Neutralization Studies with Marek’s Disease Virus and Turkey Herpesvirus

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Use of Marek’s disease virus (MDV) in a neutralization test presents several problems, which are described, making this potentially useful test difficult. To obviate these difficulties, a plaque reduction test has been designed based on cross-neutralization of turkey herpesvirus (HVT) by serum-neutralizing MDV. The technique for such a neutralization test is outlined. Kinetics of development of neutralizing antibodies in chickens inoculated with HVT and MDV are described. The neutralization test can be used to evaluate viability of HVT vaccines and the possible role of neutralizing antibodies in the protection afforded by vaccination against MDV-induced tumors.

Few studies have been reported which involve the determination of viral neutralizing antibody that develops after infection with Marek’s disease virus (MDV) or turkey herpesvirus (HVT). Such determination is desirable to evaluate the role of these antibodies in tumor formation. In addition, determination of neutralizing antibody may be useful for judging the immune status of a flock of chickens. To conduct neutralization tests, a cell-free virus preparation and a suitable indicator system are required.

The purpose of this study was to (i) investigate the suitability of using MDV and HVT in a neutralization test employing a plaque reduction technique in cell culture, (ii) standardize such a test procedure, (iii) determine titers of neutralizing antibody in chickens inoculated with HVT or MDV, and (iv) determine cross-reactivity of antiserum produced in response to MDV and HVT.

MATERIALS AND METHODS

Sources of eggs and chicks. Chicks used for the preparation of chick kidney cell (CKC) cultures and for other in vitro experiments were Line 7 (4) or specific pathogen-free (SPF) White Leghorn chickens (18). Chicken embryo fibroblast (CEF) cultures were prepared from embryonated chicken eggs obtained from the SPF flock of White Leghorn chickens negative for MDV precipitin antibodies.

Strains of virus. Unless otherwise specified, the Conn B strain of MDV was used to inoculate chicks and produce infected skins (15); other MDV strains studied included JM (20), GA (12), and Conn A (8). The FC 126 strain of turkey herpesvirus (21), obtained after the 9th passage in duck embryo cells, was propagated in CEF.

CKC cultures. Kidneys were removed from 1-week-old SPF chicks, washed in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-buffered saline to remove blood, minced, and placed in a trypsinizing flask. Cells were collected after a 5-min treatment with 0.25% trypsin, centrifuged at 500 rpm for 5 min, and then suspended in growth medium (Eagle minimal essential medium [MEM] plus 10% tryptose-phosphate broth [TPB] plus 10% calf serum). Petri dishes, 60 mm in diameter, were inoculated with 8 × 10⁶ cells per dish; confluent monolayers formed after 2 days.

CEF cultures. Nine-day-old chicken embryos were decapitated, washed in Tris-buffered saline, minced, and placed in a trypsinizing flask. Cells were collected after a 12-min treatment with 0.25% trypsin, centrifuged at 1,000 rpm for 5 min, and suspended in growth medium. Cell culture bottles (32 oz [about 0.946 liter]) were inoculated with approximately 9 × 10⁶ cells and allowed to form confluent monolayers. Secondary CEF cultures were prepared from these primary monolayers by trypsinizing the cell sheet and resuspending cells in growth medium at a concentration of 10⁶ cells per ml. Cell culture dishes (60 mm) were inoculated with 5.0 ml of this suspension; confluent monolayers grew out in 24 h.

Preparation and assay of MDV. Feather follicle tracts were removed from the skins of 4-week-old chickens infected at 1 day of age. Those positive for viral antigen in the agar gel precipitin (AGP) test (6, 9) were extracted in the sucrose-phosphate-glutamate (SPGA) buffer developed by Bovarnick et al. (3) and recommended for use with MDV by Calnek et al. (7). Feathers were cut off at the skin surface, and a 10% (wt/vol) suspension was prepared by homogenization.
for 2 min in a Sorvall mixer at 14,500 rpm and sonic treatment in a Heat-Systems model 185 sonic oscillator for 30 s at 75 to 90 W of power. Materials were held in an ice bath during homogenization and sonic treatment. Extracts were centrifuged for 10 min at 600 × g in a Sorvall RC-2B refrigerated centrifuge. In a few instances, only tips of feather shafts were used for extraction; these were processed in the same manner as the feather follicles. The 10,000 × g supernatant fluids, which were considered cell-free, were stored in liquid nitrogen.

