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Cell-Free Transmission and In Vivo Replication of Marek's Disease Virus[1]

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Marek's disease virus recovered from the feather follicle of infected chickens was found to be infectious for chickens in cell-free preparations. The virus replicated in epithelial cells of the germinative layer of the feather follicle epidermis, producing both intranuclear and round or diffuse cytoplasmic inclusion bodies in the infected cells. It was found at this site 2 weeks postinoculation and prior to the development of tumor or other gross lesions. In the nucleus, many naked and a few enveloped herpesvirions were found, whereas the cytoplasm contained predominantly enveloped herpesvirions, which were usually within the cytoplasmic inclusion bodies. Approximately 80% of the extracellular virions were enveloped. Studies with both virulent and avirulent strains of the virus revealed a relationship between virulence, contagiousness, and replication of the virus in the feather follicle.

Marek's disease (MD) is a contagious disease of chickens characterized by development of lymphoid tumors in the viscera and lymphoid infiltration of the peripheral nerves. The isolation of a herpesvirus in cell cultures inoculated with blood (11, 14) and in kidney cells (4) of infected chickens was the first significant step in establishing the etiology of the disease. Much circumstantial evidence was later obtained (1, 2, 5–7, 15, 16) which implicated this herpesvirus as the causative agent, but, due to the cell-associated nature of this virus in cell culture, definitive transmission experiments with cell-free virus could not be made. Recently, Calnek and Hitchner (3) found a viral specific immunofluorescence (IF) antigen in the feather follicle of many infected chickens. Similar IF antigens were later found by H. G. Purchase (*unpublished data*). The virus was further recovered from the feather follicle and was shown to produce the disease in chickens (B. W. Calnek et al., *unpublished data*). J. N. Beasley (*unpublished data*) also found infectious virus in sonically treated preparations of dander.

The purpose of the present study was to confirm and demonstrate cell-free transmission of the disease and study the mode of in vivo replication of the virus. Observations were also made on both the process of in vivo viral replication and associated changes in infected epithelial cells of the feather follicle.

[1] Preliminary studies were presented at the 4th International Symposium on Comparative Leukemia Research, Cherry Hill, N.J., September, 1969.

MATERIALS AND METHODS

Virus. The JM strain of MD virus (MDV) was used in this study. Chicken-propagated virus has been maintained at our laboratory (15) by serial passage of whole blood. The in vitro passaged virus was propagated in duck embryo fibroblast (DEF) cultures (14) and was either in low (2nd) passage (LP-MDV) or high (over 100) passage (HP-MDV). The LP virus was virulent for chickens, whereas the HP virus was found to be avirulent (Nazerian, *unpublished data*). A herpesvirus of turkeys (HVT), avirulent for chickens but antigenically related to MDV (Witter et al., Amer. J. Vet. Res, *in press*), was also used in these studies.

Chicken. Crossbred chicks from matings of line 15 and 7 were used for propagation of the virus and infectivity assays. Chicks from $7_2 \times 7_2$ matings were also used in one infectivity assay. Both lines of chickens are susceptible to JM strain of the virus (15).

Feather follicle samples. Ten to 12 feathers were removed from different areas of skin of inoculated or control chickens. The end of the feather shaft ordinarily enclosed within the follicle was cut and placed in 0.5 ml of distilled water. The mixture was sonically treated for 30 sec in a Di Son ultrasonic cleaner (Ultrasonic Industries, Inc., Long Island, N.Y.). Viral suspension was removed and used for electron microscope examination. For infectivity assays, similar specimens from 200 feathers of 4- to 6-week-old MD-infected chickens having gross lesions of the disease were placed in 8 ml of phosphate buffered saline (PBS) and sonically treated as above. The suspension was removed and centrifuged at 1,000 × *g* for 20 min. The supernatant fluid was carefully removed, and one portion was used for chick inoculation without any further treatment. The second portion was passed

through 0.45 μm membrane filters (Millipore Corp., Bedford, Mass.) and used for chick inoculation. After each filtration experiment, cultures of *Serratia marcescens* were passed through the filters, and the filtrates were added to tryptose phosphate buffer and incubated at 37 C. No bacterial growth was noticed in the filtrate, indicating that the filters were intact.

Chick inoculation. One-day-old chicks were inoculated intra-abdominally with 0.2 ml of inoculum per chick. Inoculum consisted of infectious blood (10^{-1} dilution in PBS), infected DEF cultures (2.5×10^5), or filtered and unfiltered viral suspension prepared from the feather follicle. Inoculated and uninoculated chicks were kept separately in Horsfall Bauer isolators for a period of 1 to 8 weeks postinoculation.

Preparation of chick kidney cultures. Kidney tissues were aseptically removed at necropsy from inoculated and control chickens and were prepared for cell culture as reported previously (5). Kidney cell cultures were maintained for 5 to 10 days and were examined periodically with the light microscope for appearance of MDV-specific microplaques (5, 17).

Negative staining. Viral suspensions in distilled water were stained with 2% phosphotungstic acid as reported previously (11) and examined with the electron microscope.

Thick and thin sectioning. Small pieces of skin were removed and fixed in 1% osmium tetroxide for 3 to 4 hr. After washing in buffer, the specimens were dehydrated in increasing concentrations of ethyl alcohol and embedded in Epon 812 (10) as reported previously (11). One-micrometer thick sections were made with a MT2 Porter Blum microtome and stained with 1% Toluidine Blue. Sections were examined with the light microscope and affected areas of the feather follicle were marked for thin sectioning. Thin sections were stained with uranyl acetate (8) and lead citrate (9) and were coated with a thin layer of evaporated carbon. All samples were examined with Elmiskop 1A electron microscope.

