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Immunofluorescence in the Study of Marek's Disease

I. Detection of Antigen in Cell Culture and an Antigenic Comparison of Eight Isolates[1]

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The indirect fluorescent-antibody (FA) test was applied to the detection of Marek's disease (MD) antigen in cell culture and antibody in the serum of birds. For the detection of antigen, sera were obtained from birds hyperimmunized with the JM strain of MD. MD antigen could be detected in the nucleus and in the cytoplasm of duck and chick embryo fibroblasts and in those of chick kidney cells infected with material known to contain the MD virus. Uninoculated cultures of chicken cells were always free of MD antigen. When chick kidney cells were infected with a stock cellular preparation of MD virus, infected cells could be detected after 24 hr with the FA test. At this time no cytopathological areas were seen by conventional light microscopy. By 7 days after infection, the same number of infected areas were detected by both methods, and the fluorescent areas coincided with the cytopathological areas. This indicates that the fluorescent areas and the areas with cytopathology are caused by the same agent. A straight-line relationship between the dilution of inoculum and the number of fluorescent or morphological foci obtained indicates that one infectious unit produced one fluorescent or morphological focus. In addition, this time sequence study confirmed the cell association of the virus and demonstrated the cell-to-cell spread of infection. Cell cultures inoculated with eight different isolates of MD were tested in all combinations with sera prepared against the same isolates. The antigens were indistinguishable from one another, indicating that either the strains are antigenically identical or there is a common antigen or contaminant in all of them so that they stained equally well. The FA test can detect MD antigen before cytopathological areas develop in cell culture; however, the small size of the area usually examined precludes its use in initial isolations in which only a small number of infectious units are present in the inoculum. MD-infected cells contain a heat-stable antigen similar to that found in herpes simplex-infected cells.

A wealth of circumstantial evidence has accumulated which incriminates a highly cell-associated herpesvirus as the etiological agent of Marek's disease (MD) (4, 12, 15, 18). The agent produces characteristic cytopathic changes in cultures of chick kidney cells, duck embryo fibroblasts, and, under special conditions, in chick embryo fibroblasts (11). Although some attempts to obtain cell-free virus have been successful, most efforts have failed. It has been impossible to obtain sufficient cell-free virus to perform neutralization tests for the identification of the virus or the detection of antibody in sera of birds.

[1] Preliminary results were reported at the 105th annual meeting of the American Veterinary Medical Association in Boston, Mass., July 1968.

Recently an antigen has been detected in infected-cell cultures which produces a line of precipitation in an agar-gel when diffused against serum from infected or recovered birds (3). This antigen can be used in the agar-gel precipitin test for the detection of antibody in birds which have been exposed to MD.

Kottaridis and Luginbuhl (10) have described a direct fluorescent-antibody (FA) test for MD antigen in cell culture. They used sera obtained from rabbits which had been hyperimmunized with extracts of infectious blood. This paper describes the application of the indirect FA test to the detection of MD antigen in cell culture and antibody in the serum of birds.

MATERIALS AND METHODS

Chickens and eggs. The inbred lines maintained at the Regional Poultry Research Laboratory were used throughout. Line 7 chickens and those produced by the cross between line 15 males and line 7 females are highly susceptible to MD, line 15 and line 15I are intermediate, and line 6 chickens are resistant to MD (5). Except where otherwise indicated the parent lines are maintained in conventional chicken houses and the flock is known to harbor MD viruses. Progeny from these chickens reared in modified Horsfall-Bauer isolators are usually free of all signs of infection.

Sources of viruses. The origin of the JM isolate of MD has been described (17). MSD 1 was obtained from T. Maag, Merck & Co., Inc., Rahway, N.J.; GA from S. Schmittle, University of Georgia (8); CONN A from R. Luginbuhl, University of Connecticut (2); CR 64 from W. Staples, Cobb Breeding Corp., Connecticut; C1 from R. Bankowski, University of California (1); RPL 39 from a field outbreak of MD in Georgia; and FC 50 from an outbreak of MD in Michigan. These isolates had been passaged 40, 1, 19, 0, 5, 1, 2, and 1 times, respectively, in chickens from the Regional Poultry Research Laboratory.

Two strains of laryngotracheitis virus were kindly supplied by R. Luginbuhl. Bryan's high titer strain of Rous sarcoma virus with Rous-associated virus$_1$ as helper [BH-RSV (RAV$_1$)] and BH-RSV (RAV$_2$) were obtained from P. K. Vogt (9).

Antigen. Kidney cultures were prepared as described by Churchill and Biggs (4) from a bird which had been inoculated with MD-infected blood and which had clinical signs and gross lesions of MD. When cytopathological areas appeared (4, 19), the cells were trypsinized and plated on fresh confluent monolayers of kidney cells prepared from uninfected birds. This procedure was repeated until an extensive cytopathological effect (CPE) was obtained, whereupon the cells were trypsinized and frozen in dimethyl sulfoxide and stored in liquid nitrogen (7).

