Replication of Venezuelan Equine Encephalomyelitis Virus In Vitro

II. Viral Growth Response to Selected Nutritional Additives in Suspension Cultures

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Received for publication 20 October 1970

An attempt was made to identify nutritional additives that influence the replication of Venezuelan equine encephalomyelitis virus in suspension cultures grown in a defined serum-free medium. Proline, serine, and choline enhanced titers of the virulent PES strain; the progeny population, however, possessed a virulence character that was somewhat different from that of the PES inocula. These nutritional supplements did not appreciably influence the titers of the attenuated 9t and 20t viral strains. When both the PES and 20t strains were employed as a mixed inoculum in culture, the presence of the latter strain appeared to interfere with the growth of the PES strain and reduced its response to the medium supplements.

Consistent with reports that Venezuelan equine encephalomyelitis (VEE) virus is capable of replicating in a wide variety of cell systems (1–3) are recent data showing that this virus reproduced to substantial titers in suspension cultures grown in serum-free media (H. R. Tribble, Jr., et al., J. Gen. Virol., in press). It was of interest, however, that higher virus titers were obtained with cell cultures grown in serum-free medium containing lactalbumin hydrolysate than in a chemically defined medium and that in cases in which serum was introduced highest titers were achieved. Although our data showed that the serum helped to prevent thermal inactivation of the virus, it was evident that it also exerted a pronounced effect on viral multiplication. These data suggested that nutritional elements within the culture may play an important role in influencing viral multiplication and that it was feasible, by using a chemically defined medium, to identify nutritional factors that may be responsible.

It is the purpose of this report to present results of a series of experiments designed to test the influence of selected nutritional factors on the replication of a virulent VEE virus strain tested previously in serum-free cultures (H. R. Tribble, Jr., et al., J. Gen. Virol., in press). Studies were also performed on attenuated strains described elsewhere (4–6).

MATERIALS AND METHODS

Cell cultures. The L-cell line employed was propagated in suspension with serum-free medium. Cultures were incubated at 35°C in a gyratory shaker (New Brunswick Scientific, Inc., New Brunswick, N.J.) operated at 126 to 130 rev/min in a rubber-stoppered 100-ml serum bottle as described elsewhere.

Medium. Serum-free lactalbumin hydrolysate (LAH) medium of Higuchi (7) with the omission of monolein and the defined medium of Nagle et al. (10), designated D medium, were used. These latter authors found that seven amino acids were nonessential but stimulatory to L-cell growth. These included glycine (60 mg/liter), L-alanine (120 mg/liter), L-serine (158 mg/liter), L-cystine (75 mg/liter), L-aspartic acid (270 mg/liter), L-glutamic acid (315 mg/liter), and L-proline (200 mg/liter). Tests were carried out without these amino acids in D medium except for cases in which they were added individually or in groups. Choline is present in D medium (1 mg/liter) and is essential for cell growth, but was tested at 10-fold concentration (10 mg/liter) because it was found to be stimulatory to cell growth at this concentration (9). These additives produced a twofold increase in cell counts during a 5- to 6-day incubation period.

Virus. The virulent large-plaque-forming PES strain was described elsewhere (6), as were the attenuated small-plaque-forming 9t and 20t strains (4–6).

Virus assays. Because three different virus strains were used in the present work, several methods of assay were needed to determine virus yields. The purpose of multiple assays, however, was not only to determine viral concentrations but also to allow for the possibility of detecting changes in virulence that might occur during viral replication in culture. The virulent PES strain used as inoculum shows high intracerebral (ic) and intraperitoneal (ip) titers that are within 1 log of each other when injection in 12- to 14-g mice is used as the method of assay. Both routes were employed as
methods of assay because progressive attenuation of strain PES often shows up as increased differences between the ic and ip titers (5, 6). The attenuated 9t strain was derived originally from the PES strain by passage in L cells (4), and the preparation used in inoculum had an ic titer of \(10^9\) to \(10^8\) mouse median intracerebral lethal dose (MICLD\(_{50}\))/ml. This strain was not lethal by the ip route. All in vivo titer values were calculated by the Reed-Muench method (12) and expressed as median lethal dose (LD\(_{50}\)) units per milliliter.

The 20t strain, which was derived from the 9t strain, was not lethal for mice by either the ic or ip route (5). Consequently, this strain was assayed only by plaque formation. In certain instances, plaque assays were performed on samples of the PES strain progeny and expressed as plaque-forming units per milliliter.

