]. The basic chloroform extracts containing amines and the acidic diethyl ether extracts containing hydroxy acids were derivatized with HFBA pyridine—ethanol to form amines and esters, respectively, as described [16, 17]. After the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines were prepared, they were dissolved in 0.1 ml of xylene–ethanol (1:1). The HFBA derivatives of hydroxy acids were dissolved in 0.1 ml of ethyl acetate. A 2-μl injection was used for analysis of all derivatives. The techniques for filling and cleaning the syringe have been described [15].

Apparatus

The derivatives were analyzed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10 mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80–100 mesh Chromosorb W HP (AW-DMCS treated) were used under conditions previously described [14, 15, 17].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS-16 revision B) accumulated data from the gas chromatograph, analyzed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid, di-*n*-butylamine, and 2-hydroxyisovaleric acid, which were added as internal standards [14, 17].

RESULTS

The results of viral replication of one representative test are shown in Table II. While all prototype viruses replicated well in LLC-MK$_2$ cells as expected, virus titers of many non-prototype virus strains did not reach 4 log. No cytopathic effects and few lysed cells were observed during infection.

The time study indicated that some change in the chemical components in the supernatant fluids in the virus-infected cell cultures was apparent as early as the second day after inoculation, but differentiation between serotypes was best on the seventh day. This was true not only of the cell cultures infected with prototype viruses but also of non-prototype strains despite the fact that the majority of the latter group did not replicate as well as the former group.

TABLE II

REPLICATION OF DENGUE VIRUS STRAINS USED IN FPEC—GLC ANALYSIS

Virus	Inoculum (log PFU/flask)*	Extracellular virus titer (log PFU/ml supernatant fluid) seven days after inoculation
DEN 1 (Hawaii)	3.6	5.9
DEN 1 (H-13806)	3.4	3.5
DEN 1 (H-23333)	3.9	2.8
DEN 1 (H-45509)	3.6	3.2
DEN 2 (New Guinea "C")	3.6	6.8
DEN 2 (H-14241)	3.5	3.4
DEN 2	3.1	2.9
DEN 3	3.3	4.3
DEN 3	3.9	3.7
DEN 3 (H-21326)	3.8	2.7
DEN 4 (H-241)	3.6	4.5
DEN 4 (H-54101)	4.0	5.8
DEN 4 (H-54157)	4.0	6.8

*PFU = plaque forming unit.

Similarly, FPEC -GLC profiles of non-prototype strains were similar to those of prototype virus within a given serotype, with two minor exceptions described below.

As shown in Fig. 1, a dramatic change occurred in the hydroxy acid components in the supernatant fluids of the infected culture (Fig. 1B) as compared with those of the control (Fig. 1A) culture. The major changes in the infected culture (Fig. 1B) were apparent in the removal of the blackened peaks 2, 7, and 8 and to a lesser degree, peaks 1, 2a, and 10. A "U" has been placed over the peaks (Fig. 1B) to indicate utilization or removal by comparison with uninfected control fluid (Fig. 1A). One new (N) peak, labelled 12 was detected in the infected sample.

As shown in Fig. 2 peaks in the profiles of four serotypes of dengue viruses differed both qualitatively and quantitatively from peaks found in the control and in the FPEC—GLC profiles between serogroups. For example, qualitatively peaks 8a, 11a, 12 and 13 were not found in the controls, and dengue serotypes 2 and 3 differed from serotypes 1 and 4 by the production of peaks 8a, 11a, and 13. Serotypes 2 and 3 differed from each other by production of peaks 8a (dengue 2) and 13 (dengue 3). Dengue serotype 1 differed from serotype 4 on the basis of utilization or removal of peak 8 and by the production in serotype 4, above that found in the controls, of peak 9.

