Immune Electron Microscopy of Avian Infectious Bronchitis Virus Serotypes†

WARD F. ODENWALD,¹ ROBERT B. JOHNSON,^{1*} WARREN W. MARQUARDT,¹ AND
FRANK M. HETRICK²

*Department of Veterinary Science,¹ and Department of Microbiology,² University of Maryland,
College Park, Maryland 20742*

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An immune electron microscopy agglutination technique in which emphasis is placed upon the importance of antigen-antibody equivalence has been developed as a possible method for the serotyping of avian infectious bronchitis viruses. The Connecticut and Massachusetts 41 serotypes were used as a model system. Stock virus concentrations were standardized by physical particle counts of virions sedimented directly onto electron microscope specimen grids. Suspensions containing approximately 150 virions per grid square were allowed to react with dilutions of homologous and heterologous antisera. Virions in these constant virus-variable serum mixtures were sedimented directly onto electron microscope specimen grids, and the relative degree of aggregation per grid was determined from the mean percent aggregation of five randomly selected grid squares. In homologous assays, regions of relative antibody excess, of equivalence, and of relative antigen excess were clearly evident. At equivalence, the mean percent aggregation was significantly higher than in the regions of relative antibody or antigen excess. In the heterologous systems, the degree of aggregation differed little from that of the virus controls containing no antiserum.

Avian infectious bronchitis is an acute, highly contagious respiratory disease of chickens which is of considerable economic importance to the poultry industry (4). A broad-spectrum vaccine giving protection against all strains of the virus has not been developed; thus, it is necessary to isolate and characterize the strains in any area where the disease is not adequately controlled. The virus neutralization test is presently considered to be the *in vitro* test of choice for serotyping. Several different host indicator systems have been used with the virus neutralization test (7, 8, 14). Interrelationships among types differ according to the methods used; hence, a satisfactory comprehensive antigenic overview of the viruses is not yet available (13).

Immune electron microscopy, a serological test which avoids the use of an indicator system and is based on the direct observation of virus-antibody interaction, has distinct advantages over a test based on indirect evidence of an *in vitro* interaction (2, 6, 11). An immune electron microscopy agglutination technique for the differentiation of avian infectious bronchitis virus (IBV) is described herein.

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in part at the 77th Annual Meeting of the American Society for Microbiology, New Orleans, La., 8-13 May 1977, and taken in part from a thesis presented by W.F.O. to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the M.S. degree, August 1977.)

MATERIALS AND METHODS

Viruses. The Connecticut (Conn) and Massachusetts 41 (Mass 41) strains of IBV used in this study have been described previously (8). Virus stocks were prepared by propagating the viruses in 9-day-old specific-pathogen-free embryonated chicken eggs. Allantoic fluid, collected 30 h post-inoculation, was centrifuged at $3,000 \times g$ for 10 min to remove erythrocytes and other cells. The supernatant fluid was further clarified by centrifugation at $12,000 \times g$ for 30 min. The infectivity titers in chicken tracheal organ cultures (10) were 3.2×10^5 median infective doses per 0.1 ml for the Conn strain and 1.0×10^7 for the Mass 41 strain.

Antisera. Type-specific antisera to the two strains were prepared in White Leghorn roosters, as described previously (9). The homologous neutralizing antibody titers or dilutions of antisera required to neutralize 100 median infective doses of virus by the tracheal culture method (8) were 1:58 for the Conn antiserum and 1:295 for the Mass 41 antiserum. Just before use, the sera were thawed, inactivated at 56°C for 30 min, diluted 1:10 in phosphate-buffered saline at pH 7.2, and fil-

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tered without prefiltration through a 0.22- μ m Swinnex-13 filter (Millipore Corp., Bedford, Mass.) to remove debris.

Preparation of the "grid stage" in centrifuge tubes and centrifuge adapters. A stage for the electron microscope specimen grid was prepared in 1-ml cellulose acetate-butyrate centrifuge tubes (E. I. Du Pont Instruments, Newtown, Conn.). A ball of modeling clay 3 mm in diameter was dropped into a clean tube and pressed into its base with a flat-ended metal rod 6 mm in diameter, so that a horizontal surface was formed. Two filter-paper disks (Whatman no. 40, ashless) 6 mm in diameter were placed over the clay surface and tapped lightly several times to secure them and to insure that the surface of the stage was at a right angle to the wall of the tube (Fig. 1A). The tubes were then stored in a plastic bag at room temperature until needed. Adapters to hold the centrifuge tubes in an SW25.1 swinging-bucket rotor in a Beckman model L preparative ultracentrifuge were machined from a solid nylon rod (Fig. 1B).