RESULTS

Recovery of the virus from the feather follicle. Three groups of chickens were used for this experiment. Chickens in groups 1 and 2 were inoculated at 1 day of age with two different preparations of the infectious blood. Chickens in group 3 were kept as uninoculated controls. At 5 weeks postinoculation all chickens were killed and examined for specific gross lesions of the disease. Kidney tissues were aseptically removed from all chickens and were individually propagated in cell culture. Samples of the feather follicle suspension from each chicken were negatively stained and examined with the electron microscope. Four of five chickens in group 1 and all chickens in group 2 had gross lesions (Table 1). None of the control chickens had gross lesions. Virus was isolated in kidney cell cultures of all inoculated chickens except one culture from group 2 that was bacterially contaminated. No virus was isolated in similar cultures from control chickens. Enveloped herpesvirions were found in the feather follicle samples from all inoculated chickens but were absent in samples from control chickens.

In another experiment 1-day old chicks were inoculated with infectious blood. At each week postinoculation for 7 weeks, 2 chickens from this group were sacrificed and examined for the presence of gross lesions. Cell cultures were prepared from kidney tissues of each bird. Sections of the feather follicle were examined with the light and electron microscope, and negatively stained preparations of the feather follicle samples from each chicken were also examined with the electron microscope (Table 2).

TABLE 1. *Correlation between the presence of MD lesions and recovery of the virus in kidney cell cultures and the feather follicle*

Group	No. of chickens	Inoculum	Gross lesion	Virus isolation in	
				Kidney culture	Feather follicle[a]
1	5	JM blood	4/5	5/5	5/5
2	5	JM blood	5/5	4/5[b]	5/5
3	4	None	0/4	0/4	0/4

[a] Determined by negative staining technique.
[b] One culture developed bacterial contamination.

TABLE 2. *Development of gross and microscopic lesions of MD and appearance of the virus in kidney tissues and the feather follicle after inoculation at 1 day of age*

Weeks postinoculation	Specific lesions		Virus isolation in		Inclusions in the feather follicle
	Gross	Micro[a]	Kidney culture[b]	Feather follicle[c]	
1	−	−	−	−	−
2	−	+	+	+	−
3	+	+	+++	++	+
4	+	+	++	++	+
5	+	+	+	++	+
6	+	+	+	+	NT[d]
7	+	+	+	+	NT

[a] Nerve lesions.
[b] Cultures with extensive number of microplaques were given 3+ score, cultures with moderate number of microplaques were given 2+ score, and cultures with few microplaques were given 1+ score.
[c] Negatively stained preparations in which herpesvirions were easily found were given 2+ score, and preparations in which herpesvirions were found after extensive examination were given 1+ score.
[d] Not tested.

At 1 week postinoculation the chickens were negative in all criteria. At 2 weeks postinoculation, the virus was recovered in kidney cell cultures, and its presence in the feather follicle samples was demonstrated by electron microscopy. Microscopic lesions were seen in the nerves. With the development of gross lesions at 3 weeks postinoculation and later, all criteria were positive. Chickens sacrificed between the 3rd and 5th weeks postinoculation all had clinical signs of the disease, and gross lesions were seen at necropsy. Samples prepared from these chickens provided a higher number of microplaques in kidney cultures and contained a higher number of herpesvirions associated with the feather follicle. Chickens sacrificed at 6 and 7 weeks postinoculation did not demonstrate obvious clinical signs. Subsequently, a lower number of microplaques in kidney cultures and a lower number of herpesvirions associated with the feather follicle were observed in samples taken from these chickens. Samples prepared from the feather follicles of different areas of skin (thigh, wing, tail, and dorsal region) all contained herpesvirions, and there was no significant difference between the number of herpesvirions found in these areas.

Cell-free transmission of the disease. In experiment 1, the infectivity of filtered and unfiltered preparations of feathers from 4-week-old inoculated chickens with signs of MD was assayed in chickens of line 15 × 7. In experiment 2, similar procedures were followed, except that the inoculum originated from a 6-week-old chicken with no obvious clinical signs and recipient chickens were of line 7_2 In both experiments a positive response was obtained in chickens inoculated with either filtered or unfiltered virus preparation, whereas there were no specific deaths or gross lesions in uninoculated controls (Table 3). In experiment 1 equal response was observed in chicks inoculated with filtered and unfiltered preparations. In experiment 2, response in chickens inoculated with filtered preparation was somewhat lower than the response in the group inoculated with unfiltered material.

TABLE 3. *Cell-free transmission of the disease*

Expt	Group	No. of chicks	Inoculum[a]	Nonspecific mortality	Specific mortality	Total gross lesion
1	1	8	None	0	0	0/8
	2	8	A	0	2	8/8
	3	8	B	0	1	8/8
2	1	10	None	1	0	0/9
	2	10	A	1	2	8/9
	3	10	B	0	0	2/10

[a] Inoculum A was virus suspension prepared from the feather follicle after sonic oscillation and centrifugation. Inoculum B was the same preparation as A in each experiment filtered through 0.45-μm membrane filter (Millipore).

