The Separation and Physicochemical Properties of the C and D Antigens of Coxsackievirus

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*J Immunol* 1965; 95:818-822; http://www.jimmunol.org/content/95/5/818
THE SEPARATION AND PHYSICOCHEMICAL PROPERTIES OF THE C AND D ANTIGENS OF COXSACKIEVIRUS

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Received for publication March 17, 1965

The purification (1, 2), physical and chemical properties (3) and serologic attributes (2, 4, 5) of the C and D components of poliomyelitis virus have received extensive study. Although there have been reasons to suspect that similar components characterize preparations of the other enteroviruses, this area of investigation has received little attention.

Two interesting problems arising out of the laboratory identification of the coxsackievirus types prompted the present study of the separable entities of the crude tissue antigens. In the first place, complement fixation tests for the type identification of the coxsackieviruses are not feasible because of the extreme heterotypic reactivity of human sera with the crude tissue culture antigen (6, 7). Secondly, it has been shown in gel-diffusion studies of human sera (8) that two lines of precipitin are formed to the crude coxsackie antigens, one of which constitutes a “group” precipitate and the other, a “specific” line demonstrable only with the infecting antigen type.

This article deals with the separation, by centrifugation in sucrose and cesium chloride gradients, of the two antigens of coxsackie B-5 virus and a comparison of their physicochemical properties to the C and D antigens of poliomyelitis virus, Type 1. The separation of the antigens of other coxsackievirus types and a more complete description of their serologic properties have been reported (9).

MATERIALS AND METHODS

Production of virus antigens. Poliomyelitis virus, Type 1 (Mahoney), and coxsackievirus, Type B-5 (Hendricks), were prepared in monolayers of HeLa cells and concentrated 100 to 200-fold by ultracentrifugation (8).

Purification by density gradient centrifugation. In order to visualize the bands it was necessary to remove much of the host material by one extraction with butanol (1). The aqueous fraction was then centrifuged in a gradient of sucrose (1) for 2 hr at 30,000 rpm in the Spincos Bucket Rotor SW-39, or in cesium chloride of initial density 1.348/ml for 22 hr at 35,000 rpm (10). The fractions were collected through a puncture in the bottom of the Lusteroid tube. Five unequal fractions, designated A, B, C, D and E were obtained from the sucrose density gradient. The cesium chloride gradient was fractionated into seven unequal aliquots, designated 1 to 7.

Complement fixation test. Hyperimmune monkey serum to poliomyelitis virus, Type 1, and coxsackievirus, Type B-5, was used in a standard complement fixation test employed for enteroviruses (11). The complement fixation titers reported in this article represent the limiting antigen dilution required to fix 2 units of complement at a serum dilution of 1:32.

Infectivity assay. Total infectivity titers were calculated as the product of TCD50/ml, determined in HeLa D tissue culture tubes (10), and the volume of the fraction in milliliters.

Electron microscopy. Preparations of the viral materials were dialyzed against 2% ammonium acetate, after which they were applied as gross droplets to grids which were subsequently shadowed with uranium.

Ultraviolet absorption analysis. Preparations of the viral components, dialyzed against 1% NaCl, were analyzed in the Beckman DK-2 automatic recording spectrophotometer.

Ribonucleic acid (RNA) analysis. The orcinol test of Umbreit et al. (12) was used.

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TABLE I

Complement fixation and infectivity titer of coxsackie, Type B-5, and poliomyelitis, Type 1, viral antigens separated by centrifugation in sucrose and cesium chloride density gradients

RESULTS

Fractionation in density gradients. The behavior of the antigens of poliomyelitis virus, Type 1, and coxsackievirus, Type B-5, in sucrose and cesium chloride gradients is seen from Table I to be very similar. In the sucrose gradient, the D antigen of both viruses possesses the principal infectivity and complement-fixing titer, while the C fractions are considerably less infectious but are nevertheless associated with appreciable complement fixation titers. The infectivity of the C fraction of both poliomyelitis Type 1 virus and coxsackie B-5 virus was reduced 2 to 4 log₁₀ units by refractionation in the same system with, however, only a negligible decrease of the complement fixation titer. Neither the infectivity nor the complement fixation titer of fraction D of both viruses is markedly affected by recentrifugation in a sucrose density gradient.

