

Isolation and Characterization of an Isolate (HN) of Marek's Disease Virus with Low Pathogenicity

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A cytopathic agent was isolated and characterized as an isolate of Marek's disease herpesvirus (MDHV) with low pathogenicity, and referred to as the HN isolate. This isolate of MDHV did not cause clinical Marek's disease (MD) or death in a highly susceptible line of chickens within 5 weeks after exposure. Gross lesions of limited extent were noted in a few of the inoculated birds. Microscopic nerve lesions in the inoculated and contact-infected birds were invariably minimal, closely resembling C-type MD lesions.

Pathogenicity of Marek's disease herpesvirus (MDHV) for chickens varies with isolates, or strains (4, 5, 11, 13, 18, 22, 23). It is also related to such factors as genetic constitution, age, and sex of birds, dose of virus, and route of exposure. Differences in susceptibility to Marek's disease (MD) in different genetic lines of chickens have been well established (4, 6, 12, 18, 22). The susceptibility of birds decreases with age (1, 4, 22), and females are more susceptible than males (4, 18). The lower the virus dose, the less likelihood of disease (2), and parenteral inoculation with the virus causes the most severe reaction (1, 4).

Rispens et al. (21) indicated in their preliminary report that strains of MDHV with low pathogenicity do exist. Biggs and Milne (3) examined 25 isolates of MDHV and divided them into three groups based on pathogenicity: those which produced acute MD, those which produced classical MD, and apathogenic isolates that produced no or only very minor histological lesions.

Zander et al. (26) described an infectious agent, referred to as the HN-1 CHV agent, which was known to be enzootic in an otherwise specific-pathogen-free (SPF) chicken flock. This agent, which was found to be present in peripheral blood, induced pocks similar to those due to MDHV on the chorioallantoic membranes when inoculated into the yolk sac of embryonated eggs. The HN-1 CHV agent spread readily through contact, and chickens preexposed either by inoculation of

the infectious blood or by contact were found to be protected under laboratory challenge and field conditions against clinical MD. Although the HN-1 CHV agent appears to be related to MDHV, it had not been characterized.

This paper reports the isolation and characterization of an isolate of MDHV with low pathogenicity (HN isolate) from chickens which had been inoculated with blood of the SPF chickens known to carry the HN-1 CHV agent.

MATERIALS AND METHODS

Fertile eggs and experimental chicks. Fertile White Leghorn eggs obtained from H & N Inc. (Redmond, Wash.) were used as a source for chicken embryo fibroblast (CEF) cell cultures. These eggs came from a commercial SPF egg supply flock free of all presently detectable poultry pathogens except the HN-1 CHV agent.

Young chickens from the Cornell S line breeder flock served as the source of chicken kidney (CK) cell cultures. White Leghorn chickens, referred to as the WSU-VS chicks, from F₃-F₄ of a White Leghorn cross (Cornell S × RPL Line 7) were used for pathogenicity studies. The WSU-VS chickens are highly susceptible to MD (7; S. Kenzy, R. F. Lapen, and J. M. Sharma, *unpublished data*). All birds used for this study came from breeder flocks maintained by the department and were hatched as described previously (8) and reared in Horsfall-Bauer type isolators.

Cell cultures. CEF cell cultures were prepared as described previously (9). Primary cultures were made in tissue culture flasks (75 cm²), and secondary cultures were made in tissue culture petri dishes (60

by 15 mm; Falcon Plastics). The growth medium (GM) for CEF cell cultures was the same as that for duck embryo fibroblast cell cultures as described by Witter et al. (25), except that fungizone (Amphotericin B; Squibb and Sons) was used (2.5 $\mu\text{g}/\text{ml}$) instead of mycostatin. Maintenance medium (MM) was the same as GM but contained 1% calf serum. Secondary CEF cell cultures (24-hr monolayers) were used for growing and assaying the HN isolate throughout this study.

The procedures and media for CK cell cultures were described previously (7).

MDHV. The Id-1 isolate (24; Kenzy, Lapen, and Sharma, unpublished data), a virulent strain of MDHV, was employed.

Isolation of the HN isolate. The HN isolate, which formed plaques in cell cultures, was isolated from broiler-type chickens which had been inoculated at day one with citrated peripheral blood of chickens from the H & N SPF flock in which the HN-1 CHV agent was known to be enzootic. These chicks were inoculated at the H & N Inc. Laboratory and brought the same day to this laboratory (through the courtesy of D. V. Zander). On arrival, the birds were immediately housed in a Horsfall-Bauer isolator and held for virus isolation and pathologic examination.

