Immunoelectron Microscopy of Chicken Anemia Agent

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Chicken anemia agent (CAA) is a virus causing severe anemia accompanied by aplasia of the bone marrow and atrophy of the lymphoid organs in young chicks [11]. CAA is thought to be classified into a new taxonomic position of the animal viruses, although virological properties of the agent have not yet been fully elucidated.

CAA particles have been observed by electron microscopy (EM) in the purified samples from infected MSB1 cells [2, 3, 6, 8]. There has been only one report on detection of CAA particles in lysates of infected MSB1 cells by immunoelectron microscopy (IEM); however, specificity of IEM was not checked [6]. We have isolated many CAA strains from field chickens in Japan [10]. This paper describes more detailed IEM studies using one of our isolates of CAA, the A2 strain [10]. Specificity of IEM used was evaluated by labeling the particle-antibody complex with colloidal gold conjugates in this report.

For purification, the cell-free culture fluid of MSB1 cells inoculated with the cloned A2 strain of CAA (CAA fluid) was collected 96 hr after inoculation. CAA infectivity and neutralizing antibody titers were measured as described previously [5].

The CAA fluid was precipitated with 40% saturated ammonium sulfate (NH_4SO_4). After centrifugation, the pellet was suspended in phosphate buffered saline (PBS), pH 7.4, followed by extraction with chloroform four times. The extracted CAA fluid was treated with 0.5% SDS for 30 min at 37°C and sedimented by centrifugation at 19,000 rpm for 24 hr at 15°C. The pellet was resuspended in a small volume of PBS. The crude purified CAA fluid was treated with 0.5% SDS again and loaded onto a cushion of 45% sucrose. After centrifugation at 45,000 rpm for 4 hr at 15°C, the pellet was resuspended in PBS at a concentration of about 1/10,000 of the volume of the starting fluid (purified CAA fluid). The cell-free culture fluid of uninfected MSB1 cells was treated in the same manners as CAA fluid (control fluid).

Chicken antiserum against the A2 strain and specific-pathogen-free (SPF) chicken serum were used for biotinylation. Biotinylation of the immunoglobulin G (IgG) fraction precipitated with 33% NH_4SO_4 was conducted by the method of Guesdon *et al.* [4]. The neutralizing antibody titers of biotinylated IgG prepared from anti-serum (CAA IgG) and from SPF chicken serum (SPF IgG) were >1:16 and <1:2, respectively.

IEM was conducted as follows: (1) In direct IEM (DIEM), the purified CAA fluid (0.2 ml) was mixed with

0.8 ml of biotinylated CAA IgG. The mixture was incubated for 90 min at 37°C followed by the further incubation overnight at 4°C. The mixture was sedimented by centrifugation at 45,000 rpm for 2 hr at 4°C. The pellet was suspended in 50 μl of PBS, and a bovine serum albumin-15 nm gold (BSA-G) conjugate (BIOCELL Research Laboratory, UK) was added to the CAA IgG-purified CAA fluid mixture as a marker. Specificity of DIEM was assessed by using biotinylated SPF IgG instead of CAA IgG. The control fluid was similarly incubated with CAA IgG and BSA-G. A collodion-coated 400 mesh grid reinforced with a thin layer of carbon was placed on the top of the mixtures, which was allowed to diffuse for 5 min. The grid was washed on drops of PBS and distilled water before negative staining with 3% phosphotungstic acid (PTA), pH 7.0; (2) DIEM using streptoavidin-gold (SA-G) conjugates was conducted in the same manner as in DIEM except that an equal volume of 5 nm SA-G conjugate (E-Y Laboratories, U.S.A.) was reacted with the mixture of biotinylated CAA IgG and purified CAA fluid for 60 min at 37°C, followed by the further incubation (overnight at 4°C). The control fluid was also reacted with biotinylated CAA IgG and SA-G conjugates.

Infectivity titers of the starting materials of CAA prior to purification were 10^7 TCID₅₀/ml. The titers of the purified CAA fluid were $10^{9.5}$ TCID₅₀/ml. In contrast, no infectivity was detected in the control fluid (<10 TCID₅₀/ml).

