Radial Immunodiffusion: a Simple and Rapid Method for Detection of Marek’s Disease Antigen(s)¹

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A qualitative radial immunodiffusion technique is described which detects antigen(s) in feathers from live or dead chickens infected with Marek’s disease herpesvirus. Antiserum, which is incorporated into a support medium, reacts with antigen(s) in the feather tip producing a radial precipitin ring. Antigen(s) was detected in 93.3% of experimentally inoculated chickens 21 days postinoculation and in 100% of infected birds subsequently tested through 6 weeks. No antigen was detectable in the feathers of un inoculated control chickens. The technique is simple and rapid to perform. Positive tests could be detected after 1 to 2 hours of incubation. Antigen detection by the radial immunodiffusion test correlated well with other criteria of infection. This technique should have application as a laboratory research tool and as an adjunct for a rapid flock diagnosis of Marek’s disease.

The most commonly used methods for detecting Marek’s disease herpesvirus (MDHV) or associated antigen(s) from diseased chickens include cell culture techniques and chicken bioassay, both of which may be combined with fluorescent antibody tests and embryo inoculation (2).

A very significant advance in the study of Marek’s disease (MD) was made with the discovery that viral antigens occurred in the superficial layers of feather follicle epithelium (4). It was subsequently shown that follicle epithelium was a source of infectious virus as well (3, 8, 9). Since the early report (4), extracts of feather follicles from infected birds have provided a ready source of antigen(s) required for serological tests in this laboratory (W. W. Marquardt, unpublished data). Several other workers have reported the preparation of antigens from feathers which could be used in agar gel precipitin (AGP) tests to detect MD antibodies (1, 6).

This report describes a simple and rapid qualitative technique to detect antigen(s) found in the feathers of living or dead chickens with MD. It is based on a modification of a quantitative method described by Mancini et al. (7).

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Materials and Methods

Virus source and culture. A MDHV preparation was obtained through the courtesy of D. A. Espeseth of this department. The MD isolate, designated CM in this laboratory, was obtained by him from M. K. Cook, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. This isolate has been found free of chick syncytial virus and RIF viruses. The CM virus (numbered 10411) was originally isolated from a flock of broiler birds at Sumter, S. C. which exhibited classical signs of MD. It produced characteristic cytopathic effects in duck embryo fibroblast (DEF) cultures. Susceptible chickens developed typical signs of MD within 34 days after intra-abdominal inoculation. Antigen prepared by freezing and thawing CM virus-infected DEF cells gave a line of identity with a cell culture antigen with the JM strain of MDHV (D. A. Espeseth, M.S. thesis, University of Maryland, College Park, 1971).

The virus was propagated in DEF cultures according to the method of Solomon et al. (10). Virus from the sixth passage in DEF cultures was stored in growth media with 7.5% dimethyl sulfoxide at −70 C and used for inoculation of chickens.

Chickens, source and inoculation. Cornell S-line embryonating eggs were obtained from R. K. Cole, Cornell University, Ithaca, N.Y. They were hatched and transferred immediately to plastic isolator units. The chickens were divided into two groups. Fifteen-day-old chickens were inoculated intra-abdominally with $7.3 \times 10^7$ plaque-forming units of CM virus. Twelve uninoculated control chickens were maintained in separate isolator units.

Feather collection. Feathers were taken from all
inoculated and control birds 3 weeks after initiation of the experiment. Feathers also were collected from birds which were paralyzed and killed during the experiment and from all survivors at the end of the 6-week experiment. Eight to 10 feathers were taken from the wing or intrascapular feather tract of each bird and placed in a labeled and capped 12 by 75-mm plastic tube (Falcon Plastics, Los Angeles, Calif.) and kept at room temperature. The test for MD antigen in feathers was conducted within 3 days after collection.

**Preparation of radial immunodiffusion (RID) plates.** Plastic tissue culture plates (35 by 10 mm; Falcon Plastics) were employed. The method of preparing antiserum-agar plates was a modification of the method described by Mancini et al. (7). The MD antiserum was obtained from MD contact exposure survivors or from chickens with a natural MD infection. These sera were positive by an AGP test conducted by a method similar to that described by Chubb and Churchill (5) and positive in a complement fixation test (W. W. Marquardt, unpublished data). The amount of antiserum to be used in the test was determined initially by making serial two-fold dilutions of antiserum and preparing antiserum-agarose plates. Tests were conducted with known positive feathers. For a qualitative test the antiserum dilution, within limits, was not critical although the size of the precipitin ring could be varied inversely with the antiserum concentration. Antiserum dilutions of 1:10 to 1:25 were used with satisfactory results.

A 0.7% solution of agarose (Nutritional Biochemicals Corp., Cleveland, Ohio) was prepared in 0.1 M phosphate-buffered saline, pH 7.2, with 8% sodium chloride or in unbuffered 8% sodium chloride. The agarose was melted and held at 56 C. In some cases 0.01% Merthiolate was added to the agarose. The required amount of antiserum was brought to 56 C, added to the agarose, and carefully mixed. A warmed pipette was used to dispense 3 ml of mixture per plate. The agarose-antiserum was allowed to harden on a flat surface, and the plates were maintained at room temperature in a humidified chamber. They were used immediately or within 3 days of preparation.

