NOTES

Immune Electron Microscopy of Rhinoviruses

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Aggregates of three rhinovirus serotypes have been readily observed by utilizing the technique of immune electron microscopy. The aggregates generally fell into three categories: those with complete virions, those with "empty" capsids, and those in which the two types of particles were mixed.

In recent years, it has been possible to study the morphology of many viruses by the electron microscope technique of negative staining (6). With this method, most viruses can be observed so easily that it is not even necessary to obtain purified suspensions as the particles are sufficiently distinctive to stand out from a background of cellular matter debris. However, the application of this technique to certain viruses, such as rubella, was disappointing even though sufficiently high-titered suspensions could be prepared. Then in 1967, a variation in the negative staining technique was applied to rubella virus (4). This comprised the addition of specific antiserum to the virus-containing preparation so that the particles would be in the form of aggregates rather than individual units; by this method it proved possible to obtain satisfactory micrographs of this virus.

Rhinoviruses, although visible in thin sections of infected cells (5, 9, 11, 12) and in purified suspensions (7, 8, 13-15), have been difficult to observe in partially purified suspensions by conventional negative staining techniques. The present paper describes the results obtained when the technique for observing rubella virus was applied to the study of 3 of the 89 described rhinovirus serotypes (1, 2). With this technique of immune electron microscopy (3), the rhinovirus particles were not only readily seen, but in addition two other morphological features were observed.

Rhinovirus type (R) 1A, strain 52992, kindly supplied by Harvey D. James, Jr., of the National Institutes of Health (NIH), Bethesda, Md., was passaged eight times in cultures of human diploid

cell strain WI38. Cells and culture fluid from the eighth passage were frozen and thawed once 3 days after infection, and stored at -70°C in 1-ml samples; the pool titered $10^{5.0}/0.2$ ml. R2 strain HGP was passaged approximately 15 times in L-132 cell cultures. Cells and fluid from the final passage were harvested 3 days after infection. The harvest was clarified by low-speed centrifugation; the pool titered $10^{7.5}/0.2$ ml. R13, strain 353, kindly supplied by Robert F. Buscho of NIH, was propagated in spinner cultures of HeLa cells by the method of Thomas et al. (16); the pool titered $10^{7.5}/0.2$ ml.

Antisera to R1A and R13, kindly supplied by Harvey D. James, Jr., of the NIH, were prepared by inoculating goats intramuscularly (im) three times with 10-fold concentrated virus suspensions. Certain inoculations were combined with Freund's complete adjuvant. R13 was genetron treated before administration. The hyperimmune R1A serum titered 1:320 versus 100 median tissue culture infective doses (TCD_{50}); the R13 serum titered 1:2560 versus 32 TCD_{50} . R2 antiserum was prepared in both guinea pigs and rabbits. The guinea pig serum was kindly supplied by H. D. James, Jr., NIH. Guinea pigs were inoculated im twice with genetron-treated virus emulsified with incomplete Freund's adjuvant; the post-immunization guinea pig serum titered 1:640 versus 100 TCD_{50} . Rabbits were inoculated intravenously with virus which had been concentrated by precipitation with saturated ammonium sulfate and purified on a 15 to 47% sucrose gradient at 25,000 rev/min for 3.5 hr. Two weeks later, a similar inoculum combined with Freund's complete adjuvant was inoculated into the foot pads. The rabbit serum titered 1:10,240 versus 100 TCD_{50} .

A 0.9-ml amount of the appropriate serum dilution was mixed with 0.1 ml of the undiluted

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virus suspension by inverting the tube several times; serum dilutions were made with phosphate-buffered saline (PBS). Controls consisting of virus suspension plus PBS alone were treated in the same manner. Routinely, the mixture was left at 4 C overnight, and the following morning it was centrifuged for 90 min at 18,000 rev/min in a Sorvall RC2B centrifuge with an SW34 fixed-angle rotor. These conditions were occasionally modified as noted later in the text or in the legends of the micrographs. The supernatant fluid was discarded, and the pellet was suspended in a drop of distilled water. The pellet suspension was mixed with an equal amount of 3% phosphotungstic acid adjusted to pH 7.0. A sample of this mixture was placed on a 400-mesh carbon-Formvar-coated grid, and excess fluid was withdrawn with a torn edge of filter paper. Grids were examined immediately after preparation in a Philips 300 electron microscope at a plate magnification of 60,000 $\times$.

FIG. 1. A single particle of R1A from a control preparation in which the R1A suspension was incubated with phosphate-buffered saline before centrifugation and further preparation for electron microscopy. Particles were found to be randomly scattered in this preparation. The particle shown here is undoubtedly virus as it exhibits a distinctive rim, but on many occasions it was impossible to decide with certainty whether a particular structure was or was not a virus particle. $\times 300,000$.