Assays were routinely conducted in CKC cultures by using two plates for each dilution of virus; dilutions were prepared in SPGA containing 0.2% ethylenediaminetetraacetic acid (EDTA) (1). Cell cultures were drained, inoculated with 0.1 ml of virus dilution, and incubated for 30 min at room temperature, after which maintenance medium was replaced. Foci were counted with an inverted microscope 8 to 10 days postinoculation.

Preparation and assay of cell-free HVT. A 90-ml amount of cell-free HVT was prepared in SPGA buffer by harvesting 60 monolayers from 100-mm dishes with a rubber spatula after 75 to 90% of cells showed lysis. Cells were resuspended in buffer, sonically treated for 15 s at 75 W, and centrifuged at 10,000 × g for 20 min. Supernatant fluid was stored in liquid nitrogen.

Assays were conducted in CEF cultures by inoculating 0.1 ml of each dilution of virus onto duplicate confluent monolayers. After incubation for 1 h at room temperature, maintenance medium was added and the plates were incubated. Eighteen hours postinoculation, monolayers were drained and an agar overlay medium (Eagle MEM plus 10% TPB plus 2% calf serum plus 0.005 M HEPES buffer [N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid] plus 0.08% I onagar No. 2) was added. Plaques, visible by 3 days, were counted at 4 to 5 days postinoculation.

Neutralization test procedure. For the neutralization test the constant virus-decreasing serum procedure, employing plaque reduction in cell culture as the indicator system, was used. HVT was used as the test virus, and a dilution containing 50 to 80 plaque-forming units (PFU)/0.1 ml was prepared in SPGA and mixed 1:1 with various serum dilutions. Controls were mixed 1:1 with diluent. Virus-serum and virus-diluent mixtures were incubated for 1 h at room temperature and then assayed in CEF cultures. Two drained monolayers were each inoculated with 0.2 ml of material and incubated for 1 h at room temperature, and then maintenance medium was replaced. Eighteen hours postinoculation, cultures were drained and the agar overlay medium was added to each plate. Plaques were counted at 5 days postinoculation. The end point was expressed as the reciprocal of the serum dilution causing a 50% plaque reduction, and significant neutralizing antibody was considered to be present when a 1:20 serum dilution caused 50% reduction (17).

Storage life: MDV extracts. Five samples of MDV were prepared from skin extracts as described, with the exception that one was the supernatant fluid of a 600 × g centrifugation rather than the usual 10,000 × g. Assays were done in CKC cultures after 1, 6, and 12 months of storage, and the loss of infectivity was determined.

Virus loss with centrifugation. Loss of infectious virus because of successive centrifugation at 2,000, 5,000, and 10,000 × g was determined by assay of supernatant fractions in CKC. Two extracts were tested.

Neutralization test curves. To determine the standard neutralization curve, titration of virus-serum mixtures was conducted at 4, 23, and 37°C. A hyperimmune HVT antiserum, which had titrated 1:160 in a previous neutralization test, was diluted 1:25, 1:125, and 1:625 and mixed with equal volumes of virus. Tests were conducted after incubation periods of 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 6 h at each of the three temperatures. As a control, negative serum diluted 1:5 was mixed with virus; infectivity was tested after 0, 0.5, 1, 2, 4, and 6 h of incubation at each temperature. This negative control serum was obtained from 8-week-old uninfected Line 7 chickens which had been held in isolator units prior to serum collection. This serum was negative for precipitin and neutralizing antibodies.

Development of neutralizing antibody. Five groups of three birds (Line 7) each were tested for maternal precipitin antibody at 1 day of age and divided into groups positive (groups 2, 4, 5) and negative (groups 1, 3) for detectable antibody. A single dose of 3,000 PFU of HVT was given intra-abdominally to each bird in groups 1 through 4 at 1 day of age. Group 5 was the uninoculated control group; groups 3 and 4 received additional inoculation of 1,000 PFU/bird at 21 and 32 days of age. Serum samples were collected each week, and equal amounts were pooled, heat inactivated at 56°C for 30 min, and tested for neutralizing antibody. Individual samples were tested each week for precipitin antibody in feather shafts by using the AGP test. At the end of the experiment, birds were killed and examined for gross lesions of MD and sections of brains were examined microscopically.

As an additional study, six birds were inoculated intra-abdominally at 1 day of age with 300 PFU of cell-free MDV skin extract virus and held in a modified Horsfall-Bauer unit. These birds were bled at 7 and 12 days, and then every 3rd day through 42 days postinoculation. Each serum sample was heat inactivated and assayed for neutralizing antibody. At the completion of the experiment, surviving birds were autopsied and examined for gross lesions.