**RID test.** About 0.5 cm of each feather tip was cut off, and the proximal end was gently embedded in the antiserum-agarose with a smooth forceps. Usually eight feather tips per bird were tested with 16 tips (two birds) on a single plate. A template was used to space the feather tips about 8 mm apart. A reference mark on the plate permitted individual bird and feather identification. A 2-mm well was cut in each plate and filled with a saline infusion control antigen which was prepared from feather tips of MD-infected chickens. The plates were incubated in a humidified chamber, right side up, at room temperature. They were observed at hourly intervals but the final reading was taken following overnight (ca. 18 hr) incubation. Tests were read with the aid of reflected light provided by a fluorescent lamp. Reading the test was facilitated by prior removal of the feather tips.

**Virus reisolation.** Virus isolation was attempted by direct kidney cell culture from birds which died or became paralyzed during the course of the 6-week experiment. Virus isolation was attempted from survivor birds, including controls, at the end of 6 weeks by inoculation of heparinized blood samples directly on monolayers of DEF cells (D. A. Espe, M.S. thesis, Univ. of Maryland, 1971). One ml of blood was placed on each of two 60 by 15-mm plastic plates of DEF cells (two plates per bird). The growth medium was as described by Solomon et al. (10) and blood was removed after 24 hr and maintenance medium added. It was changed as necessary during a 9-day observation period for cytopathic effects.

**Fluorescent antibody procedure.** Skin specimens were taken from infected chickens when they died or became paralyzed. Specimens were also collected from all infected survivors and control chickens at the end of the experiment. The specimens were frozen in dry ice-acetone and two or more 8 to 12 x 10-mm sections were cut on an International cryostat (Model CT1) and placed on glass slides. The slides were stored in acetone at -60 C until stained. Before staining by the direct fluorescent antibody (FA) procedure, the slides were briefly immersed in acetone at room temperature. The fluorescein isothiocyanate-labeled MDHV chicken antiserum used in this study was prepared by the method of Espe (D. A. Espe, M. S. thesis, Univ. of Maryland, 1971).

The slides were stained for 30 min at 37 C and rinsed for 15 min in phosphate-buffered saline. A drop of buffered glycerol was placed on the tissue section and a coverslip applied.

Slides were examined with a Lietz fluorescent microscope equipped with an Osram HBO 200 high-pressure mercury vapor lamp and a dark-field condenser. A Schott BG12 exciter filter combined with a Lietz OG1 barrier filter was employed. A bright green fluorescence in the nucleus and cytoplasm of infected cells was considered positive.

**Gross evidence of MD infection.** The development of paralysis during the course of the experiment and gross lesions observed in body organs or structures at necropsy were used as additional evidence of infection.

**RESULTS**

**RID tests.** The RID test was very simple and rapid to perform. The results were easily read with the aid of reflected light, and positive tests were observed after 1 to 2 hr of incubation.

The test appears to be a very sensitive and specific indicator for detecting antigen(s) in feather tips associated with MD. Table 1 shows the results of RID tests conducted on feathers from all birds at 3 weeks postinoculation (PI), on birds which died or became paralyzed during the experiment, and on all survivor birds killed at the end of the experiment. It can be seen that 14 of 15 infected birds (93.3%) had RID-positive feathers at 3 weeks PI, and 100% were positive thereafter. None of
TABLE 1. Detection of Marek's disease virus antigen in feather tips by radial immunodiffusion and summary of criteria for Marek's disease infection

<table>
<thead>
<tr>
<th>Test</th>
<th>RID with FT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Virus isolation from kidneys or blood (no.)</th>
<th>FA test&lt;sup&gt;b&lt;/sup&gt; on skin (no.)</th>
<th>Paralysis (no.)</th>
<th>Gross lesions at necropsy (no.)</th>
</tr>
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<tr>
<td>Infected</td>
<td>14/15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10/14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12/12&lt;sup&gt;e&lt;/sup&gt;</td>
<td>11/15</td>
<td>12/15</td>
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<tr>
<td>Controls</td>
<td>0/12</td>
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<sup>a</sup> Radial immunodiffusion (RID) performed on feather tips (FT) of all chickens at 3 weeks postinoculation (PI) and on chickens which died or were paralyzed during and at the end of the experiment (necropsy).

<sup>b</sup> Direct fluorescent antibody (FA) test.

<sup>c</sup> Number of chickens positive per number tested.

<sup>d</sup> Kidney cells from one chicken would not grow, three were negative, and one was not tested.

<sup>e</sup> Skin from three infected chickens was not tested.

the 12 uninoculated control birds had detectable RID-positive feathers at 3 weeks or at 6 weeks after initiation of the experiment.

Of 15 infected birds, 13 had MD antigen(s) in 100% of eight feathers tested at 3 weeks PI. One bird had antigen(s) in 62.5% of the feathers tested, and feathers from another bird were all negative by RID.

