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Detection and Identification by Immune Electron Microscopy of Fastidious Agents Associated with Respiratory Illness, Acute Nonbacterial Gastroenteritis, and Hepatitis A

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I. INTRODUCTION

It was in 1941 that Anderson and Stanley (10) in the United States and von Ardenne, Friedrich-Freksa, and Schramm (11) in Germany independently reported electron-microscopic studies of the reaction between tobacco mosaic virus (TMV) and its antiserum. The latter group of investigators observed that a mixture of TMV and anti-TMV rabbit
serum resulted in aggregation of the virus particles (Fig. 1), whereas by contrast such aggregation did not occur when the virus was reacted with a control normal rabbit serum (Fig. 2). Anderson and Stanley made similar observations and, in addition, demonstrated the electron-microscopic specificity of the reaction by observing the lack of such reactivity between TMV and rabbit antiserum to tomato bushy stunt virus (Fig. 3), and between tomato bushy stunt virus, and anti-TMV rabbit serum (Fig. 4). These studies were, indeed, the first demonstration of the method that was later termed immune or immuno-electron microscopy (IEM)—a technique which may be defined as the direct observation by electron microscopy of antigen–antibody interaction (8,53a).

In ensuing years, in spite of the development of electron microscopes with greater resolving power and of methods, such as negative staining
Fig. 2. Tobacco mosaic virus particles were not aggregated following reaction with a 1:1000 dilution of normal rabbit serum. Preparation treated with osmium tetroxide. [After von Ardenne et al. (11).]

(16), that greatly increased contrast, this technique was relatively underutilized (8).

II. DETECTION OF RHINOVIRUSES

My (A.Z.K.) exposure to the technique came in 1970 while a guest worker for about 6 months in the laboratory of June Almeida, a pioneer in the application of this technique (4–8), in Anthony Waterson's Department of Virology in the Royal Postgraduate Medical School in London. We were working originally on antigenic and serological studies of coronaviruses using the technique of IEM. We also applied the technique, in collaboration with E. J. Stott, to the study of rhinoviruses (46) since these agents, although visible in thin sections of infected cells (13, 36, 52, 54), and in purified preparations, (20, 31, 58–60), had been
difficult to visualize in partially purified suspensions by conventional negative staining techniques, because they do not normally grow to sufficiently high titer, do not display a very distinctive morphological appearance by negative staining, and are among the smallest viruses known.

In an experiment in which rhinoviruses 1A and 13 were incubated with either phosphate-buffered saline (PBS) or a homologous goat antiserum before centrifugation and further preparation for electron microscopy, virus particles were seen in all specimens but with greatly differing ease and certainty of recognition (46). Figure 5 shows a single particle
Fig. 4. Tobacco mosaic virus (TMV) and tomato bushy stunt virus after treatment with TMV rabbit antiserum. The TMV particles were aggregated by this serum, whereas the tomato bushy stunt virus particles were not aggregated by it. [After Mudd (67) in which the figure was reproduced after Morton (66), from the study of Anderson and Stanley (10).]

of rhinovirus 1A from a control preparation in which the virus, which had been grown in monolayer cell cultures and had a titer of $10^5$ TCID$_{50}$/0.2 ml, was reacted with PBS. The particles shown in this figure is probably the virus, but it was often difficult to determine with certainty whether or not a particular structure was a virus particle especially since even such probable virus particles were seen only infrequently. Figure 6 shows several rhinovirus 13 particles from another control preparation. The particles were present in greater number since this suspension had been prepared in spinner culture (77) and was higher titered ($10^{7.5}$ TCID$_{50}$/0.2 ml) (46). In spite of this, it was still difficult to be certain whether a particular structure was or was not a virus particle. In both of these preparations the scattered particles were distributed randomly over the grid squares. By contrast, in the virus and homologous goat antiserum mixtures, both large and small aggregates were observed,
Fig. 5. A single particle of rhinovirus 1A from a control preparation in which rhinovirus 1A was incubated with phosphate-buffered saline prior to further preparation for electron microscopy. Particles were randomly distributed, and it was difficult to determine whether certain objects were virus particles. The bar = 100 nm. [After Kapikian et al. (46).]

as shown for rhinovirus 13 in Fig. 7. The particles were no longer randomly distributed but were present as aggregates that stood out clearly from the background matter. There was no longer any doubt about the appearance of the virus since the virus–antibody reaction had resulted in the formation of distinctive aggregates that could be easily visualized, as shown also for rhinovirus 1A in Fig. 8. Optimal antibody concentration resulted in maximal clumping of particles, whereas extreme antibody excess not only tended to obscure the particles but also often hindered the formation of aggregates.

III. DETECTION OF CORONAVIRUS STRAIN 692

During these studies it occurred to me that the technique of IEM, which has the distinctive feature of increasing the sensitivity for the detection, and facilitating or enabling the recognition of a specific low-titered agent by virtue of the immunological specificity of antibody
for that very agent, might be successfully applied in a very practical way to the detection and identification of fastidious agents of human disease if a patient's convalescent serum could be utilized as the source of specific antibody in the hope that the latter would enable the detection and identification of an otherwise unidentifiable low-titered fastidious agent from that same patient. Therefore, following my return to the National Institutes of Health, we began to look for one of these fastidious agents of human disease, the coronaviruses, using IEM.

Coronaviruses have been shown in several seroepidemiologic studies to be not uncommon in both pediatric and adult populations (18, 19, 38, 40, 48, 53, 61, 62, 75) but, in spite of this, recovery of these agents has been quite rare, as only thirty-two isolations have been reported since 1965 when the first strain, B814, was recovered in human embryonic tracheal organ culture (HETOC) by Tyrrell and Bynoe (15, 38, 45,
Fig. 7. Micrograph shows particles of rhinovirus 13. The rhinovirus 13 suspension was incubated with a 1:180 dilution of rhinovirus 13 goat antiserum prior to further preparation for electron microscopy. There is now no doubt about the appearance of the virus as it has been aggregated by the specific antibody (compare with Fig. 6). The bar = 100 nm. [After Kapikian et al. (46).]

Fig. 8. Micrograph shows particles of rhinovirus 1A. The rhinovirus 1A suspension was incubated with a 1:180 dilution of rhinovirus 1A goat antiserum prior to further preparation for electron microscopy. The micrograph illustrates a large distinctive aggregate that may be observed utilizing the technique of immune electron microscopy. The bar = 100 nm. [After Kapikian et al. (46).]
Ten of the thirty-two were originally isolated in HETOC and identified by conventional electron microscopy, whereas the remainder, all of which were similar to a prototype strain designated 229E (39), were recovered with difficulty in monolayer cell cultures (15, 38, 45, 50, 68, 75a, 80). In attempting to find new methods for the detection of these fastidious agents, we adapted the technique of IEM to the study of HETOC harvests derived from washings from adults with upper respiratory tract illnesses, and by these methods recovered one of the thirty-two coronavirus strains (50). These studies (50) are described below.