A similar separation of two antigens, both of which fix complement but which differ markedly in infectivity, was also effected by centrifugation in a cesium chloride density gradient (Table I).
It was shown that Fraction D (sucrose) of both viruses was equivalent to Fraction 4 (cesium chloride) by submitting a preparation of the D component of poliomyelitis virus and coxsackie B-5 virus to recentrifugation in a cesium chloride gradient. Only one light-scattering band was observed throughout the column in Fraction 4; in addition, virtually all of the complement-fixing activity and infectivity was also associated with Fraction 4. Fraction C of poliomyelitis Type 1 virus and coxsackie B-5 virus, in the same kind of experiment, was shown to equilibrate entirely in Fraction 6.

Fraction C (sucrose) of poliomyelitis and coxsackie B-5 viruses was, however, more pure than Fraction 6 (cesium chloride) in regard to viral antigen, since the normal HeLa material, which is barely discernible in the photographs (Table I) as a diffuse light-scattering band in Fraction B of the sucrose gradient, equilibrates with Fraction 6 in the cesium chloride gradient. It was for this reason that preparations of the D and, particularly, the C fractions of coxsackieviruses for electron microscopy, ultraviolet absorption analysis, and RNA assay were first fractionated in the sucrose gradient and then submitted to repurification in the cesium density gradient. The association of the normal HeLa material with the viral antigen in Fraction 6 (see Table I) imparts an added intensity of light scattering to this band with respect to that of Fraction 4, which is not proportional either to the ratio of intensity of light scattering of Fractions C to D in the sucrose gradient or to the ratio of the complement-fixing titers of C to D or 4 to 6.

However, the separation of the two antigens of both the poliomyelitis virus and coxsackie B-5 virus with regard to infectivity was superior in the cesium chloride gradient owing to the greater separation of the bands. In addition, gel-diffusion assays have shown the greater purity of Fractions 4 and 6 of poliomyelitis virus relative to Fractions C and D from a single centrifugation in a sucrose density gradient.

The cesium chloride density gradients were centrifuged in these experiments, as noted, for 22 hr at 70,000 × G. In other separations, particularly those involved in the fractionation of the antigens of the other coxsackieviruses for a previous investigation (9), the centrifugations were performed at 70,000 × G for 16 to 20 hr. Under those circumstances the virus band, Fraction 4, is displaced slightly more toward the center of the tube and the less dense C antigen is found principally in Fraction 7 and, to a lesser degree, in Fraction 6.

The complement-fixing titers of Fractions C and 6 of three preparations of poliomyelitis virus, Types 1, 2 and 3, were found, in agreement with the report of Mayer et al. (2), to constitute only 10% of that present in Fractions D and 4. However, the complement fixation titer of Fractions C and 6 of the coxsackie B-5 virus, as shown in Table I and in an accompanying investigation (9), amount to 30 to 50% of those in Fractions D and 4.

**Effect of heating Fraction D of Coxsackie B-5 virus.** A preparation of Fraction D of coxsackievirus, Type B-5, which had been dialyzed against 0.85% NaCl, 0.1 M phosphate buffer, pH 7.0, was heated at 60°C for 20 min. Upon recentrifugation in a sucrose density gradient, the heated Fraction D retained the sedimentation velocity of Fraction D. When introduced into a 40% cesium chloride solution, the heated D fraction of coxsackie B-5 virus precipitated and denatured; the complement fixation activity and infectivity were lost. It has also been shown that the D fraction of coxsackie B-5 virus became heterotypic in complement fixation assay after heating at 60°C for 20 min (9).

**Electron micrographs.** The preparation of Fraction D (sucrose)-Fraction 4 (cesium chloride) of coxsackievirus, Type B-5, was shown to consist of spherical particles of diameter 30 ± 3 mµ (Table II). The particles comprising Fraction C (sucrose)-Fraction 6 (cesium chloride) of coxsackie B-5 virus were very similar but, like the C component of poliomyelitis virus (3), showed somewhat more flattening.