The changes in amine profile that occurred during infection are illustrated in Fig. 3. Dengue 1 was the only serotype that produced a different profile from that of the control. The significance of the reduction of peaks 1 and 5 in the dengue 1 profile (Fig. 3A), as compared with the control profile (Fig. 3B), was questionable, since peaks 1 and 5 were absent in another control (Fig. 3C) in which a different lot of growth medium was used. Further, peaks 6 and 7

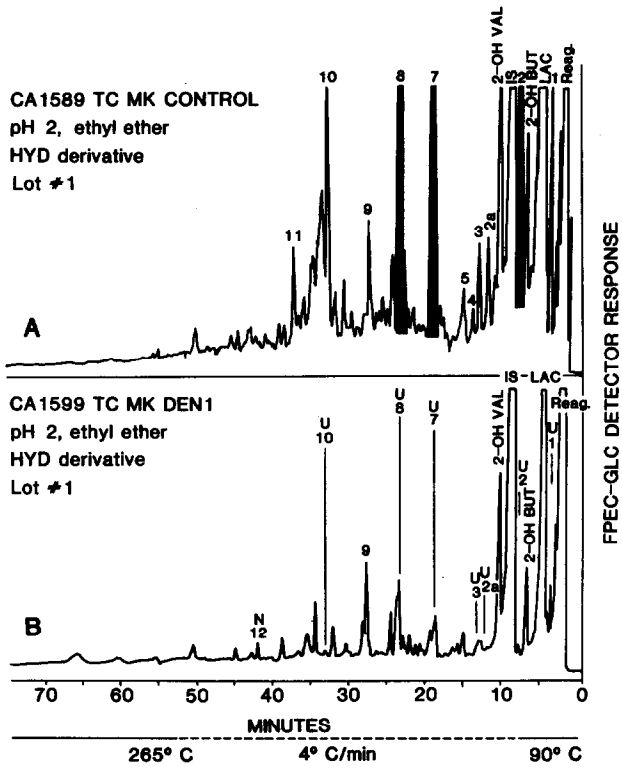

Fig. 1. FPEC—GLC traces of hydroxy acids in the supernatant fluids of (A) control and (B) dengue-infected LLC-MK_2 cell cultures. Column: OV-101. Abbreviations: TC: tissue culture; MK: LLC-MK_2 cells; HYD: heptafluorobutyric anhydride—ethanol derivatized acidic diethyl ether extracts; reag: reagent; LAC: lactic acid; 2-OH BUT: 2-hydroxybutyric acid; IS: internal standard; 2-OH VAL: 2-hydroxy valeric acid. "U" over a peak indicates utilized or removal.

(Fig. 3A) were not reproducible from lot to lot which may indicate that peaks 6 and 7 were intermediates affected by change in the medium.

Fig. 4 shows carboxylic acid profiles of normal and infected cultures. Profile differences among different lots of growth medium were again detected, as demonstrated by the lack of peaks 2 and 4 in lot 2 (compare Fig. 4A and B). The profile of Dengue 1 infected culture (Fig. 4C) is clearly distinguished from that of the control by the appearance of new peaks (3 and 6) and the reduction of peaks (1, C4, and iC5). The concentration of the internal standard (C7) shown in Fig. 4C is slightly lower than that shown for the internal standards in Fig. 4A and B. This difference in concentration as shown by other analyses and by computer evaluation which bases its concentration determination on the relationship of the size of the internal standard to a fixed molar concentration, verified the reduction of peaks 1 and iC5.

Fig. 5 shows that carboxylic acid profiles of all serotypes are different from that of the controls (Fig. 4A and B) because: (1) three peaks (1, 2, and iC5)

