# Purification and Further Characterization of an "IBV-like" Virus (Coronavirus)[1] (35074)

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The adaptation to growth in suckling mouse brain of certain "IBV-like" viruses (coronaviruses) (1) previously grown only in organ culture, resulted in the recognition of both complement-fixing (CF) and hemagglutinating (HA) antigens (1, 2). Neutralization, complement-fixation, and hemagglutination-inhibition tests were thus made available for antigenic and seroepidemiologic studies. Preliminary findings suggested that the CF and HA antigens were closely related and probably associated with the virion (2). Whether the antigens measured by these tests also occurred as soluble components or whether they consisted of more than one structural component was not determined. The present study describes a simple method for obtaining highly purified human coronaviruses from infected mouse brain and attempts to further define the nature of the CF and HA antigens.

*Materials and Methods. Virus.* "IBV-like" strain OC 43 was isolated on human embryonic tracheal organ culture ($HET_5$) and subsequently adapted to suckling mouse brain (SMB) by MacIntosh and associates (1). The strain was passaged six more times ($HET_5SMB_{13}$) in our laboratory (2). The infectivity titer of the seed virus was $10^{7.5}$ $LD_{50}$/0.02 ml.

*Production of crude antigen.* The preparation of infected suckling mouse brain antigens has been described elsewhere (2). A crude brain harvest antigen consisted of a 20% suspension of infected SMB in sterile phosphate-buffered saline (PBS, pH 7.2).

*Antigenic tests.* CF tests were performed by the standardized microtiter technique (3). HA tests were performed with PBS diluent and 0.5% adult chicken erythrocytes (4).

*Infectivity titrations.* Assays of infectivity were performed by intracerebral inoculation of 3-day-old mice. Calculations of $LD_{50}$ were made by the method of Karber (5).

*Antisera.* Immune serum was prepared in weanling mice by two intracerebral inoculations 21 days apart, followed by bleeding 7 days later (1).

*Ultracentrifugation.* Where indicated, antigen preparations were centrifuged at 23,800*g* ($P_i=4.7 \times 10^8$) for 30 min and/or 6,600*g* ($P_i=1.3 \times 10^8$) for 20 min in a Spinco[2] No. 40 rotor.

*Density gradient centrifugation.* Equilibrium centrifugation was performed in linear 25–55% sucrose gradients. A milliliter of crude or purified virus was layered on the surface and banded at 73,500*g* ($P_i=12.2 \times 10^8$) in a SW 39L rotor for 20 hr. Fractions were collected by the hole-puncture method and measured for protein by absorbance at 280 mμ and for density in an Abbe refractometer (Bausch–Lomb). Each fraction was also tested for HA, CF, and infectivity.

*Virus purification.* Intact, infectious OC 43 virus was purified by adsorption and elution from human "O" erythrocytes and by batch calcium phosphate chromatography.

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[2] Use of trade names is for identification only and does not constitute endorsement by the Public Heath Service or by the U.S. Department of Health, Education, and Welfare.

Crude antigen was freeze-thawed 4× to obtain maximum viral release and incubated for 2 hr in an ice slurry with 3× washed human "O" erythrocytes. The final RBC concentration in the mixture was 40%. The mixture was stirred intermittently and the erythrocytes were then packed by centrifugation at 0° for 5 min at 1000*g*, the supernatant fluid discarded, and the cells washed 3× in cold sterile PBS. Viral activity was eluted from the packed cells by adding an original volume of sterile PBS warmed to 37°. The mixture was incubated for 1 hr at 37° and stirred intermittently. The cells were packed by a light centrifugation and the eluate was removed and dialyzed briefly at 4° against 0.001 *M* phosphate buffer (pH 7.2). Equal volumes of eluate 0.001 *M* buffer, and fresh calcium phosphate gel prepared according to Taverne (6) and washed 7× with distilled water, were then mixed together and incubated for 1 hr at 4° for virus adsorption. Contaminating proteins were removed by washing the gel stepwise with 0.001, 0.005, 0.01, 0.05, 0.10, and 0.20 *M* phosphate buffers, all at pH 7.2. The washes were performed by briefly mixing the buffer with the packed gel, repacking the gel by centrifugation, and discarding the supernatant fluid. The virus and its associated activities were eluted from the calcium phosphate gel with 0.30 *M* phosphate buffer, pH 7.2, after 20-min incubation at 30°.

