Density of Infectious Virus and Complement-Fixing Antigens of Two Rhinovirus Strains

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ABSTRACT

DANS, P. E. (National Institute of Allergy and Infectious Diseases, Bethesda, Md.), B. R. FORSYTH, AND R. M. CHANOCK. Density of infectious virus and complement-fixing antigens of two rhinovirus strains. *J. Bacteriol.* 91:1605-1611. 1966.—Two rhinovirus serotypes (echovirus 28 and HGP) and poliovirus type 1 were banded by isopycnic centrifugation in cesium chloride. The rhinovirus virions had a density of 1.41 g/ml, whereas that of poliovirus was 1.34. Since a number of other enteroviruses also have a density of 1.34 g/ml in cesium chloride, a basic difference in density may exist between the rhinovirus and enterovirus subgroups of the picornavirus family. Whether this difference reflects differences in ribonucleic acid content or binding of cesium ions remains to be determined. In tests with echovirus 28 two peaks of CF activity were detected: one in association with the virion (1.41 g/ml), and a larger peak of lower density (1.30 g/ml). With echovirus 28 antiserum, a heterotypically reactive complement-fixing (CF) antigen was detected in the HGP virus suspension at a density less than that of the virion (1.30 g/ml). This antigen corresponded in density to the less dense CF antigen of echovirus 28.

Rhinoviruses are the agents most frequently isolated from adults with mild upper respiratory tract illness (2, 7, 19). More than 70 serologically distinct strains have been identified by the neutralization technique (7, 9, 19, 28). The rhinoviruses have been classified with the enteroviruses in the family of picornaviruses on the basis of their small size (diameter of 30 m μ or less), ether resistance, and ribonucleic acid (RNA) composition (29). Rhinoviruses differ from the enteroviruses, however, by being labile at pH 3.0 to 5.0 (14). There is one report of the density of a rhinovirus strain being "that of poliovirus (1.3)" (4).

Other than this one report, little is known of the density of the rhinoviruses. It was considered important to determine this property to better understand the relationship of rhinoviruses to other picornaviruses, and to obtain information which might be helpful in preparing multivalent vaccines for prevention of mild upper respiratory tract disease caused by these agents. We were also interested in determining the density of the antigens from infected tissue culture which fix complement with specific antiserum. This report will describe preliminary studies of two rhinovirus strains, with the use of modifications of concen-

tration and density gradient techniques previously used for the study of enteroviruses (3, 16).

MATERIALS AND METHODS

Virus strains. Strain 52992 of echovirus 28 was isolated from a throat swab specimen obtained from a marine recruit with upper respiratory illness at Camp Lejeune (2, 13). This strain was isolated in diploid fibroblast (WI-26) cultures and then passaged six times in KB tissue culture.

Strain HGP was obtained from the Common Cold Research Unit, Salisbury, Great Britain (28). It was passaged eight times in human embryonic kidney tissue culture, twice in rhesus monkey kidney tissue culture, four times in KB tissue culture, twice in human diploid fibroblast (WI-26) cultures, and once in HEp-2 tissue cultures.

Poliovirus type 1 (LSC Sabin strain, live attenuated) was passaged once in monkey kidney tissue culture and once in HEp-2 tissue culture (22).

Tissue culture. HEp-2 cells were grown in 32-oz bottles containing 75 ml of Eagle's basal medium with 100 units of penicillin per ml, 100 μ g of streptomycin per ml, and 10% calf serum. When the cell monolayer was confluent, it was washed twice with Hanks' balanced salt solution. A 75-ml amount of Eagle's minimal essential medium, with 2 mmoles of glutamine per 100 ml, 100 μ g of streptomycin per ml, 100 units of penicillin per ml, and 5% inactivated chicken serum, was then added.

Growth of virus. Each bottle was infected with 10^6 to 10^7 median tissue culture doses (TCD_{50}). The bottles were then observed daily for cytopathic effects (CPE), which were generally complete by the 3rd to 4th day. The cells and fluid were then frozen and thawed three times, and the contents of 8 to 12 bottles were pooled and clarified by centrifugation at 2,000 rev/min for 20 min at 4 C in an International PR 2 centrifuge. The supernatant fluid, which constituted the starting material, was stored at -20 C.

Concentration of virus. A 1-ml amount of octyl alcohol was added for each 500 ml of starting material, and 10-fold concentration was achieved by flash evaporation in vacuo at 25 C. Concentration of 1,000 ml required an average of 1.5 to 2 hr. The final pH of the evaporated material was 8.5. This material was centrifuged in a PR 2 International centrifuge at 2,000 rev/min for 20 min at 4 C. The supernatant fluid was then centrifuged at $93,000 \times g$ for 3 hr at 4 C. The pelleted material was resuspended in phosphate-buffered saline (pH 7.2) to 1/40th of the original volume. This material was then sonically treated in a Raytheon sonic oscillator (250 w) for 10 to 15 min at 10 kc.

