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NOTES

Detection of Coronavirus Strain 692 by Immune Electron Microscopy

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Utilization of the technique of immune electron microscopy has enabled the detection of a coronavirus in organ culture harvests derived from a washing from an adult with an acute upper respiratory tract illness; convalescent serum was the source of specific antibody.

Seroepidemiological studies have shown that coronavirus infections are not uncommon in both pediatric and adult populations (12, 14, 19, 22, 24, 27). In spite of this, however, isolation of these agents has been quite rare as the recovery of only 23 additional coronavirus isolates has been reported since the description of the first strain, B814, in 1965. Nine of the 24 originally were isolated in human embryonic tracheal organ culture (HET OC) only, whereas the remainder, all of which were similar to a prototype strain designated 229E, were recovered with difficulty in monolayer cell cultures (8, 13, 19, 25, 31, 32; J. H. Schieble, *personal communication*). In attempting to find new methods for the detection of these fastidious agents, we adapted the technique of immune electron microscopy, which had previously been employed in studies of various viruses or of virus-like agents (3a, 4-6, 9, 12a, 18, 33), to the study of HET OC harvests derived from washings from adults with acute upper respiratory tract illnesses. These studies which resulted in the recovery of coronavirus strain 692 are described below.

A washing obtained on 28 January 1966, from a 29-year-old male, National Institutes of Health (NIH) employee (study no. 692) enrolled in the "NIH Common Cold Study," was diluted as noted below and inoculated into each of two roller-tube cultures of primary rhesus monkey kidney (MK), primary human embryonic kidney (HEK), Hep2 and semicontinuous human embryonic intestine (HEI [MA177]) cells, and human diploid cell strain WI38 (19). The source and maintenance of the cultures were as described previously (19, 21). Washings in the Common Cold Study were obtained by instilling 0.85% NaCl into each nostril sequentially, with collection of the fluid emerging from the nostril and, occasionally, from the mouth also. The washing was then diluted approximately 60% with veal infusion broth containing 0.5% bovine serum albumin. Specimen 692 was inoculated "fresh" on the day of collection into each cell culture with the exception of the HEI cultures which were not available and therefore were inoculated later with an aliquot which had been frozen-and-thawed once. In addition, a blind passage was made into HEK cell cultures. Since neither cytopathic effect (CPE) nor hemadsorption (of guinea pig erythrocytes in MK cultures) was detected, the washing was carried through three passages in HET OC (15, 16) for which maintenance medium consisted of Leibovitz medium supplemented with 0.2% bovine serum albumin, 2.0 mM glutamine, and appropriate antibiotics (17, 23, 25). Strain 692 did not induce a ciliary-immobilizing effect. Harvests of days 2, 4, 6, 9, and 11 of the third OC passage were pooled, and 0.2 ml was inoculated into each of two roller tubes of the cell cultures mentioned previously, except for Hep2 which was not available on the day of inoculation. Since again neither CPE nor hemadsorption was detected, pooled harvests of the third HET OC passage were examined for the presence of virus particles by immune electron microscopy (4), utilizing the patient's convalescent serum as the source of specific antibody. An appropriate control was also examined as noted below.

Immune electron microscopy (4) was employed in the hope that virus particles, if present in OC harvests, would appear in the form of

definite aggregates, thereby enabling the observation of low-titered coronavirus suspensions which would not have been detectable by conventional techniques. A 0.1-ml amount of the HET OC harvest was incubated at room temperature for 1 hr with 0.1 ml of a 1:20 dilution of uninactivated convalescent serum obtained from patient 692, and also with 0.1 ml of 0.85% phosphate-buffered saline (PBS). An 0.8-ml amount of PBS was then added to each, and the mixtures were centrifuged at 17,000 rev/min for 90 minutes in a Sorvall RC 2 centrifuge with an SW34 fixed-angle rotor (4, 18). The supernatant fluid was carefully discarded. The pellet or sediment, or inapparent residue was suspended with a few drops of distilled water, stained with 3% phosphotungstic acid (*p*H 7.2), and placed on a 400-mesh Formvar-carbon-coated grid, with the excess fluid being removed with the edge of a filter paper disc. The grid was examined at a magnification of approximately 40,000 with a Siemens Elmiskop 1A electron microscope. In this technique, a definite pellet or sediment was usually present in serum-containing mixtures but not usually in serum-free mixtures after the centrifugation procedure.