Cross-neutralization studies. Antisera were prepared against HVT and the GA, JM, Con A, and Con B strains of MDV by inoculating day-old chicks with cell-free extracts of each virus. Five chicks were inoculated with each virus strain. To prevent cross-infection, birds which received HVT, GA, and JM virus were placed in isolated facilities that had never been used to house chickens or conduct MD research. Birds receiving Con A and Con B were placed in modified Horsfall-Bauer units located in buildings in which studies had been conducted with only that respective virus. Serum samples were collected at 6 to 8 weeks postinoculation and tested for precipitin antibody with the AGP test by using skin extract from...
chickens inoculated with Conn B as the soluble antigen. Serum pools were prepared for each virus strain from the AGP-positive samples, inactivated at 56 C, and tested for neutralizing antibody. Antiserum prepared against HVT was tested for comparable neutralization of HVT and MDV.

**RESULTS**

**Virus assay.** A total of 35 MDV skin extracts was prepared and titered during this study; the highest titer obtained was 12,000 PFU/ml, but the majority of extracts titered less than 1,000 PFU/ml. Thus, titers of MDV obtained from skin extracts were generally too low for use as standard virus for the neutralization test. Therefore, a pool of cell-free HVT was prepared and used for neutralization tests. This preparation, when diluted 1:25, consistently produced 60 to 80 PFU/0.1 ml. HVT was titered in each test at dilutions of 1:5, 1:25, 1:250, and 1:500, and plaques were counted after the agar was lifted off and the cellular monolayer was stained with crystal violet. Two different types of plaques, a focus of refractile rounded cells (Fig. 1) and a “classic” plaque with a central clear area surrounded by rounded refractile cells, were observed (Fig. 2). The latter type was often produced during agar removal—when heavily infected cells remained attached to the agar as it was removed, resulting in a hole in the monolayer (14). However, holes were also observed without removal of agar.

**Storage life.** Extract 1 had the highest virus titer of the five MDV preparations tested. It contained 12,300 PFU/ml after 1 month of storage, 1,700 PFU after 6 months of storage, and showed a complete loss of titer after 12 months of storage. Although the other four extracts had lower initial virus titers, they showed a similar pattern of loss of titer during storage, i.e., at least 95% of the original titer after 12 months. In contrast, the HVT stock preparation showed no deterioration after 1 year of storage in liquid nitrogen.

**Centrifugation loss.** Supernatant fluids from MDV extracts 1 and 2 had titers of 9,000 and 2,000 PFU/ml, respectively, after centrifugation at 600 x g. The number of PFUs decreased 72 to 76% after centrifugation of the 600 x g supernatant fluid at 10,000 x g; centrifugation of a 600 x g supernatant fluid at 2,000 x g was sufficient to reduce virus titer by 51%.

**Standard curve for virus neutralization.** Standard curves obtained for neutralization reactions conducted at various temperatures showed that, with hyperimmune HVT antiserum diluted 1:25, peak neutralization, as indicated by percentage of plaque reduction, occurred in 3 h at 4 C, 1 h at 23 C, and 0.5 h at 37 C (Fig. 3-5). The 1:125 serum dilution followed approximately the same pattern, but the corresponding reductions in PFU were lower for each assay period. At 37 C the 1:625 dilution of specific antiserum and the 1:5 dilution of normal serum caused reductions as high as 41% after 6 h of incubation, but reduction did not reach the level of 50% which was considered the minimal indication of neutralization.

**Neutralizing antibody in chickens inoculated with HVT.** By the second week after inoculation with HVT, the mean neutralizing titer for all groups was similar, i.e., 1:20. By week 5 it was clear that titers were dependent on dose of virus administered, with groups receiving three inoculations showing considerably higher mean neutralizing titers (Fig. 6, 7). Decay of maternal antibody among the control birds had occurred largely by week 3. At the end of the experimental period of 7 weeks, no appreciable difference in titer was seen between groups with or without maternal antibody at the start of the immunization schedule (Fig. 6, 7).
FIG. 3. Turkey herpesvirus neutralization curve. Normal serum (diluted 1:5) and hyperimmune antiserum (diluted 1:25, 1:125, and 1:625) were reacted for various time periods at 4°C with an equal volume containing the test dose of virus, then assayed on CEF cultures.

FIG. 4. Turkey herpesvirus neutralization curve. Normal serum (diluted 1:5) and hyperimmune antiserum (diluted 1:25, 1:125, and 1:625) were reacted for various time periods at 23°C with an equal volume containing the test dose of virus, then assayed on CEF cultures.