Viral replication in the feather follicle of chickens inoculated with virulent and avirulent strains of MDV. Since attenuated strains of MDV and HVT were found to be poorly transmitted to chickens exposed by direct contact, experiments were undertaken to investigate whether this poor transmission was due to inability of these strains to replicate in the feather follicle. Four groups of chicks were used in this experiment. Fifty per cent of the chicks in groups 1, 2, and 3 were inoculated intra-abdominally with 5×10^3 PFU of LP-MDV, 5×10^4 PFU of HP-MDV, and 5×10^4 PFU of HVT per chick, respectively. The other half of each group of chickens was kept in contact with the respective inoculated chickens. Approximately 50% of the inoculated and contact chickens in each group were removed and examined at 4 weeks postinoculation and the remainder were removed and examined at 8 weeks. All chickens were examined for gross lesions of the disease, development of specific microplaques in cultures established from their kidney tissues, and the presence of herpesvirions in their feather follicle samples (Table 4).

A high proportion of LP-MDV inoculated and contact chickens had gross lesions at both 4 and 8 weeks postinoculation, indicating the virulence and contagiousness of this virus. The overall incidence of gross lesions was 90% in inoculated and 66% in contact chickens. All other chickens from other groups examined 4 or 8 weeks postinoculation lacked gross lesions. Virus was isolated in kidney cultures at 4 weeks postinoculation from 100% of inoculated chickens in all three groups and contact chickens in group 1. Contact chickens in groups 2 and 3 were all negative. At 8 weeks postinoculation virus was isolated from 100% of inoculated chickens in all three groups, 66% of contact chickens in group 1, 20% in group 2, and only 16% in group 3. The overall incidence of virus isolation was 100% in inoculated chickens in all three groups and 83, 8, and 8% in contact chickens in groups 1, 2, and 3, respectively. Electron microscope findings of herpesvirions in the feather follicle samples were positive in 100% of inoculated and 33% of contact chickens in group 1 examined 4 weeks postinoculation and negative in all other groups. At 8 week postinoculation, electron microscope examinations were positive in 100% of inoculated and 66% of contact chickens in group 1 and negative in all other groups. The overall incidence of herpesvirions in the feather follicle was 100% in inoculated, 50% in contact chickens in group 1, and 0% in all other groups.

TABLE 4. *Replication of virulent and avirulent strains of MDV in the feather follicle of experimentally infected chickens*

Groups	No. chicks	Treatment	Inoculum	4 Weeks			8 Weeks			Summary[a]		
				Gross lesions	Virus isolation in		Gross lesions	Virus isolation in		Virulence	Virus in follicle sample	Contact transmission
					Kidney culture	Follicle sample		Kidney culture	Follicle sample			
1	12	Inoculated	LP-MDV	5/5	5/5	5/5	3/4	4/4	4/4	+++	+++	+++
	12	Contact exposed	None	2/6[b]	6/6	2/6	6/6	4/6	4/6			
2	12	Inoculated	HP-MDV	0/4	4/4	0/4	0/8	8/8	0/8	−	−	±
	12	Contact exposed	None	0/7	0/7	0/7	0/5	1/5	0/5			
3	12	Inoculated	HVT	0/6	6/6	0/6	0/5	5/5	0/5	−	−	±
	12	Contact exposed	None	0/6	0/6	0/6	0/6	1/6	0/6			
4	5	None	None				0/5	0/5	0/5	−	−	−

[a] A 3+ score indicates 75 to 100%, a ± score indicates less than 10%, and a − score indicates 0% response by each virus.

[b] Four chickens in this group were accidentally destroyed before gross diagnosis was made.

Light microscopy. Cytopathological changes in the feather follicle were restricted to stratum germinativum of the follicle epidermis (Fig. 1). This stratum is composed of several rows of epithelial cells and is further subdivided to stratum transivativum, stratum intermedium, and stratum basale (A. M. Lucas, *personal communication*). Cells in stratum basale and stratum intermedium were moderately affected and slightly misplaced, but viral-specific cytopathological changes were observed only in epithelial cells of stratum transivativum. A generalized cytoplasmic and nuclear degeneration was observed in most of these cells. Nuclei in infected cells were slightly smaller than the nuclei of adjacent cells. Chromatin material was usually marginated, and many nuclei had herpes-type inclusion bodies (Fig. 2). These inclusions were separated from the nucleolus and the nuclear membrane by a clear margin. A generalized granulation in the cytoplasm was also noticed. Many of these cells contained round or irregular cytoplasmic inclusions (Fig. 2). All chickens examined at 3, 4, and 5 weeks postinoculation contained these inclusion bodies in epithelial cells of at least one of three follicles examined.

Electron microscopy. Electron microscopic examination of the feather follicle also showed that viral specific cytopathological changes were restricted to cells in stratum transivativum (Fig. 3). Basal cells and three to four adjacent inner layers of epithelial cells appeared normal, whereas a generalized cytoplasmic and nuclear degeneration was often noticed in cells in three to four rows of the outermost layer of the follicle epidermis. The degenerative change appeared to originate in the inner cells and became more extensive in the outermost row of cells. Cytopathological changes were first seen in the nucleus. These changes included margination of the chromatin material and appearance of the inclusion bodies typical of herpesvirus infection (Fig. 4). These inclusion bodies were granular in structure, occupied the central portion of the nucleus, and seemed to displace the nucleoli and chromatin material to the periphery. Naked and occasionally enveloped herpesvirions were found either within the inclusion body or in the peripheral regions of the nucleus. In later stages of infection, many inclusion bodies developed in the cytoplasm and largely replaced the normal cytoplasmic components. These inclusions were often round (Fig. 3 and 6), but sometimes they had an irregular shape (Fig. 5) and occupied a major portion of the cytoplasm. These inclusions were very homogeneous except for the presence of herpesvirions, all of which were enveloped. The nuclear membrane was usually ruptured at this stage and the nuclear content was spilled into the cytoplasm.