PES virus was tested with the media shown in Tables 1 and 2 a minimum of five times. The 9t and 20t strains were tested as shown at least twice; tests with either of these strains were accompanied by parallel tests with the PES strain. Viral assays were performed on samples obtained from duplicate cultures at 24-hr intervals for four consecutive days postinoculation. Only the 24-hr values are shown in the tables because maximal titers uniformly occurred at that time.

**RESULTS**

The first series of experiments was designed to determine whether amino acids that previously were found to be nonessential but stimulatory to L-cell growth (10) could influence viral replication. The results of this approach revealed only alanine, proline, and serine as showing evidence of VEE (PES) viral titer enhancement.

In the experiments that follow, alanine, proline, serine, and a 10-fold increase in the concentration of choline were tested further as supplements for D medium. Tests with LAH medium were included to verify previous information and to provide a comparative medium for studies on viral growth. Two attenuated strains of VEE virus, 9t and 20t, were tested in addition to the virulent PES strain.

Virus titers obtained in cell cultures grown in LAH and in various modifications of the D medium are compared in Tables 1 and 2. When the methods of viral assay included titrations by both the ic and ip routes, the values in parentheses represent the difference between the two titers. Because titers obtained with D medium plus proline and serine and D medium plus choline were comparable, data from these experiments were combined for presentation in Table 1.

The data in Table 1 show that, compared with D medium, replication of the nonattenuated PES strain was increased in cells grown in LAH medium and in D medium containing proline and serine or 10-fold increases in choline or in D medium containing proline and serine and 10-fold increases in choline. Enhanced proliferation of the new PES viral progeny by nutritional additives was more apparent when assays were performed in mice by ic injection than by the ip route. In contrast to results with the PES strain, attenuated strains 9t and 20t showed little evidence of increased viral reproduction resulting from the nutritional additives. Further observations concerning this phenomenon are presented in Table 2.

Table 2 contains data showing replication of the large-plaque virulent PES and the small-plaque attenuated 20t strains inoculated singly or as a mixed inoculum in serum-free cultures. Both strains were tested in defined medium with proline, serine, alanine, and increased choline. Consistent with the data in Table 1, it can be seen that PES strain replication was increased in the presence of the additives, especially in D medium fortified with both choline and the three amino acids, and that yields of the virus type most obviously enhanced was the ic-lethal type. Growth of the 20t strain was not enhanced in medium containing the nutritional additives.
Table 2. Replication of large-plaque virulent (PES) and small-plaque attenuated (20t) strains of Venezuelan equine encephalomyelitis virus in L cells grown in chemically supplemented defined medium

| Suspending medium | PES* | 20t* (PFU) | PES and 20t* |
|-------------------|------|------------|--------------|
|                   | PFU  | MICLD₅₀ | MIPLD₅₀ | PFU  | MICLD₅₀ | MIPLD₅₀ |
| D . . . . . . . .  | 7.2 (L) | 7.9 | 7.3 (0.6)* | 7.9 (S) | 6.5 (L) | 6.9 |
| DPSA . . . . . .  | 7.7 (L) | 8.8 | 7.9 (0.9) | 8.2 (S) | 8.5 (S) | 7.0 |
| DPSAC . . . . .  | 8.5 (L) | 10.5 | 8.6 (1.9) | 8.4 (S) | 8.1 (S) | 8.3 |

* L cells grown and infected in defined medium (D) supplemented with proline (P), serine (S), alanine (A), and 10-fold increases in choline (C).  
* PES strain progeny were assayed in mice by the intracerebral (MICLD₅₀) and intraperitoneal (MIPLD₅₀) routes and by formation of plaques, all of which were large (>2 mm). PFU, plaque-forming unit.  
* The 20t strain progeny, not lethal for mice, were assayed by formation of plaques, all of which were small (0.5 to 1 mm).  
* PES and 20t virus strains were introduced into culture as a mixed inoculum. Virus assays were performed in mice and plaques. PES viral growth was discerned by intracerebral and intraperitoneal lethality in mice and by the appearance of large (L) plaques; 20t progeny were assayed by the appearance of small (S) plaques.  
* Numerical values in parentheses represent the differences between the MICLD₅₀ and MIPLD₅₀ titers.