All samples of serum or feather tip samples from individual birds were tested and found to be negative for precipitin antibody or antigen. Birds autopsied did not exhibit any gross lesions of MD, but histopathological examination of brains from these birds revealed that ½ of the HVT-inoculated birds and ⅔ of contact controls had brain lesions similar to those produced
by MDV, although generally the degree of perivascular cuffing with mononuclear cells was minimal.

Neutralizing antibody in chickens inoculated with MDV. Birds inoculated with MDV developed precipitin antibody by 30 days postinoculation (Table 1). By 36 days postinoculation all birds exhibited symptoms characteristic of Marek's disease, i.e., depression, ruffled feathers, and paralysis. Two birds died during the experiment and at necropsy had lymphomas of the testes, kidneys, liver, and spleen. No gross lesions were observed among surviving birds autopsied at the end of the experiment.
Cross-neutralization comparisons. Serum from chickens given a single dose of the Conn A, Conn B, JM, and HVT virus isolates all had about the same neutralizing activity, i.e., the 1:20 dilution reduced the plaque count by 50% or more. Antiserum to the GA isolate and hyperimmune HVT antiserum had higher titers of 40 and 160, respectively (Fig. 8).

In the two trials in which HVT antiserum was reacted against MDV (Table 2), the 1:20 dilution of hyperimmune antiserum neutralized 100% of the virus, and the same dilution of serum (from chickens receiving only one dose of HVT) neutralized 75% of the virus. Although normal serum caused some reduction in focus count, it did not approach the 50% level used as the arbitrary criterion for neutralization. The hyperimmune serum exhibited neutralizing activity at a 1:100 dilution also, but not at 1:200.

DISCUSSION

Three procedures have been used to prepare

Table 1. Development of neutralizing antibody in birds inoculated at 1 day of age with Marek's disease skin extract virus

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a Days postinoculation.

b p, First sample which was positive for precipitin antibody; all subsequent samples were also positive.

c Indicates bird died prior to the day samples were taken.

Fig. 8. Plaque reduction curves obtained with convalescent antiserum from the groups of chickens inoculated with HVT and GA, JM, Conn A, and Conn B strains of MDV; hyperimmune antiserum prepared by multiple inoculation of chickens with HVT; and a serum sample from uninoculated chickens.
cell-free virus extracts of MDV. Cook and Sears (10) used a demineralized water treatment to extract virus from cell cultures. Evans et al. (13) obtained cell-free virus from chicken dander by sonic treatment for 5 min and differential centrifugation followed by filtration. Calnek and associates (5, 6), who first described cytoplasmic enveloped herpesvirus particles in epithelial cells of feather follicles from MDV-infected birds, also have extracted MDV from skin feather tracts by homogenization, sonic treatment, and centrifugation. HVT is readily extracted from infected cell cultures by sonic treatment and centrifugation (7). With these procedures, relatively high-titer virus preparations can be prepared and stored with relatively little deterioration.

Extracts of MDV and HVT used in this study are described as cell-free preparations. Since centrifugation at 6,000 × g is sufficient to sediment nuclei, membranes, and most other cellular components, and all virus extracts were 10,000 × g supernatant fluids, the cell-free classification is justifiable. In addition, Carrozza (Ph.D. thesis, University of Connecticut, Storrs, 1972) viewed 10,000 × g supernatant fluids of similar material with the electron microscope and found no intact cells. The sonic treatment also reduces the likelihood of intact cells being present in the supernatant fluid.

Cell culture provides a reliable procedure for in vitro assay of both HVT and MDV. Results obtained with MDV assay in this investigation generally support those by Addlunger and Calnek (1). The results indicate that cell culture is a fairly sensitive assay system. EDTA (0.2%) was included in the SPGA buffer, and this resulted in a fivefold increase in virus titer over SPGA alone. This effect is possibly due to the action of EDTA, a chelating agent, on the cell membrane, enhancing adsorption and/or penetration of the virus.

Development and standardization of the plaque reduction test for MD was based on the recommendations of Lennette and Schmidt (16), including a dose test virus of 100 PFU/0.1 ml, a 1:1 mixture of the virus with the serum dilution, and a virus-serum incubation period of 1 h at room temperature. After incubation, cell cultures were infected with 0.2 ml of the virus-serum or virus-diluent sample. Such a procedure represents a constant virus-decreasing serum method, and serum end points are represented by the reciprocal of the serum dilution causing a 50% plaque reduction.