When the chicks were 4 weeks of age, heparinized peripheral blood from each of two birds was inoculated (0.1 ml) into each of three dishes of secondary CEF and primary CK monolayers which were fed with 5 ml of the respective MM. After 24 hr, the medium and inoculum (blood) were drained off, and each culture was fed with fresh MM and then at 2- to 3-day intervals. Control cell cultures were treated similarly, but no blood was inoculated. All cell cultures were examined daily for CPE until the seventh day postinoculation.

Cloning the HN isolate. CEF cell cultures exhibiting typical cytopathic effects (plaques) were overlaid with a 0.8% Noble agar (Difco) in GM. After 2 hr, agar over a well-isolated plaque was punched out, and the well was filled with trypsin-ethylenediaminetetraacetic acid (EDTA) and incubated at 37 C for 20 min. The detached cells were then aspirated with a capillary pipette and inoculated onto a normal CEF monolayer, the MM being replaced after 24 hr and then at 2- to 3-day intervals. Two more successive clonings were performed in a similar manner.

After three cycles of cloning, the infected CEF cell cultures of the second CEF passage were dispersed with trypsin-EDTA, sedimented, and then suspended in GM (2×10^7 cells/ml) containing 10% calf serum and 10% dimethylsulfoxide. The cell suspension, which was then slowly frozen (approximately at a drop of 1 C per min) and stored in liquid nitrogen, served as the source of the HN isolate for characterization and pathogenicity studies. The cloned HN isolate had now been passaged seven times in CEF cell cultures during the initial isolation, earlier passages, and subsequent cloning (three cycles) and twice after completion of cloning.

Assay of the HN isolate. The isolate (infected

CEF cell suspension) was inoculated (0.1 ml) into secondary CEF monolayers (24 hr culture), three dishes per sample or per dilution. The inoculated cultures were immediately fed with 5 ml of MM which was replaced with fresh MM after 24 hr, and then at 2- to 3-day intervals. Plaques were counted at 5 days postinoculation, and the average number of the plaque counts from three replica dishes was used to estimate the number of plaque-forming units per ml of the undiluted material.

Characterization of the HN isolate. Since the HN isolate in cell cultures was highly suggestive of MDHV, the cloned isolate was examined for cytopathology in cell cultures, cell association of infectivity, effects of rapid freezing and thawing on infectivity, the type of nucleic acid, and the antigenic relationship to MDHV.

For cytopathologic studies, infected CEF and CK cell cultures grown on cover slips were stained at 7 days after inoculation with May-Grünwald-Giemsa stain.

Cell association of the infectivity of the HN isolate was examined by assaying the culture fluids and dispersed cells of the same infected CEF cell cultures. Culture fluids harvested and pooled (10 ml) from two dishes of the infected CEF cultures (610 and 620 plaques, respectively) were centrifuged at 4 C for two cycles at $2,000 \times g$ for 20 min. The supernatant fluids were then inoculated, 0.5 ml (1/10 of the culture fluids of one infected dish) into each of three replica dishes of normal CEF monolayers, to assay for the isolate. Dispersed cells from the same two infected dishes were pooled, sedimented, and resuspended in 20 ml of GM. This infected cell suspension was then assayed for the isolate by inoculation into CEF monolayers, 0.1 ml (1/100 of the cells of one dish) into each of three replica dishes. The same infected cell suspension was also used for rapid freezing and thawing and for determination of nucleic acid type of the agent.

Effects of rapid freezing and thawing on the infectivity of the HN isolate were studied. A 1-ml amount of the infected cell suspension was subjected to three cycles of rapid freezing (by using a mixture of dry ice and absolute ethanol) and thawing at 37 C. The treated and nontreated cell suspensions were then assayed, respectively, for the isolate.