When the purified CAA fluid was reacted with SPF IgG, single spherical or hexagonal particles were scattered, although no aggregation of the particles occurred (Fig. 1). The mean diameter of the single particles was 20 nm (19 to 26 nm). Individual capsomers on surface of the particles were generally indistinct, although stain-filled hollows were seen on the surface. Rarely, particles with 10 peripheral protrusions were noted. Smaller ring-like particles (11 nm in mean diameter) were also present (Fig. 1).

With CAA IgG, on the other hand, aggregates of spherical or hexagonal particles which were surrounded by strands or fringes suggestive of antibody were seen (Fig. 2). Particles in the aggregates averaged 21 nm (19 to 25 nm) in diameter. Stain-filled particles were rarely seen in the aggregates. Although smaller particles were also present in the preparations, no aggregates of these particles were observed.

When the control fluid was reacted with CAA IgG, no single particle or aggregate was noted.

To examine the specificity of IEM, SA-G conjugates were added to the mixture of purified CAA fluid with

Fig. 1. A single spherical particle with surface protrusions in the purified CAA fluid reacted with IgG from an SPF chicken. No aggregates of particles are detectable. An arrow shows a smaller ring-like particle. $\times 210,000$.

Fig. 2. Spherical or hexagonal particles aggregated by IgG antibody to CAA in the purified CAA fluid. Strands or fringes suggestive of antibody surrounding the particles are seen. $\times 150,000$.

biotinylated antibody. The aggregates of particles were specifically labeled with SA-G conjugates (Fig. 3). Gold particles were seen around and between the particles. Rarely, a few smaller particles were mingled in the aggregates or attached to the rim of the aggregates. Very rarely, aggregates consisting of several smaller particles were noted; however, no particles were labeled with SA-G conjugates. When the purified CAA fluid was reacted with biotinylated SPF IgG and SA-G conjugates, no aggregates of particles or single particles labeled with or without the conjugates were observed.

The morphology of single particles of CAA observed by us seemed to be similar to that reported by other worker groups [2, 3, 6, 8]. However, single particles present in our preparations were limited in number despite high titers of CAA in the purified sample.

In this report, the spherical or hexagonal particles

Fig. 3. Specific gold labeling of aggregates in the purified CAA fluid reacted with biotinylated IgG antibody to CAA followed by SA-G conjugates. $\times 120,000$.

observed in the purified CAA fluid were specifically aggregated by homologous chicken antibody to CAA. CAA particles were surrounded by materials suggestive of antibody. Virus particles may be nonspecifically aggregated by many reasons [7]. Specificity of the method used was confirmed by additional specific reactions between biotinylated CAA IgG and streptoavidin gold conjugates or by using SPF IgG. Furthermore, no particles were detectable in the control fluid by IEM.

The smaller particles (11 nm in mean diameter) were also present in the purified CAA fluid. These smaller particles were quite similar to those reported by McNulty *et al.* [6]. They also found such particles in control preparations from uninfected MSB1 cells [6]. Although significance of the smaller particles in CAA infections is unclear, antibody did not aggregate these particles in the present experiment. Therefore these particles seem not to be directly related to CAA itself.

McNulty *et al.* [6] reported large clumps of particles aggregated by 2A9 monoclonal antibody in the purified sample derived from lysates of infected MSB1 cell. However, they failed to find CAA particles in ultrathin sections of infected cells by IEM with the same monoclonal antibody [6]. We could not find particles suggestive of CAA in ultrathin sections of MSB1 cells infected with our isolate (unpublished data). The reasons why CAA is not found in infected cells are unknown.

In staining with PTA in the present study, the mean diameter of our isolate was 20 nm (19–26 nm). The diameter of particles observed by EM was different by workers [2, 3, 6, 8]. It was suggested that such differences in size may be attributable to the different staining or calibration methods [2].

Protein A-gold conjugates have been successfully used as probes for detection of antigen-antibody complexes [1]. The use of biotinylated antibody and avidin-gold conjugates seems to be very beneficial to IEM studies particularly on avian viruses, since protein A does not react with

avian IgG.

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