Primary chick kidney cultures were prepared and grown on cover slips (11 by 22 mm) placed in 60-mm plastic disposable petri dishes and they were infected with various sources of MD virus. At intervals or when a clearly visible CPE was present, cover slips were removed, rinsed in phosphate-buffered saline (PBS), and fixed by immersion in acetone at 4 C for 2 min. The cover slips were dried under an air blower and stored at 4 C until used within the next few days.

Primary chick embryo and duck embryo fibroblast cultures were prepared as described previously (15, 16). Chick embryo fibroblasts infected with the JM strain of MD were obtained from K. Nazerian.

Antisera. Line 6 chickens were reared in modified Horsfall-Bauer isolators. At 8 weeks of age, a blood sample was obtained to confirm the absence of antibody. They were then inoculated intraperitoneally with 0.5 ml of fresh whole blood obtained from a chicken with clinical and gross signs of MD. Simultaneously, 0.5 ml of complete Freund's adjuvant was inoculated into the breast muscle. Birds were bled 2 weeks later for the cross-fluorescence studies in Table 1. In other instances in which a high titer of antibody was required, the above procedure was repeated 3 times at 2-week intervals and birds were exsanguinated 2 weeks after the last inoculation. All sera were heat-inactivated at 56 C for 30 min and clarified by centrifugation at approximately 1,000 $\times g$ for 5 min before dilution and application in the FA test.

Fluorescein-conjugated antichicken globulin. Conjugates from various commercial sources were tested by the following procedure. Spleen sections from freshly killed, 6- to 10-week-old chicks from any source available were cut at a 6 μm-thickness on a cryostat (Lab-tek; B. C. Ames Co., Waltham, Mass.). They were immediately fixed in acetone at -10 C for 2 min and then air dried. After moistening with PBS, the cover slips were flooded with twofold dilutions of various commercial fluorescein-labelled antichicken globulins and allowed to react for 30 min at room temperature and then washed in PBS for 15 min. The sections were then permanently mounted (6) and examined under a Leitz fluorescence microscope with a BG 12 excitor filter and an OG 1 barrier filter. Dilutions of the conjugate which stained the globulin-producing cells in the spleen with the least amount of nonspecific fluorescence of the surrounding tissue were selected for use in the indirect FA test. Conjugates from various commercial and laboratory sources were found to differ greatly in quality. The same dilution of the best conjugate was found to give the most satisfactory results in the indirect test by using MD virus-infected cell cultures as antigen and reacting this antigen with globulin from recovered serum and then with the antiglobulin as described below.

Staining procedure. Cover slips were divided into 1 to 4 areas with water-repellent ink and were attached horizontally to the top of rubber stoppers with adhesive tape. They were flooded with PBS for a few seconds, and then dilutions of serum were placed on the different areas of the cover slip. The stoppers were carefully placed around the periphery of a plastic beaker so that the cover slips pointed toward the center. The tightly covered beaker contained sufficient PBS, which was stirred continuously on a magnetic stirrer to keep the atmosphere humidified. After incubation at room temperature for 30 min, they were submerged in PBS and rinsed by gentle stirring for 15 min. The PBS was removed from the beaker with a vacuum device, and the cover slips were covered with an appropriate dilution of fluorescein-labelled antichicken gamma globulin and allowed to react for 30 minutes. The cover slips were again submerged for 15 min in PBS, removed from the stoppers, dipped in distilled water, and mounted on glass slides in 90% glycerol and 10% PBS, in Elvanol (13) or in Unimount (6).

Terminology. Cytopathological areas observed under conventional light microscopy are referred to as morphological foci, whereas those observed after FA staining are referred to as fluorescent foci.

RESULTS

Development of antigen. Monolayers of chick kidney cells on cover slips were infected with a stock preparation of JM-infected chick kidney cells. At 1, 3, 5, and 7 days after infection, cover slips were removed, fixed, and stained with the indirect FA technique in which sera prepared against the JM isolate were used, and they were examined under the fluorescence microscope. Uninfected cultures were similarly treated.

On the 1st day after infection, with conventional light microscopy, many rounded refractile cells could be seen attached to the monolayer, but they could not be recognized as morphological foci. Upon FA staining, however, some of the cells fluoresced very brightly. Many of them were spherical (Fig. 1), others had thin processes extending from them, and a few were flattened and resembled the surrounding kidney cells which were normal in shape but contained antigen (Fig. 2). There were many groups of cells which were morphologically indistinguishable from the surrounding cells but contained brightly staining antigens (Fig. 3 and 5). These areas could not have been recognized as cytopathological areas by conventional light microscopy.

