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Pathogenicity and Antigenicity of Clones from Strains of Marek's Disease Virus and the Herpesvirus of Turkeys[1]

H. G. PURCHASE,[2] B. R. BURMESTER, AND C. H. CUNNINGHAM

Regional Poultry Research Laboratory, Animal Science Research Division, Agricultural Research Service, East Lansing, Michigan 48823 and Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

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Virus was extracted by filtration from chicken embryo fibroblast cultures infected with the JM, high passage JM(JMHP), GA, and RPL39 strains of Marek's disease virus (MDV) and from the herpesvirus of turkeys (HVT) and purified by cloning. The plaques produced by clones of HVT, JMHP, and other MDV strains differed in morphology from one another. Clones of MDV varied greatly in pathogenicity for chickens, but JMHP and HVT were nonpathogenic. Two pathogenic clones of JM virus and a clone of JMHP virus lacked the A precipitin antigen present in all other clones tested. All clones had at least one B antigen in common. HVT and MDV clones with and without the A precipitin antigen could be distinguished from each other by the indirect fluorescent antibody test. Changes in virus-host cell relationships, loss of pathogenicity, and loss of the A antigens were independent events.

Marek's disease herpesvirus (MDV) antigen and antibody have been detected by the agar-gel precipitin test (4, 16) and by both the direct and indirect fluorescent-antibody tests (17, 18, 20, 22). Initial examination of several laboratory and field isolates of MDV failed to reveal any antigenic differences between them. However, there was no indication of the purity of the strains used, and a common contaminant could have accounted for the serological cross-reactions.

An antigenic change has been described in MDV that was passaged many times in chicken kidney cell culture (7). During the 20th to 30th passages in cell culture, the virus lost an antigen which was usually found in the supernatant fluids of cultures infected with the original strain. Furthermore, the subcultured virus had become apathogenic for chickens, its growth characteristics in cell culture had become altered, and larger plaques containing larger syncytia occurred in a shorter time than with the original virus. A similar attenuation and morphologic change of the cytopathological areas (plaques) occurred on passage of the JM strain of MDV (13).

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Witter et al. (25) described a nonpathogenic herpesvirus isolated from turkeys (HVT). The HVT produced larger plaques than MDV and was antigenically similar but could be distinguished from it by the indirect fluorescent-antibody test. Vaccination with HVT protected chickens against subsequent challenge with MDV (15). A similar herpesvirus isolated from turkeys (10) has given similar protection (D. P. Anderson, *unpublished data*).

Variants of herpes simplex virus could be distinguished from one another by the cytopathic effects they produced (9, 14, 21), the distribution and intensity of fluorescent staining in cell culture (11), and the size of the pocks produced on chorioallantoic membranes of chicken embryos (12). Similar differences in cytopathic effect were described for variants of B virus (*Herpesvirus simiae*), and there was a relationship between plaque size and virulence for rabbits (2). This virus could be distinguished from herpes simplex by the direct or indirect fluorescent-antibody tests (1). Passage of pseudorabies virus in cell culture altered the size of plaques produced and decreased the pathogenicity for rabbits (23). From the above, it appeared likely that variants of MDV might exist and that characteristics used to detect the variants might be useful in distinguishing MDV from HVT.

The present paper describes the purification by cloning of three strains of MDV and one of HVT, their pathogenicity in chickens, and antigenic differences by the indirect fluorescent-antibody and agar-gel precipitin tests. A description of the plaques produced by the clones and of host-range studies will be presented in another communication.

MATERIALS AND METHODS

Source of viruses. Blood or tumor cells from 3- to 6-week-old chicks infected at 1 day of age with the JM, RPL39, and GA strains of MDV were used (17). A strain of JM virus (JMHP) which had been passaged more than 45 times in chicken embryo fibroblast (CEF) and duck embryo fibroblast (DEF) culture was kindly supplied by K. Nazerian (13). The FC126 isolate of HVT used in thse studies has been described (25).

Chickens and embryos. Line 15×7 chickens (8) and line 1900 embryos were from the Single Comb White Leghorn flocks reared at the Regional Poultry Research Laboratory. Line 1900 are commercial chickens of C/O phenotype reared under pathogen-free conditions. They are free of lymphoid leukosis viruses and MDV (H. A. Stone, *unpublished data*). Embyronated duck eggs were obtained from Truslow Farms Inc., Chestertown, Md.

Cell cultures. Primary CEF and DEF cultures were prepared from 10- and 14-day-old embryos, respectively, as previously described (26) and grown in medium F10 and 199 (Grand Island Biological Co., Grand Island, N.Y.) with 5% calf serum (Colorado Serum Co., Denver, Colo.). The chicken kidney cells were prepared from birds under 4 weeks of age and were cultured in Eagle's basal medium with 5% fetal bovine serum (26; Grand Island Biological Co.).

Propagation and cloning procedures. Secondary DEF cultures were inoculated with blood or tumor cells from birds infected with the JM, RPL39, and GA strains of MDV. The cells were passaged one to three times until extensive cytopathologic effects had developed. Because CEF cultures were shown to produce more cell-free virus than DEF cultures (13), the viruses were adapted to grow in CEF cells. They were first propagated in a mixture of equal numbers of CEF and DEF cells in culture. When extensive cytopathologic effects had developed, usually two to six passages, the cells were passed onto pure CEF cultures. At each subsequent passage on CEF cultures, an attempt was made to extract filterable virus as described below.