Subsequent RID tests on these infected birds resulted in 12 of 15 birds with MD antigen(s) in 100% of the eight feathers tested. One bird had antigen(s) in 87.5%, another bird 75%, and one bird had antigen in 25% of the feathers tested. The latter bird was dead and partially decomposed when feathers were collected.

**Virus isolation.** The results of virus isolation from infected and control chickens are also shown in Table 1. Virus was reisolated from kidneys or blood in 10 of 14 infected birds cultured. Kidney cells from one bird would not grow. Virus isolation was not made from three infected chickens nor from any of the 12 control chickens.

**FA test.** The results of FA tests on follicular epithelium from infected and control chickens are shown in Table 1. It can be seen that 12 out of 12 (100%) of the infected birds gave a positive FA test whereas none of the 12 control birds was positive by this test.

**Other criteria of infection.** Eleven of 15 infected birds (73.3%) exhibited paralysis of one or both legs sometime during the 6-week experiment (Table 1). None of the 12 control chickens showed signs of paralysis.

At necropsy 12 of 15 infected chickens (80%) had gross lesions of MD (Table 1). The gross lesions consisted of tumors or enlargement of one or several body organs or structures. Tumors were found in the kidneys, heart, proventriculus, liver, lung, and pectoral muscle. The gonads were enlarged to several times their normal size, and the sciatic nerve was frequently enlarged as judged by loss of striations. None of these lesions were observed in any of the 12 control chickens.

**DISCUSSION**

During the course of MD, the virus responsible for the disease is located in the skin follicular epithelium as well as other target organs. The RID test described was capable of detecting antigen(s) in the feather tips of live or dead birds associated with this infection. This sensitive technique is quite simple to perform, and results can be obtained rapidly. It was possible to detect positive feathers by 1 hr after the tips were embedded in the antiserum-agar. This could be of value from a diagnostic standpoint by permitting a rapid positive diagnosis. Weak reactions may not be detectable this early. Therefore, it is best to allow 12 to 18 hr for incubation, after which period no detectable qualitative changes were found.

Removal of the feather tips at completion of the test facilitated the final reading by increasing the contrast, particularly when feather tips produced a small precipitin ring. Variation in precipitin ring diameter was observed on the same plate and appeared to be related to the diameter of the feather tips which were not uniform. It could also be a reflection of antigen concentration, being higher in the larger tips. Diffusion proceeds until an optimal proportion between antigen and antiserum was reached. The size of the ring does not present a problem for a qualitative test of this type because the ring is either present or absent.

Ninety-three per cent of the infected birds had detectable antigen in their feathers by 3 weeks postinfection. It has been observed that MD antigen(s) could be detected in feathers by
RID as early as 12 days postinfection (W. W. Marquardt, unpublished data). This is somewhat later than the immunofluorescent-staining antigen reported by Purchase (9). He was able to detect immunofluorescent antigen in follicular epithelium by 5 days PI.

All of the infected birds gave positive RID tests subsequent to the initial test at 3 weeks PI. None of the control chickens gave positive tests by RID at any time. This indicates that the RID test is specifically measuring antigen(s) associated with MD. The greater the number of feathers used in the test, the greater the assurance of detecting the MD antigen in an individual bird. However, on a flock basis assuming all or nearly all birds are infected, the number of feathers tested per bird does not seem critical in making a flock diagnosis.

The apparent lack of antigen in the feather tips of one inoculated chicken at 3 weeks PI was verified by retesting another series of eight feathers. This suggests that the pathogenesis of infection occurs at different rates in different chickens. Antigen was detected in the feather tips of this bird when the experiment was terminated, and the virus was also isolated. If the initial dose of inoculum was ineffective in establishing an infection in this bird, perhaps infection was established through contact with virus-shedding cage mates. Contact transmission from virus-shedding infected birds has been observed to occur in a period of just over 2 weeks (W. W. Marquardt, unpublished data).

Virus isolation of DEF cells was achieved from 71.4% of the infected birds with positive RID tests. Thus, it appears that evidence of infection by RID is a more sensitive indicator than virus isolation. The kidney cells of one dead bird would not grow, presumably because nearly all normal kidney cells were replaced by tumor cells.

The RID and FA tests were in good agreement. All birds positive by the FA test were also positive by RID. One bird positive by these tests was not paralyzed and did not have gross lesions at necropsy.

The CM virus used in this study produces a high rate of paralysis (73.3%) and gross lesions (80%) in genetically susceptible S-line chickens. However, the RID test appears to be a more reliable criterion of MD infection than either of these two parameters.

The simplicity and specificity of the RID test and the rapidity in which it can be conducted make it a valuable technique in laboratory investigations of MD. It should also find utility in rapid flock diagnosis of MD and may serve as a valuable adjunct in the differential diagnosis of lymphoid leukemia and MD. The lymphoid leukemia viruses are not known to invade the skin but may provoke other similar pathological changes.

This technique offers an advantage over antibody detection in that it should detect MD infections in immunologically incompetent birds too young to respond or, perhaps, in those birds which cannot respond because of an impaired immunological system.

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LITERATURE CITED