By 5 and 7 days postinoculation, progressively more morphological foci were visible than at 3 days, and, on close examination, some larger refractile cells could be seen. Some fluorescent foci consisted mainly of rounded refractile cells (Fig. 6), and others contained one or more polykaryocytes (Fig. 7). Among different foci, the proportion of rounded cells to polykaryocytes varied. By the 7th day postinoculation, nearly all the fluorescent foci also contained cells with cytopathology (i.e., they coincided with morphological foci).

Both cytoplasmic and nuclear staining was observed, and it was not possible to determine which antigen appeared first. The staining in the nucleus was usually diffuse (Fig. 2 and 3) and did not obscure the unstained nucleolus. There was usually a nonstaining halo around the brightly stained "intranuclear inclusion" (Fig. 3 and 9). A diffuse staining was most common in the cytoplasm, although some cells also contained brightly staining, irregular granules (Fig. 9). The rounded cells in the center of the focus stained the most brightly and the intensity decreased centrifugally (Fig. 6).

At no time during these experiments did uninfected chick kidney cultures stain (Fig. 8).

In another experiment similar to that described above, chick kidney cultures were prepared directly from JM-infected birds showing clinical signs of MD. Antigen was first detected on the 2nd day after preparation of the cultures. The development of staining and cytopathology progressed as described above.

Staining of MD antigen in duck and chick embryo fibroblasts. Antigen in duck embryo fibroblasts infected with the JM isolate of MD stained brightly. There was a diffuse nuclear antigen and a diffuse and irregularly granular cytoplasmic antigen. In addition, many cells in both the infected and normal duck embryo fibroblast cultures contained small, uniform, spherical cytoplasmic granules which tended to obscure the specific stain in the infected cultures.

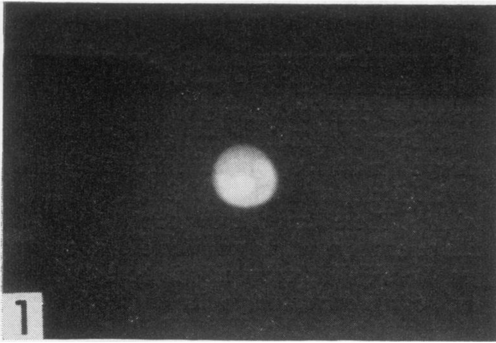
Chick embryo fibroblasts infected with MD contained a diffuse nuclear and cytoplasmic antigen, and granular cytoplasmic antigen could be easily detected in infected cells (Fig. 4).

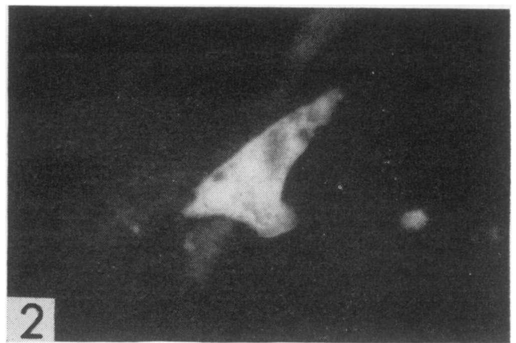
Staining of heterologous antigens. Chick kidney cultures on cover slips were infected with two strains of infectious laryngotracheitis virus, BH-RSV (RAV_1), BH-RSV (RAV_2), and JM isolate of MD, and chick embryo fibroblasts on cover slips were infected with BH-RSV (RAV_1) and BH-RSV (RAV_2). They were fixed and stained in the indirect FA test with a JM antiserum. There was no fluorescent staining in any of these cultures, except in those infected with the JM isolate of MD which showed bright specific fluorescence.

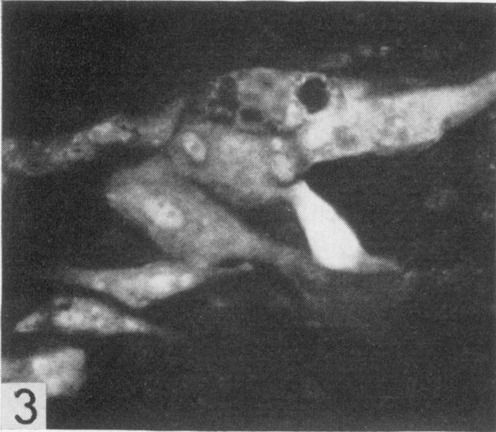
Controls within the indirect FA test. When saline or serum from uninfected birds replaced the anti-MD serum in the first step of the indirect test, no staining was obtained