The JMHP and HVT produced cytopathologic changes in CEF, and filterable virus was extracted from them.

Filtration and cloning. The growth medium on heavily infected CEF cultures was replaced with antibiotic-free medium 24 hr before extraction. When nearly all of the cells in the cultures had become morphologically altered and there were large numbers of rounded cells floating in the medium, but before holes appeared in the monolayer, the cells were scraped off the plate into the supernatant medium. This suspension was kept on ice and sonically treated with the Bronwell Biosonic oscillator (Will Scientific Inc., Rochester, N.Y.) by using the small-needle probe at 70% of maximal intensity for 10 sec, a procedure which will disrupt over 99% of the cells. The extract was then centrifuged at approximately 2,000 × *g* for 5 min. The supernatant fluid was decanted into a fresh tube, and a few drops of a turbid, 3-times washed, 24-hr-old culture of *Seratia marcessens* was added. The fluid was then filtered through a 0.45-μm membrane filter (Swinnex, Millipore Corp., Bedford, Mass.) which had been pretreated with fetal bovine serum (24).

The integrity of the filter was ascertained by bacteriological sterility tests of the first few drops after the void volume of the filter had been expressed and the last few drops after filtration. The filtrate, 1 ml per plate, was used to inoculate CEF, DEF, and CK cultures grown in 60-mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). Growth medium was added to the cultures 4 hr after inoculation, and it was changed every 2 days thereafter. Cultures were examined daily for cytopathologic changes.

When plaques consisting of about 10 or more rounded refractile cells had appeared, the cultures were overlaid with growth medium containing 1% agar. Holes, approximately 2 mm in diameter, were cut with a sterile cork borer through the agar above the plaques. These areas and a few surrounding cells were removed from the petri dish with a few drops of 0.05% trypsin and placed in 0.5 ml of calf serum until they were used to inoculate DEF cultures. These clones were propagated in DEF cultures until a sufficient stock was obtained, usually two to three passages, at which time the cells were preserved in liquid nitrogen.

Pathogenicity of clones. Dilutions of virus-infected cells containing 1,000 plaque-forming units (PFU) per 0.2 ml were inoculated intra-abdominally into 1-day-old line 15×7 chicks which were then reared in modified stainless-steel Horsfall-Bauer isolators. All chickens dying during the experiment were necropsied. All survivors were exsanguinated and necropsied at 10 weeks of age. When a diagnosis could not be made on gross examination, portions of the left and right brachial and sciatic plexuses, the celiac nerve, and a gonad were removed for histopathologic examination.

Fluorescent-antibody and agar-gel precipitin tests. The procedures used for the indirect fluorescent-antibody test have been described (17). Cell monolayers infected with each clone were prepared by inoculating confluent CK cultures on cover slips (11 by 22 mm) in petri dishes with approximately 100 PFU per cover slip. The cover slips were removed and fixed when early cytopathologic changes were visible, i.e., at 1 to 3 days after inoculation.

Reagents for the agar-gel precipitin tests were prepared from the stocks of cloned viruses after two additional passages in DEF cultures (20). When there were advanced cytopathologic changes, the supernatant fluid and cells were harvested. The supernatant medium was centrifuged at 1,500 × *g* for 10 min to remove most of the cells and then concentrated approximately 50 times by precipitation twice, with 80%

and then 60% saturated ammonium sulfate. The final precipitate, suspended in distilled water, is referred to as supernatant reagent.

Cells were scraped off of the petri dish and suspended in phosphate-buffered saline (*p*H 7.4) at a concentration of approximately 2×10^7 cells per ml. They were placed on ice and sonically treated for 30 sec with the small probe of a Biosonic oscillator at 70% of maximum intensity. This material is referred to as cell reagent. Both the supernatant and cell reagents were stored at −20 C until just before use.

Agar-gel precipitin tests were performed as previously described (16, 20) with the following modifications. A glass slide (25 by 75 mm) was painted with a thin layer of a 0.5% agar containing 0.05% glycerine and dried rapidly on a warm (70 to 90 C) surface. A second slide was placed above the coated slide and separated from it by two layers of electrical tape and agar (0.5%, 1%, or 2% in phosphate-buffered 8% NaCl solution) was poured between them. After the agar had solidified, the uncoated slide was removed and a plexiglas template (Bolab Inc., Reading, Mass.) with a flat polished lower surface lightly coated with silicone grease was placed on the agar. The holes in the template were then filled with 50 μliters of antigen or serum. Incubation was at room temperature for 72 hr.

Sera used in both the indirect fluorescent-antibody and agar-gel precipitin tests were from the survivors of the pathogenicity tests. The hyperimmune sera were the same as those used previously (17).

RESULTS

Filtration and cloning of viruses. Of the 50 attempts to filter the viruses, only 18 (36%) were successful (Table 1). The JMHP and HVT were filtrable in 4 of 5 (80%) attempts, whereas the low-passage JM, RPL39, and GA strains yielded filtrable virus in 14 of 45 (32%) attempts. There was less virus (1.3 to 14.7 PFU/ml produced by the low-passage strains than by the JMHP (56.5 PFU/ml) and HVT (250 PFU/ml.). Of the eight filtration experiments performed with MDV which had been passed between 4 and 10 times in cell culture, none was successful. However, sporadic successes were obtained with viruses between the 11th and 18th passages.