Reaction of the HET OC harvest with the patient's convalescent serum resulted in the appearance of both large and small aggregates (Fig. 1-3). The particles, which morphologically appeared to be heavily coated with antibody, were not randomly distributed but were present almost exclusively as groups which stood out clearly from the surrounding matter. Two of the particles in Fig. 3 appeared to be penetrated by the stain, with a suggestion of at least one "hole" in the envelope of each of these particles, possibly due to the action of complement in conjunction with specific antibody as previously described (3-5, 7, 9, 10a). It was of interest that a single particle was observed which appeared to be covered with little, if any, antibody (Fig. 4). Examination of the strain 692-PBS mixture which had been treated the same as the virus-serum mixture in the same experiment revealed no coronavirus particles, but only predominantly round or oval-shaped matter (Fig. 5-8). In a later experiment, examination of a mixture of a 1:20 dilution of the patient's convalescent serum and PBS by immune electron microscopy, as described above, did not reveal any coronavirus particles.

Additional studies were performed to determine whether strain 692 was related to coronaviruses 229E and OC43, the only known distinct coronavirus "serotypes" of human origin which we are able to propagate in cell cultures in this laboratory (11, 13, 19, 20). A serological test was performed by immune electron microscopy, as described above, using the presence or absence of aggregation as the measure of serological reactivity (1, 2, 4). In this test, strain 692 was incubated with (i) a 1:20 dilution of the convalescent serum of patient 692, (ii) a 1:20 dilution of 229E guinea pig serum (neutralization titer of 1:80 versus 100 mean tissue culture doses [TCD_{50}]), (iii) a 1:20 dilution of OC43 mouse serum, lot 1571 (neutralization titer of 1:640 versus 320 TCD_{50}), and (iv) PBS. Although less numerous than in the initial experiment, antigen-antibody aggregates were again observed in the virus suspension incubated with the convalescent serum, whereas no such aggregates were observed in any of the other preparations, indicating that by this test system strain 692 did not appear to be related serologically to strains 229E or OC43.

Further tests were done to determine the pathogenicity of virus 692 for suckling mice since OC43 virus has been found to induce encephalitis in suckling mice (24). Strain 692 was inoculated intracranially and intraperitoneally into two litters of NIH general-purpose mice. After 19 days, the mice showed no signs of illness and were sacrificed, and the heads were stored at −70 C. Subsequently, a 20% suspension of the brain harvests from the sacrificed mice were inoculated into two litters of suckling mice, and illness was not observed over a 26-day period.

Serological studies of the patient's paired sera were difficult to interpret and were inconclusive since the acute serum may have been pooled shortly after collection with another patient's acute serum (by error of the serum-processing laboratory). Cell culture neutralization tests in which absence of CPE (for 229E virus) and absence of hemadsorption (for OC43 virus) were the indicators of neutralization as previously described (13, 19, 20) revealed that the convalescent serum titered 1:8 versus 10 TCD_{50} of strain 229E (acute was 1:8) and 1:512 versus 100 TCD_{50} of strain OC43 (acute was 1:128). Such titers are consistent with epidemiological studies in a college population in which neutralizing antibody titers to OC43 virus were at consistently higher levels than those to 229E virus (*unpublished studies*).

Attempts to adapt the HET OC harvests to various cell cultures are underway and, if successful, the antigenic relationship, if any, of strain 692 with 229E and OC43 (or OC38, an identical agent), and to strains LP and EVS