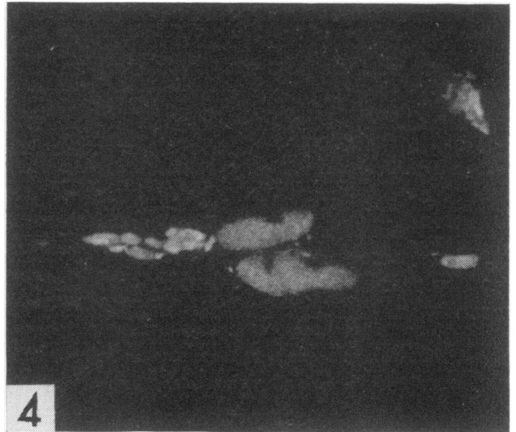
The specificity of the indirect test was also examined by absorbing a positive serum with MD antigen. Antigen was prepared from JM-infected duck embryo fibroblasts and chick kidney cells by a method similar to that described by Chubb and Churchill (3). A similar batch of antigen was prepared from normal duck embryo fibroblasts and chick kidney cells. A 1:10 dilution of a positive serum was added to an equal quantity of MD cell antigen, normal cell antigen, and saline in separate tubes. The tubes were incubated at room temperature with intermittent agitation for 2 hr and centrifuged at 3,000 $\times$ g for 30 min; the supernatant fluid was used in the indirect FA test to stain positive JM antigen grown on cover slips. The brightness of staining was scored from 0 to 4 plus. Both MD-cell antigens absorbed out the MD antibody from the serum, and only a 1 plus staining was obtained, whereas the normal cell antigens and the saline left the antibody which gave a 4 plus fluorescence of the MD antigen.

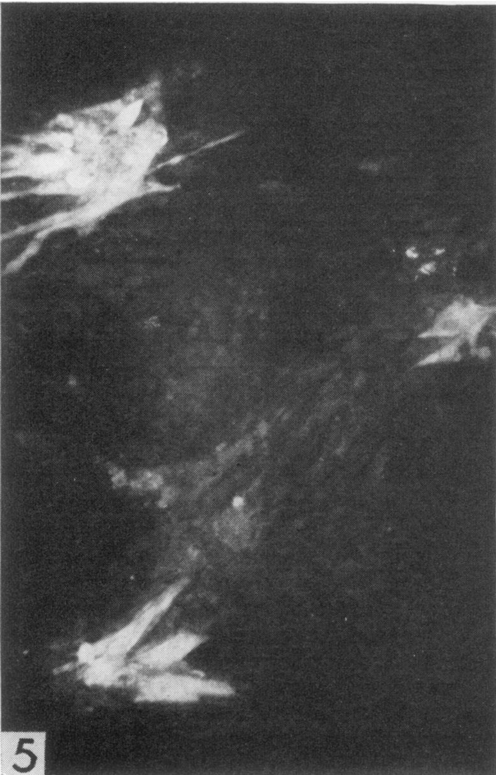
Comparison of sensitivity of FA and TC tests for antigen. The supernatant medium from confluent chick kidney cultures growing on cover slips was replaced with 5 ml of 1/3 log dilutions of a stock of JM-infected kidney cells. After 1, 3, 5,











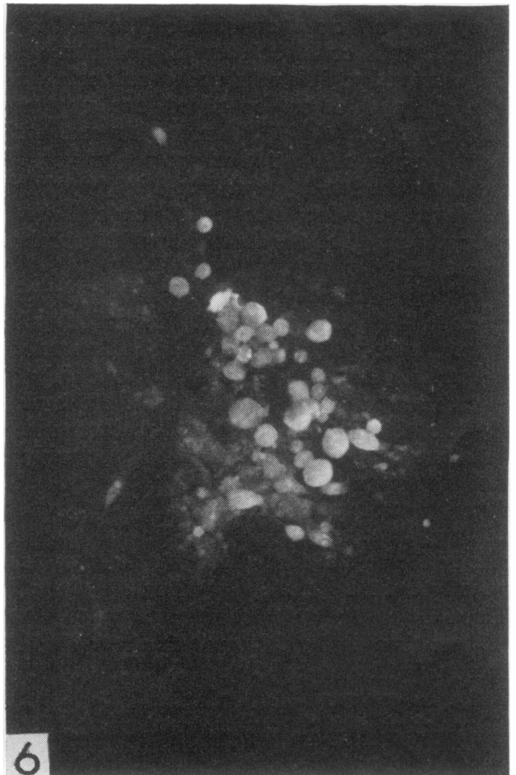


FIG. 1–6

and 7 days of incubation, cover slips were removed and stained by the indirect FA technique with antisera prepared against the JM isolate. Replicate plates without cover slips were examined with an inverted conventional light microscope for cytopathological areas (morphological foci). The numbers of foci per 100 mm^2 of surface area as observed by each method were plotted (Fig. 11).

All infected viable cells could be detected by the FA technique on the day after infecting the culture. However, they were easier to count on the 3rd day after infection when the fluorescent foci were larger. It was not until the 7th day that most fluorescent foci had developed cytopathology which could be seen under the fluorescence microscope as a rounding and retracting of cells and as the presence of polykaryocytes. At this time, there were individual, rounded, fluorescent cells attached to the monolayer between the large fluorescent foci. They were similar to the cells seen at 1 day after infection and were probably cells which had been washed off the foci and were initiating secondary foci.