A diluted nasal-nasopharyngeal washing from an NIH employee (study #692), enrolled in our “common cold study,” was inoculated into roller tube cell cultures of primary rhesus monkey kidney (MK), primary human embryonic kidney, Hep-2, semicontinuous human embryonic intestine, and cultures of human diploid cell strain WI38. Since neither cytopathic effect (CPE) nor hemadsorption (in MK cell cultures) were observed, the diluted washing was carried through three passages in HETOC. Harvests from various days of the third passage were pooled and inoculated into the roller tube cell cultures previously mentioned, except for Hep-2. Since again neither CPE nor hemadsorption were observed, the pooled harvests of the third HETOC passage were examined for the presence of virus particles by IEM utilizing the patient’s convalescent serum as the source of specific antibody in the hope that virus particles, if present, would appear in the form of aggregates thereby enabling the visualization of a low-titered coronavirus suspension that might not have been detectable without reaction with the specific antibody in the convalescent serum.

Briefly, the procedure involved incubation of 0.1 ml of the organ culture harvest for 1 hour at room temperature with 0.1 ml of a 1:20 dilution of the patient’s uninactivated convalescent serum, and also with 0.1 ml of PBS (the latter as a control to the IEM procedure). Then, 0.8 ml of PBS was added to each tube and the mixture centrifuged at 17,000 rpm for 90 minutes in a Sorvall RC2 centrifuge with an SS34 fixed-angle rotor. The supernatant fluid was discarded, the pellet or residue re-suspended with a few drops of distilled water, stained with 3% PTA, placed on a 400-mesh Formvar carbon-coated grid, and the excess fluid withdrawn with filter paper. The grid was examined at a magnification of approximately 44,000, with a Siemens Elmiskop 1A electron microscope.

Reaction of the organ culture harvest with the patient’s convalescent serum resulted in the appearance of both large and small aggregates of coronavirus-like particles which appeared to be heavily coated with antibody (Fig. 9A, B, and C). The particles were not randomly distributed but were present almost exclusively as groups that stood out clearly from the surrounding matter. Interestingly, a single particle that ap-
Fig. 9. (A–C) Large and small aggregates of coronavirus strain 692. The human embryonic tracheal organ culture harvest derived from a washing from a patient in the NIH common cold study was incubated with a 1:20 dilution of the patient’s convalescent serum prior to further preparation for electron microscopy. Particles heavily coated with antibody were not randomly distributed but were present almost
peared to be covered with little, if any, antibody (Fig. 9D) was also observed. Examination of the HETOC harvest–PBS mixture did not reveal any coronavirus particles but only predominantly round or oval-shaped structures (Fig. 10A–D). Additional studies were made by IEM to determine if this coronavirus strain, designated as 692, was related to coronaviruses 229E and OC43, the only known distinct coronaviruses of human origin that we have been able to propagate in either cell cultures or an animal system or both in our laboratory (17, 39, 45, 48, 49). In serological tests by IEM in which the presence or absence of aggregation with different sera was used as the measure of serological reactivity (4, 5, 8), aggregates, although less numerous than in the initial experiment, were observed following incubation of strain 692 with the homologous convalescent serum, but not with specific animal antisera to the other two coronaviruses, indicating that by this test system strain 692 was not related serologically to strains OC43 and 229E (50). Thus, the technique of IEM not only enabled the detection of a fastidious coronavirus but also demonstrated its serological distinctness.

IV. IDENTIFICATION OF AN AGENT ASSOCIATED WITH ACUTE NONBACTERIAL GASTROENTERITIS

We also attempted to utilize the IEM technique for the identification of fastidious agents of acute infectious nonbacterial gastroenteritis, since, in spite of intensive efforts, an etiological agent for this syndrome had not been found (12). This self-limited disease, which is characterized by a spectrum of clinical symptoms which may include vomiting, diarrhea, and abdominal pain, or a combination thereof, affects a broad segment of the population and was the second most common disease experience in the Cleveland family study (12, 23–25, 35). The disease, which has been given various descriptive names, such as winter vomiting disease and epidemic diarrhea and vomiting, was transmitted to volunteers in the 1940s and 1950s, and again more recently in the 1970s, but all attempts to cultivate and characterize definitively an etiological agent in vitro have failed (12, 21, 26, 27, 30, 33–36, 43, 55, 72, 76). In one of these studies, by Dolin et al. (27), a filtrate prepared from a rectal swab specimen from an adult who developed a secondary case of acute nonbacterial gastroenteritis during an outbreak in Norwalk,
Fig. 10. (A–D) The human embryonic tracheal organ culture harvest from a washing from patient 692 was incubated with phosphate-buffered saline prior to further preparation for electron microscopy in the same experiment described for Fig. 9. No coronavirus particles were observed, only predominantly round or oval-shaped objects. The bar = 100 nm. [After Kapikian et al. (50).]
Ohio (3), induced the typical illness in two of three volunteers following its oral administration (27). It was serially passaged two additional times in volunteers by Dolin et al. (26, 27) and again induced the typical illness. Characterization studies (26) in volunteers revealed that the infectious agent in Norwalk outbreak-derived filtrates was less than 66 nm in diameter and probably less than 36 nm, and, in addition, was not inactivated by ether, acid, or heating at 60°C for 30 minutes. However, attempts to cultivate the infectious agent in cell cultures and animals were uniformly unsuccessful, and attempts in human embryonic intestinal organ cultures, inconclusive (12, 26).

In an attempt to detect these fastidious, presumably viral gastroenteritis agents, we adapted the technique of IEM to the study of stool filtrates derived from the Norwalk outbreak (51). A 2% second human passage Norwalk-derived stool filtrate (designated 8FIIa) was used as antigen in the IEM studies (51). It had been filtered through a 1200- and a 450-nm membrane filter and was known to contain an infectious agent since it had induced gastroenteritis in 6 of 10 volunteers, but extensive attempts to recover or detect an etiological agent by conventional techniques were unsuccessful (83). We, therefore, examined this filtrate for the presence of virus particles by IEM utilizing, as in the coronavirus study, a volunteer's convalescent serum as the source of specific antibody; the serum was obtained from an experimentally infected volunteer who had developed the typical illness. This approach was taken in the hope that virus particles would appear in the form of aggregates thereby enabling the visualization and the recognition of a viral agent present in low titer. The IEM technique was similar to that outlined previously in the coronavirus study except for minor modifications (51).