Density gradient centrifugation. A 6.6-ml amount of the sonic-treated material was mixed with 5.4 ml of a saturated solution of cesium chloride (density, 1.9) and 3 ml of 10% human or bovine serum albumin. Samples (5 ml) of this mixture were added to cellulose nitrate tubes which were then placed in the cups of a SW-39 swinging-bucket rotor. Mineral oil was used as an overlay. Centrifugation was then carried out at $111,000 \times g$ for approximately 70 hr at 4 C.

After centrifugation, the tubes were examined for the presence of bands, by use of transmitted light. Any visible bands were noted and measured. Fractions of 0.25 or 0.5 ml were collected after puncturing the bottom of the tube.

Density measurement. A 100- μ liter amount of each fraction was weighed in a volumetric micropipette. All densities were recorded in grams per milliliter.

Infectivity assay. A 0.1-ml amount of each fraction was diluted 10-fold in Hanks' balanced salt solution containing 0.5% gelatin. Titrations were carried out in WI-26 or WI-38 roller-tube cultures maintained with half-strength Eagle's medium and half-strength medium 199 with 100 units of penicillin, 100 μ g of streptomycin per ml, 2 mmoles of glutamine, and 2% calf serum. Four cultures were inoculated with each decimal dilution, and the titer in TCD_{50} was calculated by the formula of Reed and Muench (21). In certain instances the plaque technique was used to assay infectivity. This always followed a preliminary infectivity assay in which four roller-tube cultures of human diploid fibroblast were used for each decimal dilution. Based upon this titer, a single dilution calculated to contain 20 to 30 plaque-forming units (PFU) was used to inoculate three human diploid fibroblast petri dish cultures. The mean number of plaques on the three replicate petri dish cultures was used to calculate infectivity titer. An agar overlay was used which contained 400 mg of diethylaminoethyl (DEAE)-dextran per ml.

Samples of the original tissue culture harvest mate-

rial, flash-evaporated specimens, resuspended pellet, and supernatant fluid after ultracentrifugation were also assayed for infectivity.

Complement-fixation (CF) test. A 1:5 dilution of each fraction was made in phosphate-buffered saline. These were placed in cellulose tubing and dialyzed overnight at 4 C against isotonic sodium chloride solution to eliminate anticomplementary effects of cesium chloride. The CF test was performed with 0.025-ml volumes of antigen and antiserum in microtiter plates, by use of 1.7 units of complement and 4 units of antibody (26). The sera were: NIH Reference Reagent monkey antiserum (echovirus 28-2060) made by inoculating virus grown in monkey kidney tissue culture into monkeys, and rabbit antiserum for type 1 poliovirus kindly supplied by Leon Levintow (24). Neither serum fixed complement with a suspension of uninfected KB cells. All values were expressed in CF units per milliliter. The reciprocal of the highest dilution which produced 75 to 100% fixation was considered as the number of CF units in 0.025 ml.

RESULTS

Density of the virion and CF antigens of echovirus 28. To determine the density and CF activity of the virion and of other virus-specific antigens, it was necessary to concentrate a large amount of infected tissue culture fluid to a convenient working volume. A 1-liter amount of infected tissue culture fluid with a titer of $10^{6.2}$ infectious particles per milliliter was concentrated 10-fold by flash evaporation. In the experiment shown in Table 1, this procedure resulted in an approximate threefold loss of infectivity, and a one-third loss of CF activity. A 30-fold loss in infectivity occurred during centrifugation at $93,000 \times g$ for 3 hr, whereas no loss in CF activity was noted. This suggests that a significant proportion of the virions were rendered noninfectious by our method of sedimentation; however, particles so affected still fixed complement in the presence of specific antibody. In addition, a significant quantity of CF antigen was detected in the supernatant fluid. This suggests that particles other than the virion (empty capsids or capsomeres) were present in the supernatant fluid.

The resuspended pellet was then mixed with cesium chloride and albumin and subjected to density gradient centrifugation. We had previously determined that echovirus 28 was stable in cesium chloride, but, to insure stability, a 10% solution of human or bovine serum albumin was added. Fractions of 0.5 ml were removed from the gradient by the drop collection method. After centrifugation at $110,000 \times g$ for 70 hr at 4 C, 18% of the input infectivity was recovered from the gradient. As shown in Fig. 1, the peak infectivity was present in fractions of density 1.39 and 1.42. The infectivity of the peak frac-

TABLE 1. Recovery of infectivity and CF antigens during concentration and density gradient centrifugation of rhinovirus echovirus 28