Morphological foci were first detectable on the 3rd day after infection when they consisted of small groups of six or more rounded, refractile cells, often with adjacent, fusiform, refractile cells. The foci increased in size and numbers until they reached a maximum at about the 7th day (Fig. 12).

There is a linear relationship between the fluorescent foci and the dilution of inoculum and a similar relationship between the morphological foci and the dilution of inoculum (Fig. 11). The lines for the fluorescent foci and for the morphological foci seen at 3, 5, and 7 days after infection are parallel.

Detection of MD herpesvirus in field samples. Blood samples were obtained from different field flocks and used to inoculate replicate plates of chick kidney cells. One plate contained a cover slip which was removed between the 4th and 7th day after inoculation and stained by the indirect FA test with JM antiserum. The other plate was examined for morphological foci between the 10th and 21st day postinoculation. MD herpesvirus was detected in 21 (72.4%) of the 29 samples. Of these, 7 (33.3%) produced morphological and fluorescent foci, whereas 14 (66.6%) produced only morphological foci; no fluorescent foci were detected on the cover slip. There were no samples which produced only fluorescent foci, and eight samples (27.6%) were negative by both tests.

In order to increase the number of infectious units per culture, and thus increase the likelihood of a focus occurring on a cover slip, cultures were passaged to fresh confluent monolayers of kidney cells 7 days after inoculation with blood. An additional 43 field samples were examined by this method. Only 15 (34.9%) contained MD herpesvirus. Of these, eight (53.3%) produced morphological and fluorescent foci, whereas seven (46.7%) produced only morphological foci. Twenty-seven samples (62.8%) were negative by both tests, and one sample (2.5%) had fluorescent foci on the cover slip but no morphological foci on the petri dish. In this instance, a replicate sample of blood was inoculated into line 15 × 7 chickens and lesions of MD were produced. None of the 10 samples obtained from isolated control birds and examined by this method produced either morphological or fluorescent foci in cell culture.

Antigenic relationship between isolates of MD. Antigen and antibody for the indirect FA test were prepared from the same inoculum source. Serum from each immunized bird was used in the indirect FA test on each of the antigens. The brightness of fluorescence was scored from 0 to 4 plus by two observers, and the average score for each group of antisera was referred to as the staining index (Table 1).

Birds inoculated with the GA isolate failed to produce antibody and died of MD shortly after being bled for antibody. The staining index of the antibody against homologous antigen was

FIG. 1. *Chick kidney monolayer one day after inoculation with a stock of MD-infected chick kidney cells. Single, spherical cell stains. Ca.* × *380.*

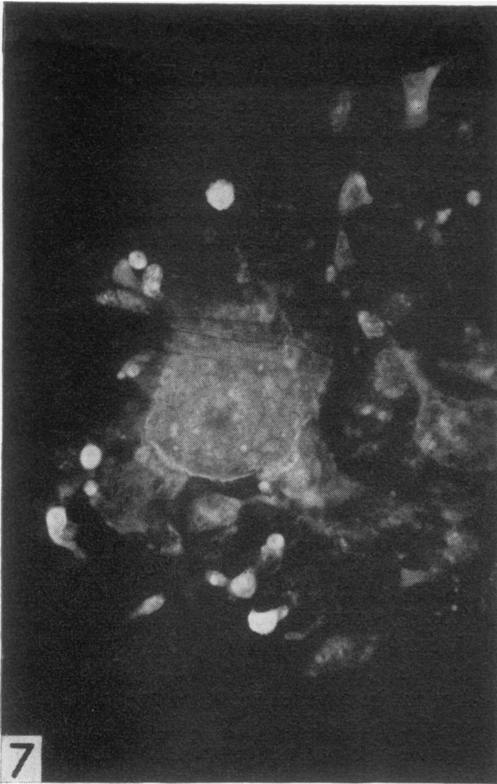
FIG. 2. *Chick kidney monolayer one day after inoculation with a stock of MD-infected CK cells. Single, flattened cell with an intranuclear inclusion stains. Ca.* × *380.*