Morphology of the virus. Naked virions in the nucleus (Fig. 4 and 8) had the general morphology of other herpesviruses. Immature naked virions were found within the nucleus. Some had eccentrically located nucleoids and some seemed empty. In more mature virions the nucleoid seemed to fill the entire capsid (Fig. 7). Mature virions were close to the nuclear membrane and

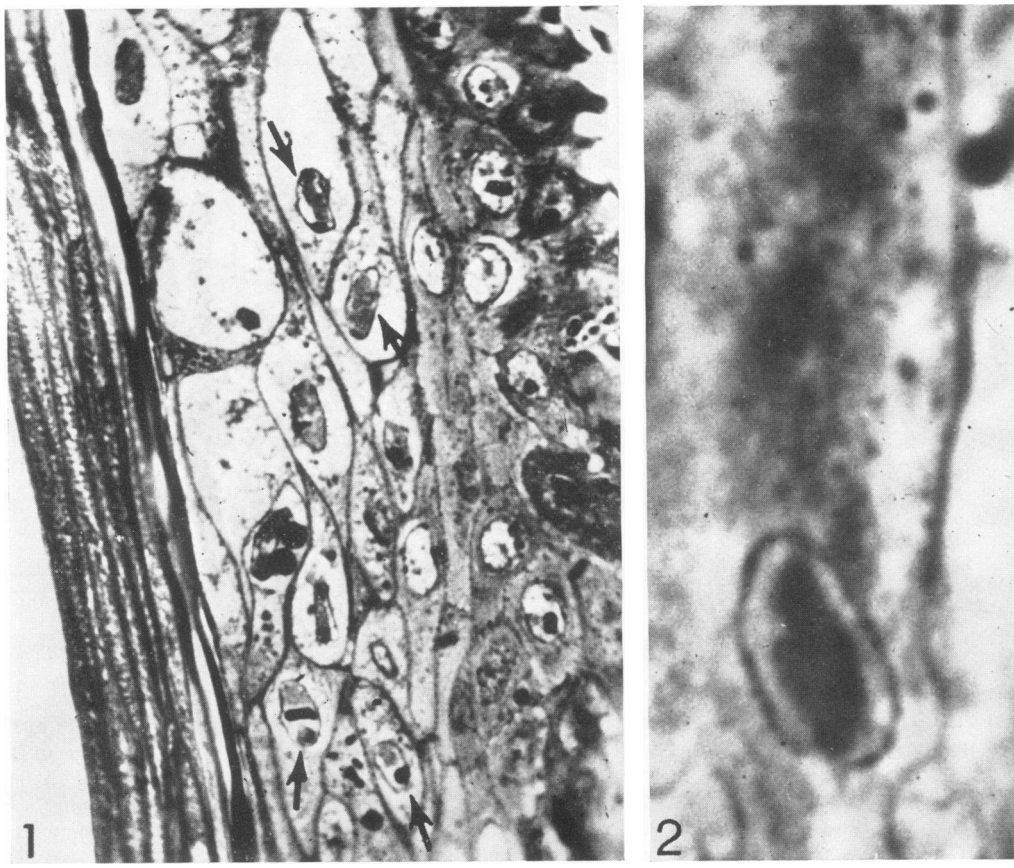


FIG. 1. *Light micrograph of portion of a feather follicle of an infected chicken. Many intranuclear inclusion bodies (arrows) are seen in epithelial cells (stratum transivativum) of the follicle epidermis. Cells in the basal layer and in the intermediary layer (to the right) do not show any signs of viral infection. Stratum corneum of the follicle epidermis is seen in the left side of the micrograph.* × *900.*

FIG. 2. *Light photomicrograph of portion of an infected epithelial cell in the feather follicle. A distinct inclusion body is seen in the nucleus in which chromatin material is clearly marginated. A diffuse cytoplasmic inclusion body is also seen immediately above the nucleus.* × *3,700.*

seemed to obtain an envelope by budding through this membrane (Fig. 7). Enveloped virions within the nuclear vesicles (Fig. 8) or immediately outside the nucleus measured 150 to 170 nm in diameter. The envelope was closely attached to the capsid. The majority of enveloped virions were found within cytoplasmic inclusion bodies and, in this location, had a slightly different morphology. They measured 200 to 250 nm in diameter (Fig. 9). The envelope was swollen, and the nucleocapsid was often eccentrically located within the envelope. The space between the nucleocapsid and the envelope was filled with a fine granular material similar to the material which composed the cytoplasmic inclusion body. Sometimes two nucleocapsids were found in one envelope (Fig. 9).

The extracellular virions examined by the negative staining technique were more similar (Fig. 10) to the enveloped virions within the cytoplasmic inclusion bodies than were those few enveloped virions found in the nucleus. Approximately 80% of the extracellular virions were enveloped. Most of these were ruptured when examined in negatively stained preparations (Fig. 10–12) and were penetrated by potassium phosphotungstate. The capsid was composed of typical hollow centered capsomeres (Fig. 12) and was surrounded by a fine filamentous material (Fig. 11 and 12).

