Chromatography of Venezuelan Equine Encephalomyelitis Virus Strains on Calcium Phosphate

CARL E. PEDERSEN, JR., DONALD R. SLOCUM, AND NEIL H. LEVITT
U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

Received for publication 4 March 1972

Column chromatography of selected Venezuelan equine encephalomyelitis (VEE) viruses on calcium phosphate gel offered a simple and reproducible method for examination of biochemical characteristics and relatedness of strains within the VEE complex. Members of antigenic subgroup I demonstrated a series of elution profiles within a narrow range of 0.22 to 0.25 M phosphate buffer. Members of antigenic subgroups II, III, and IV differed substantially among themselves and viruses of antigenic subgroup I. These differences in elution behavior may contribute to understanding of observed differences in biological behavior and antigenic variation among VEE viruses.

The absorptive capacity of calcium phosphate in columns, employed initially as a simple and useful chromatographic tool for the purification of influenza virus (11), has since been adapted for use with herpesviruses (12) and adenoviruses (9). This procedure has recently been used to demonstrate different elution profiles among members of the group B arboviruses (10), vaccine and progeny strains of polioviruses (6), and plaque variants of mengovirus (7), encephalomyocarditis (2), and Sindbis viruses (1). Although the Venezuelan equine encephalomyelitis (VEE) complex represents a group of viruses exhibiting similar biological behavior, subtle antigenic variation among these viruses has been observed (14). The present study describes results obtained by calcium phosphate chromatography of selected strains of VEE virus in an attempt to examine unique properties possessed by members of this complex.

MATERIALS AND METHODS

Viruses. The viruses used in this study (Table 1) are representative of the VEE antigenic subgroups defined by Young and Johnson (14) by employing the kinetic hemagglutination inhibition technique.

Concentration of virus. Virus seed stocks were grown in 18-hr-old primary duck or chick embryo cells maintained with Medium 199 containing Earle's balanced salt solution and 0.5% human serum albumin. After 18 hr at 37 C the maintenance medium was pooled, and cell debris was removed by centrifugation at 600 × g. Stock virus preparations were concentrated and partially purified without pelleting by a modification of the method of Gruber (5). Prior to ion exchange chromatography 500-ml virus preparations were dialyzed overnight against 5 liters of distilled water. The virus was then applied to a column (4.5 by 45.0 cm) containing 15 g (dry weight) of charged diethylaminoethyl-cellulose equilibrated with 0.01 M NaCl and 0.01 M Na2HPO4 buffer, pH 7.2. The column was washed with 500 ml of phosphate buffer, and the virus was eluted with tri(hydroxymethyl)aminomethane (Tris)-saline sodium succinate buffer (TSS; 0.7 M sodium chloride, 0.08 M sodium succinate, and 0.05 M Tris) at pH 10.0. Twenty 5.0-ml fractions were collected, and tubes containing peak viral activity were pooled and then banded on 5.0 ml of 45% sucrose in TSS at 65,000 × g for 2.5 hr. The virus band was harvested and dialyzed overnight against 0.005 M phosphate buffer, pH 7.4, prior to chromatography on calcium phosphate.

Chromatographic technique. The brushite form of calcium phosphate (CaHPO4·2H2O) was prepared from 0.5 M CaCl2 and 0.5 M Na2HPO4, as described by Burness (2). The gel was stored at 4 C in 0.005 M phosphate buffer, pH 7.4, until used. Columns were prepared by pouring 75 ml of the slurry into a glass column (2.0 by 50.0 cm). After a 15-min settling period the gel was washed with 0.005 M phosphate buffer until the surface position was constant (routinely, a 25-ml packed volume). The column was checked for channeling or skewing with a band of brom cresol purple, and the amount of eluate collected before the first drop of dye appeared was considered the void volume. A 1-ml amount of the concentrated, partially purified virus (ca. 1014.5 plaque-forming units/ml) was then added to the gel, and a linear gradient of elution buffer (0.1 to 0.6 M phosphate buffer, pH 7.4) was applied. The flow rate of
the column was 0.5 to 1.0 ml/min, and 5.0-ml fractions were collected. The infectious virus titer of each fraction was determined by plaque assay.

Plaque assay. Plaque assays were performed essentially as described by Dubos and Vogt (4). Serial 10-fold dilutions of the column fractions were prepared in phosphate-buffered saline containing 1.0% normal rabbit serum, penicillin (100 units/ml), and streptomycin (50 μg/ml). A 0.1-ml amount was added to 25-cm² flasks and allowed to adsorb for 1 hr at 37°C. The cells were then overlaid with 5.0 ml of Medium 199 containing Earle’s balanced salt solution, 3.0% calf serum, 1.0% L-norarg, penicillin (100 units/ml), and streptomycin (50 μg/ml). After incubation at 37°C for 48 hr, the cells were overlaid with 2.0 ml of neutral red (0.1 mg/ml) in Hank's balanced salt solution to facilitate reading of plaques.

Hemagglutination technique. Hemagglutination tests were performed by the method of Clarke and Casals (3).