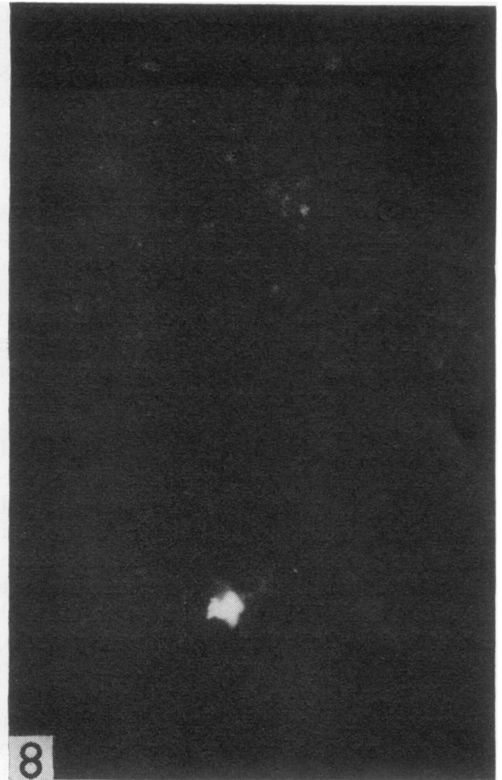
FIG. 3. *Chick kidney monolayer one day after inoculation with a stock of MD-infected CK cells. A group of flattened cells with diffuse and granular cytoplasmic antigen and intranuclear inclusions. Ca.* × *380.*

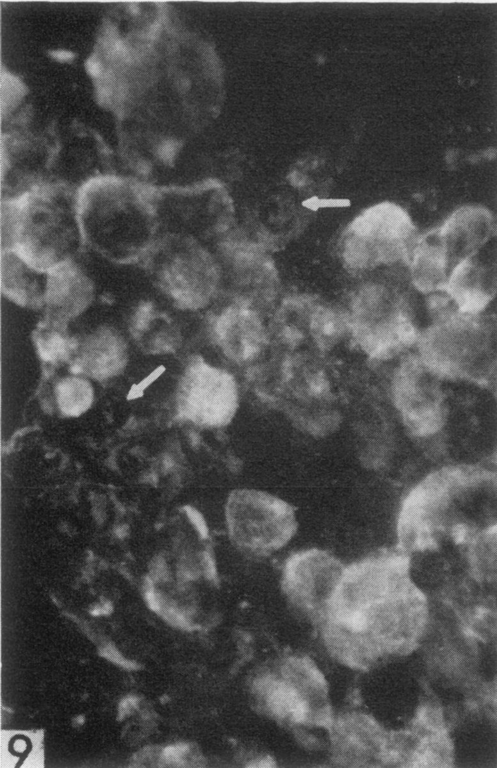
FIG. 4. *Chick embryo fibroblast cultures infected with JM strain of MD virus. The diffuse nuclear antigen and the irregular cytoplasmic granules stain brightly. Ca.* × *250.*

FIG. 5. *Chick kidney monolayer at 1 day after inoculation with a stock of MD-infected CK cells (same monolayer as Fig. 1–3). Three fluorescent foci can be clearly seen. Ca.* × *130.*

FIG. 6. *Chick kidney monolayer 5 days after infection with a stock of MD-infected CK cells. A fluorescent (and morphological) focus composed of rounded cells. Ca.* × *130.*







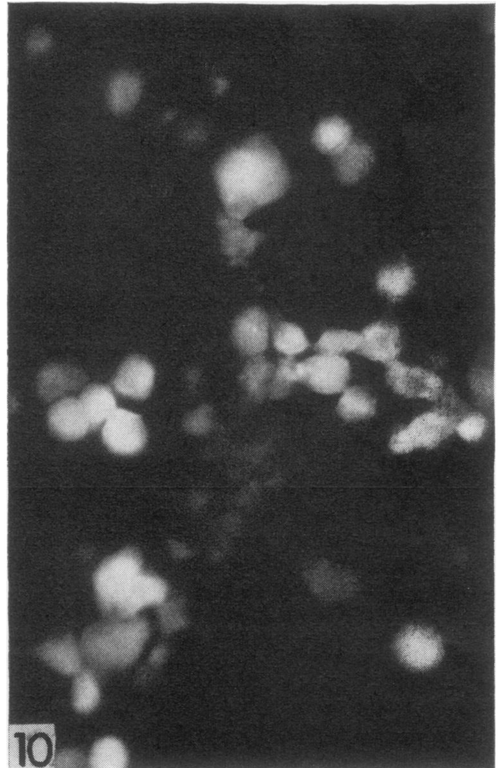


FIG. 7–10

TABLE 1. *Antigenic relationship among eight isolates of MD*

Antibody produced to	No. of Chickens per group	Antigens								$\bar{X}^a$	$\bar{\bar{X}}^b$
		JM	MSD1	GA	CONN A	CR64	C1	RPL39	FC50		
JM	4	**3.0**[c]	2.0	2.8	1.3	1.5	2.3	2.0	2.1	2.1	1.9
MSD1	3	1.5	**0.5**	1.8	0.7	1.0	1.0	0.8	1.2	1.1	1.6
GA	2	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	0.0	0.0	2.0
CONN A	4	1.5	1.3	1.9	**1.3**	1.0	1.8	1.5	1.4	1.5	1.6
CR64	4	3.8	2.8	3.3	3.3	**2.0**	3.5	3.6	2.8	3.1	1.3
C1	4	2.0	2.1	1.8	2.3	1.4	**1.6**	2.1	1.6	1.9	1.7
RPL39	3	2.3	2.3	2.5	2.2	1.8	1.8	**2.2**	1.5	2.1	1.7
FC50	4	1.4	1.8	1.9	1.6	1.4	1.4	1.4	**1.0**	1.5	1.5

[a] Mean staining index of antibody, i.e. mean of the scores for one group of antisera against all eight antigens. Sera from eight control birds had a mean index of 0.0.

