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Immunoferritin Studies of Marek's Disease Virus Directed Intracellular and Membrane Antigens

By

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With 5 Figures

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Summary

Intracellular and membrane antigens of Marek's disease virus (MDV) were studied by immunofluorescence and immunoferritin techniques. Sera from chickens surviving the disease and rabbits hyperimmunized with purified virus had antibodies to the envelope and the capsid of the virion and virus directed membrane antigens detected on the surface of infected cells. Indirect evidence suggests that the membrane antigen is related to the structural proteins of the virus.

1. Introduction

Marek's disease (MD) is a neoplastic disease of chickens caused by a herpesvirus (4, 9). Naturally infected and experimentally inoculated chickens develop antibodies against the virus, which can be detected by immunoprecipitin (IP) (3) and immunofluorescence tests (IF) (10). A new membrane antigen (MA) was also detected on the surface of MDV infected cells (1, 2). The specificity of these antigen-antibody reactions to the structural components of the virus, however, was not determined.

The purpose of the present study was to investigate the specificity of the MDV antigen-antibody reactions in relation to the structural antigens of the virion.

2. Materials and Methods

2.1. Virus

The cloned preparations (11) of the GA (5) strain of MDV was used in this study. The virus was propagated in duck embryo fibroblast (DEF) cultures. Infected DEF's

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were suspended in growth medium (M 199—F 10 mixture) (14) containing 10% DMSO and 10% calf serum and were stored in liquid nitrogen.

2.2. Cell Cultures

Primary DEF cultures for propagation of virus were prepared as described previously (15). Chick kidney (CK) cultures were prepared from 1- to 2-week-old chicks (16). CK cultures were used for the study of MDV directed intracellular and membrane antigens (MA).

2.3. Antisera

Day-old chicks from Line 15-I flock maintained in isolation at this laboratory were inoculated with 1×10^5 DEF cells previously infected with the GA strain of MDV. Chicks were reared in isolation and at 10 weeks postinoculation sera were collected from all survivors. The pool of such sera was twice adsorbed with duck embryo powder. Sera from uninoculated chickens were used as control. Anti-MDV antibodies were prepared in rabbits by injecting 1 mg of partially purified (8) MDV in 0.5 ml of virus buffer mixed with equal amount of Freund's adjuvant into the foot-pad.

Further intramuscular injections were carried out at 2-week intervals for 2 months using 1 mg of purified virus in the inoculum. Serum was collected from these rabbits and was twice adsorbed with duck embryo powder. Both chicken sera and rabbit sera were specific to MDV by IF test.

2.4. Immunfluorescence Technique

The indirect IF test described previously (10) was used. Monolayer cultures of CK cells on 11×22 mm coverslips were infected with 100 plaque forming units (PFU) of MDV. After the appearance of morphologically distinguishable microplaques coverslip cultures were stained for detection of the intracellular antigens and the MA. For detection of the intracellular antigens, the cultures were fixed in cold acetone prior to treatment with MDV specific antiserum whereas for detection of the MA, cultures were directly treated with the antiserum without any fixation. Cultures were treated with appropriate antiserum for 30 minutes at room temperature, were washed in PBS and treated with fluorescein-conjugated anti-chicken or anti-rabbit gamma globulin for 15 minutes at room temperature. The conjugates were horse anti-chicken and goat anti-rabbit gamma globulin (Roboz Surgical Instrument Co., Inc., Washington, D.C.). Cultures were washed several times in PBS following each reaction. Stained cultures were mounted in Elvanol (12) and examined with a Leitz dark field fluorescence microscope.