DISCUSSION

Specificity of the virus to the disease. Calnek and Hitchner (3) found an IF antigen in the

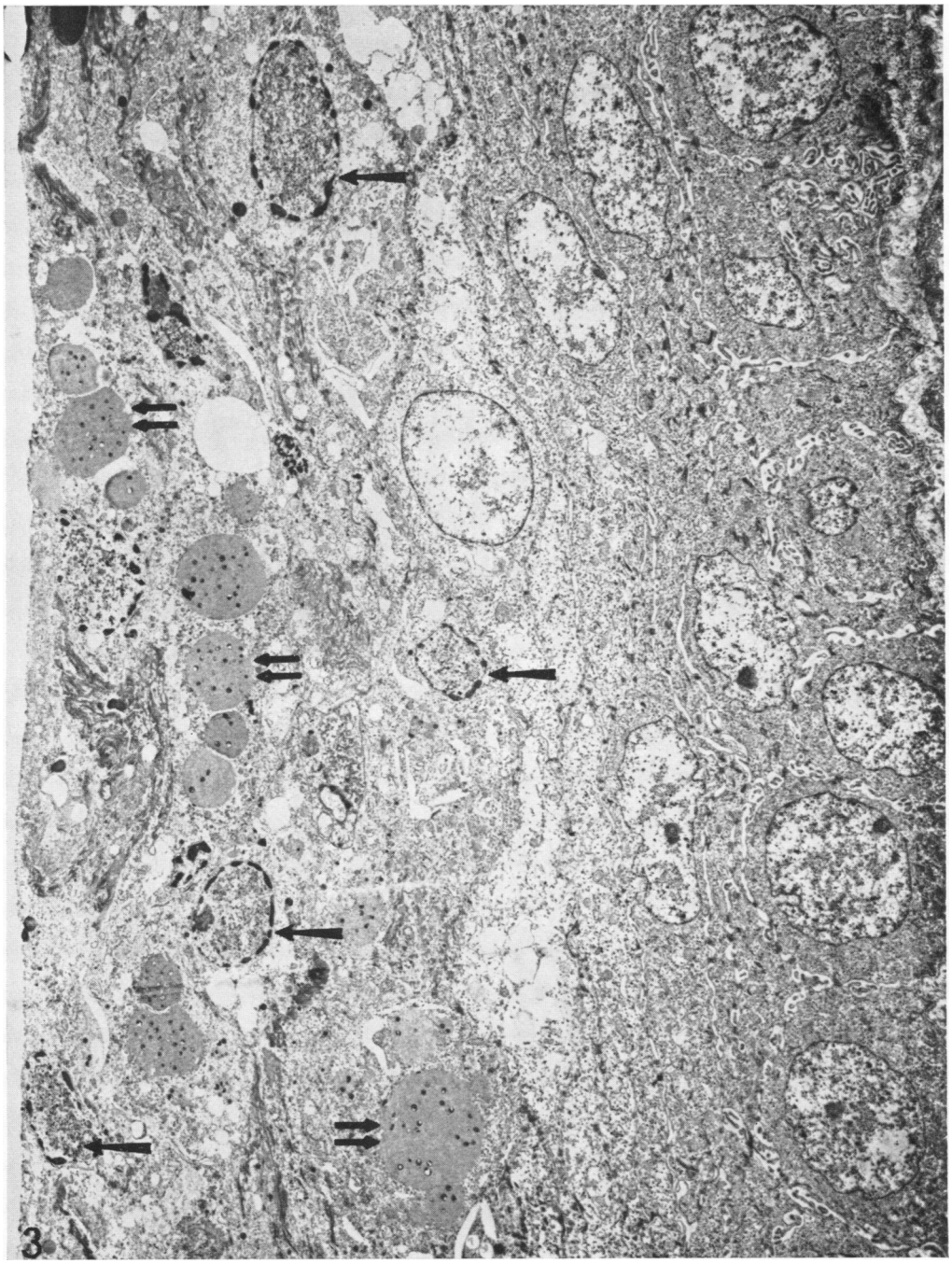
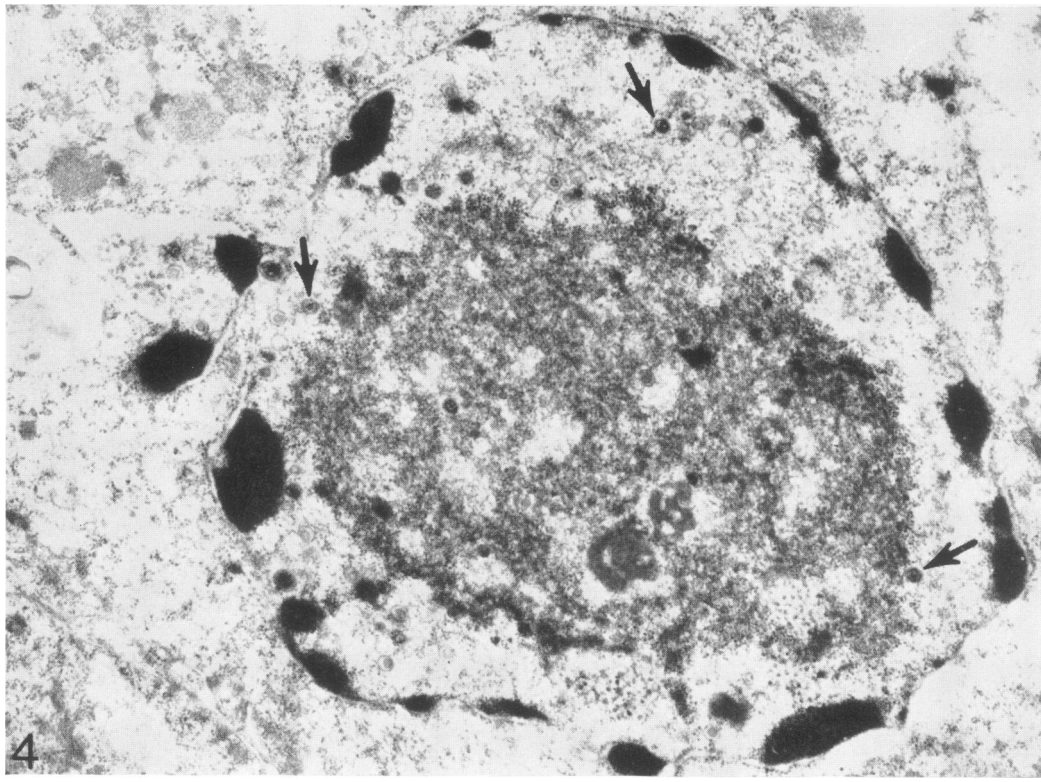
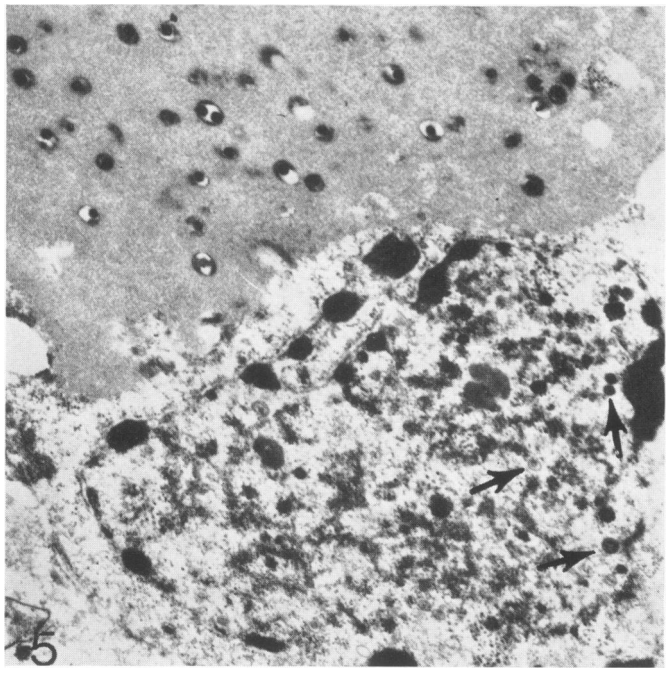