[b] Mean brightness of antigen, i.e. mean of the scores for all groups of antisera against one antigen. Control antigen had a mean brightness of 0.0.

[c] Values in boldface are average scores (staining indexes) for each group of antisera; the brightness of fluorescence was scored from 0 to 4 plus by two observers.

higher than the mean staining index against all antigens in two instances (JM and RPL 39), but was lower than the mean staining index in the others. The mean brightness of the antigen was similar in each instance.

Agreement between observers. In the above test, two observers examined each preparation and scored the brightness of fluorescence from 0 to 4 plus, independently (Table 2). Of the 224 observations, 143 (63.8%) were in full agreement, 79 (35.2%) deviated by a score of 1 plus and 2 (0.9%) deviated by a score of 2 plus. There was full agreement among the 64 observations with sera from control birds and among the 28 observations with control antigen. There was a 98.4% agreement among positives and negatives.

Heat stability of MD antigen. Two cover slips on which JM-infected chick kidney cells had been grown were fixed as described above. One was placed in distilled water and boiled for 90 min. They were then stained in the indirect FA test.

There was bright nuclear and cytoplasmic staining in the unboiled cover slip (Fig. 9). After being boiled for 90 min, the cells shrank slightly but there was no decrease in the intensity of staining (Fig. 10).

DISCUSSION

When chick kidney cells were infected with a stock cellular preparation of the JM isolate, fluorescent cells could be detected after 24 hr, but at this time no morphological foci were seen. By 7 days after infection, the same number of infected areas were detected by both methods and the fluorescent foci coincided with the morphological foci. A straight-line relationship between the dilution of inoculum and the number of fluorescent or cytopathic areas obtained indicates that one infectious unit produced one fluorescent or morphological focus. Thus the antigens detected in cell culture by the indirect FA test are induced by the virus which causes the characteristic cytopathology.

The indirect FA test as described here is highly specific since MD antisera did not stain cultures infected with other poultry pathogens. In addition the antibody could be absorbed from the serum by MD-infected chick or duck cells but not by uninfected cells. Control uninfected cultures developed neither cytopathic areas nor fluorescent-staining foci.

Antigen could be detected in both the nucleus and the cytoplasm of duck and chicken cells and its morphology and distribution were similar to those described for herpes simplex although no small nuclear granules were seen (14).

The origin of the FA-staining cytoplasmic granules in duck embryo fibroblast cultures is

FIG. 7. *Chick kidney monolayer 7 days after infection with a stock of MD-infected CK cells. A fluorescent (and morphological) focus composed mainly of polykaryocytes. Ca. × 130.*

FIG. 8. *Uninfected chick kidney monolayer fixed and stained at the same time as that in Fig. 7. Note areas of rounded epithelioid cells at top of picture which do not stain. The fluorescing artifact can be easily distinguished from MD antigen. Ca. × 130.*

FIG. 9. *Chick kidney monolayer with a large proportion of MD-virus-infected cells. Arrows show diffuse and irregularly granular nuclear staining. Ca. × 320.*

FIG. 10. *Chick kidney monolayer identical to that in Fig. 9 but boiled for 90 min in distilled water. Ca. × 320.*

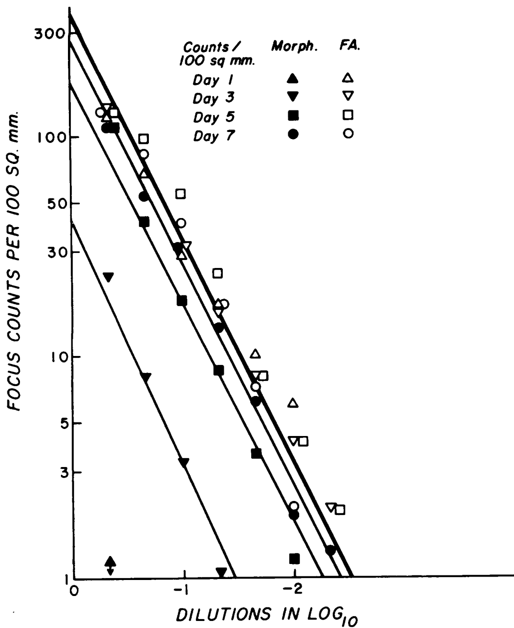


FIG. 11. *Numbers of foci detected morphologically and by FA in cultures at various times after inoculation with dilutions of MD-infected cells.*