2.5. Immunoferritin Technique

The CK monolayer cultures in 100 mm Falcon plastic Petri dishes were inoculated with a high dose of virus to insure the infection of at least 50% of the cells in the monolayer. Both direct and indirect conjugation techniques were applied for the study of MDV antigens in CK cultures. Horse spleen ferritin (Miles Laboratories) was recrystalized and conjugated to appropriate gamma globulin fractions according to the method of HOWE *et al.* (7). Anti-MDV chicken gamma globulin and anti-MDV rabbit gamma globulin were conjugated for the direct technique and horse anti-chicken gamma globulin and goat anti-rabbit gamma globulin were conjugated for the indirect technique. Unfixed cultures were applied for the study of the membrane antigen. Cultures used for detection of the intracellular antigens were fixed in 5% formaldehyde in PBS for 10 minutes, then washed 3 times in PBS and were mixed with appropriate antibody preparation (ferritin-conjugated anti-MDV gamma globulin fraction for the direct test and appropriate serum against each virus in the indirect test). The mixture was freeze-thawed once and was incubated for 1 hour at 37° C. Monolayers were then scraped off and washed 3 times in PBS and centrifuged to obtain a pellet. In the indirect test the samples were further reacted with the appropriate ferritin conjugate for 30 minutes at 37° C and washed three times in PBS. All samples were then fixed in 1% osmium tetroxide for 1 hour and prepared for electron microscopy as described earlier (9). Thin sections were stained with uranyl acetate and lead citrate and were examined with an Elmiskop 1A Siemens electron microscope.

3. Results

3.1. Detection of the Membrane Antigen of MDV

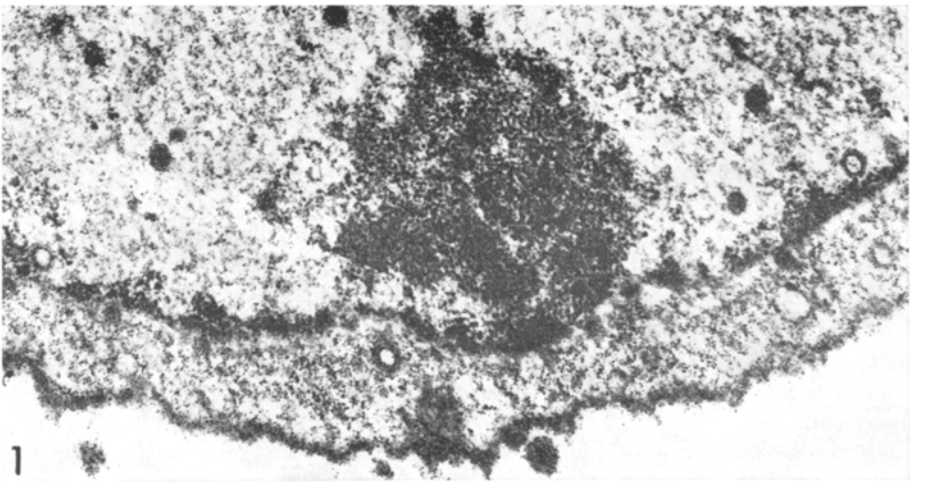
Unfixed CK coverslip cultures infected with the GA strain of MDV were indirectly stained by IF technique. Sera from chickens surviving Marek's disease by the 10th week post-inoculation and from hyperimmune rabbits both stained the membrane antigen. Both sera had a titer of approximately 1:40 for the membrane antigen. Control chicken serum and the preimmunization rabbit sera did not react with the membrane antigen. The membrane antigen was found mostly on rounded cells but was also seen on the membrane of flat and fusiform cells in the area of the microplaque. The distribution of the antigen over the cell membrane was not uniform in all positive cells. In some cells the antigen was evenly distributed over the entire surface whereas in other cells the distribution of the antigen was discontinuous.

Examination of unfixed cells treated with ferritin-conjugated antibody showed the tagging of the cell membrane in rounded cells with ferritin granules (Fig. 1). Most cells positive for the membrane antigen also contained virus particles. However, occasionally some cells had a heavy ferritin tagging but did not contain any virus particles. Nor did these cells show any other features associated with the replication of the virus; such as, margination of the nuclear chromatin, nuclear inclusions, and cell degeneration. Uninoculated control cells treated with positive serum or infected cells treated with control serum did not react in this test.