Reaction of 0.4 ml of the stool filtrate with 0.1 ml of a 1:10 dilution of a convalescent serum resulted in the appearance of aggregates of viruslike particles (Fig. 11). The particles, which were heavily coated with antibody, were not randomly distributed but were present as groups that stood out clearly from the surrounding matter and could be easily differentiated from other round structures or other groups of round structures devoid of antibody in the stool filtrate. Morphologically, the antibody-coated particles resembled the picorna- or parvoviruses. In stool filtrate–PBS control preparations, particles or groups of particles without or with little apparent antibody were seen only rarely in comparison; their significance would have been difficult to evaluate without previous experience from examining similar but heavily coated particles aggregated by antibody. The particles measured approximately 27 nm in their shortest and 32 nm in their longest diameters.

We used this stool filtrate, which had readily detectable particles by
Fig. 11. Aggregates observed following (a) incubation of 0.4 ml of the 8FIIa stool filtrate with 0.1 ml of a 1:10 dilution of convalescent serum from volunteer A who developed gastroenteritis following challenge with the Norwalk filtrate and (b) further preparation for electron microscopy. (A) Aggregate observed in the initial 8FIIa experiment (incubation of serum-stool filtrate 1 hour) and (B–D) aggregates observed in the second 8FIIa experiment (incubation of serum-stool filtrate 11 hours). The particles are coated with antibody. The bar = 100 nm. [Fig. 11B from Kapikian et al. (51).]
IEM, to examine several groups of paired sera for antibody to this virus-like antigen in order to determine if the particle was related to gastroenteritis. In a manner similar to that described for the coronavirus studies, 0.2 ml of a 1:5 dilution of uninactivated serum was incubated with 0.8 ml of the stool filtrate and prepared for IEM. Antibody evaluations were made on coded specimens in order to eliminate the possibility of biased interpretation. Routinely, five squares on each grid were examined and the relative concentration of antibody in each serum specimen was estimated by judging the amount of antibody coating the aggregated particles on a 0-4+ scale. Three or more particles in a group were considered to constitute an aggregate. A rating of 0 (i.e., no antibody) was assigned when no aggregates were seen, whereas a 4+ rating indicated that the aggregated particles were so heavily coated with antibody that they were almost obscured. Single and paired particles that were heavily coated with antibody, thereby enabling their easy recognition, were observed along with aggregates in some convalescent sera which had high levels of antibody. Ratings of 1+, 2+, and 3+ indicated the presence of antibody but in lesser amounts than that rated 4+. A 1+ change in antibody rating between paired sera was considered to be significant. Examples of aggregates scored as 1+ and 4+ are shown in Fig. 12. Serum of volunteer A was scored as 1+ since the particles were well-defined and appeared to be covered with very little antibody, whereas that of volunteer B was scored as 4+ since the particles so heavily coated with antibody were almost obscured (51). Heavily coated particles were usually found in small aggregates (or as single or paired units), whereas those with less antibody usually formed larger aggregates. An example of what is considered to be a significant seroresponse, as observed by IEM, with a single volunteer’s paired sera is shown in Fig. 13.

As seen in the Table 1, all 4 volunteers who developed gastroenteritis following administration of a second human passage Norwalk stool filtrate developed serological evidence of infection to the 27-nm particle as determined by IEM. A fifth volunteer, who did not develop illness, did not demonstrate a significant antibody response to this agent. Representative aggregates observed by IEM with this volunteer’s pre- and postinoculation sera (rated 1+ and 1-2+, respectively) are shown in Fig. 14. Aggregates were not observed in PBS–Norwalk stool filtrate mixtures when such controls were utilized in certain of these serological studies (Table 1).

Although all 4 volunteers who developed illness after challenge with the stool filtrate developed serological evidence of infection, it was possible that the observed particles might represent a virus not related to the etiological agent of the Norwalk outbreak, since it was conceivable
that an adventitious agent could either have been present in the stool of the patient from the original Norwalk outbreak or could have been acquired during passage through volunteers. We examined this possibility by testing paired sera from 6 individuals from the original Norwalk outbreak. Four were primary cases, 1 a secondary case, and 1 a contact who did not become ill. [Serum samples were kindly supplied by Drs. Hatch, Adler and Zickl (3).] As shown in Table 1, 3 of the 5 individuals with naturally acquired Norwalk gastroenteritis developed an increase in antibody to the 27-nm particle as determined in tests of paired sera by IEM, whereas the 2 who did not show such an increase demonstrated a high level of antibody in both acute and convalescent sera. The acute
Fig. 13. (A) An aggregate observed after incubation of 0.8 ml of Norwalk (8FIIa) stool filtrate with 0.2 ml of a 1:5 dilution of a volunteer's prechallenge serum and further preparation for electron microscopy. This volunteer developed gastroenteritis following challenge with a second passage Norwalk filtrate which had been heated for 30 minutes at 60°C (26). The quantity of antibody on the particles in this aggregate was rated 1-2-2+ and this prechallenge serum was given an overall rating of 1-2+. (B) A single particle and (C) three single particles observed after incubating 0.8 ml of the Norwalk (8FIIa) stool filtrate with 0.2 ml of a dilution of the volunteer's postchallenge convalescent serum and further preparation for EM. These particles are very heavily coated with antibody. The quantity of antibody on these particles was rated 4+ and the serum was given an overall rating of 4+ also. The difference in the quantity of antibody coating the particles in the prechallenge and postchallenge sera of this volunteer is clearly evident. The bar = 100 nm and applies to A, B, and C.