None of the uninfected control cells had cytopathic changes or yielded filtrable virus.

Morphology of plaques in CK cells. The appearance of plaques produced in CK cultures by all of the clones except JMHP and HVT was similar to that previously described (5–7, 26). Infected cells became rounded and highly refractile and were sometimes multilayered. In each plaque, the number of spherical refractile cells increased with time so that it resembled a "bunch of grapes." However, the plaques rarely exceeded 1 mm in diameter, even in cultures 14 days postinoculation.

The plaques produced by JMHP were similar to those described previously (7, 13). They appeared 1 to 2 days earlier, were larger than those produced by low-passage virus, and contained larger spheroidal, highly refractile syncytia. Cells lysed, became detached, or both, so that plaques developed a hole in the center. This occurred more frequently with plaques induced by JMHP than with those induced by low-passage virus.

Plaques produced by HVT could be detected as early as 2 to 3 days after inoculation (25). After 10 days, the plaques were 1.5 to 2 mm and occasionally 3 mm in diameter. In their earlier stages of development, plaques consisted of a few polygonal refractile cells. Later, the cells in the center were lysed and cells to the periphery became more rounded and refractile. Syncytia spread out over the surface of the petri dish and were flattened. The syncytia and the rounded cells were less refractile than those of the JMHP or low-passage viruses.

Pathogenicity of cloned viruses. To compare the pathogenicity of different clones from a single strain of MDV, the seven clones from the JM

TABLE 1. *Filtration and cloning of strains of Marek's disease virus (MDV) and the herpesvirus of turkeys (HVT)*

Source	No. successful/ no. attempted	Avg and range of plaques/ml of filtrate	Maximum no.[a]	Clones	
				No. examined	Final passage level
JM (MDV)	5/17	1.3 (1–10)	10	7	11–13
JMHP (MDV)	3/4	56.5 (1–270)	20	3	13 (+41)[b]
RPL39 (MDV)	4/16	5.0 (7–11)	9	4	15–17
GA (MDV)	5/12	16.7 (1–80)	11	7	15–16
HVT	1/1	250 (250)	250	4	3 (+7)[c]
Control	0/18	0			

[a] Maximum number of plaques on plate from which clones were picked.
[b] Previously passaged 41 times in chick and duck embryo fibroblasts.
[c] Previously passaged 7 times in duck embryo fibroblasts.

TABLE 2. *Response of 15×7 chickens to strains of Marek's disease virus (MDV) and to the herpesvirus of turkeys (HVT) before and after cloning*

Time of response	Strain and clone no.	PFU inoculated	Expt no.	Per cent MD response			Per cent with visceral tumors	MLPD[c]	Antigen
				Dead	Gross[a]	Total[b]			
Before cloning	JM[d]		24		96		22		A, B
	GA[d]		16		81		62		A, B
	RPL39		20	85	90	100	85	40	A, B
	JMHP		10	0	0	0	NA[e]	NA	B
	HVT	10,000	20	0	0	0	NA	NA	A, B
After cloning	JM19	1,210	10	80	90	90	44	56	A, B
	GA	460	10	60	70	70	100	49	A, B
	JM31	330	10	60	70	70	33	40	B
	JM30	330	10	50	80	90	44	47	B
	JM35	1,050	10	40	70	70	14	27	A, B
	RPL39	1,090	9	33	89	89	63	35	A, B
	JM34	920	10	20	70	80	13	45	A, B
	JM36	400	10	0	30	30	33	NA	A, B
	JM32	370	10	0	20	20	50	NA	A, B
	JMHP	1,020	10	0	0	0	NA	NA	B
	HVT	900	9	0	0	0	NA	NA	A, B
	Control	0	9	0	0	0	NA	NA	None

[a] Includes those birds that died plus those that had gross lesions of MD at termination of the experiment.

[b] As in *a*, but also includes those with histologic lesions.

[c] MLPD, median latent period to death.

[d] Birds were killed at 6 weeks postinoculation and examined for visceral lesions. These data kindly supplied by R. L. Witter.

[e] NA, not applicable.

strain were selected. In addition, single clones from the JMHP, GA, RPL39, and HVT strains were also used. The different clones derived from the JM strain killed 80% (JM 19) to 0% (JMHP) of the chickens and induced from 90 to 0% lesions, respectively (Table 2). The clones of the GA and RPL39 strains produced an intermediate level of mortality and proportion of lesions. However, these strains produced many more visceral lesions than any of the clones of JM virus. In addition to tumors of the gonad, over half the tumors produced by the GA and RPL39 strains occurred in the liver, lung, kidney, heart, and muscle; those produced by the clones of the JM strain occurred almost exclusively in the gonad. The median latent period to death was not directly related to the degree of mortality or gross or microscopic lesions. The HVT was nonpathogenic in these tests.

Antigenic analysis by the agar-gel precipitin test. Antigen preparations were the supernatant and the cell reagents from each clone and from uninfected cells, calf serum, and Tryptose phosphate broth. They were tested in the agar-gel precipitin test against sera from two birds inoculated with each clone, against selected hyperimmune sera, and against sera from uninoculated control birds of the same source and age. Reagents from uninfected cells, calf serum, and Tryptose phosphate broth did not react with any of the sera, and the sera from uninoculated birds did not react with any antigen. Clone reagents tested against positive sera produced as many as six different precipitin lines. The strongest line of precipitation produced by most supernatant reagents represented an antigen referred to by Churchill et al. (7) as antigen A. This antigen was not present in high-passage viruses and was also absent from the JMHP supernatant and cell reagents. In addition, this line of precipitation did not occur with JM30 and JM31 but was present in HVT reagents. All clones, including the HVT, appeared to have at least one other antigen in common, the B antigen which was probably similar to that described by Churchill (7). Although there were often multiple lines of precipitation in this region, no one line could be consistently identified, so they were referred to as B-antigen lines to distinguish them from the A-antigen lines.