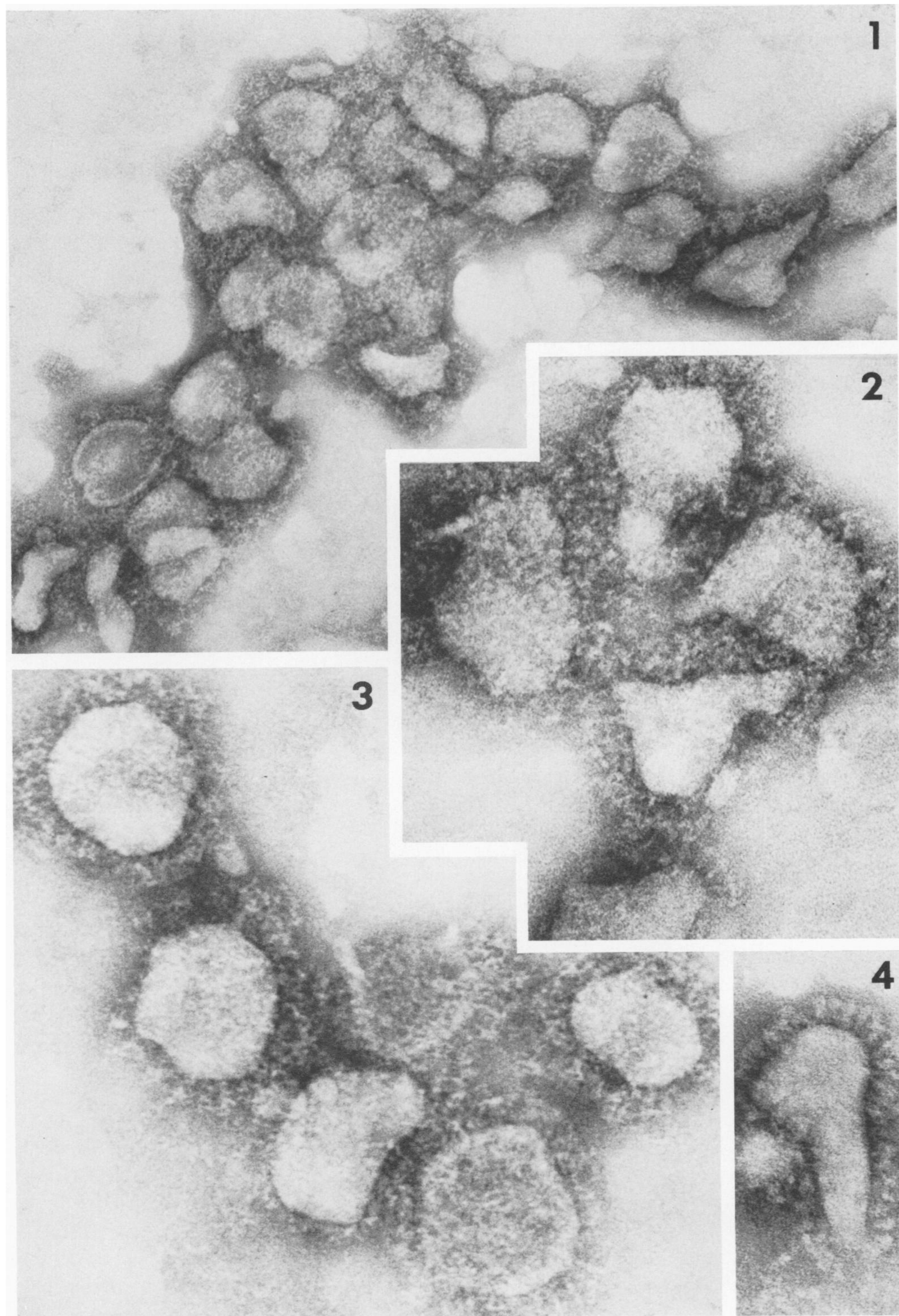

FIG. 1–3. *Large and small aggregates of coronavirus strain 692. The HET OC harvest derived from a washing from patient 692 was incubated with a 1:20 dilution of the patient's convalescent serum prior to centrifugation and further preparation for electron microscopy. The particles which morphologically appeared to be heavily coated with antibody were not randomly distributed but were present almost exclusively as groups which stood out clearly from the surrounding matter. Two of the particles in Fig. 3 appear to be penetrated by the stain and there is a suggestion of at least one "hole" in the envelope of each of these two particles. ×135,840 for Fig. 1; ×226,400 for Fig. 2 and 3.*

FIG. 4. *A single coronavirus strain 692 particle was also found on the same grid on which Fig. 1–3 were observed. This particle appears to be covered with little, if any, antibody. ×226,400.*