Step	Prepn	Total virus infectivity		Total CF activity	
		PFU (log ₁₀)	Per cent of starting material	CF antigen units (log ₁₀)	Per cent of starting material
1	Starting material	9.2	100	4.8	100
2	Supernatant fluid (2,000 rev/min) of flash-evaporated concentrate (10 ×)	8.7	33	4.6	63
3	Resuspended pellet after ultracentrifugation at 93,000 × g	7.7	3.3	4.3	33
3	Supernatant fluid after ultracentrifugation at 93,000 × g	6.4	0.2	4.3	33
4	Fractions from density gradient centrifugation	6.9	0.6	4.0	16

tions shown in Fig. 1 ranged from $10^{5.1}$ to $10^{5.2}$ PFU per fraction. The infectivity of the peak fractions in the two other tubes ranged from $10^{5.8}$ to $10^{6.2}$.

CF activity was recovered in two peaks: approximately 30% (or 1,200 CF units per ml) was associated with the infectivity peak, and 60% (or 2,400 units per ml) was present in two fractions with an average density of 1.30. Approximately one-half of the input CF activity was recovered from the gradient (Table 1).

Figure 2 illustrates the results obtained during a confirmatory experiment with echovirus 28. Fractions in this experiment were collected by the drop method in volumes of 0.25 ml; 99% of the infectivity was present in three fractions of density 1.38, 1.42, and 1.44, the average density being 1.41. The infectivity titers were $10^{3.8}$ to $10^{4.8}$ PFU per fraction. The CF activity was present in two peaks—one associated with the infectivity peak (16% = 800 CF units per ml) and the other (75% = 3,200 CF units) detected in two fractions with densities 1.29 and 1.31 (average, 1.30).

Uninfected HEP-2 tissue culture control material was concentrated and centrifuged in a cesium chloride gradient, by use of the same procedure outlined above. None of the density gradient fractions fixed complement with the echovirus 28 monkey antiserum used in these experiments.

Density of the virion and CF antigens of HGP. The density of a number of enteroviruses in cesium chloride has been found to be approximately 1.34 (Table 2). For this reason, we decided to determine the density of another rhinovirus (HGP) to ascertain whether echovirus 28 was unusual in having a high density in cesium chloride. The same procedure outlined for echovirus 28 was carried out with HGP virus. After density gradient centrifugation, 0.25-ml fractions

FIG. 1. Density of infectious virus and CF antigen of rhinovirus echovirus 28 in cesium chloride.

were collected; 99% of the infectivity was recovered in three fractions of densities from 1.38 to 1.43 (Fig. 3). The maximal infectivity was in a fraction with a density of 1.41. A second experiment with HGP virus yielded similar results.

CF activity was determined with heterotypic hyperimmune antiserum (echovirus 28), because homotypic antiserum with CF antibodies was not available. CF activity was recovered in three fractions of density 1.28 to 1.31 (average, 1.30).

The peak fraction had a density of 1.31 and contained 1,600 CF units per ml.

Density of virion and CF antigen of poliovirus 1. To insure the comparability of our method with that of other investigators, we determined the density of poliovirus by use of the experimental

FIG. 2. Density of infectious virus and CF antigen of rhinovirus echovirus 28 in cesium chloride.

technique employed for echovirus 28 and HGP viruses (Fig. 4). Echovirus 28 and poliovirus type 1 were mixed individually with cesium chloride and albumin, placed in separate tubes, and centrifuged simultaneously in the same rotor. The peak infectivity of echovirus 28 was detected in a fraction with a density of 1.42, and that of poliovirus in two fractions of density 1.34 and 1.31. In a subsequent experiment, echovirus 28 infectivity was recovered in fractions with densities of 1.40 and 1.42, whereas poliovirus was recovered in fractions with densities of 1.33 and 1.35. In both experiments, as noted previously, the CF activity of echovirus 28 was found in two peaks, one corresponding to the density of the virion (1.41) and the other to a density of most proteins in cesium chloride (1.30). In contrast, the CF activity of poliovirus was detected in a single peak with a density of 1.29 to 1.34. Echovirus 28 fractions did not react in the CF test with poliovirus antiserum, or vice versa.

DISCUSSION

Rhinoviruses and enteroviruses constitute the major subgroups of the family of picornaviruses (11). The observation that rhinoviruses are labile at pH 3.0 to 5.0, whereas enteroviruses are stable in this pH range, has been used as the basis for separating these two subgroups (14, 29). Until

TABLE 2. Reported density of picornaviruses in cesium chloride

Group	Virus	Density	Reference
Enterovirus	Poliovirus I	1.33	16
		1.34	5, 17, 23
	Poliovirus II	1.34	17, 23
		1.32	20
	Poliovirus III	1.34	23
	Echovirus 4	1.35	12
	Echovirus 7	1.34	23
		1.36	18
	Echovirus 12	1.29 ^a	8
		1.33 ^b	8
	Echovirus 19	1.29 ^c	5
		1.34 ^b	5
	Coxsackievirus A-9	1.34	17
	Coxsackievirus A-10	1.34	17
Rhinovirus	Echovirus 28	1.41	— ^d
	HGP	1.41	— ^d
FMDV	Group A	1.43	27

^a Empty capsid.