3.2. Intracellular Antigens of MDV

Cells infected with MDV had IF antigens in the cytoplasm and the nuclei. Sera from MDV infected chickens and hyperimmunized rabbits reacted with both cytoplasmic and nuclear antigens. These sera had a titer for intracellular antigens 32 times higher than that for the membrane antigen. The nuclear antigen, when present, was brighter than the cytoplasmic antigen and appeared similar in morphology to the nuclear inclusions. The cytoplasmic antigens, however, were mostly diffused and bright granular antigens could only be seen in certain parts of the cytoplasm.

Similar results were obtained with both direct and indirect immunoferritin techniques but less ferritin granules were attached to viral capsids in the direct test than in the indirect test. This could be due to the low concentration of virus specific antibody and a weak conjugation reaction. Therefore, the indirect test was applied in most of the experiments. Extensive tagging of the cytoplasmic and nuclear material was observed only between MDV infected CK cells and the anti-MDV chicken or rabbit serum. Some tagging was observed when infected cells were treated with control serum but this was always very weak and was, therefore, considered nonspecific. Positive specific ferritin tagging was found both in the cytoplasm and the nucleus. In the cytoplasm extensive tagging of the smooth cytoplasmic membrane and the enveloped virions was observed. The nuclear membrane and the nucleoplasm were also tagged. However, viral capsids were tagged more than any other membranes or any particles. In the indirect tests a layer of electron dense material was observed immediately surrounding the capsid, and ferritin granules were attached to this layer. Most likely this layer was the anti-