Determination of nucleic acid form of the HN isolate was done by using a deoxyribonucleic acid inhibitor. Six replica dishes of secondary CEF monolayers were fed with MM, three dishes with MM containing 5-iododeoxyuridine (IUDR; Calbiochem, Los Angeles) at a concentration of 100 $\mu\text{g}/\text{ml}$ of MM, and the other three dishes without IUDR. Each of these six dishes was then inoculated with 0.1 ml of the same infected cell suspension. In parallel with this, B₁ strain of Newcastle disease virus (NDV) was inoculated into six separate replica dishes, three dishes with IUDR and three without IUDR. After 5 days of incubation, cell cultures were removed from the dishes, pooled according to IUDR treatment, and resuspended in 3 ml of GM, respectively, to be assayed for the isolate. The culture fluids of NDV-inoculated cell cultures were harvested at 3 days

postinoculation, pooled according to IUDR treatment, and titrated for NDV in CEF cell cultures. Titration end points of NDV, expressed as TCID₅₀, were determined by the Reed-Muench method (20).

Antigenic relationship of the HN isolate to MDHV was examined by the micro agar-gel precipitin test (15) and by an indirect fluorescent antibody test (17, 19, 20). The agar-gel precipitin antigens of the HN isolate were prepared from heavily infected CEF cell cultures as described for MDHV antigen preparation (15). Similarly, MDHV antigens were prepared from CK cell cultures infected with MDHV (Id-1 isolate) that exhibited more than 80% CPE. Chicken anti-MD (Id-1) serum was kindly provided by R. F. Lapen, who described the preparation of the immune serum (14). Both chicken anti-MD serum and fluorescein-conjugated horse anti-chicken globulin (Roboz Surgical Instrument Co., Inc., Washington, D. C.) were diluted 1:20 with phosphate-buffered saline for indirect fluorescent antibody staining.

Pathogenicity of the HN isolate for chicks. The WSU-VS chickens from the same hatch were randomly allocated into four groups, A through D (Table 2). Each group consisted of 15 chickens, except group D, which had 8 untreated chickens as controls. Ten chickens from each of groups A and B were each inoculated intraabdominally at 3 days of age with 16 and 166 plaque-forming units of the HN isolate, respectively. Ten chicks of group C were inoculated similarly with 181 plaque-forming units of MDHV (Id-1 isolate). Five uninoculated chickens served as contact cagemates for each of groups A, B, and C. Each group was housed in separate HB isolators which were kept sealed until the termination of the experiment.

At 5 weeks post-exposure, a peripheral blood sample (heparinized) was taken from each survivor for virus isolation before necropsy. Isolation of the HN isolate or MDHV was done by inoculating the blood sample into CEF monolayers as described above. For histologic examination, brachial and sciatic plexuses were collected, fixed in 10% Formalin, and processed for microscopic examination.

RESULTS

Isolation of the HN isolate. CPE began to appear in both CEF and CK cell cultures at 5 days postinoculation of heparinized blood from the two birds which had been inoculated with blood of chickens from the H & N SPF flock in which the HN-1 CHV agent was known to be enzootic. Blood samples from each of the two birds induced morphologically similar plaques in the respective cell cultures. The CPE was characterized by formation of plaques composed primarily of round refractile cells resembling herpesvirus plaques. Non-inoculated control cell cultures were free of any noticeable CPE.

The plaques in CK cell cultures were small (less than 0.5 mm) and compact, and were

comprised primarily of small round refractile cells closely resembling microplaques of MDHV (10). The plaques in CEF cell cultures were larger (1 to 2 mm in diameter) and less compact, and were comprised of refractile cells of variable sizes and shapes. The plaques contained, in addition to small round refractile cells, giant round cells which appeared either refractile or dark, and occasional giant bizarre-shaped refractile cells (Fig. 1A).

Plaques also developed in primary CK cell cultures prepared from pooled kidneys of the two H & N birds. These plaques were morphologically indistinguishable from those observed in CK cell cultures inoculated with blood of these birds.

The same cytopathic agent was reisolated in CK and CEF cell cultures by inoculation of the trypsinized kidney cell suspension from these two birds. The kidney cells, prepared at the time of primary CK cell cultures, were suspended in GM containing 10% calf serum and 10% dimethylsulfoxide slowly frozen and stored at -70 C for 4 weeks.

Similar plaques could be reproduced in normal CK and CEF cell cultures by inoculation of cells (1/100 of one dish) but not culture fluids (1/10 of one dish) of the infected cell culture. Similar passages of cells from uninoculated control cell cultures did not result in any cytopathic effects.