FIG. 3. *Low-magnification electron micrograph of epidermis of the feather follicle of an infected chicken. Viral-specific changes are seen only in the outermost three to four layers of cells of epidermis. Intranuclear inclusion bodies are seen in many of the infected cells (single arrows). Chromatin material is clearly marginated in these nuclei. Many round and irregularly shaped cytoplasmic inclusion bodies are also seen in infected cells (double arrows). These inclusion bodies contain a large number of virions.* × *6,000.*





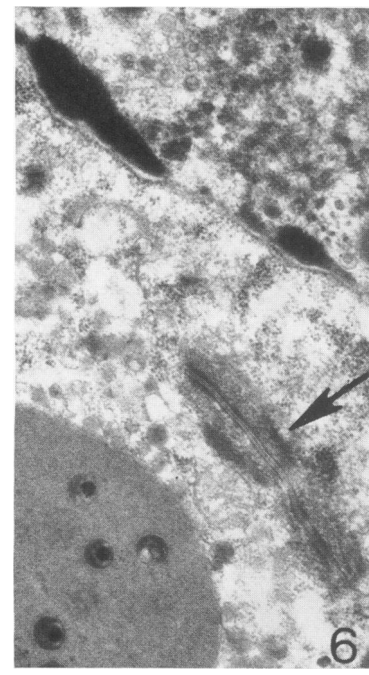


FIG. 4. *Portion of an infected cell. A distinct inclusion body is seen in the nucleus. This inclusion body is composed of a highly granular substance. Naked herpesvirions are seen inside the inclusion body and in the periphery of the nucleus (arrows).* × *23,000.*

FIG. 5. *Portion of an infected cell with a massive perinuclear inclusion body in the cytoplasm. Naked virions are seen in the nucleus and enveloped virions are only seen in the cytoplasmic inclusion body.* × *20,000.*

FIG. 6. *Portion of an infected cell. Several enveloped virions are seen in part of a round cytoplasmic inclusion seen in the lower left corner of the micrograph. Desmosomes (arrow) and cytoplasmic bridges were commonly seen between the infected cells.* × *30,000.*