*Methods of virus treatment.* Crude and purified virus preparations were treated with aqueous solutions of sodium lauryl sulfate (SLS), sodium deoxycholate (DOC), and beta-propiolactone (BPL) (7); trypsin (8); ether (9); and ether–Tween 80 (10) at final concentrations and under conditions indicated.

*Ouchterlony test.* Radial double-diffusion tests were carried out in 0.75% barbital-buffered agarose on 1 × 3 in. slides. Six peripheral wells and one center well, all 2-mm diameter and 1 mm deep were punched 5 mm apart, center to center. The wells were filled 3× during the first 12 hr. The test was held for 3 days at 24° and photographed by indirect light.

*Immunoelectrophoresis.* Tests were carried out in 1% agarose on slides precoated with 0.1% Noble agar (11). The samples were electrophoresed at 250 V, 17 mA, for 4 hr at 4° in a 0.10 *M* barbital buffer, pH 8.6. After addition of antiserum, slides were incubated for 2 days at room temperature for development of precipitin arcs.

*Electron microscopy.* Viral test preparations were mixed with 2% sodium tungstosilicate at pH 7.0 and sprayed on carbon-coated grids. The grids were examined in a Philips EM-300 electron microscope operating at 80 kV.

TABLE I. Separation of OC 43 from Mouse Brain Tissue by Differential Centrifugation.

| Sample[a] | Titer[b] HA | Titer[b] CF | Infectivity ($\log_{10}LD_{50}$/0.02 ml) | Protein ($\mu$g/ml) |
|---|---|---|---|---|
| Crude | 640 | 8/32[c] | 6.8 | 5660 |
| 6600*g*, 20 min, $P_i = 1.3 \times 10^8$: | | | | |
| Supernatant | 640 | 8/32 | 6.2 | 3548 |
| Pellet | 40 | neg[d] | 0.4 | 2139 |
| 23,800*g*, 30 min, $P_i = 4.7 \times 10^8$: | | | | |
| Supernatant | <10 | neg | 0 | 3280 |
| Pellet | 640 | 8/32 | 6.0 | 2422 |

[a] Centrifugation data includes the gravitational force (*g*) at average radius of tube, time at this force but not including deceleration time (approx. 30 min), and performance index ($P_i$). Pellets were resuspended in PBS to original volume for testing.

[b] Titer expressed as reciprocal of endpoint dilution.

[c] Optimal antigen dilution (numerator) with optimal antiserum dilution (denominator) in a CF antigen block titration.

[d] <2/<4.

*Results.* Most viruses in the size range of coronaviruses can be pelleted at approximately 26,000*g* and thereby separated from lighter (usually soluble) components. Centrifugation at 23,800*g* of OC 43 crude mouse brain harvests sedimented all infectivity, CF and HA activity, which suggests that each of these properties was associated with either the intact virus or structures of similar size (Table I).

Further support for these findings was obtained through equilibrium density gradient centrifugation studies. Crude virus material in 25–55% sucrose gradients was separated into two distinct bands containing high infectivity, CF and HA titers (Fig. 1). The first of these bands, located in the 1.18- to 1.20-g/cm$^3$ region of the gradient, was fairly sharp and was revealed by electron microscopy to contain predominantly single virions. Intact OC 43 virus was therefore determined to have a buoyant density in sucrose of 1.19 g/cm$^3$. The second band, found in the 1.11- to 1.15-g/cm$^3$ area, also contained all three biological activities but in slightly less concentrations, and was found by electron microscopy to be composed of tissue-bound or tissue-trapped virus. There was no evi-

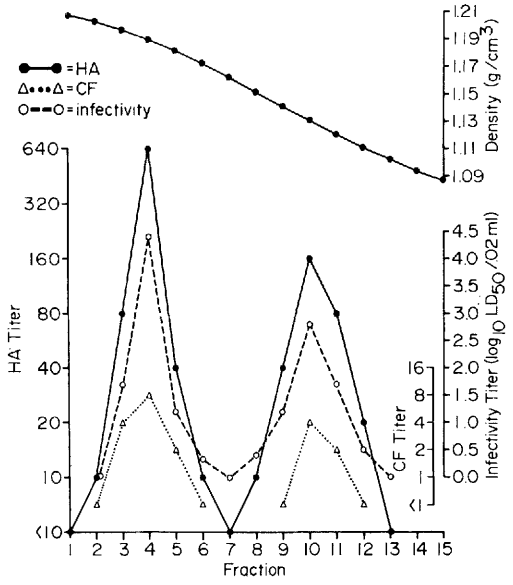


FIG. 1. Equilibrium centrifugation of crude OC 43 suspension on 25–55% sucrose gradients.