All supernatant reagents produced strong A-antigen lines. However, by using selected sera, the B and other antigens could also be detected in most of these reagents. The cell reagents were

more variable. Some (e.g., JM19 and GA) had A, B, and additional antigens; others (e.g., JM34 and JM35) lacked the A antigen.

To confirm and extend the above observations, the following reagents and sera were selected for use in subsequent tests. (i) The A antigens (supernatant reagents from JM19 and GA) gave strong A lines and very weak B and other lines. (ii) The B antigen (JMHP cell reagent) gave no A precipitin line with any antiserum. (iii) the AB antigens (cell reagents from RPL39, GA, and JM32) gave multiple strong precipitin lines. (iv) The A antisera (sera from birds infected with JM19 and JM32) gave strong A lines when reacted with an A antigen and weak or no B lines when reacted with a B antigen. (v) The B antisera (sera from birds infected with JM30 and JM731) gave no reaction with A antigen and strong reactions with B antigens and were prepared against clones which lacked the A antigen.

All clones were examined for the presence of the A and B antigens. RPL39, JM19, GA, JM32, and JM34 had A antigens and JMHP, JM30, and JM31 lacked these antigens (Fig. 1, 2). Sometimes the A antigen produced two lines of precipitation, both of which were absent from the clones lacking the A antigen (Fig. 1). The HVT had one antigen in common with the other clones, but another was absent or differed (Fig. 1, 7, 8). All clones had at least one B antigen in common (Fig. 3, 4). All MDV clones which had A antigen were serologically indistinguishable.

To confirm that JM30 and J731 lacked the A antigen, sera from birds inoculated with these clones were examined for antibody to the A antigen (Fig. 5, 6). None of the sera had antibody to the A antigen.

The HVT supernatant and cell reagents were examined in more detail for identity of their antigens with the A and B antigens of the MDV. In both supernatant and cell preparations, one of the two A antigens gave a line of identity and the second gave a reaction of partial identity as there was a spur on the HVT antigen side (Fig. 7, 8). The HVT cell reagent (Fig. 7) had three antigens, at least one of which was in common with one of the B antigens of MDV. The supernatant preparation reacted much more weakly with the B antiserum, and only one precipitin line could be identified (Fig. 8). The A antigen appeared to be of lower molecular weight than the precipitating globulin in the antiserum since the line was concave towards the serum well. In addition, it formed an equally strong line in a supporting medium containing 2 or 0.5% agar, indicating that it diffused readily through these matrices. The B antigen had a molecular weight similar to that of the precipitating antibody since it usually produced a straight line. It reacted more strongly in 0.5% agar than in 2% agar.

The A-antigen line was nearly always closer to the serum well than the other lines.

Antigenic analysis by the indirect fluorescent-antibody test. Uninfected cells and cells infected with each clone were analyzed by the indirect fluorescent-antibody test with the same sera as were used above. Uninfected control cells did not become stained with any serum, and control sera did not stain any of the cell preparations. A good cross-reaction was obtained between cells infected with all MDV clones and sera from chickens which survived inoculation with the clones. When JM30-, JM31-, and JMHP-infected cells were reacted with homologous antisera, the rounded refractile cells in the plaques stained intensely, whereas the surrounding flattened cells adjacent to them stained very poorly or not at all (Fig. 9). When cells infected with other clones were treated with homologous antisera which stained antigen intensely, the rounded cells in the plaques were surrounded by morphologically normal cells whose cytoplasm contained a bright diffuse or very finely granular stain (Fig. 10). The number of flattened cells staining in this way varied in different plaques and cell preparations because of the different stages of development of the plaques. The flattened cells could not be easily identified by using sera which stained the rounded cells less brightly. When MDV-infected cells were treated with antiserum to HVT, the morphologically altered cells stained weakly, and it was not possible to distinguish between the MDV clones. When HVT-infected cells were treated with homologous antiserum, antigen could be demonstrated in both the nucleus and the cytoplasm in the morphologically altered cells and in a broad band of morphologically normal cells surrounding them. There was frequently a perinuclear ring of brightly stained coarse spherical particles, diffuse, very fine granular antigen-containing particles, or both. When similar cells were reacted with antiserum to any of the MDV clones, only the nuclear antigen stained and then at a much lower intensity than with homologous antiserum. When the nuclear staining was particularly bright, a faint diffuse cytoplasmic antigen could also be detected in the morphologically altered cells but usually not in the broad band of cells surrounding them in which the nuclei were very prominent. Thus, antibody against MDV could be readily distinguished from antibody against HVT.