When inoculated CEF and CK cell cultures were stained with May-Grünwald-Giemsa at 7 days postinoculation, refractile cells of plaques stained intensively without discernible cell structure, and multinucleated cells were common in both CK and CEF cell cultures. Those giant round or bizarre-shaped refractile cells observed within plaques of unstained cultures were found to be multinucleated cells when stained. However, intranuclear inclusions were not observed in the early cell culture passages.

Characterization of the HN isolate. The infectivity of the agent was cell-associated and completely destroyed by three cycles of rapid freezing and thawing of infected cells. The culture fluids had no plaque-forming activity when 1/10 of the culture fluids of one infected dish at the seventh day of incubation was assayed for the agent, but 1/100 of the cells of the same infected culture induced 298 plaques 5 days after inoculation into CEF monolayers. The same infected cell suspension (298 plaque-forming units per 0.1 ml) lost all its plaque-forming activity when subjected to three cycles of freezing and thawing (Table 1).

The replication of the HN isolate was signif-

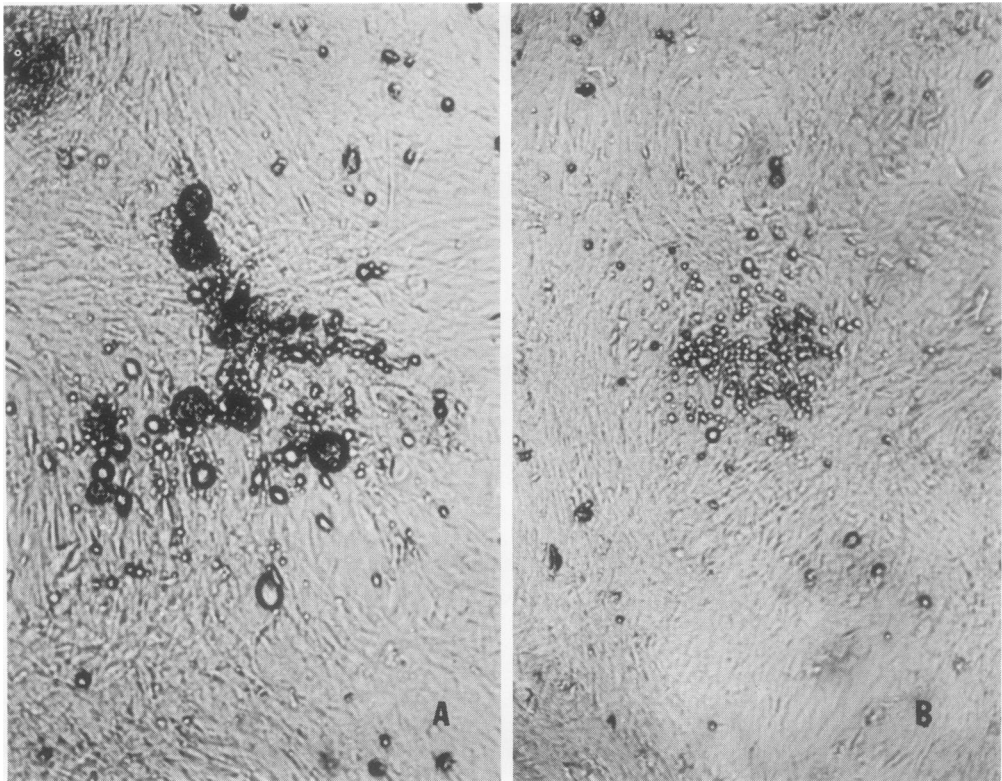


FIG. 1. Plaques of the HN (A) and Id-1 isolates (B) of Marek's disease herpesvirus in CEF cell cultures (7 days after infection). Unstained cell culture. $\times 100$.

TABLE 1. *In vitro* characteristics of the HN isolate

Property	Materials treated and assayed for the HN isolate	Titer of the HN isolate in PFU ^a
Cell association of infectivity	HN isolate-infected CEF cell cultures Culture fluids, 0.5 ml (1/10 of the culture fluids of one dish)	0, 0, 0 ^b
	Dispersed cells, 0.1 ml (1/100 of the cells of one dish) Before rapid freezing and thawing After rapid freezing and thawing three times	322, 277, 294 0, 0, 0
Effect of IUDR ^c	HN isolate-infected CEF cell cultures IUDR-treated, dispersed cells, 0.1 ml	0, 1, 0
	Not-treated dispersed cells, 0.1 ml	244, 238, 218
	NDV-infected CEF cell cultures IUDR-treated, culture fluids	5.6 ^d
	Non-treated, culture fluids	5.8

^a Plaque-forming-units.