Phase sera were not collected until several days after the onset of disease and this could explain our failure to detect a response in 2 of the patients. The contact who did not become ill did not exhibit a serum response. It is of interest that among the seroresponders was donor (J) of the original
Table 1. Serological Evidence of Infection Detected by Immune Electron Microscopy: Study of Volunteers with Experimentally Induced Illness and Individuals in Original Norwalk Outbreaka

| Category                        | Inoculum                  | Gastrointestinal illness | Serum  | Amount of antibody present on particles | No. of aggregates observed in five squares |
|--------------------------------|----------------------------|--------------------------|--------|-----------------------------------------|------------------------------------------|
| Experimentally infected volunteers | Second passage stool filtrate (8FII)c | Individual 6            | Postchallenge | 1-2                                     | 4                                        |
|                                 |                            | Individual J             | Postchallenge | 2-3                                     | 4                                        |
|                                 |                            | Individual L             | Postchallenge | 1-2                                     | 3                                        |
| Naturally occurring illness (Norwalk, Ohio, outbreak) | Individual A               | Prechallenge             | Postchallenge | 4                                        | ca. 15                                    |
|                                 |                            | Individual B             | Postchallenge | 4                                        | 10                                       |
|                                 |                            | Individual C             | Postchallenge | 1-2                                     | 3                                        |
|                                 |                            | Individual D             | Postchallenge | 4                                        | 3d                                      |
|                                 |                            | Individual E             | Postchallenge | 2-3                                     | 4                                        |
| Contact (Norwalk, Ohio, outbreak) | Individual F                | Acute                    | Convalescent | 3                                       | 15                                       |
|                                 |                            | Individual G             | Convalescent | 4                                       | 8                                        |
|                                 |                            | Individual H             | Convalescent | 2                                       | 5                                        |
|                                 |                            | Individual I             | Convalescent | 3-4                                     | 8                                        |
|                                 |                            | Individual J             | Convalescent | <1                                      | 3                                        |
| Experimentally infected volunteers | Filtrate from rectal swab derived from stool of individual J | Prechallenge | Postchallenge | 1-2                                     | 2                                        |
|                                 |                            | Postchallenge            | 4-5                                             |

† After incubation with individual serum and further preparation for EM.

a After Kapikian et al. (51).

b In experiments with sera from individuals A, B, C, D, E, F, and K, filtrate 8FIIa was also incubated with phosphate-buffered saline as control, and no aggregates were observed.

c Filtrates 8FII and 8FIIa derived from stools obtained from same individual during same illness.

d Six squares counted.

e Eight squares counted.

f Primary case.

g Secondary case.
2. DETECTION OF FASTIDIOUS AGENTS BY IMMUNE EM

Fig. 14. Aggregates observed after (1) incubation of 0.8 ml of Norwalk SFIIa stool filtrate with 0.2 ml of a 1:5 dilution of prechallenge serum (A) and a 1:5 dilution of postchallenge serum (B) from volunteer E who did not develop illness following challenge with the Norwalk filtrate and (2) further preparation for electron microscopy. No significant change in antibody was observed between the pre- and postchallenge sera. The prechallenge serum was rated 1+ and the postchallenge, 1–2+. [After Kapikian et al. (51).]

rectal swab specimen which had been used to induce illness in 2 of the 3 volunteers in the 1971 study (27). We examined by IEM the prechallenge and convalescent sera of these 2 volunteers and found that 1 developed an increase in antibody whereas the other did not. The development of a significant increase in antibody by 3 of 5 individuals with naturally induced illness suggested strongly that the 27-nm particle was not an adventitious agent acquired during passage in volunteers and, furthermore, indicated that this viruslike agent had also infected patients in the Norwalk outbreak.

Finally, we investigated the possibility that gastrointestinal disease might stimulate the observed antibody responses by a nonspecific mechanism. In another study, volunteers were first administered either a stool filtrate containing an infectious agent from a secondary case of gastroenteritis that had occurred in Honolulu, Hawaii, or from a secondary case that had occurred in Montgomery County, Maryland (83). Those volunteers who became ill after primary challenge were later rechallenged with the same agent and homologous immunity was demon-
Table 2. Serological Evidence of Infection Detected by Immune Electron Microscopy: Study of Sequentially Challenged Volunteers

| Serum sequence tested [prechallenge (Pre) and days postchallenge (indicated by number)] | Estimated quantity of antibody associated with aggregates of 27-nm particles<sup>a-c</sup> |
|---|---|
| 1st challenge | 2nd challenge | 3rd challenge | Volunteer C | Volunteer D |
| Pre | — | — | 2 | 2<sup>d</sup> |
| 27 | — | — | 1–2 | 3 |
| 52 | 4 | — | 1 | 2–3<sup>e</sup> |
| 83 | 35 | Pre | 1–2<sup>f</sup> | 2–3<sup>d</sup> |
| 104 | 56 | 21–25 | 4<sup>g</sup> | 4 |

<sup>a</sup> After Kapikian et al. (51).

<sup>b</sup> In these experiments, stool filtrate 8FIIa was also incubated with phosphate-buffered saline as control, and no aggregates were observed.

<sup>c</sup> Volunteers C and D developed gastroenteritis after first and third challenges. Volunteer C challenge sequence: H filtrate, H filtrate, 8FIIa filtrate. Volunteer D challenge sequence: MC filtrate, MC filtrate, 8FIIa filtrate [H and MC filtrates were each derived from a secondary case of gastroenteritis in Honolulu (H), Hawaii, and in Montgomery County (MC), Maryland, respectively].

<sup>d</sup> Eight squares counted.

<sup>e</sup> Nine squares counted.

<sup>f</sup> Plasma utilized since serum not available.

<sup>g</sup> Six squares counted.

Stratified. Subsequently, challenge with a filtrate derived from the Norwalk outbreak produced disease, indicating that the infectious agent in the filtrate derived from the Hawaii and from the Maryland cases differed from that of Norwalk. Sequential sera from 2 volunteers who underwent the sequence of the three challenges just described were studied by IEM with the Norwalk filtrate (8FIIa) again as antigen (Table 2). The volunteer who received the filtrate derived from the Hawaii case failed to develop an increase in antibody following gastroenteritis induced by the primary challenge, but did develop a significant response after illness induced by the Norwalk filtrate, suggesting that the IEM seroresponse was specific. The other volunteer developed an increase in antibody after gastroenteritis induced by primary challenge, and a further increase after illness induced by the Norwalk filtrate, suggesting that the infectious agents in the Maryland and Norwalk filtrates may have been antigenically related (51).