DISCUSSION

Infectious cell-free virus could be obtained inconsistently from low-passage MDV-infected

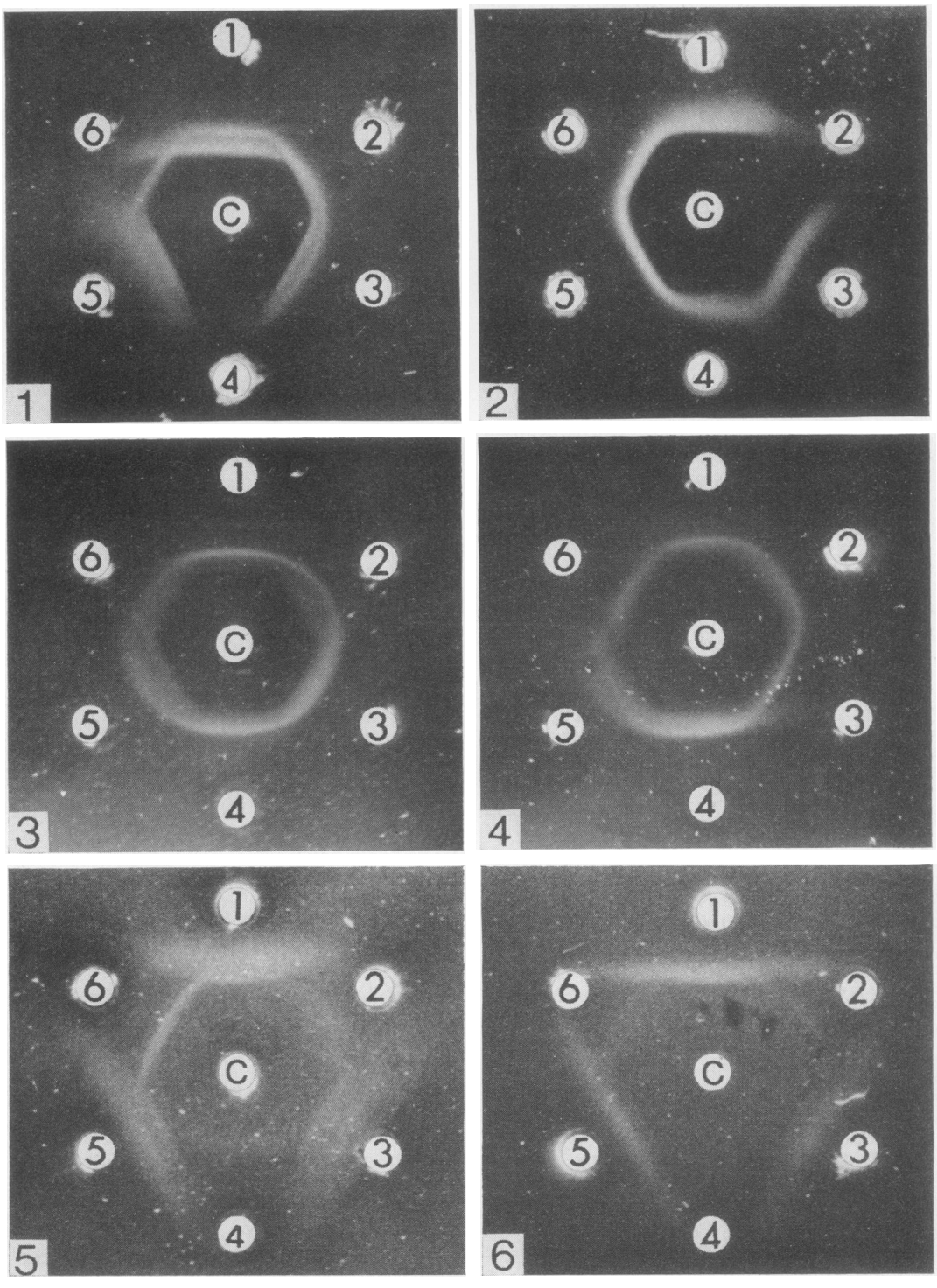
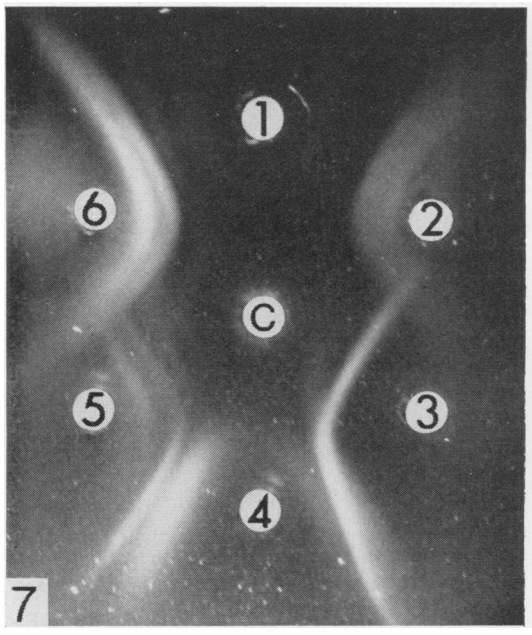
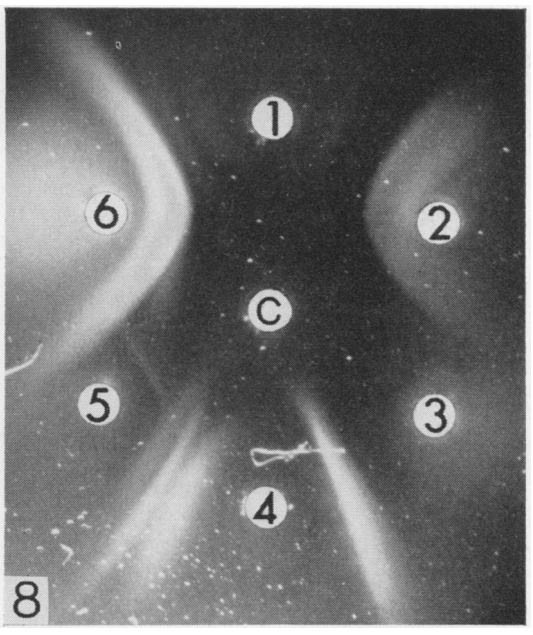
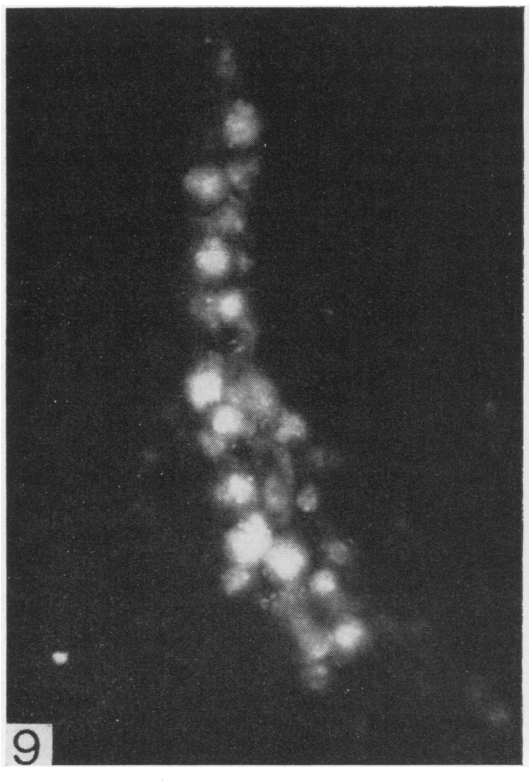