^b Number of plaques on each of three replica dishes.

^c 5-Iododeoxyuridine.

^d Log₁₀ TCID₅₀.

icantly inhibited by the presence of IUDR in the culture medium, as indicated by 99.9% reduction in the number of plaques compared with that of control cultures without IUDR treatment. The replication of NDV tested in parallel was not significantly affected (Table 1).

Plaque formation and cytopathology in CEF and CK cell cultures inoculated with the cloned HN isolate were the same as those observed on the primary isolation and in the early passages. Intracellular inclusions (type A) were not observed in infected CEF cell cultures until 13 serial passages after cloning.

The plaque morphology in CEF cell cultures of the HN isolate was characteristically different from that of the Id-1 isolate of MDHV. The Id-1 isolate induced plaques in CEF monolayers when inoculated with heparinized blood of chickens which had been infected either by inoculation or by contact infection (*unpublished data*). The plaques of the Id-1 isolate of MDHV were smaller and more compact compared with those of the HN isolate and consisted of small, round, refractile cells relatively uniform in size and shape (Fig. 1B). Such giant cells observed within plaques of the HN isolate have never been observed in the plaques of the Id-1 isolate of MDHV.

Chicken anti-MD serum produced precipitin lines when tested against the antigens of the HN isolate and MDHV (Id-1 isolate) by micro-agar-gel precipitin test. With each antigen, a single precipitin line was produced, and the line of the HN isolate antigens was contiguous with the line of MDHV antigens, indicating antigenic similarity or identity (or both) between the HN isolate and MDHV (Fig. 2).

The CEF cell cultures of the cloned HN isolate exhibited specific fluorescence which was virtually limited to those round cells (both small and giant) composing the plaque, when stained by indirect fluorescent antibody using chicken anti-MD serum and conjugated horse anti-chicken globulin (Fig. 3).

The HN isolate, thus, had all the *in vitro* characteristics of MDHV, excluding the marked differences in plaque morphology, and appeared antigenically similar or identical to MDHV (Id-1 isolate).

Pathogenicity of the HN isolate for chickens. The chickens inoculated with the HN isolate did not show any clinical signs of MD, whereas most of the birds inoculated with MDHV (Id-1 isolate) exhibited clinical signs of MD, including death. Necropsy and histologic findings in these birds are summarized in Table 2.

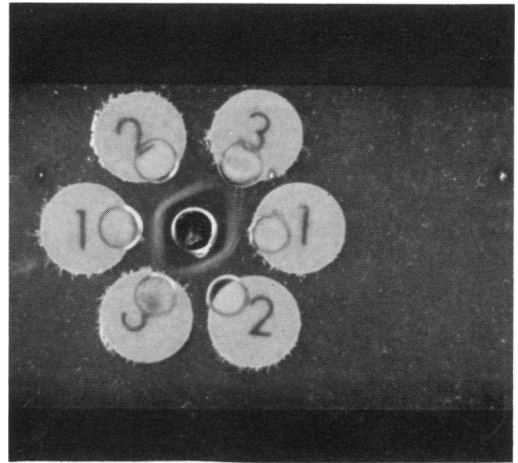


FIG. 2. Micro agar gel precipitin reactions of the Id-1 (1), HN isolates (2), and cultured normal chicken kidney cell antigens (3) against anti-Marek's disease serum (center well).

Six of the 10 chicks inoculated with MDHV (181 plaque-forming units per bird) died of MD within 5 weeks after exposure, and gross lesions were noted in all the survivors, including the contact-exposed cagemates. Siblings of the same hatch, each inoculated with only 16 PFU of the HN isolate, did not develop any visible gross lesions, however, one of the contacts had a single feather follicle lesion of cutaneous MD. Of 10 chicks inoculated with the larger dose of the isolate (166 plaque-forming units per bird), two developed limited gross visceral lesions and one exhibited minimal lesions in feather follicles.

Microscopic nerve lesions were present in the majority of the birds inoculated with the HN isolate as well as in their contact cagemates. These lesions were invariably very mild and were characterized by infiltrations of a few lymphoid cells closely resembling C-type MD lesions (16; *see* Fig. 4A). There was no difference in the nature and extent of nerve lesions in the inoculated birds and their contact cagemates within the same group or between the groups inoculated with small or larger doses of the HN isolate. In most nerve lesions, the lymphoid cells appeared to be degenerating as shown by pyknosis or karyorrhexis of their nuclei.