TABLE II. Purification of OC 43 Virus.[a]

| Step | Activity titer[b] | | | Recovery[c] (cumulative %) | Protein (μg/ml) | Protein purification (cumulative fold decrease) | Purification factor (cumulative) |
|---|---|---|---|---|---|---|---|
| | Infectivity in SMB ($\log_{10}LD_{50}$/0.02 ml) | CF | Total HA units[c] | | | | |
| Crude 20% SMB | 7.0 | 8/32 | 3840 | 100 | 5710 | — | 1 |
| RBC eluate | 5.5 | 4/32 | 3200 | 83 | 235 | 24 | 20 |
| $CaHPO_4$ eluate | 5.1 | 4/32 | 3220 | 84 | 1.3 | 4400 | 3700 |

[a] All values are averages of at least three runs.
[b] Titer expressed as reciprocal of endpoint dilution.
[c] Recovery was the percentage of total units recovered. Total units = volume × activity units/ml, based on an HA titer of 1:640 as equal to 100 units/ml.
[d] The cumulative purification factor was the protein purification (protein content of fraction divided by protein content of crude starting material) × per cent recovery.

dence of any biological activity outside of these two bands.

Ouchterlony tests with crude brain harvests against mouse antisera and convalescent human sera revealed only a single diffuse band near the antigen well. This band probably represents intact virions which, like other large viruses (herpes, adenovirus), do not readily diffuse in agar. Each of the above procedures suggested that soluble antigenic components of OC 43 were not produced in measurable quantity in mouse brain.

Previous observations had shown that "IBV-like" virus strains OC 38 and OC 43 were easily eluted from human "O" erythrocytes after the agglutinated cells were transferred from 4° to room temperature (2). A slight modification of this technique and adsorption to and elution from calcium phosphate gel provided a simple and efficient method for obtaining purified virus (Table II). Less than 2-fold HA or CF activity was lost during the two steps, resulting in an 84% yield, a 4400-fold decrease in total protein, and an overall enrichment factor of 3700.

The purified virus was examined by immunodiffusion and immunoelectrophoresis using hyperimmune mouse serum. No lines other