RESULTS

Column chromatography utilizing calcium phosphate provided a simple method for evaluating members of the VEE complex. In our procedure 1.0-ml samples of virus applied to 25 ml of packed gel provided reproducible chromatographic separation, good flow rates, and routine recovery of at least 90% of the virus. Figure 1 illustrates the results of chromatography with the TC-83 vaccine strain of VEE virus. This pattern is representative of the antigenic subgroup I VEE viruses examined. Both virus and hemagglutinin activity eluted in a sharp peak which was associated with a minor peak of optical density. The phosphate molarity, which corresponded with maximal infectivity of VEE viruses, is shown in Table 2. We found that antigenic subgroup I varieties elicited virus peaks in a very narrow range which suggests an intimate biochemical relationship.

Members of antigenic subgroups II, III, and IV exhibited unique profiles. Examination of Fe 3-7c (subgroup II) exhibited two peaks of virus infectivity (Fig. 2), which suggested a differentiation of plaque size variants. Figure 3 represents the combined elution profiles of Mucambo, Pixuna, and Mena II viruses. These patterns indicate different surface properties among these strains. Mena II is a member of subgroup Ie, whereas Mucambo and Pixuna belong to subgroups III and IV, respectively.

DISCUSSION

The VEE complex represents a large number of serologically related viruses distinguished primarily by geographic origin, source of the isolate, and by their effect on various animal species. A degree of cross-protection and similar biological behavior offer evidence of interrelatedness between members of this complex (8, 13). Antigenic variation, possibly due to geographic distribution or evolution of the viruses, serves to distinguish the strains (14). Adsorption to and subsequent differential elution of virus populations from calcium phosphate gels most likely involves properties unique to each prototype virus, which may be a reflection of the charge of the viral mem-

### TABLE 1. Venezuelan equine encephalomyelitis viruses studied

| Antigenic subgroup | Virus strain | Geographic origin | Year isolated | Original host | Source |
|--------------------|--------------|-------------------|---------------|---------------|--------|
| Ia                 | Trinidad     | Trinidad          | 1943          | Donkey brain  | USAMRIID* |
| Ib                 | TC-83 (vaccine) | Maryland          | 1961          | Guinea pig heart cells | USAMRIID |
|                    | Ica          | Peru              | 1946          | Equine        | USAMRIID |
|                    | 9859         | Texas             | 1971          | Mosquito (Porophora confinnis) | USAMRIID |
| Ic                 | P-676        | Venezuela         | 1963          | Human         | MARU* |
| Id                 | 3880         | Panama            | 1961          | Human         | MARU |
| Ie                 | Mena II      | Panama            | 1962          | Human         | MARU |
| II                 | Fe 3-7c      | Florida           | 1963          | Mosquito (Culex [Melanocoton] spp.) | YARU* |
| III                | Mucambo      | Brazil            | 1954          | Monkey (Cebus apella) | ATCC |
| IV                 | Pixuna (BeAn8) | Brazil           | 1961          | Mosquito (Anopheles nimbue) | ATCC |

* Based on classification of Young and Johnson (14).
* U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Md.
* Middle America Research Unit, Balboa Heights, Canal Zone.
* Yale Arbovirus Research Unit, New Haven, Conn.
* American Type Culture Collection, Rockville, Md.

### Table 2.

| Subgroup | Virus strain | Geographic origin | Year isolated | Original host | Source |
|----------|--------------|-------------------|---------------|---------------|--------|
| Ia       | TC-83        | Trinidad          | 1943          | Donkey brain  | USAMRIID* |
| Ib       | TC-83 (vaccine) | Maryland          | 1961          | Guinea pig heart cells | USAMRIID |
| Ic       | P-676        | Venezuela         | 1963          | Human         | MARU* |
| Id       | 3880         | Panama            | 1961          | Human         | MARU |
| Ie       | Mena II      | Panama            | 1962          | Human         | MARU |
| II       | Fe 3-7c      | Florida           | 1963          | Mosquito (Culex [Melanocoton] spp.) | YARU* |
| III      | Mucambo      | Brazil            | 1954          | Monkey (Cebus apella) | ATCC |
| IV       | Pixuna (BeAn8) | Brazil           | 1961          | Mosquito (Anopheles nimbue) | ATCC |
Calcium phosphate chromatography was selected for purification of selected members of the VEE complex to study their biochemical properties. We observed that VEE viruses were amenable to elution from the gel by a linear gradient (0.1 to 0.6 M) of phosphate buffer, pH 7.4. The virus strains examined were eluted within a range of 0.19 to 0.36 M phosphate. Among the members of antigenic subgroup I, virus peaks were detected within very narrow limits (0.22 to 0.25 M). This finding reinforces the serological relatedness of these viruses (14). Spertzel and McKinney (in press) have reported that a Ib strain was responsible for the outbreak of VEE among equines in the United States during 1971. We found that a VEE isolate from mosquitoes collected in Texas during the epizootic eluted in the same manner as the subgroup I VEE viruses. In utilizing our chromatographic technique we have not succeeded in clearly differentiating the attenuated TC-83 strain from the parental, virulent Ia (Trinidad) strain.