**Ultraviolet absorption spectra and RNA analysis.** The high content of 22% RNA in Fraction D-Fraction 4 of coxsackievirus, Type B-5, was shown to consist of spherical particles of diameter 30 ± 3 mµ (Table II). The particles comprising Fraction C (sucrose)-Fraction 6 (cesium chloride) of coxsackie B-5 virus were very similar but, like the C component of poliomyelitis virus (3), showed somewhat more flattening.

**Other enteroviruses.** Similar components have also been noted in preparations of echoviruses, Types 7 and 11.
TABLE II

Physicochemical properties of the C and D antigens of coxsackie, Type B-5, and poliomyelitis, Type 1, viruses

<table>
<thead>
<tr>
<th>Property</th>
<th>Component D</th>
<th>Component C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cox. B-5</td>
<td>Polio 1</td>
</tr>
<tr>
<td>RNA</td>
<td>22%</td>
<td>24%</td>
</tr>
<tr>
<td>Principal absorption band</td>
<td>282 mμ</td>
<td>260 mμ</td>
</tr>
<tr>
<td>OD260 mμ/OD280 mμ</td>
<td>1.68</td>
<td>1.61</td>
</tr>
<tr>
<td>Particle diameter</td>
<td>30 ± 3 mμ</td>
<td>28 ± 3 mμ</td>
</tr>
<tr>
<td></td>
<td>30 ± 3 mμ</td>
<td>30 ± 3 mμ</td>
</tr>
<tr>
<td>Packed arrays</td>
<td>40 ± 4 mμ</td>
<td>45 ± 6 mμ</td>
</tr>
<tr>
<td>Single particles</td>
<td></td>
<td>40 ± 3 mμ</td>
</tr>
</tbody>
</table>

* Reference (3).

DISCUSSION

The lower density of the C component of the poliomyelitis and coxsackieviruses, due to the absence, or negligible amount, of RNA, results in a clear-cut separation of this antigen from the denser D component in the cesium chloride gradient. It was also observed that the C components banded sharply in the cesium chloride gradient, indicating no appreciable spread in the density of the C antigens of both poliomyelitis, Type 1, and coxsackie B-5 viruses.

Centrifugation in cesium chloride density gradient was shown to be superior to centrifugation in sucrose density gradient for the routine preparation of C and D antigens of poliomyelitis and coxsackie B-5 viruses for serologic purposes. The cesium chloride gradient is simpler to prepare although it requires a longer running time in the ultracentrifuge. However, in one centrifugation, the separation of the infectivity and the antigens is more clear-cut in the cesium chloride density gradient than in the sucrose gradient.

The association of large amounts of complement-fixing activity with the C fraction of coxsackie B-5 virus has several implications with regard to recent serologic findings. The heterotypic response of human sera to tissue culture antigens of coxsackieviruses is probably due to the presence of large amounts of the C fraction which has been shown to be heterotypic in both complement fixation and gel-diffusion studies.

The demonstration that heating of the D fraction of coxsackie B-5 virus does not affect its sedimentation velocity, but does change its serologic specificity (9), is in agreement with earlier findings pertaining to the D antigen of poliomyelitis virus. It is interesting that heated D component of coxsackie B-5 virus as well as poliomyelitis virus denatures when exposed to the high concentration of cesium chloride, a phenomenon which probably relates to some "damage" to the virus particles at 60°C.

Occasionally the Fraction 4 of some of the coxsackieviruses (9) has given rise to both "group" and "type" specific lines in gel-diffusion assay. This finding may not reflect so much on the efficiency of the fractionation as it might result from the presence of "damaged" antigens in Fraction 4.

SUMMARY

The two antigens of coxsackievirus, Type B-5, separated by centrifugation in sucrose and cesium chloride density gradients, were shown to be very similar to the C and D antigens of poliomyelitis virus in their infectivity, complement-fixing properties, particle size and shape, and nucleic acid content. Certain practical aspects of the efficiency of separation of the C and D antigens in sucrose and cesium chloride density gradients were also discussed.

REFERENCES