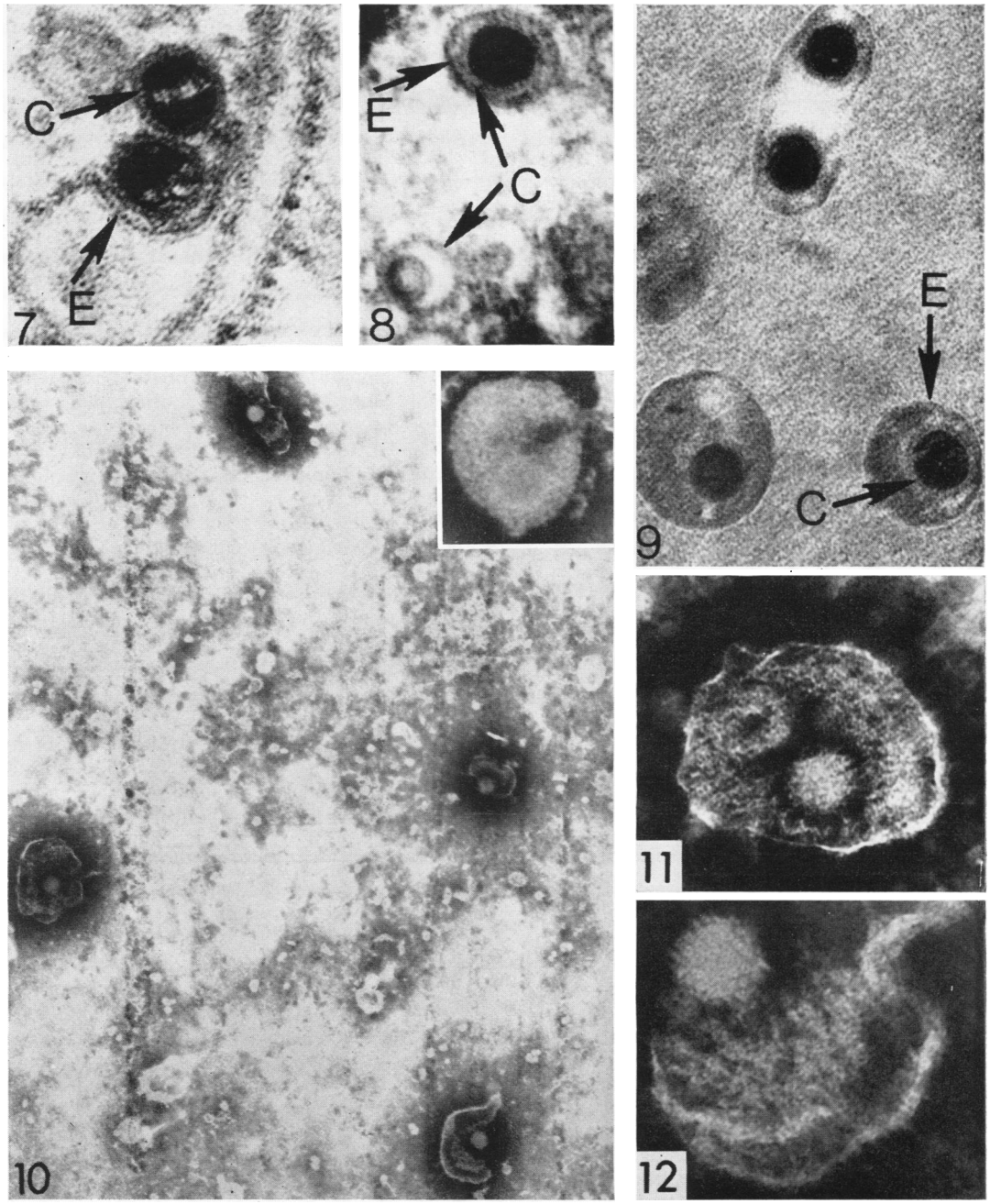


FIG. 7. *Micrograph showing three virions near the nuclear membrane. One of these virions is budding into a nuclear vesicle. Symbols: C, capsid; E, envelope.* × *140,000.*

FIG. 8. *Several naked and an enveloped virion are seen in portion of a nucleus. Nucleoid is small and eccentrically located in naked virions but fills the whole capsid in enveloped virions. There is little space between capsid (C) and the envelope (E) in the intranuclear enveloped virion.* × *120,000.*

FIG. 9. *Several enveloped virions are seen in portion of a cytoplasmic inclusion body. These are larger in size and contain a dense material besides the nucleocapsid. This dense material is similar to the content of the cytoplasmic inclusion body. In the virion located at the lower right corner of the micrograph another coat or envelope is seen covering the capsid (C).* × *90,000.*

FIG. 10. *Negatively stained preparation of the extracellular virions. All four virions seen in this micrograph are enveloped. The envelope is partially ruptured in most of the virions.* × *20,000. Virion seen in the inset is not ruptured and is not penetrated by the stain. Nucleocapsid is poorly stained and is seen in the lower portion of the virion.* × *90,000. Note the resemblance of this virion to the one seen in the lower left corner of Fig. 9.*

FIG. 11 and 12. *Details of viral structure are seen in these negatively stained virions. The envelope is partially ruptured in both virions and reveals a filamentous material (Fig. 11,* × *90,000) and a nucleocapsid composed of hollow centered capsomeres (Fig. 12,* × *140,000).*

feather follicle of a high proportion of MDV-infected chickens. The presence of herpesvirions in the feather follicles of MD infected chickens was found in this experiment to be specific and related to the disease as demonstrated by their presence in all the infected chickens (more than 40 examined) and their absence in all uninoculated control chickens (more than 9 examined). This specificity is further shown by cell-free transmission of the disease described by Calnek et al. (*unpublished data*) and reported here. Successful cell-free transmission of the disease was obtained in both experiments undertaken in these studies. A higher response was obtained in the first experiment than in the second experiment. This could be due to a low concentration of virus in the inoculum used in the second experiment, as it was obtained from a 6-week-old infected chicken with no clinical signs but with typical gross lesions and also because of short duration of the experiment (4 weeks as compared to 6 weeks in experiment 1). Inoculum for the first experiment was obtained from a 4-week-old infected chicken with both clinical signs and gross lesions of the disease. Other information presented in this paper indicates that more herpesvirions were found in samples taken 3 to 5 weeks post-inoculation from clinically sick chickens than older chickens with no clinical signs of the disease. Epidemiological studies (Witter, *unpublished data*) have also shown that the disease is highly contagious in similar periods postinoculation, perhaps due to a higher concentration of the virus in the feather follicle.