The Fe 3-7c prototype virus (antigenic subgroup II) gave an elution pattern which exhibited a bifurcated peak. We have found that this virus population consisted of approximately equal numbers of small and large plaque-forming viruses (unpublished data). The first peak (0.19 M) consisted primarily of large plaque variants while the second peak (0.30 M) consisted primarily of small plaque variants. This pattern is consistent with data presented by other investigators (1, 2, 7) studying plaque variants. Studies are currently underway in this laboratory to further characterize these variants.

Mucambo and Pixuna viruses exhibit substantial differences from other members of the VEE virus complex. Although Scherer and Pancake (8) found cross-protection in hamsters inoculated with Mucambo, Pixuna, and a Mexican isolate of VEE, Young and Johnson (13) showed that Mucambo and Pixuna do not confer complete protection to rodents subsequently challenged with a member of antigenic group I (3880); in addition, these investigators also reported that the ecology of Mucambo and
Table 2. Phosphate molarity of peak virus infectivity

| Antigenic subgroup | Virus strain | Fraction no. | Phosphate molarity |
|-------------------|--------------|--------------|--------------------|
| Ia                 | Trinidad     | 20           | 0.25               |
| Ib                 | TC-83        | 18           | 0.23               |
| Ic                 | Ica          | 20           | 0.25               |
| Ic                 | 9859         | 20           | 0.25               |
| Id                 | P-676        | 19           | 0.24               |
| Ie                 | 3880         | 17           | 0.22               |
| IIp                | Mena II      | 18           | 0.23               |
| III                | Fe 3-7c      | 14           | 0.19               |
| III                | Mucambo      | 14           | 0.23               |
| IV                 | Pixuna       | 27           | 0.32               |

Antigenic groupings are based on studies by Young and Johnson (14).

Two peaks of virus infectivity have been observed.

Pixuna differed from other members of the VEE virus complex. The molarity of phosphate at which viruses elute appears to differ according to the nature of the virus tested (11). Our results suggest that Mucambo and Pixuna do indeed differ from the viruses of serological subgroup I. The difference might reside in protein composition, possibly in terms of amino acid sequence or unique amino acids (1, 2); however, the exact nature of this difference, as expressed by elution on calcium phosphate, remains to be elucidated.

We have extended the use of calcium phosphate chromatography to the study of the VEE complex of group A arboviruses. Elution characteristics of members of this complex offer another means to investigate the interrelationships of these viruses. In addition the virus product obtained from a linear elution gradient may be utilized for detailed physical and chemical analyses.

LITERATURE CITED

1. Bose, H. R., G. Z. Carl, and B. P. Sagik. 1970. Separation of Sindbis virus plaque-type variants by calcium phosphate chromatography. Arch. Gesamte Virusforsch. 29:83-89.
2. Burness, A. T. H. 1967. Separation of plaque-type variants of encephalomyocarditis virus by chromatography on calcium phosphate. J. Virol. 1:308-316.
3. Clarke, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer. J. Trop. Med. Hyg. 7: 561-569.
4. Dulbecco, R., and M. Vogt. 1954. One step growth curve
of Western equine encephalitis on chicken embryo cells grown in vitro and analysis of virus yields from single cells. J. Exp. Med. 99:183-199.

5. Gruber, J. 1970. Purification, concentration, and inactivation of Venezuelan equine encephalitis virus. Appl. Microbiol. 20:427-432.

6. Ozaki, Y., A. B. Diwan, M. Takizawa, and J. L. Melnick. 1965. Chromatography of poliovirus on calcium phosphate and its application to the identification of vaccine progeny strains. J. Bacteriol. 89:603-610.

7. Scraba, D. G., P. Hostvedt, and J. S. Colter. 1969. Physical and chemical studies of mengo virus variants. II. Chromatographic behavior and chemical composition. Can. J. Biochem. 47:165-171.

8. Sherer, W. F., and B. A. Pancake. 1970. Cross protection among viruses of the Venezuelan equine encephalitis complex in hamsters. Amer. J. Epidemiol. 91:225-229.

9. Simon, M. 1962. Chromatography of adenoviruses on calcium phosphate columns. Acta Virol. (Prague) 6:302-308.

10. Smith, C. E. G., and D. Holt. 1961. Chromatography of arthropod borne viruses on calcium phosphate columns. Bull. W.H.O. 24:749-759.

11. Taverne, J., J. H. Marshall, and F. Fulton. 1968. The purification and concentration of viruses and virus soluble antigens on calcium phosphate. J. Gen. Microbiol. 19:451-461.

12. Taverne, J., and P. Widly. 1959. Purification on herpes simplex virus by chromatography of calcium phosphate. Nature (London) 184:155-156.

13. Young, N. A., and K. M. Johnson. 1969. Viruses of the Venezuelan equine encephalomyelitis complex. Infection and cross challenge of rodents with VEE, Mu-cambo, and Fixuna viruses. Amer. J. Trop. Med. Hyg. 18:280-289.

14. Young, H. A., and K. M. Johnson. 1969. Antigenic variants of Venezuelan equine encephalitis virus: their geographic distribution and epidemiologic significance. Amer. J. Epidemiol. 89:286-307.