FIG. 1–6. *A and B antigens of clones of MDV and HVT.* FIG. 1. *A antigen of different clones of MDV and HVT. The center well contains the A antiserum (against JM32), and wells 1 to 6 contain supernatant reagents from RPL39, JM19, GA, JM31, JM19, and HVT. (1% agar).* FIG. 2. *A antigen of different clones of MDV. The center well contains the A antiserum (against JM32), and wells 1 to 6 contain supernatant reagents from JM19, JM30, JM32, JM34, JM35, and JM36. (1% agar).* FIG. 3. *B antigen of different clones of MDV and HVT. The center well contains the B antiserum (against JM30), and wells 1 to 6 contain cell reagents from RPL39, JM19, GA, JM31, JM19, and HVT. (0.5% agar).* FIG. 4. *B antigen of different clones of MDV and HVT. The center well contains the B antiserum (against JM30), and wells 1 to 6 contain cell reagents from RPL39, JMHP, HVT, GA, JM32, and HVT. (0.5% agar).* FIG. 5 *and* 6. *Absence of anti-A antibody among birds inoculated with JM30 and JM31. The center well contains the AB antigen (RPL 39 cell reagent); wells 1, 3, and 5 contain A antiserum (against JM32); and wells 2, 4, and 6 contain sera from birds inoculated with JM30 (Fig. 5) and JM31 (Fig. 6).*







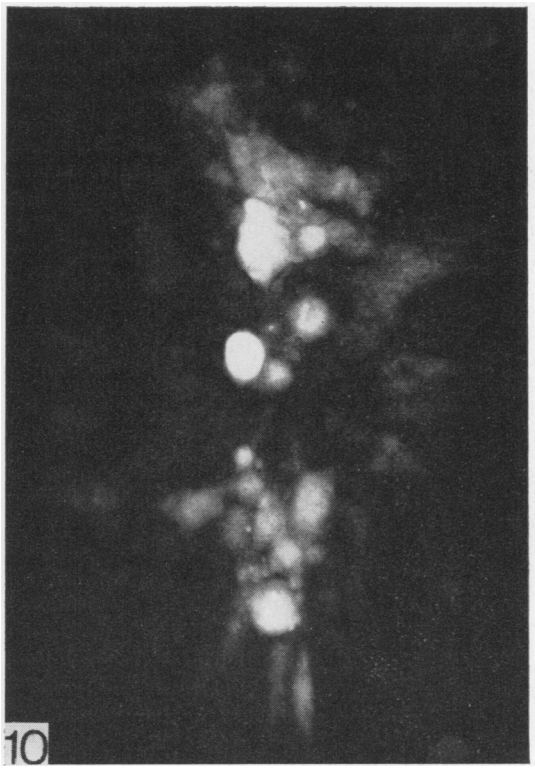


FIG. 7 and 8. *Relationship between HVT antigens and MDV antigens. The center well contains HVT cell reagent (Fig. 7) and HVT supernatant reagent (Fig. 8); well 1, A antigen (GA supernatant reagent); well 2, A antiserum (against JM19); well 3, B antiserum (against JM30); well 4, B antigen (JMHP cell reagent); well 5, B antiserum from a different bird than well 3 (against JM30); and well 6, A antiserum (against JM32). (0.5% Agar).*

FIG. 9. *Antigen prepared from clone JM30 reacted in the indirect fluorescent antibody test with homologous antiserum. Note that only the rounded cells stain. × 300.*

FIG. 10. *Antigen prepared from clone JM35 reacted in the indirect fluorescent-antibody test with serum against RPL39. Note that the cells of normal morphology surrounding the rounded refractile cells have a diffuse or finely granular cytoplasmic antigen. × 300.*

CEF cultures. Although there were minor variations in culture conditions, in stage of development of cytopathologic alterations at the time of harvest, and in the method of harvest, none of them could be directly linked to the success or failure of a particular filtration experiment. It appeared to be necessary to have optimal conditions so that there was a maximum yield of infectious virus to offset the inefficiency of the extraction and filtration procedures. As reported previously (13, 25), the JMHP and HVT generally yielded virus in larger amounts than the low-passage strains.