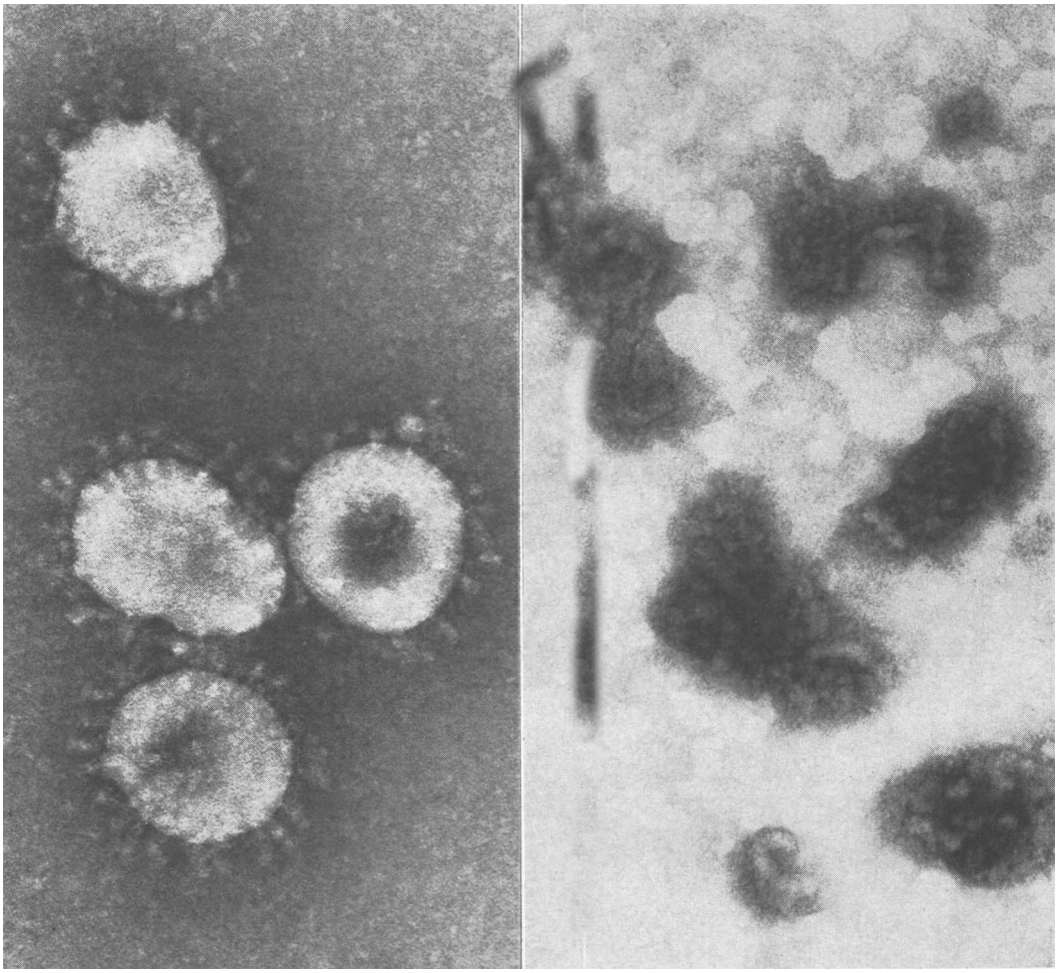

FIG. 2. A. Electron micrograph of 4400-fold purified OC 43 virus (×225,000). B. Electron micrograph of purified OC 43 virus treated with 20% ether overnight. Note disrupted and filamentous membranes and dense areas of collapsed viral aggregates (×147,000).

TABLE III. Effect of Various Chemical and Physical Treatments on the HA, CF, and Infectivity Titers of Crude and Purified Antigens.

| Treatment | Incubation | Temp. | Antigen | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Crude | | | Purified | | |
| | | | HA | CF | Inf.[a] | HA | CF | Inf. |
| None | | | 640 | 8/32[b] | $10^{7.5}$ | 320 | 4/32 | $10^{7.0}$ |
| SLS, 1% | 30 min | 37° | 640 | 8/32 | No | 20 | —[c] | No |
| | 2 hr | | 640 | 8/32 | No | <10 | Neg[d] | No |
| DOC .1% | 30 min | | 640 | 4/16 | No | 10 | 2/16 | No |
| BPL, .01% | 2 hr | | 640 | 8/32[e] | No | 80 | 2/32 | No |
| .1% | 3 hr | | 640 | — | No | 10 | — | No |
| Trypsin, 1% | 2 hr | | 160 | 8/32 | No | 10 | Neg | No |
| | 3 hr | | 80 | 8/8 | No | 10 | — | No |
| Ether, 20% | 5 min | 25° | 80 | 8/32[e] | No | 80 | 4/16[e] | No |
| | 2 hr | 4° | 10 | 8/32[e] | No | 10 | 2/4 | No |
| | 18 hr | | 10 | 8/32 | No | <10 | 2/4 | No |
| Ether, 50% | 18 hr | | 10 | 4/16 | No | <10 | Neg | No |
| Tween 80, 1% + ether, 20% | 2 hr | | 160 | 8/32 | No | 40 | 2/32[e] | No |
| | 18 hr | | 160 | 8/32 | No | 10 | — | No |
| Tween 80, .1% + ether, 50% | 18 hr | | 20 | 8/16[e] | No | 10 | 4/16 | No |
| Heat | 30 min | 56° | 320 | 8/32 | No | 160 | 4/16[e] | No |
| | 1 hr | | 320 | 8/32 | No | 160 | 2/16 | No |

[a] <undiluted, with exception of controls.

[b] Titer expressed as reciprocal of endpoint dilution.

[c] Lytic, anticomplementary, or prozone activity.

[d] <2/<4.

[e] Centrifuged 23,800$g$ for 30 min. Supernatant negative for HA and CF. All biological activity recovered in pellet reconstituted to original volume in PBS.

than the diffuse zone near the antigen wells were observed in either test. Equilibrium centrifugation of samples of the purified virus on 25–55% sucrose gradients yielded a single very sharp band in the 1.19 g/cm$^3$ area of the gradient. This band was identical in biological activity and morphology to the 1.19 g/cm$^3$ band observed with the crude virus (Fig. 1). Electron microscopic studies of the purified virus revealed a uniform population of intact virions against a very clean background (Fig. 2A). The virions were observed to be pleomorphic or roughly spherical spiked particles, 90–120 m$\mu$ in diameter, with occasional virions ranging up to 145 m$\mu$ in size.