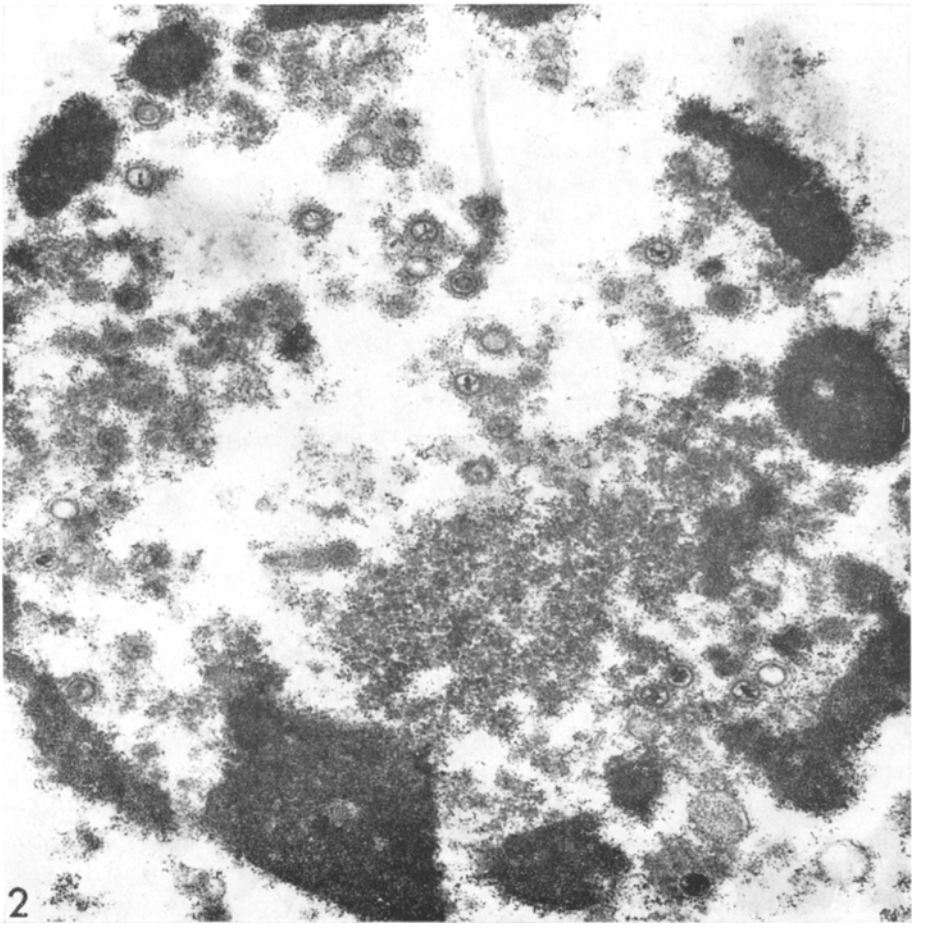


Fig. 1. Indirect immunoferritin test. A CK cell infected with MDV and reacted with anti-MDV serum prior to fixation
A heavy layer of antibody-ferritin conjugate is observed on the surface of the cell membrane. Unlabeled nucleocapsids are seen in the nucleus. A nucleocapsid laying outside of the cell is also labeled. × 25,000

Fig. 2. Indirect immunoferritin test. Part of a MDV infected CK cells treated with anti-MDV serum after fixation
Specific labeling of the nucleocapsids is clearly seen. Light labeling of the nuclear matrix is also seen. × 30,000

viral specific antibody (Figs. 2, 3, 4). In addition to the tagging of the capsid, some small granules in the nucleus were also tagged with ferritin particles (Fig. 4). The envelop virions, when present, were also heavily tagged with ferritin (Fig. 5).