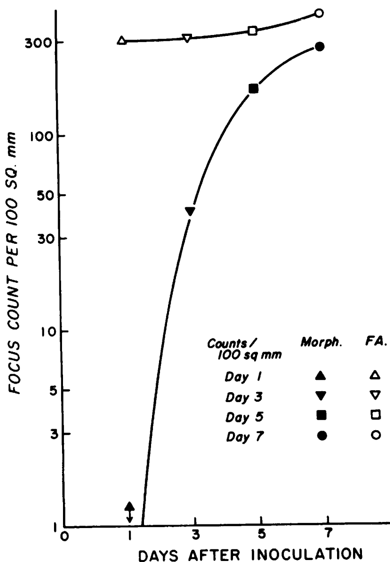


FIG. 12. *Numbers of foci detected morphologically and by FA in cultures at various times after inoculation.*

unknown, but they could be easily distinguished from the MD antigen by their morphology and distribution. Since they were present in all cultures examined, duck embryo fibroblasts were only rarely used for antigen.

TABLE 2. *Number of sera examined by each observer which had the brightness indicated*

Score 1	Score 2				
	0[a]	1	2	3	4
0	**54**[b]	3			
1	2	**24**	14		
2		9	**24**	17	
3			11	**34**	6
4			2	17	**7**

[a] Brightness of fluorescence was scored from 0 to 4 plus.

[b] The 64 observations with sera from control birds and 28 observations with control antigen were in full agreement and have been omitted from this table. Values in boldface are in agreement.

MD herpesvirus-infected cells possess an antigen which is not destroyed by boiling for 90 min. In this respect MD herpesvirus is similar to herpes simplex (14).

Cell culture antigens appeared about 3 days before the CPE could be detected, but eventually nearly all the fluorescent foci developed into morphological foci. In this respect, the FA test is as sensitive as that depending on morphological foci for detecting infectious virus; however, it suffers the disadvantage that only a small area (in this case 100 mm^2) is usually examined. Morphological foci may occur on the petri dish and not on the cover slip. This possibility accounts for the observations in which morphological foci but not fluorescent foci were detected. In an attempt to increase the sensitivity of the FA test, I passaged cultures to fresh kidney cells. This increased the efficiency of recovery from 33.3 to 53.3%. Larger cover slips could be used, but the time required to scan them for fluorescent foci is much greater than is required to examine a petri dish for morphological foci. This precludes the use of the FA test in initial isolations in which only a small number of infectious units are present in the inocula.

Approximately the same number of fluorescent foci were detected at 1 day after infection of chick kidney cultures as were detected at 7 days after infection. Since all cultures were maintained under liquid media, this indicates that there was very little, if any, spread of virus through the media. The slight increase in the number of foci at 5 and 7 days after infection is probably due to secondary foci originating from infected cells which had drifted loose from cytopathological areas. These individual cells were clearly visible when stained with FA.

The fluorescence on day 1 probably reflects the

presence of infected cells in the inoculum. It is possible that the foci originated from division of the infected cells added to the culture rather than by infection of surrounding cells. This is unlikely since infected cells do not propagate in continuous culture and slough off from the monolayer and die as the culture gets older. Also the morphology of the foci produced is characteristic of the recipient monolayer and not of the donor cells (19). In mature foci, there was a gradation of staining from very bright staining in the center of a focus to just detectable staining at the periphery. If the entire focus had originated by division of an infected cell, one would expect a focus of cells containing approximately the same amount of antigen as was seen at 1 day after infection (Fig. 3 and 5). The gradation of staining from the center outward indicates that the infection is spreading from cell to cell in a centrifugal direction (Fig. 6). These findings confirm the highly cell-associated nature of this virus and demonstrate that infection is transmitted from infected to adjacent cells. Convincing proof of cell to cell transmission awaits studies on the mechanism of focus formation.

Of the eight isolates studied in these experiments, seven could not be distinguished from one another by the indirect FA test. No conclusions can be drawn with regard to the eighth isolate since the chickens that were inoculated did not produce antibody to this isolate. This may have been because the chickens succumbed to MD before they could produce antibody. In another experiment (*unpublished data*) chickens produced antibody to this isolate and it stained JM antigen well. These results indicate that either the eight isolates are antigenically identical or that there is a common antigen or contaminant in all stocks of the isolates.

The indirect FA test can be considered to be fairly objective. Both observers had considerable experience with the indirect FA test and the observations were made independently; there was excellent agreement between them.

ACKNOWLEDGMENTS

The author wishes to acknowledge the skilled technical assistance of C. A. Hunt and P. A. Frank.

ADDENDUM IN PROOF

Recent electron microscopic studies have demonstrated that all cells containing antigen demonstrable by the FA test also contain herpesvirus and that cells which do not contain antigen do not contain herpesvirus particles (K. Nazerian and H. G. Purchase, *manuscript in preparation*).

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