The data from our studies suggested that the 27-nm Norwalk particle was the etiological agent of Norwalk gastroenteritis. Additional evidence to support this view has emerged from recent studies in our laboratory,
in which the technique of IEM was used to determine shedding patterns of the Norwalk agent (78). These studies revealed by IEM the presence of the 27-nm Norwalk particle in stool filtrates derived from 11 of 23 volunteers who developed illness following challenge with Norwalk outbreak-derived stool filtrates. A total of seventy-seven stool filtrates were studied by IEM using a single convalescent serum known to contain a high level of antibody to the Norwalk particle. It was noteworthy that the 27-nm particle was observed in none of 12 specimens obtained prior to the onset of illness, in 26 of 54 collected during the first 72 hours after the onset of illness, and in only 2 of 11 obtained 72 hours following the onset of illness. Tabulation of the total number of particles observed by IEM in five grid squares in stool filtrates derived from serial stool samples from 8 particle-positive volunteers revealed that the shedding of the 27-nm particle was maximal at the onset of experimental illness and shortly thereafter (Fig. 15). Three of the particle posi-

![Fig. 15. Shedding pattern of the 27-nm Norwalk particle in stool filtrates derived from stool specimens from 8 volunteers experimentally infected with Norwalk filtrates. [From Thornhill et al. (78).]](image-url)
Fig. 16. Aggregates observed after incubation of 0.8 ml of Norwalk (8FIIa) stool filtrate with 0.2 ml of a 1:5 dilution of immune human serum globulin followed by further preparation for electron microscopy. These particles were heavily coated with antibody. The quantity of antibody on these particles was rated as 4+ for A, and 3-4+ for B, C, and D with an overall rating of 3-4+ for the 1:5 dilution of the immune human serum globulin. The bar = 100 nm and applies to A–D.

It is of interest that in a further study by IEM we found that incubation of the 8FIIa Norwalk filtrate with a 1:5 dilution of an aliquot from a preparation of commercial immune human serum globulin resulted in the appearance of particles very heavily coated with antibody as shown in Fig. 16, indicating that antibody to this or a related agent must occur more than rarely in the general population.

We have also recently shown that 5 volunteers, who developed gastroenteritis following oral administration of the “W” gastroenteritis agent (69) of Clarke et al. (21), did not develop serological evidence of infection by IEM to the 8FIIa Norwalk agent indicating these two agents are probably not related serologically; 2 had developed the illness in volun-
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Immuneelectron studies by Clarke et al. (21), and 3 in such studies by our laboratory in collaboration with the University of Maryland (85). In addition, in another study from our laboratory in which IEM was utilized, the successful transmission of the Norwalk particle to chimpanzees was demonstrated (84). Although illness did not occur, serological evidence of infection with the 27-nm particle was shown by IEM.

In additional studies, in which we collaborated with Dr. John Gerin, we determined the buoyant density of the 27-nm particle in cesium chloride by examining each of sixteen gradient fractions by IEM using an appropriate human serum to aggregate the particles (47). The total number of particles in the gradient was determined and the number in each fraction expressed as a percentage of the total number of particles; the highest percentage of particles was seen in Fraction 4 which had a mean density of 1.403 (Fig. 17). Examples of some of the aggregates observed in Fraction 4 and in Fraction 5, which had the second highest percentage of particles, are shown in Fig. 18. In two additional isopycnic banding experiments in cesium chloride, the peak fractions were found by IEM to be at densities of 1.376 and 1.413 gm/cm³, respectively. On the basis of its buoyant density, it appears that the Norwalk agent is not an enterovirus, the latter having a lower buoyant density in cesium chloride (74). Although the Norwalk agent has a density

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**Fig. 17.** Isopycnic banding of the 27-nm 8FI1a Norwalk particle in a cesium chloride density gradient. Quantitation of particle recovery was by IEM. [From Kapikian et al. (47).]
Fig. 18. Aggregates observed after incubation of 0.8 ml of a 1:3.3 dilution of each of Fractions 4 and 5 with 0.2 ml of a 1:5 dilution of prechallenge serum from volunteer E (see Table 1). (A, B) Aggregates observed in Fraction 4; (C) an aggregate in Fraction 5. [After Kapikian et al. (47).]

similar to that of a rhinovirus in cesium chloride, it can also be excluded from this subgroup of the picornaviruses since rhinoviruses are acid-labile, whereas an infectious stool filtrate derived from the Norwalk outbreak has been shown by Dolin et al. to be acid stable (26, 37, 44, 74, 79). Although the nucleic acid content of the Norwalk particle has not yet been determined, it appears that this agent, as previously suggested by Dolin et al. from volunteer studies (26), is parvovirus-like
since it not only has a similar buoyant density in cesium chloride but also shares other properties with this group such as morphology, and, as shown in volunteer studies cited above, ether, acid, and relative heat stability (26, 41).

V. IDENTIFICATION OF AN AGENT ASSOCIATED WITH HEPATITIS A

At the conclusion of this phase of the gastroenteritis studies, we began to look by IEM for the fastidious agent of hepatitis A in stool filtrates obtained from different sources utilizing techniques similar to those in the gastroenteritis studies. Stool specimens for the study described here (29) were supplied under code as normal saline extracts by Dr. Dean Gibson of the Armed Forces Institute of Pathology. These extracts consisted of the supernatant fluids of approximately 20% fecal suspensions in normal saline which had been shaken for homogenization and then centrifuged at low speed (32). The original specimens had been obtained from each of 4 adult volunteers, from the Joliet, Illinois studies, who developed hepatitis after receiving either orally or parenterally the MS-1 or MS-1-derived strain of hepatitis A virus (14, 56). The extracts, which were derived from stools obtained prior to challenge and during the acute phase of illness from each volunteer, were further diluted to a 2% suspension and passed through a 450-nm membrane filter. All of the stool filtrates were examined by IEM using a volunteer's convalescent phase serum as a source of specific antibody for reasons similar to those in the gastroenteritis studies; commercial immune human serum globulin was also used to examine two pairs of these filtrates.

With our previously described approach, we found small viruslike particles measuring approximately 27 nm in diameter in one of two stool filtrates derived from stools from 2 volunteers in the acute phase of the disease, after the filtrates had been incubated with either a volunteer's convalescent phase serum or with human immune serum globulin (29). No significant particles were observed in two stool filtrates derived from prechallenge stools of these two volunteers, after incubation of the filtrates with the same convalescent serum or immune serum globulin as above. The 27-nm particles stood out clearly from the surrounding matter and were easily differentiated from other numerous spherical objects usually found in stool filtrates because the former occurred either as single particles heavily coated with antibody or as particles in groups aggregated by antibody (Fig. 19). Later in the study, we found similar appearing particles, which were much fewer in number and occurred as single particles, in one of two stool filtrates derived from stools of
Fig. 19. Hepatitis A viruslike particles observed after 1 ml of the 2% stool filtrate was incubated with 0.1 ml of a 1:10 dilution of a convalescent serum or a 1:20 dilution of immune human serum globulin followed by further preparation for electron microscopy. An aggregate (A), a single particle (B), and a doublet (C) observed following incubation of the stool filtrate with a convalescent serum from a patient from the Joliet, Illinois, study. The particles are heavily coated with antibody. The quantity of antibody in this serum to the hepatitis A particle was rated 3–4+. (D, E, F, and G) Hepatitis A, viruslike particles observed following incubation of 1 ml of the stool filtrate with immune serum globulin. The particles are heavily coated with antibody also. The quantity of antibody in this lot of immune serum globulin to the hepatitis A particle was also scored 3–4+. The bar = 100 nm and applies to A–G. [Micrographs A, B, F, G from Feinstone et al. (29).]