MDV was isolated in kidney cultures and was demonstrated in the feather follicle extract of inoculated chickens 2 weeks postinoculation, before the development of clinical signs or gross lesions of the disease. More herpesvirions were found in the feather follicle samples from chickens examined 3 to 5 weeks postinoculation. The number of virions at this site decreased at 6 and 7 weeks postinoculation, but they were present in chickens examined up to 8 weeks postinoculation. Perhaps infected chickens continue to replicate and release the virus in the feather follicle indefinitely.

Although most of the tissues of infected chickens can produce the disease by inoculation into recipient chicks and they have been shown to produce MDV-specific microplaques in cell culture (17), the virus has been highly cell associated in these tissues and only rarely have been morphologically intact virions found in these tissues (13; K. Nazerian, *unpublished data*), which were invariably immature and did not have the envelope. Whereas immature herpesvirions were found in only two gonad tumors and in lymphoid infiltrations of one nerve (K. Nazerian, *unpublished data*) of more than 100 such specimens examined with the electron microscope, all the infected chickens examined in these experiments contained herpesvirions in their feather follicle samples. MDV, therefore, may exist in infected chickens in three different forms. (i) In most of the tissues, the virus or viral genome exists in nonpermissive cells and morphologically is not distinguishable; (ii) immature virus occasionally is produced in lymphoid tumor cells and epithelial cells of kidney and other organs but is defective and noninfectious; and (iii) the virus replicates in epithelial cells of the feather follicle and produces mature infectious virions. This concept is extended by finding (3; H. G. Purchase, *unpublished data*) in certain tissues (except lymphoid tumor cells), such as feather follicle epithelium, bursa, kidney, thymus, etc., viral-specific immunofluorescent and precipitin antigens and also, in some cases, accompanying cytolytic changes, both of which may be directly related to virus replication (H. G. Purchase, *unpublished data*).

Natural transmission of the virus. Spread of the virus from chicken to chicken may be related to the degree in which it replicates in the feather follicle. Results presented in this paper demonstrate a positive correlation between the presence of the virus in the feather follicle and its spread to contact chickens. The virulent strain of the virus was easily found in the feather follicle and was also easily transmitted to contact chickens. The avirulent strains were very poorly transmitted to contact chickens, and samples of the feather follicle of chickens inoculated with these strains did not contain any virions. The low incidence of virus transmission that occurred in chickens exposed to avirulent strains could be due to a low concentration of the virus in the feather follicle, undetectable by the method used, or may have been caused by another unknown means of transmission. However, the good correlation established between the presence of the virus in the feather follicle and contact transmission of the virus supports previous studies showing indirect transmission of the disease through the air and indicates that the feather follicle is a major port of exit of virulent virus.

Morphological studies on virus replication. Light and electron microscopic studies showed that viral replication takes place in three to four of the outermost layers of epithelial cells in the feather follicle epidermis. In no instance were virus specific changes noticed in skin epidermis, feather epidermis, or in the lymphoid cells occasionally infiltrated into the follicle dermis. This is in agreement with the location of viral-specific IF antigens (3; Purchase, *unpublished data*). MDV, therefore, seems to have a selective affinity to

replicate and mature in epithelial cells of the feather follicle.

Of particular significance was the occurrence of a large number of cytoplasmic inclusion bodies in infected epithelial cells. These inclusion bodies have not been found in other tissues of MDV-infected chickens (13; K. Nazerian, *unpublished data*). In infected cell cultures such inclusion bodies were either absent (5, 11, 12) or occasionally seen (1). Since, in the feather follicle, mature infectious forms of the virus were often associated with these bodies, they might be directly related to and essential for synthesis of cell-free virus. Extracellular virions were quite similar to virions found in the cytoplasmic inclusion bodies and both contained a dense filamentous material similar to the material found in the inclusion bodies. The nature of this material is not known, but perhaps it is the glycoprotein component of the virus responsible for penetration into a new cell.

Similar to other herpesviruses, the nucleocapsid was released into the cytoplasm by budding through the nuclear membrane. However, a considerable change in shape of the envelope and its content occurred in the cytoplasm. It is not known whether an additional envelope was acquired by the virus in the cytoplasm or the envelope obtained at the nuclear membrane was enlarged, perhaps because of penetration by some material while in the cytoplasmic inclusion bodies. The role of envelope in ability of the virus to express its infectivity is further strengthened by the findings reported here that approximately 80% of the extracellular virions from the feather follicle had the envelope and remained infectious, whereas extracellular virus prepared in cell culture rarely had the envelope and was not infectious (1, 5, 11, 12). Among several herpesviruses isolated from human and animal malignancy MDV is the only one proven to cause neoplasia.

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