Extracted virus was filtered through a 0.45-μm filter to remove clumps of virus and to increase the probability that each cytopathologic area would develop from a single infectious particle. The average pore diameter was only slightly greater than the diameter of an enveloped virion. MDV is entirely cell-associated (6), thus contamination of a clone by released virus is highly unlikely although there is a danger from floating infected cells. To reduce the danger, clones of low-passage MDV were selected as soon after infection as possible from plates containing 11 plaques or less. The cloning procedure was not repeated because additional passages of MDV in cell culture would have further modified the virus clones. The clones prepared from JM virus were different from one another, indicating that a selection had taken place. Thus, it is likely that in most instances the procedure was sufficiently exact to ensure isolation of the progeny of a single infectious particle.

The cloning procedure for JMHP and HVT was less rigorous since clones were selected from plates with a larger number of plaques. However, conclusions derived from the comparison of these viruses with the low-passage MDV are not as dependent on the success of the cloning procedure.

Some of the characteristics of the original strains were present in the cloned preparations. Thus, the RPL39 and GA clones produced a large proportion and wide distribution of visceral tumors characteristic of these strains, whereas the pathogenic JM clones produced a low incidence of visceral tumors, almost exclusively of the gonad. The JMHP and HVT clones remained nonpathogenic.

The pathogenicity of different clones of the JM strain of MDV varied. The most pathogenic was JM19 which induced lesions in 90% of the birds, and the least pathogenic was the JMHP which did not induce any lesions. There are two possible explanations for this variation, namely that the original stock of JM virus was a mixture of many genetically different viruses which varied in pathogenicity or that a change of pathogenicity occurred during passage of the virus. Strains that vary widely in pathogenicity have been observed (19), and it is quite possible that unpurified stocks would contain more than one virus. The variable responses obtained with different unpurified stocks of the same strain and the variation in pathogenicity with passage from chicken to chicken which has been well documented (3) could be explained by an alteration in the relative amounts of the more pathogenic and less pathogenic strains in a given stock. However, loss of pathogenicity may also occur during passage in cell culture. The contribution of cell culture passage to the lack of pathogenicity of some of the clones can not be estimated.

The viruses studied could be divided into three groups on the basis of their antigenic properties. First, there are those with the A and other antigens: JM19, JM32, JM34, JM35, JM36, RPL39, and GA. These clones were serologically indistinguishable. Second, there are those which lacked the A antigen, such as clones JM30, JM31, and JMHP. It is not known whether viruses of this group occur naturally or whether they are products of cell culture passage. Third, HVT has an antigen in common with at least one component of the A antigen of the first group of MDV strains. Because of the close proximity of the precipitin lines produced by the two components of the A antigen, they often appeared as a single line. The HVT had at least one B component in common with the MDV. It is possible that reagents could be developed which would distinguish between HVT and the other viruses in the agar-gel precipitin tests.

JMHP produced cytopathologic changes in a shorter time than the low-passage clones of MDV, and the plaques were of different morphology. It is unlikely that virus similar to JMHP could have been present in the original stock since it would easily have been recognized and since none of the low-passage clones produced plaques of this type. Thus, the change in morphological appearance and rate of growth of the plaques can be attributed to passage in cell culture.

Loss of pathogenicity during passage in cell culture or innate lack of pathogenicity are unrelated to the presence or absence of the A antigen. Thus relatively pathogenic clones with (JM19) and without (JM31) the A antigen and relatively nonpathogenic clones of the same strain with (JM32) and without (JMHP) the A antigens have been isolated. Similarly, the change in appearance of the plaques and increase in rate of growth characteristic of JMHP are not related to loss of the A antigen since JM30 and JM31 were morphologically indistinguishable from the other low-

passage clones yet lacked the A antigen. Thus, the above observations indicate that, although passage in cell culture may be responsible for loss of pathogenicity, loss of the A antigen, and change in virus-host cell relationship, these three events are independent of one another. Also, only in the case of the change in virus-host cell relationship can the alteration be attributed entirely to passage in cell culture.

Differences between viruses with and without the A antigen could also be observed by using the indirect fluorescent-antibody test. However, it was not possible to differentiate between these viruses consistently because of variations in the stages of development of the plaques in different preparations and in the amount of antibody in the sera. In plaques produced by clones with the A antigen, a diffuse antigen in the cytoplasm of infected, morphologically normal cells surrounding the plaques stained with homologous antiserum. Cells in close proximity to plaques induced by viruses without the A antigen did not stain with their homologous antisera and in these plaques only the rounded refractile cells stained. Since the virus infection spreads centrifugally by cell-to-cell contact, the cells around the periphery of a plaque would be the most recently infected. Therefore, it appears that the A antigen is produced earlier in the cycle of infection than the other antigens, an observation confirmed by sequential harvesting of antigen for the agar-gel precipitin test (Okazaki and Purchase, *unpublished data*).