2 other volunteers in the acute phase of the disease, after the filtrates had been incubated with the same convalescent serum as above. Again, no significant particles were found in filtrates derived from prechallenge stool specimens from these 2 volunteers, after the filtrates had been incubated with the same convalescent serum as above.

Using the initially positive stool filtrate as antigen for serological studies by IEM, we examined several sets of paired sera for antibody to this viruslike agent in order to determine if it was related to hepatitis
A. Usually, 0.1 ml of a 1:10 dilution of an uninactivated serum was mixed with 0.9 ml of the stool filtrate and examined by IEM with minor modifications of the technique used in the gastroenteritis studies. The relative concentration of antibody in each serum specimen was again estimated by scoring the amount of antibody coating the particle on a 0 to 4+ scale. One difference between the 8FIIa gastroenteritis and hepatitis A filtrates was the latter contained fewer particles and, therefore, in the presence of antibody, the hepatitis A antigen occurred frequently as individual particles coated with antibody and less often in the form of antigen–antibody aggregates. However, although the particles were fewer, the rating for antibody was somewhat simplified since antibody-coated single particles were generally more quickly visible, since they appeared to be randomly distributed whereas aggregates generally were not. As before, antibody evaluation of paired sera were made on coded specimens in order to eliminate the possibility of biased interpretation.

Four paired sera from experimentally infected hepatitis A individuals from the Joliet, Illinois study (14) and 2 paired sera from experimentally infected hepatitis A individuals from the Willowbrook, New York study (56) were examined by this method. As shown in Table 3, all 6 of these experimentally infected hepatitis A patients developed serological evidence of infection with the 27-nm, hepatitis A, viruslike particle as determined by IEM. However, since the hepatitis A containing inoculum used in both experimental studies was derived from the same source (MS-1), it was conceivable the seroresponses might have been due to an unknown adventitious agent in the original MS-1 inoculum and thus may have had no relationship to hepatitis A. We examined this possibility by testing paired sera from 3 individuals who developed hepatitis A in a small naturally occurring hepatitis A outbreak in American Samoa in 1972 (73) and, in addition, from 3 individuals who developed this disease during a naturally occurring hepatitis A outbreak in Massachusetts in 1969—the Holy Cross College football team outbreak (65, 82). We found, as shown in Table 3, that all 6 developed serological evidence of infection to the 27-nm hepatitis A particle by IEM, thereby suggesting strongly that the 27-nm particle was not an adventitious agent. Some of the heavily coated particles observed following reaction of the stool filtrate with a convalescent serum from the American Samoan and a convalescent serum from the Massachusetts outbreaks are shown in Figs. 20 and 21.

In further experiments designed to determine whether a serological relationship existed between the hepatitis A particle and hepatitis B antigens, we found, as shown in Table 3, that (a) none of the 12 seroreactants to the hepatitis A antigen developed a serological response
Fig. 20. A small aggregate of three particles plus a single particle (A) and three single particles (B) observed following (a) incubation of 0.9 ml of the stool filtrate with 0.1 ml of a 1:10 dilution of convalescent serum from a patient who developed hepatitis during the naturally occurring outbreak in American Samoa and (b) further preparation for electron microscopy. The particles are clearly covered with antibody. The ready recognition both of the antibody-coated particles in the aggregate and of the antibody-coated single particles illustrates clearly the use of the technique of immune electron microscopy in these studies. The bar = 100 nm and applies to A and B.

...to hepatitis B antigen by radioimmunoassay (57); (b) 2 individuals with hepatitis B demonstrated a serological response to hepatitis B antigen by radioimmunoassay but did not develop a seroresponse to the hepatitis A antigen by IEM; (c) neither guinea pig hyperimmune serum to hepatitis B antigen nor guinea pig serum to the core of the Dane particle reacted with the hepatitis A antigen by IEM (22, 42, 71). Therefore, we could not detect a relationship between the hepatitis A antigen and hepatitis B antigens (29).

We also studied the possibility of an antigenic relationship between the hepatitis A and Norwalk particles by IEM since both were not only similar morphologically but also since infectious hepatitis A serum and infectious Norwalk stool filtrates have been shown by Provost et al. (70) and Dolin et al. (26), respectively, to be ether, acid, and relatively heat stable in infectivity studies. We could not demonstrate a serological response by IEM to the Norwalk particle in paired sera
Fig. 21. Two aggregates observed following (a) incubation of the stool filtrate with 0.1 ml of a dilution of approximately 1:10 of a convalescent serum of a patient who developed hepatitis during the naturally occurring outbreak among members of the Holy Cross College football team and (b) further preparation for electron microscopy. The quantity of antibody on these particles was rated 3-4+. The bar in A and B = 100 nm. [Micrograph A after Feinstone et al. (29).]

of 2 volunteers with experimentally induced hepatitis A (and with seroreponses to the hepatitis A antigen), nor could we demonstrate a serological response by IEM to the hepatitis A antigen in paired sera from 2 volunteers with experimentally induced Norwalk gastroenteritis (and with seroresponses to the Norwalk agent), indicating that the two agents were probably not serologically related.

In a previous study, a virus was recovered from cell cultures inoculated with specimens from volunteers in the Illinois study (63). This virus was later shown to be a parvovirus and to be related to the latent rat virus complex of agents (64). Although this isolate was most likely a contaminant unrelated to hepatitis A, the possibility of its association with the hepatitis A particle was examined with guinea pig hyperimmune serum to the Kilham strain of latent rat virus and rat virus antigen grown in cell culture. In a reciprocal cross-IEM study done under code, convalescent phase hepatitis A serum from a Joliet volunteer did not react with rat virus antigen but did with its homologous hepatitis A antigen, and the hyperimmune rat virus serum did not react with the hepatitis A antigen but did with its homologous rat virus antigen. Thus, it could be concluded that the hepatitis A antigen was not related to rat virus, and, in addition, the lack of an etiological relationship between rat virus and hepatitis A was confirmed.
Table 3. Antibody to Antigens Associated with Hepatitis A, Hepatitis B, and Norwalk Gastroenteritis in Paired Sera from Patients with These Diseases