Initially, MDV extracts from infected skins was planned for use as the standard virus antigen for the plaque reduction test. However, MDV was unsatisfactory as a test virus because titers deteriorated rapidly on storage, even in liquid nitrogen. Of the 35 extracts prepared over an 8-month period, only 5 had suitable titers. Part of the reason for the loss of MDV infectivity might have been due to the g force used for clarification since extracts clarified at 600 to 1,000 × g maintained infectivity. In one report, MDV in a 1,000 × g supernatant fluid was found attached to membranes and also contained in nuclei (2), and membranes and nuclei may exert a protective and adherent effect on the virus. Since centrifugation at 10,000 × g would remove the nuclei and much of the membrane fraction, the remaining virus may not survive storage. This may also explain why so much virus was lost during centrifugation of the 600 × g supernatant fluid, since at 10,000 × g, virus attached to membranes, nuclei were sedimented, and possibly unattached virus remained in the suspension. Calnek and Addlunger (5) have reported that 600 × g supernatant fluids of MDV did not decrease in titer after 210 days of storage at 65 C. However, with 10,000 × g supernatant fluids, titers showed a considerable decrease after 180 days of storage.

For conducting cell culture studies, the use of HVT has many advantages over MDV. It can be extracted easily from infected CEF cultures by sonic treatment and centrifugation and can be stored in liquid nitrogen for up to 1 year with no appreciable loss in titer. EDTA does not enhance HVT titers and can be eliminated from the diluent (19). Plaques form 3 days after infection of the cells and can be counted, without the use of a microscope, 4 to 5 days postinoculation. Thus, the test is much faster and easier to read than when MDV is used as the indicator virus. Finally, the fact that HVT can be propagated in CEF cultures is a distinct

### Table 2. Neutralization of cell-free MD skin extract virus with antiserum prepared in chickens against HVT

<table>
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<th>Serum dilution</th>
<th>PFU/ml Trial 1</th>
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<td>220</td>
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<tr>
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<td>Virus + HI HVT serum</td>
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advantage since these cells are much easier to prepare and handle than CKC cultures, and there is less danger of adventitious infection with latent MDV.

The plaque reduction test, employing HVT as the test virus, was used to conduct a number of neutralization studies. The effect of temperature on rate of neutralization was studied by conducting tests at 4, 23, and 37°C. As expected, the rate of neutralization decreased as the temperature at which the virus-serum mixtures were incubated was lowered. Based on the results of this work, it would appear that the following incubation conditions are satisfactory for conducting this test: 30 min at 37°C, 60 min at 23°C, and 3 to 4 h at 4°C. For all of the other studies reported in this work, an incubation period of 60 min at room temperature (23°C) was used.

To avoid virus clumping and insure dispersion, the virus suspension must be thoroughly mixed, and for that purpose the Vortex-Genie mixer used in this study proved adequate. The number of infectious virus particles in the test dose should be kept between 50 and 100 to provide a convenient number of plaques for counting when determining serum titers. Plaque distribution was uniform throughout the plate with the methods described.

A critical component of the agar overlay medium used for this test is HEPES buffer, which is particularly efficient in stabilizing pH (11). In tests conducted without this buffer, the pH of the medium turned alkaline, possibly because of alterations of CO₂ content in the incubator, thus preventing or inhibiting plaque formation.

Birds inoculated with HVT and held in strict isolation were tested weekly for precipitin antibody and skin antigen; all remained negative for both tests throughout the 7 weeks of the experiment. In tests for precipitin antibody, serum samples were only reacted against the MD-soluble skin antigen because all attempts to prepare a soluble antigen from HVT-infected cell cultures were unsuccessful. This may have been due to the antigenicity of this virus preparation or to some other factor not identified in this study. In spite of this, birds developed significant neutralizing titers for HVT within 3 weeks postinoculation.

Results of cross-neutralization tests suggest strongly that an antigenic relationship exists between HVT and the various MDV strains tested. Patterns of plaque reduction with antiserum to MDV strains closely followed that of the HVT antiserum, and titers were similar. This determination correlates with results of other studies which have shown an antigenic relationship between MDV and HVT precipitin and fluorescent antibodies (21). This relationship allowed the use of HVT as the indicator virus in this neutralization study. Unfortunately, due to difficulties encountered with MDV, it was not possible to determine the antigenic relationships among the MDV strains. This would, of course, require suitable cell-free virus preparations for each MDV strain to be tested. Although it is known that HVT antiserum neutralizes MDV and vice versa, the actual mechanism of protection afforded by HVT vaccines has still not been defined.

The plaque reduction test, as described in this investigation, represents an excellent tool for the study of neutralizing antibody development in MDV- and HVT-infected chickens and invites further investigation of the role of such antibodies in the protective action of HVT vaccines.

ACKNOWLEDGMENT

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LITERATURE CITED