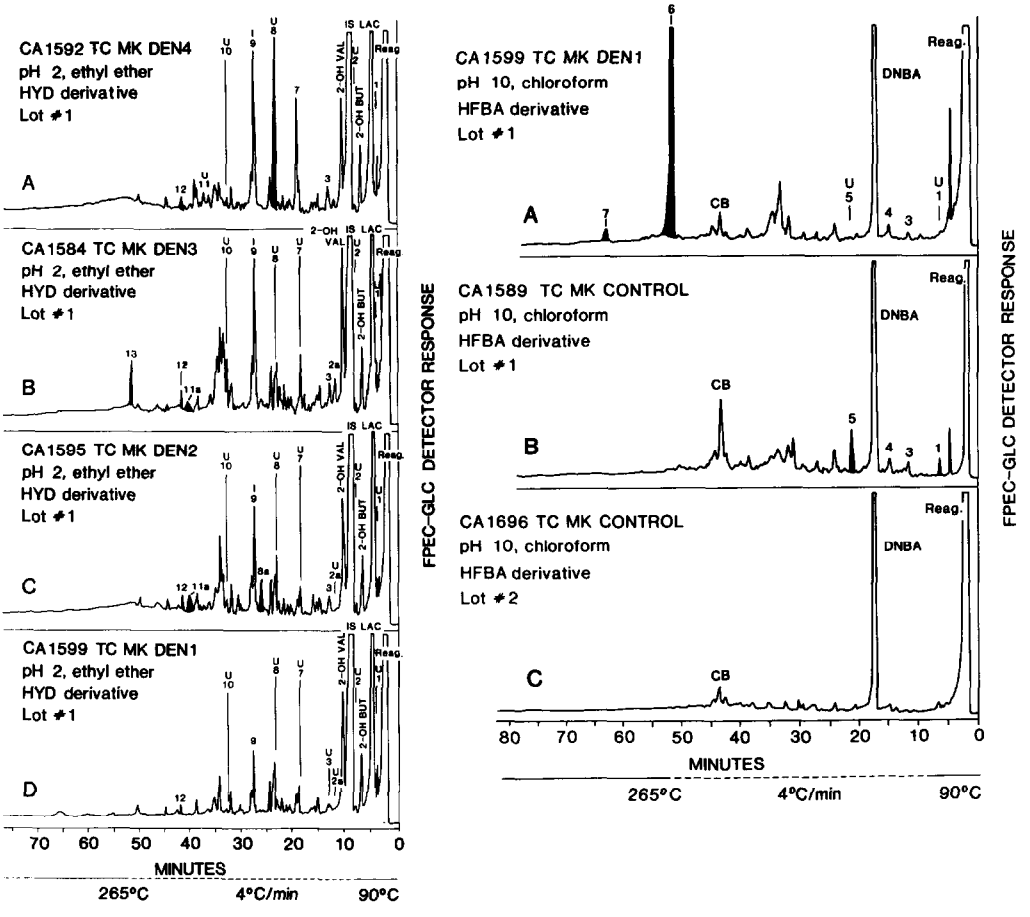

Fig. 2. FPEC—GLC traces of hydroxy acids in the supernatant fluids of LLC-MK$_2$ cell cultures infected with four dengue serotypes. Column: OV-101. Abbreviations: "I" over a peak indicates that the peak was increased over that found in the control. For other abbreviations, see Fig. 1.

Fig. 3. FPEC—GLC traces of amines in the supernatant fluids of two sets of normal (B, C) and a dengue virus-infected LLC-MK$_2$ (A) cell cultures. Column: OV-101. Abbreviations: HFBA: Heptafluorobutyric anhydride; DNBA: internal standard, di-*n*-butylamine; CB: column bleed. For other abbreviations, see Fig. 1.

in the control profile were consistently reduced; and (2) a new peak was present (peak 6) that was not detected in the control. Dengue 1 profile for carboxylic acids was further distinguished from the profiles of the other serotypes by a new peak 3 not shared by the others.