Physical particle titration. Physical particle titrations were performed with the virus stocks to standardize their concentrations. Phosphate-buffered saline, filtered through a 0.22- μ m Swinnex-25 filter, was used as the diluent throughout these experiments. Virus dilutions were incubated at 35°C for 25 min, and then 0.8 ml of each dilution was transferred to 1-ml centrifuge tubes. The surface of carbon-Formvar-coated 400-mesh copper grids was rendered hydrophilic by touching the grid to a drop of Zephiran Chloride (1:750 dilution in distilled water). The excess Zephiran Chloride was removed immediately by touching the side of each grid to the edge of a tissue paper. The grids were then submerged below the meniscus of the virus dilution and allowed to settle, carbon side up, onto the prepared surface. The tubes were placed in prechilled (4°C) adapters and centrifuged at 44,000 $\times g$ for 20 min. The supernatant fluid was carefully removed by gentle aspiration with a Pasteur pipette, the tip of which was kept just below the meniscus. One drop of 2% sodium phosphotungstic acid, pH 7.0, was then placed on top of the grid with a fine-tipped Pasteur pipette. After 1 min the excess stain was removed from the side of the grid with a Pasteur pipette. The centrifuge tube was then cut open with a scalpel just above the grid stage (Fig. 1A), and the grid was removed. Grids were stored in a desiccator over calcium chloride until they could be examined in the electron microscope.

Electron microscope examination. Virus particle counts were made with an Hitachi HU-11B electron microscope equipped with a 40- μ m objective aperture. An accelerating voltage of 75 kV was used for viewing the grids. The screen magnification was $\times 37,000$. Five squares in each grid were selected for counting by manipulating the specimen stage control knob in a random manner. The mean total number of virions per grid square was determined for each virus dilution. Approximately 150 virions per grid square was the standard virus concentration for the immune electron microscopy agglutination experiments.

Immune electron microscopy agglutination. Homologous and heterologous assays were conducted with dilutions of the treated sera. A 0.5-ml portion of

FIG. 1. Materials used to sediment virus onto specimen grids. (A) Centrifuge tubes, each containing the grid stage. The tube in the vertical position has been cut open to facilitate removal of the specimen grid. (B) Nylon adapters holding the centrifuge tubes. Adapters fit in the SW25.1 Beckman swinging-bucket rotor.

each serum dilution was mixed with an equal volume of the standard virus dilution and incubated at 35°C for 25 min; then 0.8 ml of each dilution was transferred to a centrifuge tube. The remaining procedures were identical to those described for the physical particle titrations. The mean percent aggregation per grid square was calculated for those virions on the five randomly selected grid squares in each of the grids examined from the virus controls (containing no se-

rum) and from the constant virus-variable serum mixtures. Two or more closely associated virions were considered to be aggregated. Student's *t* distribution (15) was used to calculate 95% confidence limits for the mean percent aggregation per grid square observed in the virus controls and in each of the constant virus-variable serum mixtures ($N = 6$) (Fig. 2 and 3).

RESULTS

Physical particle titrations. The standard concentration of 150 virions per grid square was obtained with virus dilutions of 1:250 for the Conn and 1:300 for the Mass 41 virus stocks. Two morphological differences in the virus populations were apparent between the two serotypes. Ruptured virions and virions lacking complete coronas were present more frequently in the Conn preparations than in the Mass 41 preparations (Fig. 4 and 5).

Immune electron microscopy agglutination. The results of homologous assays for both viruses (Fig. 2 and 3) confirmed the need for antibody-antigen equivalence for maximum sensitivity in immune electron microscopy agglutination, as found by other investigators (1, 5, 12). As the degree of homologous antibody excess decreases, the percent aggregation increases sharply until a peak is reached at equivalence. As the dilution of the antiserum is continued, the resulting antigen excess causes a sharp drop in percent aggregation. The aggregation in the

FIG. 2. *Conn* and *Mass 41* virus aggregation by dilutions of *Conn* antiserum. Symbols: $\blacktriangle$, homologous assay; $\triangle$, heterologous assay with *Mass 41* virus. Vertical bars represent 95% confidence limits. The aggregation in the virus controls is outside the y-axis.