^b Infectious virus.

^c Noninfectious hemagglutinin.

^d This report.

FIG. 3. Density of infectious virus and CF antigen of rhinovirus HGP in cesium chloride.

now, acid lability has been the only unique biophysical property of the rhinovirus subgroup of picornaviruses. This report describes another biophysical property that may distinguish rhinoviruses from enteroviruses. The two rhinoviruses studied, echovirus 28 and HGP, had a density of 1.41 g/ml, in cesium chloride, whereas all reported enteroviruses have a density of 1.32 to 1.36 g/ml in this medium (Table 2; 5, 6, 8, 12, 16-18, 20, 23). This difference in density should be confirmed and extended to include other rhinoviruses and enteroviruses before it can be said to represent a basic biophysical difference.

The estimates of rhinovirus density obtained in these experiments were based upon analyses of virus concentrates containing only 3% of the original virus infectivity. During subsequent experiments in which approximately 50% of the virus infectivity was recovered after concentration, the same density (range, 1.39 to 1.41 g/ml) was obtained for the echovirus 28 virion. For this reason, the values reported in this paper probably represent reliable estimates of the density of the original viral population rather than that of a minority population of stable particles.

The difference in densities between the two rhinoviruses studied and a number of enteroviruses may represent differences in nucleic acid or protein content. Since these viruses consist of RNA and protein, an increased percentage of RNA could result in a higher density (31). However, a difference in density may represent a different degree of interaction between viruses of these two subgroups and cesium chloride. The binding of water and cesium ions by viruses is known to alter their densities (31). In this regard, it is of interest that the density of poliovirus in cesium

FIG. 4. Densities in cesium chloride of infectious poliovirus and rhinovirus echovirus 28 after centrifugation simultaneously.

chloride (1.34) is very close to its calculated hydrated density (1.32), indicating that there is no appreciable binding of cesium ions (23). Experiments involving isopycnic banding in other density gradient materials and direct chemical analysis of purified rhinoviruses are required before the basis for the high density of these viruses can be understood.

The high density recorded for the two rhinovirus strains (1.41) is quite close to the density of foot-and-mouth disease virus (FMDV) in cesium chloride (1.43) (27). This, coupled with the fact that this virus is also labile at pH 3.0 to 5.0, suggests the possibility that rhinoviruses are more closely related to this subgroup of picornaviruses than to the enteroviruses (1). Further investigations should be carried out to determine whether other relationships exist which may permit these subgroups to be classified together.

As noted above, a considerable loss of infectivity occurred during the concentration procedure. In contrast, less CF activity was lost during the various stages of concentration and

density gradient centrifugation. The major loss of infectivity occurred during centrifugation of the flash-evaporated suspension at $93,000 \times g$. In the suspension concentrated by flash evaporation, approximately 50% of the CF activity was associated with particles which sedimented at $93,000 \times g$ (3 hr). The remainder of the CF activity was associated with particles having a lower sedimentation coefficient. Possibly the latter activity represents capsomeres or capsomere subunits produced in excess by infected cells or released from virions disrupted during the course of flash evaporation and centrifugation, or both.

In the density gradient studies, only those particles which sedimented at $93,000 \times g$ (3 hr) were analyzed. Assay of the fractions from the gradient indicated that CF activity of the echovirus 28 suspension was associated with particles of two distinct densities. One peak of activity was associated with the virion at a density of 1.41 g/ml, whereas the other peak had a density of 1.30 g/ml. Less than 1% of the total infectivity was recovered in the lighter peak, the density of which corresponds to that of most proteins in cesium chloride. Possibly the activity at the lower density represents empty capsids or capsomeres separated from capsids during gradient centrifugation.

Antibodies in echovirus 28 monkey antiserum reacted in CF tests with both the dense (1.41 g/ml) and less dense (1.30 g/ml) antigens of the homologous virus. In contrast, the serum reacted only with a corresponding light (1.30 g/ml) antigen of the heterotypic HGP virus. CF activity was not detected in association with the virion, but it is unlikely that this failure can be attributed to insufficient material, since the HGP virus preparations contained approximately the same quantity of infectious virus and less dense CF antigen as did the echovirus 28 preparations. This suggests that the dense and less dense antigens may exhibit a different specificity, the latter being group-specific, whereas the former is type-specific. Such differences in serological specificity have been observed for "infectious bottom component" and "noninfectious top component" of a number of different enteroviruses and FMDV (10, 15, 25, 30).

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