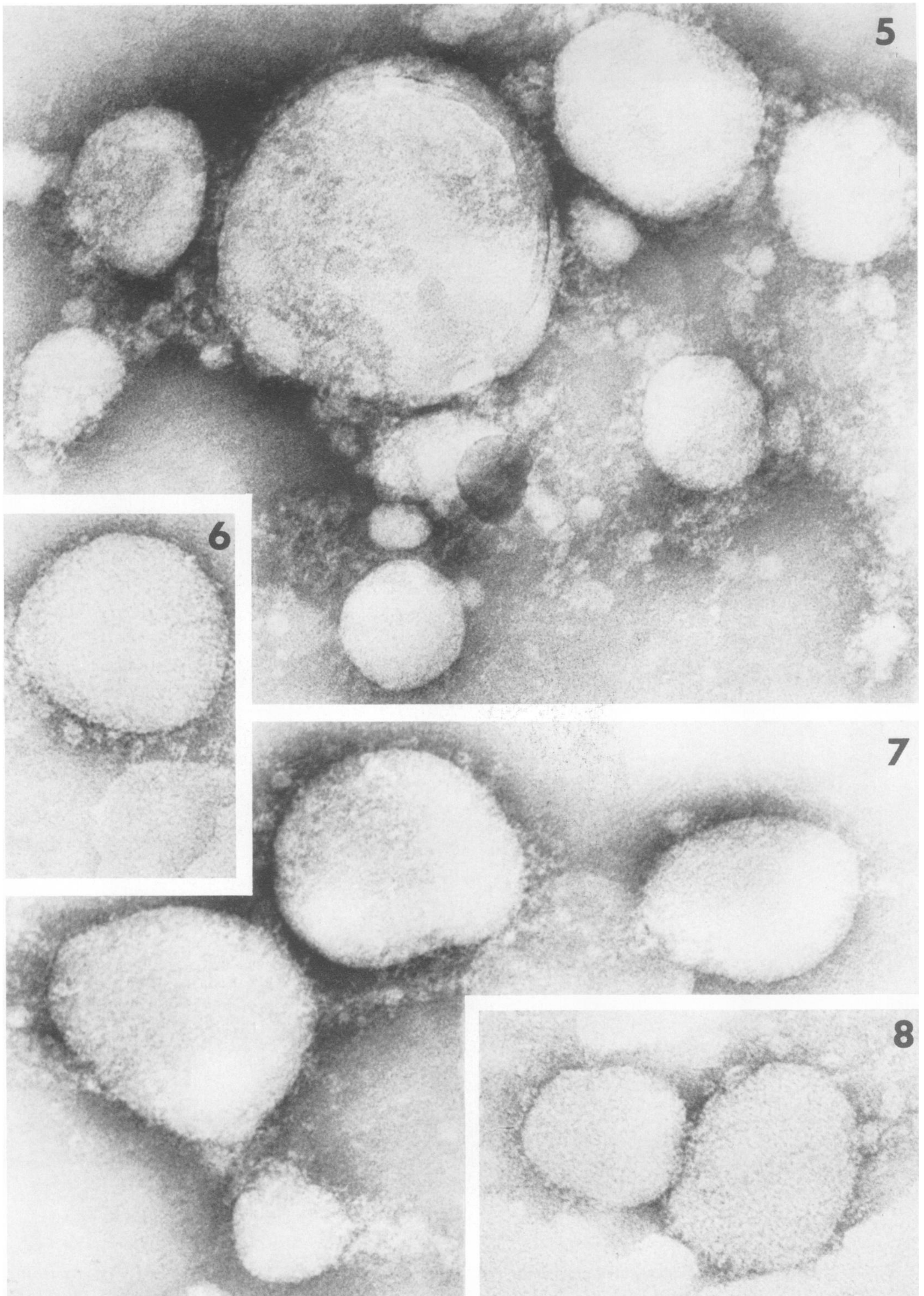

FIG. 5–8. *The same HET OC harvest derived from a washing from patient 692 was also incubated with PBS prior to centrifugation and further preparation for electron microscopy in the same experiment described for Fig. 1–4. No coronavirus particles were observed, only predominantly round or oval-shaped matter as shown in Fig. 5–8. ×226,400.*

(the former closely related to strain 229E; the latter's antigenic relationships are not yet reported), could then be studied by conventional cell culture neutralization tests (8-10, 26, 30, 32). The relationship of strain 692 to OC16, OC37, OC44, and OC48 (none of which have been successfully grown in any cell monolayer cultures), and B814 (which has not been able to be grown in cell monolayer cultures in this laboratory) must await the availability of suitable cell cultures for the propagation of these fastidious agents (11, 25, 26, 31).

The technique of immune electron microscopy has enabled us to detect a coronavirus in HET OC harvests derived from a washing obtained from a patient with an acute upper respiratory tract illness. The application of this technique to the study of OC harvests offers considerable promise for the detection of fastidious agents—such as those coronaviruses which are recoverable only in OC—which may be present in such low titers that they would not be detectable by conventional electron microscopic techniques. Also the technique of immune electron microscopy, which has been utilized for detecting particles in serum (33) and in fecal extracts (12a), offers promise for detecting, in cell culture harvests and in specimens such as nasal and pharyngeal washings, agents which may be noncytopathogenic and/or too low titered and/or too small in size to permit observation by conventional techniques. In addition, by utilization of a patient's convalescent serum as the source of specific antibody, immune electron microscopy may be helpful in determining whether a fastidious isolate was actually derived from the patient or was a laboratory contaminant.

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