In an experiment in which rhinoviruses 1A and 13 were incubated with either PBS or homologous goat antiserum and subsequently examined by electron microscopy as described above, virus particles were visible in all specimens but with greatly differing ease of recognition. Those in which the virus suspension had been mixed with PBS alone displayed randomly scattered particles that appeared as in Fig. 1 for R1A and Fig. 2 for R13. More particles were evident in the higher titered R13 preparation. Since rhinoviruses do not display a very distinctive morphology by negative staining, there may have been more particles than were recognized, but to avoid confusion with small protein aggregates only those particles which displayed distinctive virus form were considered to be rhinoviruses.

Reaction of a 0.1-ml suspension of each of the two rhinovirus strains with 0.9 ml of a 1:180

FIG. 2. Several R13 particles from a control preparation in which the R13 suspension was incubated with PBS before centrifugation and further preparation for electron microscopy. Since the R13 suspension was higher titered than the R1A suspension, particles were present in greater number than in Fig. 1; however, it was still difficult to be certain whether a particular structure was a virus particle. For example, the particle that is furthest left in this micrograph is not spherical and is somewhat larger than the other particles shown in this micrograph; it may or may not be a rhinovirus particle. $\times 300,000$.

FIG. 3. This micrograph again shows particles of R13. However, this time the R13 suspension was incubated with a 1:180 dilution of goat antiserum prior to centrifugation and further preparation for electron microscopy. (To be compared with Fig. 2.) There is now no doubt about the appearance of the virus as it has been complexed with anti-body to form both large and small aggregates that can be observed easily; both "full" and "empty" particles are present. $\times 250,000$.

FIG. 4. Produced as Fig. 3, except with the addition of a 1:180 dilution of R1A goat antiserum to a R1A suspension. (To be compared with Fig. 1.) The micrograph illustrates the large distinctive aggregates obtained by immune electron microscopy; both "empty" and "full" particles are present. $\times 250,000$.

FIG. 5. An R1A aggregate. The R1A suspension which had been incubated with homotypic goat antiserum was centrifuged for only 0.5 hr at 12,000 rev/min before further preparation for electron microscopy. The group shown here, with one exception, is made up of "full" particles. $\times 300,000$.

dilution of each homotypic goat antiserum resulted in the appearance of both large and small aggregates (Fig. 3 and 4). The particles were no longer randomly distributed but were present as groups or aggregates which stood out clearly from the surrounding background matter. It was noteworthy that, in an additional experiment in which 0.1 ml of the R1A suspension was incubated with 0.9 ml of a 1:180 dilution of homotypic goat antiserum for about 2 hr at 37 C, aggregates could be observed after centrifugation of the virus-serum mixtures for only 0.5 hr at 12,000 rev/min (Fig. 5).

R2 aggregates, similar to those described above for R1A and R13, were observed when homotypic rabbit antiserum (incubated with R2 at room temperature [RT] for 1 hr) and homotypic guinea pig antiserum (incubated with R2 at RT for several seconds, 60 or 80 min) were used.

The amount of antibody present influenced the definition of the virus particles. Optimal antibody concentrations resulted in maximal clumping of

FIG. 6. An R1A aggregate comprised completely of "empty" particles. A 0.1-ml amount of an R1A suspension was incubated with 0.1 ml of a 1:32 dilution of inactivated (56 C; 30 min) homologous goat antiserum overnight followed by the addition of 0.8 ml PBS prior to centrifugation and further preparation for electron microscopy. Only "empty" particles were present in this preparation. $\times 300,000$.

FIG. 7. Another portion of the aggregate shown in Fig. 6. Some of the particles appear to be disintegrating within the aggregate. $\times 300,000$.

particles, whereas high concentrations of anti-serum not only frequently obscured the particles but also often hindered the formation of aggregates.

Examination of virus aggregates revealed that there was a reasonably distinct division into aggregates containing complete ("full") virus particles as determined by their white appearance in the negative stain, and aggregates containing incomplete ("empty") particles distinguished by their ring appearance, suggesting that rhinoviruses may display distinct antigenic properties depending on whether they are full or empty as previously described for poliovirus type 1 (10). On some occasions an entire specimen was found to consist of empty particles (Fig. 6). However, in those specimens in which there were both full and empty aggregates, the division was not completely clear-cut, as mixed complexes containing both full and empty particles were also found. The aggregates of full particles were more easily seen than the aggregates of empty particles. Individual virus particles measured approximately 27 nm in diameter and displayed a smooth outer surface in which subunits could not be resolved. In addition, it was noteworthy that some of the empty particles within certain antigen-antibody complexes appeared to be disintegrating (Fig. 4, 7).

Two possible explanations are (i) that the virus itself was extremely unstable, and the disintegrating particles were preserved by the stabilizing effect of adsorbed antibody and were contained in what could be called a protective complex, or (ii) that the presence of antibody on the rhinovirus capsid may itself have produced strain on the capsid leading to its disintegration.

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