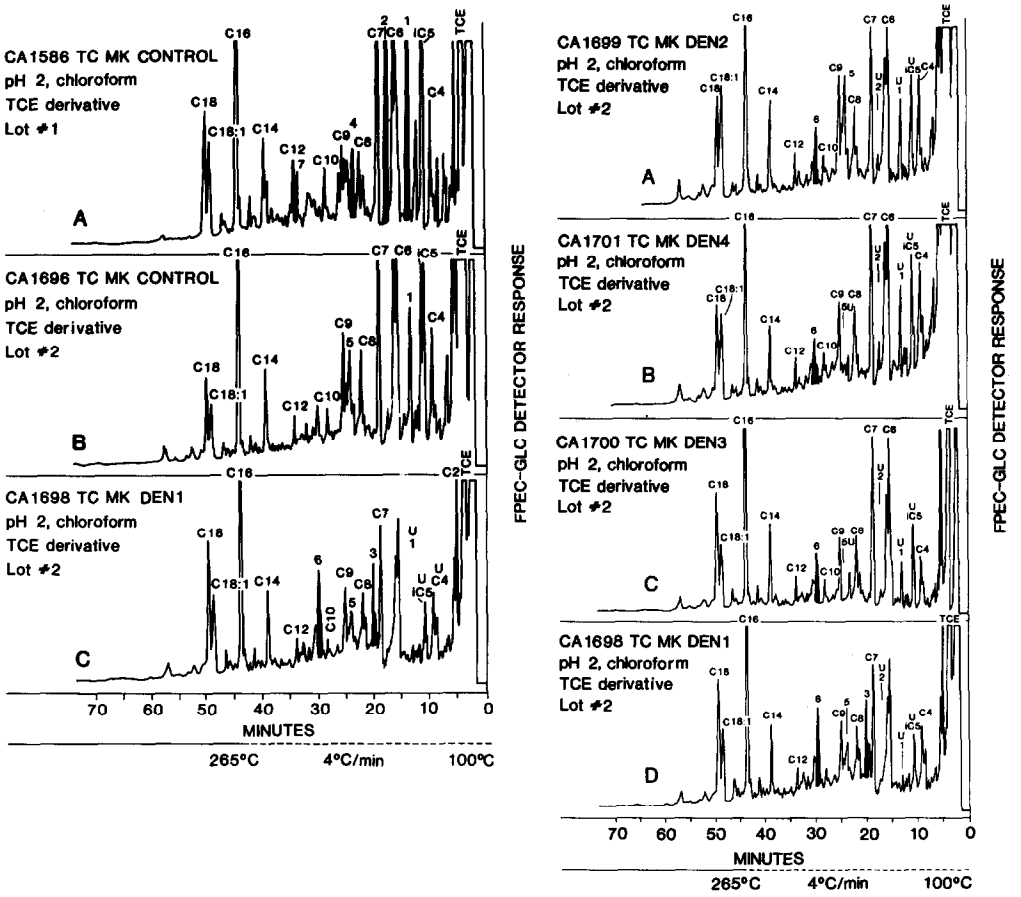

Fig. 4. FPEC—GLC traces of carboxylic acids and alcohols in the supernatant fluids of two sets of normal (A, B) and a dengue virus-infected LLC-MK_2 (C) cell cultures. Abbreviations: TCE: trichloroethanol; C7: internal standard. The letter C followed by a number indicates a saturated straight chain carboxylic acid with the number of carbon atoms indicated by the number. The letter "i" indicates "iso"; and the use of a colon between two numbers indicates unsaturation. For other abbreviations, see Fig. 1.

Fig. 5. FPEC—GLC chromatograms of carboxylic acids and alcohols in the supernatant fluids of LLC-MK_2 cell cultures infected with four dengue serotypes. Column: OV-101. For abbreviations, see Figs. 1 and 4.

DISCUSSION

The results from our studies clearly show that the profiles of metabolites in LLC-MK_2 cell cultures infected with dengue viruses are different from those of uninfected control cultures. Further, the metabolism is altered in a sufficiently different manner to produce FPEC—GLC profiles which show that genetically different viruses can alter the cellular metabolism in a manner that makes distinction by FPEC—GLC possible. Among the compounds studied, hydroxy

acids appear to be the most promising indicators of type differences, since each serotype produced a distinct profile.

FPEC—GLC profiles of dengue strains of a given serotype were homogeneous enough to make classification possible, as long as tests were performed with the same lot of growth medium. Minor variations detected in two lots of media probably were derived from different lots of FBS employed, since all other constituents of the media came from the same lots of ingredients. In a blind coded test only two strains, a dengue 1 (H-13806) and a dengue 3 (PR-6), had different FPEC—GLC profiles from those produced by prototype viruses, dengue 1 (Hawaii) and dengue 3 (H-87), respectively. It is of interest to note that the dengue 3 (PR-6) strain was isolated in Puerto Rico and was demonstrated to be different serologically from the prototype dengue 3 (H-87) which was originally isolated in Southeast Asia [18]. The dengue 1 (H-13806) strain was isolated during the first documented dengue 1 pandemic in the Caribbean.

The main variations between the FPEC—GLC profiles obtained from control cell cultures grown in different lots of growth medium seem to be caused by inherent differences between lots of bovine sera. For this reason, the use of cells grown with serum-free media would appear to be better for FPEC—GLC analysis. An alternative would be the use of predetermined reference serotypes which were cultured and tested by FPEC—GLC with each new lot of medium.

The present study adds support to previous findings [7, 9] that different viral diseases seem to be capable of altering the host cell metabolism or body response in different ways, and possibly offers a way to study changes in cellular metabolism caused by viral infections. If so, these differences may be useful as an aid in the identification of several types of viral diseases including dengue fever. The ultimate dengue diagnostic scheme would involve FPEC—GLC detection of dengue infection through analysis of human body fluids, and preliminary studies indicate further research in this area could be fruitful.

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