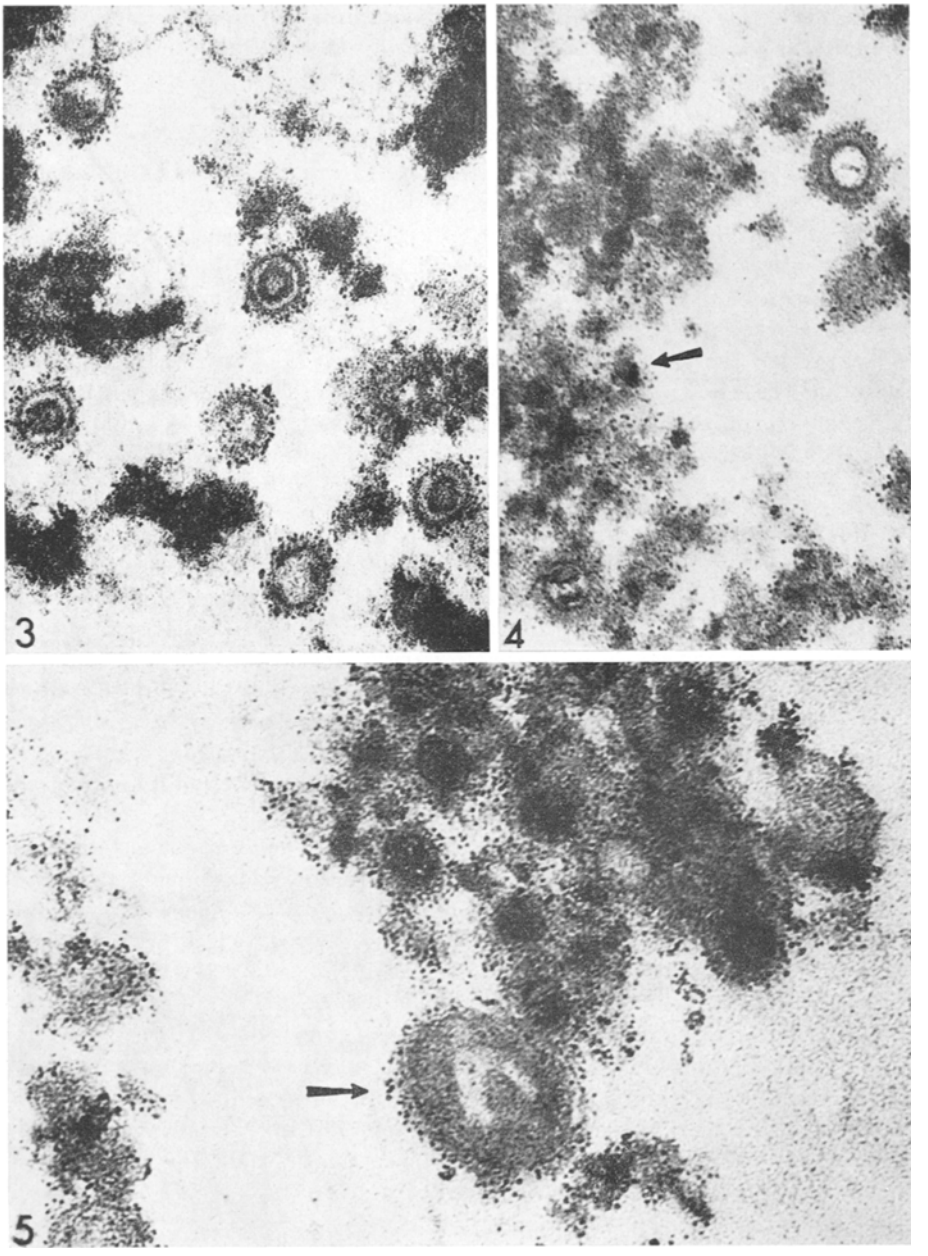


Fig. 3. Indirect immunoferritin test. Higher magnification of MDV infected CK cells reacted with anti-MDV serum Labeling of the nucleocapsids is heavier than any other structure in the cell. × 100,000

Fig. 4. Indirect immunoferritin test. A similar preparation as in Figure 3 showing labeling of the nucleocapsids and small nuclear particles (arrows). × 100,000

Fig. 5. Indirect immunoferritin test. A group of nucleocapsids and a single enveloped virion (arrow) in an MDV infected CK culture. Both viral capsids and viral envelope are labeled with anti-MDV serum. × 100,000

4. Discussion

4.1. The Membrane Antigen

CHEN and PURCHASE (2) first reported the presence of a new antigen on plasma membrane of MDV infected cells. The membrane antigen is specific to MDV infection but it is not clear if this antigen is identical with structural components of the virion. However, sera from rabbits hyperimmunized against purified virus reacted strongly against MDV directed membrane antigen. This may indirectly suggest a similarity between the membrane antigen and the virion components. Although the possibility of purified virus preparations being contaminated with traces of the membrane antigen and resulting in production of antibodies to this antigen can not be excluded. The resemblance of HSV directed membrane antigen and Epstein-Barr (6) virus to their respective envelop antigen is already demonstrated (13, 14).

The great majority of cells with membrane antigen also contained virus particles either in the nucleus or in the nucleus and the cytoplasm. Occasionally some cells possessed the membrane antigen but were free from any virus particles or any other changes associated with virus replication. The exact explanation for this phenomenon is lacking. However, these cells may have only been abortively infected in which case certain features of virus replication may not occur.

4.2. Intracellular Antigens

Previous studies (10) by immunofluorescence tests showed an abundance of virus specific antigens in the cytoplasm either as a fine powdery mass of anitgen or as distinct granules. Experiments reported here showed similar results both in IF test and in immunoferritin test. In the immunoferritin test, the entire membrane system in the cytoplasm was tagged with ferritin labeled antibody. No tagging of the large cytoplasmic vesicles or the mitochondria was observed. Antigens present in the cytoplasm could be non-structural and not related to the structural proteins of the virion since not a large number of virus particles were found in the cytoplasm. On the other hand, they may be structural proteins produced in excess and accumulated in the cytoplasm.

The tagging of the nuclear membrane and the nucleoplasm was also observed but virus particles were tagged heavier than any other particle or membrane. Both naked nucleocapsids and enveloped virions were tagged with ferritin labeled antibody. Also some particles 35-40 nm in diameter were tagged with antibody conjugate. These particles have been found in the nucleus of MDV infected cells and may be structurally related to the virion.

Both chicken and rabbit sera contained specific antibodies to virus capsid and envelope antigens. Antibodies to these antigens may be different but differentiation could not be attempted because monospecific antibodies were not available.

These experiments demonstrate that chicken and rabbit sera positive in IF test contain antibodies against the structural proteins of MDV. The IF test, therefore, may be detecting viral structural antigens.

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