As our results indicated that PES viral replication was enhanced in cultures supplemented with nutritional additives and attenuated virus was not, the question arose as to whether nutritional supplements might be employed to "select" virus particles by encouraging the reproduction of these types in a mixed-virus environment. In tests of this type with VEE virus, growth of PES virus can be discerned by assays for lethality in mice by the ic and ip routes and by formation of large plaques, in contrast to 20t virus, which is not lethal for mice and which forms small plaques.

Data on the right side of Table 2 show that when PES and 20t strains were inoculated together in either nonfortified or fortified D media in suspension cultures, titers of the former were suppressed. This was most evident in medium containing all additives when ic titrations were used to assay viral growth. A PES titer of only 10⁷·³ MICLD₅₀ was obtained with the combined inoculum, but, when PES was inoculated singly (left side Table 2), a titer of 10⁷·⁸ was obtained. Furthermore, the ip titer of PES virus (10⁷·⁹ LD₅₀) and the large-plaque titer (10⁷·³) were lower in fortified D medium cultures with a mixed inoculum than were the PES titers obtained alone; when employed singly, PES inoculum produced an ip titer of 10⁷·⁸ and a plaque titer of 10⁸·¹. In contrast to the response of the PES virus, 20t virus showed little or no evidence of being adversely affected by the presence of PES virus.

DISCUSSION

Previous work showed that, although the PES strain replicated to high titers in suspension cell cultures grown in defined medium, higher titers of virus were obtained in LAH medium with or without serum (H. R. Tribble, Jr., et al., J. Gen. Virol., in press). The defined medium in the work mentioned above contained seven amino acids that were stimulatory but not essential to the growth of cells. In addition, unpublished results have shown that the PES virus replicated to higher titers with the nonessential amino acids than without them. All of these data suggested that nutritional factors other than those vital to cellular reproduction could play an important role in viral replication.

In the first series of tests, only three of the seven nonessential amino acids were stimulatory to viral growth. These were alanine, proline, and serine. Later, the effect of alanine, which had been marginal, could not be confirmed. Proline and serine, however, were confirmed repeatedly as stimulators for viral replication, which was most pronounced when they were combined. Choline enhanced viral titers that could be improved when the choline was combined with proline and serine.
The fact that viral concentrations increased as much as 10- to 100-fold in 24 hr with the nutritional additives, whereas cell proliferation increased 2-fold or less in 5 to 6 days, indicates that the increase in cell growth was not responsible for the enhanced virus yield.

The question arises as to whether nutritional factors could conceivably influence the selection of attenuated particle types that consistently have been observed during passage of VEE virus in L-cell cultures (4–6; H. J. Hearn and H. R. Tribble, Jr., Bacteriol. Proc., p. 104, 1965). In answer to this, it is interesting to note that the nutritional supplements did not necessarily induce a general increase in virus yield but rather an ic-virulent particle. One possible explanation of these data might be that perhaps preliminary steps toward the attenuation of the virus had begun, as manifested by the enhancement of only the ic-virulent virus; one of the prominent virulence characters of several attenuated VEE strains has been the relative loss of ip lethality with the retention of a substantial ic lethality (4–6, 8; H. J. Hearn and H. R. Tribble, Jr., Bacteriol. Proc., p. 104, 1965). This hypothesis, however, is not consistent with the fact that nutritional supplements appeared to provide no appreciable stimulatory effect on the growth of the attenuated 9t and 20t strains.

When it became apparent that growth of the PES strain was enhanced whereas the attenuated 9t and 20t strains were affected very little or not at all by the nutritional additives that we employed, a series of tests was performed to determine if such medium supplements might provide a selective advantage in vitro for the virulent strain in a heterogeneous viral environment. In a previous study (5), when the PES and 20t strains were introduced as a mixed inoculum into L-cell monolayers, maintained in medium containing serum, the latter multiplied more efficiently than did the former. This effect presumably was implemented by an interference by the 20t virus with the PES virus. By using a mixed inoculum in the present studies, once again it appeared that the 20t virus interfered with the PES type. It is interesting to note that suppression of the PES strain was most marked in the presence of all of the additives, whereas titers of the 20t strain continued to remain unaffected.

Although nutritional factors that may encourage the selection of attenuated virus have not been identified thus far, the data presented here do not rule out the possibility that perhaps other as yet unidentified cell nutrients could favor the selection of attenuated 9t- or 20t-type particles. Further work is needed to search for and identify medium components and, possibly, cell types that could accomplish this.

ACKNOWLEDGMENTS

We thank Kiyoshi Higuchi for careful review and thoughtful criticisms during preparation of the manuscript and Francis Foreman for able technical assistance.

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