The microscopic nerve lesions in chickens inoculated with MDHV (Id-1 isolate) and in their contact cagemates were characterized by intensive infiltrations of lymphoid cells, many of which were blast-type cells. The lesions in almost all birds, inoculated or contact exposed, were A or B type MD lesions (16; *see*

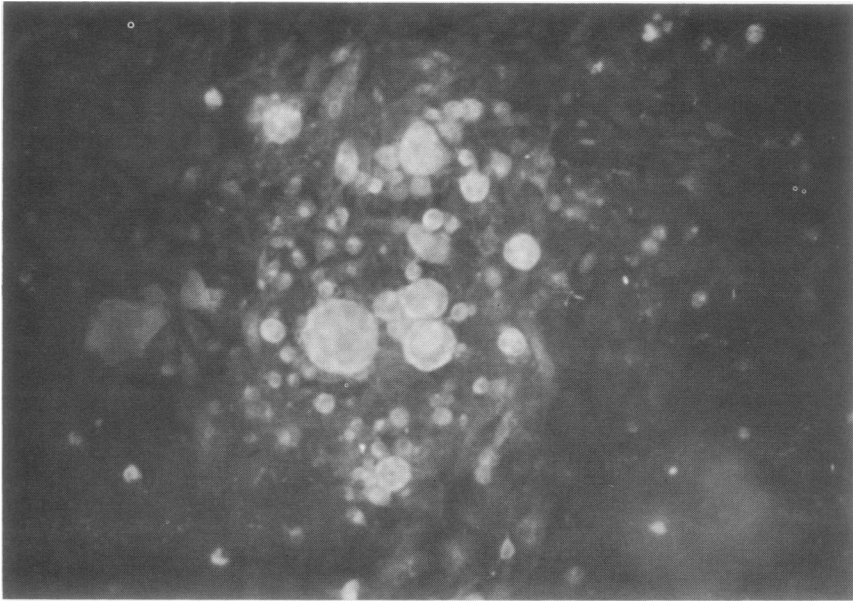


FIG. 3. Indirect fluorescent staining of a plaque of the HN isolate of Marek's disease herpesvirus with anti-Marek's disease serum. Note the specific fluorescence virtually limited to those round cells (small and large) composing the plaque. $\times 160$.

TABLE 2. Gross and microscopic lesions in chickens inoculated with the HN and Id-1 isolates of Marek's disease herpesvirus (MDHV)

Group	No. of birds	Treatment	MD death	Gross lesions present in survivors				Microscopic nerve lesions of MD
				Tumors	Enlarged nerves	Swollen feather follicles	Total with lesions	
A	10	Inoculation of HN isolate 16 PFU ^b /bird	0/10 ^a	0/10	0/10	0/10	0/10	8/10
	5	Contact cagemates	(1)/5 ^c	0/4	0/4	1/4	1/4	3/4
B	10	Inoculation of HN isolate 166 PFU/bird	(2)/10 ^c	2/8	0/8	3/8	1/8	6/8
	5	Contact cagemates	0/5	0/5	0/5	0/5	0/5	5/5
C	10	Inoculation of MDHV (Id-1) 181 PFU/bird	6/10 ^d	2/4	1/4	4/4	3/4	4/4
	5	Contact cagemates	0/5	3/5	4/5	5/5	4/5	5/5
D	8	None	0/8	0/8	0/8	0/8	0/8	0/8

^a Number of birds positive/no. of birds treated or examined.

^b Plaque-forming units.

^c Non-specific death within a week.

^d These birds were observed to be paralyzed before death but to maintain cage isolation were not removed.

Fig. 4B).

Each of the inoculated and contact cagemates of groups A, B, and C was viremic with the respective virus, but none of the control chickens (group D) was viremic.