Preparations of crude and purified virus were subjected to various chemical and physical treatments to determine if degradation of virions under certain conditions might release soluble CF or HA subunits (Table III). All methods of treatment rendered both the crude and purified suspensions noninfectious, but none produced an increase in CF or HA titer. Treatment of the crude harvest with trypsin or ether by any procedure produced an appreciable decrease in HA titer; treatment with SLS, DOC, BPL, or heat did not. Although the crude CF antigen was more stable than the HA, exposure to trypsin for 3 hr resulted in a significant decrease in CF titer.

The purified virus suspension was more susceptible to treatment by any method than was the crude control suspension. All treatments except heat reduced the HA titer 4-fold or greater. All treatments also had some effect on the purified virus CF titer, but DOC, BPL, short-term exposure to ether, or

heat resulted in only a minimal loss. Electron microscopy of the pellet from ether-treated purified virus revealed disrupted virus particles or aggregates (Fig. 2B).

The greater stability of the crude virus antigen after treatment was not unexpected and appeared to be related to the presence of extraneous mouse brain material. The virus was inherently less stable in the purified form even on storage. Crude virus suspensions were observed to retain their serologic activity and infectivity titers after storage at —70° or —20° for 1–2 months. Suspensions of the purified virus in phosphate-buffered saline lost most activity when stored under the same conditions.

Although several of the treatment methods with both crude and purified antigens resulted in a differential loss of HA activity and raised the possibility that the remaining CF titers might represent soluble components, this was found not to be the case. The residual HA and CF activity could be readily removed by centrifugation at 23,000*g*.

*Discussion.* Examination of crude virus harvests by differential and equilibrium centrifugation, immunodiffusion, and immunoelectrophoresis demonstrated the CF and HA activity to be associated with the virion or particles of similar size and density. No soluble antigenic components were detected. Treatment of crude and purified antigen with a variety of substances known to be effective in disrupting other viruses also failed to produce soluble CF or HA components in sufficient quantity to be detected by the methods employed. Most treatment procedures resulted in a decrease in antigenic activity suggesting either denaturation of critical antigenic sites or loss of available sites through aggregation and fusion of virions, or both.

Our results do not agree with those of Tevethia and Cunningham (12) who used avian IBV strains. In their studies, precipitating antigen remained in the supernatant fluid after differential centrifugation of IBV strain 42 at 109,000*g*, and soluble antigen was also released after treatment with ether. Immunodiffusion tests revealed at least three virus-specific soluble antigens, and two antigens could be separated from the virus on the basis of buoyant densities. Also, Corbo and Cunningham (13) and Biswal *et al.* (14) using trypsin or ether were able to demonstrate hemagglutinins with avian IBV. In our studies application of trypsin or 20% ether to crude or purified "IBV-like" virus resulted in a severe loss of HA activity. The discrepancy between our results and those described above may reflect differences in the host systems employed rather than in the basic structure of the virion.

The demonstration of soluble antigen by gel-diffusion tests requires both concentrated antigen and potent antisera. We have no way of determining antiserum potency for an unknown antigen, but the sera employed contained high levels of CF and HI antibody. Our results do not imply that soluble antigens were absent but rather that such structures were not present in high concentrations in mouse brain and were not readily released by the treatment procedures utilized. However, our results clearly suggest that the antigens active in the conventional CF and HA tests are closely associated with the virion and may be related structures on its surface. Although the presence of soluble components remains to be determined, a systematic investigation of other techniques for releasing viral subunits is now made possible through the simple purification procedures which are described in this paper.

*Summary.* Examination of crude mouse brain harvest of coronavirus strain OC 43 by ultracentrifugation, sucrose density gradients, immunodiffusion, and immunoelectrophoresis demonstrated that the antigens active in the conventional CF and HA tests were associated with the virion. No soluble antigenic components were detected. Purification of the virus was achieved by adsorption and elution from human "O" erythrocytes followed by batch calcium phosphate chromatography. Studies with the purified virus confirmed our findings with the crude mouse brain harvest. Treatment of crude and purified antigen with a limited number of disrupting substances failed to yield soluble CF or HA

subunits, but a systematic investigation of other techniques for releasing viral subunits is now made possible through the simple purification procedures described in this report.

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