FIG. 3. *Conn* and *Mass 41* virus aggregation by dilutions of *Mass 41* antiserum. Symbols: $\bullet$, homologous assay; $\circ$, heterologous assay with *Conn* virus. Vertical bars represent 95% confidence limits. The aggregation in the virus controls is outside the y-axis.

virus controls is shown to the left of the y-axis in Fig. 2 and 3.

Antibody excess is also characterized by antibody halos surrounding the viral peplomers projecting from the virions. The extent of antibody coating varied among virions, apparently being dependent upon the amount of peplomer coverage on the virion. Fuzzy antibody halos were observed only in those areas of the virion which possessed the peplomers (see Fig. 7). These observations confirm those of others (3). Small aggregates, ranging from 2 to 50 virions, were observed in the region of antibody excess. As the antibody concentration decreased, the density of the antibody halos surrounding the peplomers decreased. Viral aggregates in the equivalence regions ranged from 2 to >700 virions, with the majority of the aggregates consisting of <100 virions (Fig. 6 and 7). Further decreases in the antibody concentration resulted in a decrease in aggregation and an increase in the number of virions having little or no antibody attached to their peplomers.

In the heterologous assays there was no significant increase in aggregation above that observed in the virus controls. Virions present either singly or in small aggregates had no apparent antibody involvement.

In preliminary experiments with normal serum and homologous or heterologous antiserum below a 1:100 dilution, the degree of viral aggre-

FIG. 4. *Conn virus control. Arrow indicates a ruptured virion. Note the pleomorphic nature of the virions. Bar = 300 nm.*

gation or the amount of antibody coating could not be accurately assessed. The excessive debris seen at these antiserum concentrations camouflaged the virions. A reduced mean number of virions, compared with the mean number of

virions in the virus controls (without serum), was found whether normal serum, homologous antiserum, or heterologous antiserum was used.

Assays were performed with two coded IBV samples to further test the method as a means

FIG. 5. *Mass 41 virus control. Bar = 300 nm.*

of differentiating serotypes. The standard virus concentrations of the unknowns were assayed with antiserum dilutions from the relative antibody excess and equivalence regions of the Conn and Mass 41 homologous assays. Unknown no. 1 was antigenically indistinguishable from the

Mass 41 serotype. Unknown no. 2 failed to show any significant increase in aggregation. Unknown no. 1 was Mass 41, and unknown no. 2 was the JMK strain which is antigenically distinct from Conn and Mass 41 by the virus neutralization test.

FIG. 6. *Reaction of Mass 41 virus with homologous antiserum in the region of virus-antibody equivalence. Bar = 600 nm.*

DISCUSSION

With optimal concentrations of virus and antibody, immune electron microscopy agglutination may be used for the rapid and unequivocal identification of the Conn and Mass 41 serotypes

of IBV. There appears to be no reason to believe that the method cannot be extended for the identification of other serotypes as well.

Standardization of the relative virus concentration by physical particle titrations enables

FIG. 7. Typical immune aggregate from the equivalence region of the Conn homologous assay. Note the variability among the aggregated virions with respect to the degree of antibody coating. Bar = 200 nm.

one to assess the morphological characteristics of the virus, to determine the relative degree of viral aggregation, and to detect contamination by morphologically different viruses. These advantages, along with the elimination of the biological variables inherent in infectivity titrations, afford a more accurate means of standardizing the antigenic concentrations of the stock viruses.

The curves resulting from homologous assay

data were different for the two viruses. The shape of the curve is dependent on several variables: (i) the number of antigenic determinants present on the surface of the virions, (ii) the concentration and type of antibody molecules directed against these determinants, and (iii) the controlled experimental variables, such as the time and temperature of incubation. The mean percent aggregation in the equivalence region of

the Conn homologous assays was significantly lower than that of the Mass 41 homologous assay. This may be due to the lower degree of peplomer coverage on the Conn virions. Virions possessing fewer surface antigenic determinants are less likely to participate in viral aggregates formed by cross-linking antibody molecules.

The technique should lend itself to the rapid identification of unknown IBV isolates, because after the standardization of reagents, the actual examination is rapid and can be carried out with several samples simultaneously.

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