DISCUSSION

The HN isolate was identified as an isolate of MDHV with low pathogenicity. Excluding the marked differences in plaque morphology,

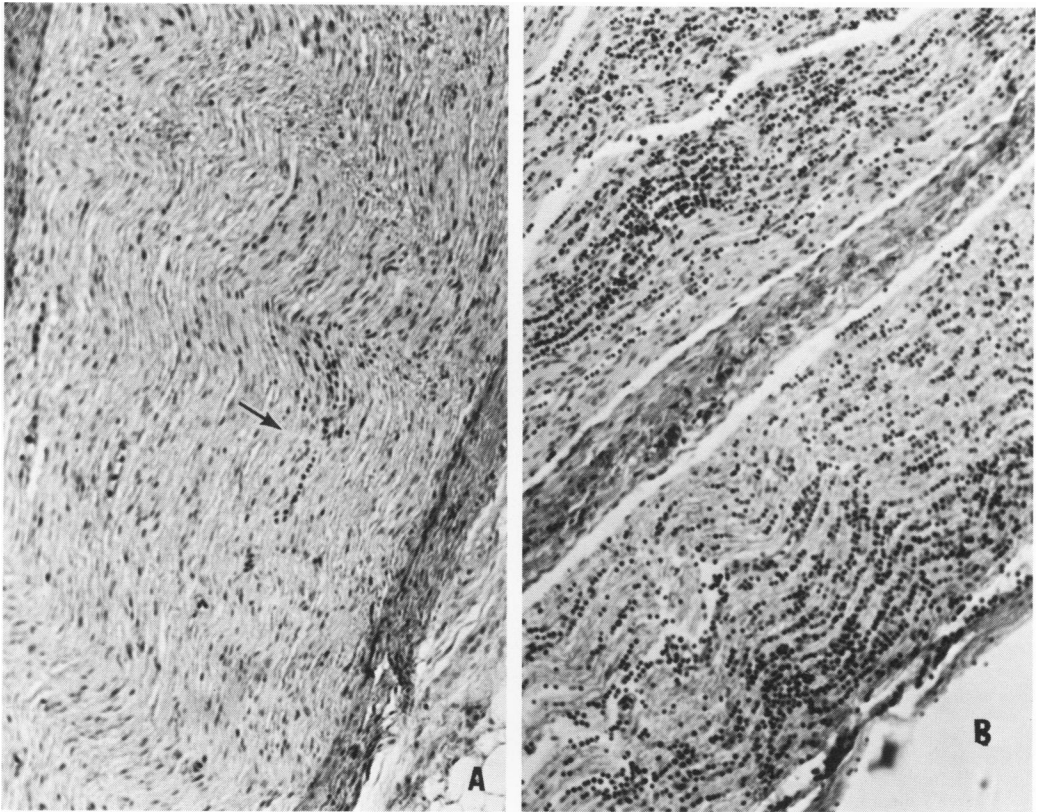


FIG. 4. Microscopic lesions of nerves of chickens inoculated with the HN (A, arrow) and Id-1 isolates (B) of Marek's disease herpesvirus, 5 weeks after inoculation. H & E stain. $\times 160$.

the isolate had all the in vitro characteristics of MDHV and appeared antigenically similar to MDHV (Id-1 isolate). The isolate did not cause clinical MD or death but produced minimal gross and microscopic MD lesions in chickens highly susceptible to MD, whereas an acute strain of MDHV caused a high MD mortality and pronounced gross lesions in all inoculated and contact exposed cagemates.

It was impossible to determine whether the HN isolate of MDHV was the same as the HN-1 CHV agent described by Zander et al. (26), since the latter agent had not been characterized. However, it was very likely that these two agents were the same. In a subsequent trial, a cytopathic agent was isolated from a sample of citrated blood taken from chickens of the same SPF flock from which the HN isolate originated. This cytopathic agent had the same in vitro growth and physicochemical characteristics as the HN isolate.

Biggs and Milne (3) recognized two sizes of

plaques when they grew 25 field isolates of MDHV in CK cell cultures. They found that the majority of the small plaque isolates were apathogenic or caused classical MD, whereas most of the normal plaque isolates produced acute MD. In this observation, the HN isolate of low pathogenicity produced larger plaques in CEF cell cultures, whereas the Id-1 isolate, a highly pathogenic isolate, formed smaller plaques. The difference in plaque size and morphology between these two isolates was not significant when grown in CK cell cultures.

It was interesting to note that the HN and Id-1 isolates of MDHV induced plaques of different morphological characteristics on primary isolation in CEF cell cultures from peripheral blood of infected chickens. This might provide a tool for studying the in vivo interaction between MDHV isolates of low and high pathogenicity. It would also be interesting to determine if the plaque type of MDHV might serve as a biological marker of oncogenicity.

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