| Patient | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P |
|---------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|         | First | Second | First | Second | First | Second | First | Second |
| A [3]   | 0     | 3-4   | -    | -     | 1     | 1     |
| B [4]   | 0     | 3-4   | -    | -     | 1     | 1     |
| C [5]   | 0     | 1-2   | -    | -     | NT    | NT    |
| D [6]   | 0     | 3-4   | -    | -     | NT    | NT    |
| E [1]   | 0     | 1-2   | -    | -     | NT    | NT    |
| F [2]   | 0     | 1-2   | +    | +     | NT    | NT    |
| G [10]  | 0     | 2     | -    | -     | NT    | NT    |
| H [11]  | 0     | 3     | -    | -     | NT    | NT    |
| I [12]  | 1-2 (acute) | 3-4   | -    | -     | NT    | NT    |
| J [7]   | 0-1 (acute) | 3-4   | -    | -     | NT    | NT    |
| K [8]   | 0 (acute) | 3     | -    | -     | NT    | NT    |
| L [9]   | 0 (acute) | 3     | -    | -     | NT    | NT    |
| M [13]  | 0     | 0     | -    | +     | NT    | NT    |
| N [14]  | 0     | 0     | -    | +     | NT    | NT    |
| O [15]  | 0     | 0     | -    | -     | 1     | 4     |
| P [16]  | 2-3   | 2-3   | -    | -     | 1     | 4     |

* After Feinstone et al. (29).

* Antibody to the hepatitis A antigen was detected by immune electron microscopy (IEM) and was rated on a 0-4 scale, depending on the amount of antibody coating the particles. Antibody to the gastroenteritis antigen was determined in the same way. Antibody to hepatitis B antigen was measured by radioimmunoassay (RIA). The first serum of each serum pair listed was obtained prior to exposure, except in the case of four sera that were obtained during the acute phase of illness (acute). The second serum of each pair was obtained approximately 1-6 months after the onset of illness. Sera were tested by IEM at an initial dilution of 1:10 except for sera from patients J,K,L which were tested at approximately 1:10 and sera from patients E and F which were tested at 1:15. Sera were tested by RIA at a dilution of 1:4 or 1:10. Abbreviations: NT, not tested; -, negative; +, positive.

* Numbers in brackets indicate previous designation of patient in Feinstone et al. (29).

* No antibody increase.
We also tested another lot of immune human serum globulin for antibody to the hepatitis A antigen by IEM and found it to contain less antibody (2+) than the previously studied strongly positive (3-4+) lot (see Fig. 19). Such tests may prove useful in the potency standardization of immune serum globulin used for the prevention of hepatitis A.

The data from these IEM studies suggest that the 27-nm particle visualized in a stool filtrate derived from a patient with hepatitis A is the etiological agent of this disease. In addition, the detection of this agent in the stools of hepatitis A patients and the development of a serological technique with which to determine antibody to it provide a way to diagnose and study hepatitis A.

In collaboration with Dr. John Gerin, we attempted further to characterize the hepatitis A viruslike particle by determining its buoyant density in cesium chloride utilizing IEM to observe the distribution of particles in diluted fractions of the density gradient using minor modifications of the method employed previously for the Norwalk particle (28, 47). A convalescent serum known to contain antibody to the hepatitis A particle (29) from a patient with naturally acquired hepatitis A in American Samoa was used in the IEM studies (28). We studied original, approximately 20% stool extracts that had been shown, after ten-fold dilution and filtration, to be positive for the hepatitis A particle in studies by IEM as previously described (29). By this method we studied a stool extract derived from a volunteer during the acute phase of illness and found the peak fraction of the 27-nm particle to be at a buoyant density of approximately 1.41 gm/cm³ in cesium chloride, as shown in Fig. 22. Figure 23 shows an aggregate of hepatitis A particles observed by IEM at a mean density in cesium chloride of about 1.4 gm/cm³ (Fraction 4) in a previous isopycnic banding study of a stool extract from another volunteer in which only four consecutive fractions, 3-6, were reexamined in a repeat IEM study, and the peak number of particles was found in Fraction 4. (Details of the initial IEM study are described in the legend of Fig. 23.) The density of the hepatitis A particle of approximately 1.4 gm/cm³ is consistent with that of a parvovirus or a rhinovirus but not an enterovirus (37, 41, 44, 74, 79). Although the antigen has a density similar to a rhinovirus, it can also be excluded from this subgroup of the picornaviruses, since rhinoviruses are acid-labile, whereas an infectious hepatitis A serum has been shown by Provost et al. to be acid-stable in studies in marmosets (37, 44, 70, 74, 79). Although the nucleic acid content of the hepatitis A particle has not yet been determined, it appears that this agent is parvovirus-like since it not only has a similar buoyant density in cesium chloride but also shares other properties with the parvoviruses such as morphology, and, as shown
**Fig. 22.** Isopycnic banding of the hepatitis A viruslike particle in a cesium chloride density gradient. Quantitation of particle recovery was by IEM. [From Feinstone et al. (28).]

by Provost et al. in studies in marmosets with an infectious hepatitis A serum, acid, ether, and relative heat stability (41, 70).

**VI. CONCLUSION**

We have attempted to show the use of the technique of IEM for the detection of fastidious agents of human disease. For Norwalk gastroenteritis and hepatitis A, the technique enabled not only the definitive visualization and identification for the first time of viruslike particles from individuals with these diseases but also enabled us to demonstrate for the first time the serological association of an identifiable viruslike agent with each of these diseases. It is noteworthy that this was accomplished without benefit of any cell culture or animal system. I am certain that this technique will have many other applications, some practical, such as the typing of viruses directly after recovery or isolation, the latter having already been shown by Anderson and Doane (9) to be possible with certain enteroviruses. We have also recently been able to type a rhinovirus by IEM by reacting the cell culture isolate with
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Fig. 23. An aggregate of hepatitis A particles observed by immune electron microscopy (IEM) at a mean density of about 1.4 gm/cm³ in cesium chloride. The particles in the diluted fraction (Fraction 4) have been aggregated by a 1:10 dilution of a late convalescent serum from a patient with naturally acquired hepatitis A. Only four consecutive fractions, 3–6, of the gradient from this initial isopycnic banding experiment were reexamined in a repeat IEM study, and the peak fraction of particles was found in Fraction 4. [On initial examination of twelve fractions (2–13) by IEM, only 50 particles were seen: 6 in Fraction 3; 19 in Fraction 5; and 25 in Fraction 10. Fractions 4 and 11 could not be examined satisfactorily because of poor preparations.] The bar = 100 nm.

serum pools containing antibody to the eighty-nine numbered rhinoviruses plus the subtype (1, 2), followed by individual testing by IEM of the isolate with each serum in the pool that aggregated the virus. However, the most rewarding and exciting use of this technique will be in its application in the quest for the multitude of fastidious agents of human disease that cannot be cultivated or cannot be cultivated effectively in any in vitro or in vivo system.
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DISCUSSION