As has been reported elsewhere (15, 25), HVT can be distinguished from MDV by the intensity and distribution of staining antigen in HVT- and MDV-infected cells. The pattern of staining was consistent for the sera prepared against all clones of MDV studied. A similar difference in the appearance of nuclear and cytoplasmic antigen of *Herpesvirus hominis* types 1- and 2-infected cells stained in the direct fluorescent-antibody test was reported by Nahmias et al (11). They could use the test to distinguish between the two strains of virus.

LITERATURE CITED

1. Benda, R. 1966. Attempt to differentiate B virus from herpes simplex virus by the fluorescent antibody technique. Acta Virol. **10**:348–353.
2. Benda, R., and J. Cinatl. 1966. Isolation of two plaque variants from the prototype strain of B virus (herpes simiae). Acta Virol. **10**:178.
3. Biggs, P. M., and L. N. Payne. 1967. Studies on Marek's disease 1. Experimental transmission. J. Nat. Cancer Inst. **39**:237–280.
4. Chubb, R. C., and A. E. Churchill. 1968. Precipitating antibodies associated with Marek's disease. Vet. Rec. **83**:4–7.
5. Churchill, A. E. 1968. Herpes-type virus isolated in cell culture from tumors of chickens with Marek's disease. I. Studies in cell culture. J. Nat. Cancer Inst. **41**:939–950.
6. Churchill, A. E. and P. M. Biggs. 1967. Agent of Marek's disease in tissue culture. Nature (London) **215**:528–530.
7. Churchill, A. E., R. E. Chubb, and W. Baxendale. 1969. The attenuation, with loss of oncogenicity, of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J. Gen. Virol. **4**:557–564.
8. Crittenden, L. B. 1968. Avian tumor viruses: prospects for control. World's Poultry Sci. J. **24**:18–36.
9. Hoggan, M. D., and B. Roizman. 1959. The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. Amer. J. Hyg. **70**:208–219.
10. Kawamura, M., D. J. King, and D. P. Anderson. 1969. A herpesvirus isolated from kidney cell culture of normal turkeys. Avian Dis. **13**:853–863.
11. Nahmias, A. J., W. T. Chiang, I. Del Buono, and A. Duffey. 1969. Typing of herpesvirus hominis strains by a direct immunofluorescent technique. Proc. Soc. Exp. Biol. Med. **132**:386–390.
12. Nahmias, A. J., W. R. Dowdle, Z. M. Naib, A. Highsmith, R. W. Harwell, and W. E. Josey. 1968. Relation of pock size on chorioallantoic membrane to antigenic type of herpesvirus hominis. Proc. Soc. Exp. Biol. Med. **127**: 1022–1028.
13. Nazerian, K. 1970. Attenuation of Marek's disease virus and study of its properties in two different cell cultures. J. Nat. Cancer Inst. **44**:1257–1267.
14. Nii, S., and J. Kamahora. 1963. Pathological changes induced by herpes simplex virus in the chorioallantoic membranes of hatching eggs. Biken J. **6**:205–241.
15. Okazaki, W., H. G. Purchase, and B. R. Burmester. 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys (HVT). Avian Dis. **141**:413–429.
16. Okazaki, W., H. G. Purchase, and L. Noll. 1970. Effect of different conditions on precipitation in agar between Marek's disease antigen and antibody. Avian Dis. **14**:532–537.
17. Purchase, H. G. 1969. Immunofluorescence in the study of Marek's disease. I. Detection of antigen in cell culture and an antigenic comparison of eight isolates. J. Virol. **3**:557–563.
18. Purchase, H. G. 1970. Virus-specific immunofluorescent and precipitin antigen and cell-free virus in the tissues of birds infected with Marek's disease. Cancer Res. **30**:1898–1908.
19. Purchase, H. G., and P. M. Biggs. 1967. Characterization of five isolates of Marek's disease. Res. Vet. Sci. **8**:440–449.
20. Purchase, H. G., and G. H. Burgoyne. 1970. Immunofluorescence in the study of Marek's disease: detection of antibody. Amer. J. Vet. Res. **31**:117–123.
21. Roizman, B., and L. Aurelian. 1965. Abortive infection of canine cells by herpes simplex virus. I. Characterization of viral progeny from co-operative infection with mutants differing in capacity to multiply in canine cells. J. Mol. Biol. **11**:528–558.
22. Spencer, J. L., and B. W. Calnek. 1970. Marek's disease: application of immunofluorescence for detection of antigen and antibody. Amer. J. Vet. Res. **31**:345–358.
23. Svobodova-Somogyiova, J. 1968. Pseudorabies virus with lost virulence for rabbits after intramuscular inoculation, obtained from persistently infected cultures. Acta Virol. **12**:285.
24. Ver, B. A., J. L. Melnick, and C. Wallis. 1968. Efficient filtration and sizing of viruses with membrane filters. J. Virol. **2**:21–25.
25. Witter, R. L., K. Nazerian, H. G. Purchase, and G. H. Burgoyne. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Amer. J. Vet. Res. **31**:525–538.
26. Witter, R. L., J. J. Solomon, and G. H. Burgoyne. 1969. Cell culture techniques for primary isolation of Marek's disease-associated herpesvirus. Avian Dis. **13**:101–118.