Dr. O'CONNOR: The elegant results reported by Dr. Kapikian prompt me to report that another technique besides immune electron microscopy is now available for this kind of study. Dr. Ralph deBlois and his associates at General Electric, Schenectady, have developed a new method for sizing and counting virus particles—the electrical resistance pulse analysis (ERP). In the ERP technique, virus particles in fluid flowing through a microcylindrical pore (made by General Electric Nucleopore process in a plastic sheet) are detected by the disturbance that they produce in an electrical voltage applied across the pore. Individual virus particles are sized and counted. The technique essentially involves an extension of the existing Coulter counter to particles of virus size. Viruses at concentrations >10^8/ml can be sized and counted in times of approximately 1 minute. To date the technique has been applied to polyomavirus, T phages, mouse leukemia virus, and mouse mammary tumor virus. Dr. Sam Mayassi and his associates at Charles Pfizer and Co., Maywood, have used this technique for sizing and counting virus particles routinely in production of oncornavirus stocks. They have further demonstrated that addition of specific antiserum causes formation of aggregates that are detected in the resistance pulse analyzer. Although occasional plugging of the pore by virus–antibody complexes still presents mechanical difficulties, this problem appears solvable. This technique does appear amenable to applications in detection of viruses and virus–antibody complexes. The foregoing results by Dr. deBlois and associates and by Dr. Mayassi and associates were executed under the Molecular Control Program of the National Cancer Institute and will be reported at the spring meeting of the American Association for Cancer Research in Houston.

Dr. KAPIKIAN: We reported in our original gastroenteritis study by immune electron microscopy that it took a median time of approximately 1 hour to examine a specimen in order to assign an antibody rating. It doesn’t take us quite as long now. In the hepatitis A studies, such ratings took less time because the hepatitis antigen we used in the original serologic studies contained fewer particles than the Norwalk 8FIla gastroenteritis antigen and therefore in the presence of specific antibody the hepatitis A particles were more randomly distributed as they occurred more often as antibody-coated single particles. In general, serological studies such as these by immune electron microscopy are quite time consuming; the system you mention would certainly be a great boon to us all if applicable for such serological studies.
DR. MELNICK:
1. Two distinct viruses have been passed in marmosets from hepatitis A patients, one by Deinhardt's group and a second by Hilleman's group. Have you examined paired sera from marmosets from these two laboratories?
2. From the acute sera of three of the Joliet volunteers, we have reported the isolation of a morphologically similar virus in the ill-fated Detroit-6 cells—at a time when they showed "hepatitis A susceptibility." On serial passage it appeared that the viruses were replaced with a contaminating rodent parvovirus. It would be of interest to examine the sera of the volunteers to determine if these sera were also positive for your virus by immune electron microscopy.

DR. KAPIKIAN: First, in response to the second question, Drs. Feinstone, Purcell, and I have not studied any of the sera from the Joliet study specifically for the purpose of trying to detect hepatitis A particles by immune electron microscopy as yet. We hope to do such studies soon. Second, we have not examined marmoset sera for antibody to the hepatitis A particle from the sources that you mentioned.

DR. KOCH: Can your method be used to get an estimate on the number of antibody molecules bound to one virus particle?

DR. KAPIKIAN: No. I don't think the technique enables such an estimate to be made. We can get a general idea of the amount of antibody coating a particle and assign a rating to it. Occasionally we see what we think is a bridging antibody molecule between two particles, but because of the limits of resolution of electron microscopy, I don't think we could estimate the number of such small molecules on a virus particle with the technique of immune electron microscopy as we use it.

DR. HALL: I would like to compliment Dr. Kapikian and his colleagues on their very beautiful work. Using fecal and serum samples obtained by Dr. David Madden (NINDS, NIH) during an outbreak of hepatitis A at the Lynchburg (Virginia) Training School and Hospital, Dr. H. E. Bond and I have been looking by immune electron microscopy at a naturally occurring epidemic of hepatitis A. We feel that we have found some particles similar to those described by Dr. Kapikian but require further studies before we can draw any firm conclusion about their distribution.

DR. KAPIKIAN: Drs. Feinstone, Purcell, and I recently collaborated with Dr. D. Madden, who provided paired serum specimens from patients who developed hepatitis during this Lynchburg outbreak, to determine if this hepatitis outbreak was associated serologically with the 27-nm hepatitis A particle. In a study under code of paired sera from each of 2 patients who developed hepatitis during this outbreak, it was found that both patients developed a significant serological response to the hepatitis A particle by immune electron microscopy.

DR. LENNETTE: My comments are impelled by the passing remark of the chairman concerning the potential applicability of the techniques described by Dr. Kapikian to practical problems of biomedicine.

An important example that almost immediately comes to mind is the rapid identification of variolavirus by the immuno-electron-microscopic approach described by Dr. Kapikian and the new techniques described by Dr. O'Connor.

Public health officials in this country are always on the alert for possible introduction of smallpox virus and its rapid identification, which is not always feasible under
present circumstances. This is a matter of some importance since it is the word from
the laboratory that determines whether or not the costly machinery of a vaccination
campaign is to be put into motion. The need for an accurate, as well as a rapid,
diagnosis is obvious.

As many of you are aware, vaccination against smallpox is no longer a requirement
in this country, this action being based on the long-recognized fact that the number
of adverse reactions to vaccination has outnumbered by far the incidence of the
actual disease. Unless smallpox as a disease is completely eradicated, we will face
the increasingly important problem of an increasing population of smallpox suscepti-
bles and, hence, the need for a rapid method for recognizing and identifying the
virus should a suspected case of variola be introduced into the country. Immunelec-
tron microscopy offers such a diagnostic aid (not only for smallpox but also for
other viral diseases), and I take occasion to emphasize here that the electron
microscope is no longer an instrument basic solely to the laboratory engaged
in sophisticated or esoteric research, but is one that has now found, and will continue
to find, pragmatic applications in the diagnostic public health and clinical virology
laboratories.

DR. KAPIKIAN: Yes. I think the technique of immune electron microscopy could
have practical applications in the rapid diagnosis of certain diseases, among them
smallpox. Its use could assist in determining the etiology of certain vesicular lesions
since with specific antiserum an agent present in vesicular fluid could be identified.
In addition, if the viral agent in a vesicle was too low titered to be visualized by
conventional electron microscopy, the technique of immune electron microscopy might
enable its visualization and with this, its identification.