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Induction of Deoxyribonucleic Acid Synthesis and the Oncogenicity of Marek's Disease Virus

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Leukocytes recovered from birds inoculated with an oncogenic strain of Marek's disease virus synthesized deoxyribonucleic acid in vitro at a rate 7.9 times that of leukocytes from normal birds or birds inoculated with the nononcogenic herpesvirus of turkeys. A close relationship was observed between the level of in vitro deoxyribonucleic acid synthesis and in vivo tumor formation.

Marek's disease (MD) is a lymphoproliferative disease of domestic chickens. The affected birds display nerve lesions and lymphoid tumors. The causative agent has been well established as a herpesvirus (1, 8) whose deoxyribonucleic acid (DNA) molecular weight was estimated at approximately 10^8 (7).

Several DNA-containing oncogenic viruses have been shown to stimulate cellular DNA synthesis (3-5, 13). Because MD virus (MDV) is an oncogenic DNA virus (1), it remains to be demonstrated that induction of DNA synthesis follows MDV infection. Since it is not possible to infect cells with cell-free MDV, DNA synthesis in leukocytes from infected and uninfected birds was compared. This paper presents data on DNA synthesis in leukocytes from MDV-infected chickens and reports in vitro DNA induction sequentially monitored in the course of tumor formation.

MATERIALS AND METHODS

Medium and viruses. Medium 199, without thymine but supplemented with 1% dialyzed fetal calf serum, 100 units of penicillin, and 100 μg of streptomycin sulfate per ml, was used as the growth medium for leukocytes. ^{3}H-thymidine (16.0 Ci/mmole) and ^{14}C-thymidine (57 mCi/mmole) were obtained from Schwarz/Mann, Orangeburg, N.Y. The GA strain of MDV (12) and the FC126 herpesvirus of turkeys (HVT) (10, 14) were propagated in primary duck embryo fibroblast cultures as previously described (6).

Animal inoculation. Day-old chickens of the Regional Poultry Research Laboratory inbred line, 15×7, $7_2 \times 7_2$, were used (H. A. Stone, Theor. Appl. Genet., *in press*). They were inoculated intra-abdominally with cell-associated MDV or HVT at a standard dosage of 10^5 plaque-forming units per 0.2 ml per chick. Both uninoculated and inoculated chickens were maintained in a Horsfall Bauer isolator and kept under observation. Blood samples were taken from these birds at either weekly or biweekly intervals.

Preparation of cells for culture. Blood (1 to 2 ml) from each bird was carefully drawn by heart puncture into 2.5-ml plastic syringes containing 0.2 ml of heparin. Samples from each group were pooled separately, and leukocytes were selected from other cells by an albumin flotation technique (11). The leukocytes were collected and washed once with phosphate-buffered saline and sedimented at $400 \times g$ for 10 min at 4 C in a PR-6 International Equipment Co. centrifuge. The viable cells were counted by the trypan blue exclusion test. The leukocytes were then diluted in growth medium to a final concentration of 2.5×10^6 viable cells per ml and incubated at 37 C. ^{3}H-thymidine or ^{14}C-thymidine was added immediately after suspension of leukocytes.

Assaying of samples. Two triplicate sets of samples were taken at hourly intervals from the labeled leukocyte culturing flasks. To one set of samples, 3 ml of 10% cold trichloroacetic acid was added. The trichloroacetic acid-insoluble material was collected on Whatman glass filters (GF/C). The filters were dried and placed in liquid scintillation vials containing 5 ml of 0.4% 2,5-bis[2-(5-tert-butylbenzoxazolyl)]-thiophene in toluene. The radioactivity of the filter was measured in a Beckman LS-100 scintillation counter. The cells in the second set of samples were collected by centrifugation, and their DNA content was then determined by the diphenylamine test (2).

Isopycnic centrifugation of DNA in cesium chloride solution. Approximately 10^7 leukocytes from infected and uninfected birds were labeled for 15 hr with ^{3}H- and ^{14}C-thymidine (1 to 2 mCi/ml), respectively, harvested, and washed with phosphate-buffered saline. They were lysed together with 2% sarkosyl and 0.5% sodium dodecyl sulfate at 60 C. The lysate was mixed with a CsCl solution to an initial density of 1.710 g/cm^3 and then centrifuged to equilibrium in a Spinco SW39 rotor at 35,000 rev/min at 20 C for 48 hr. In some experiments DNA was analyzed in a model E analytical ultracentrifuge as described in the legend to Fig. 3.

Virus reisolation, DNA synthesis, and gross lesions. Birds inoculated with MDV and HVT were examined for visceral tumors at 2 to 4 weeks postinoculation, i.e., when a large proportion infected with MDV showed symptoms characteristic of MD. In the course of examination, the birds were first bled and then autopsied. Leukocytes from individual birds were recovered, and approximately 10^7 to 2×10^7 cells were seeded on secondary duck embryo fibroblast for reisolation of viruses. Incorporation of ^{3}H-thymidine in leukocytes from each bird was studied, and examination of tumors in the visceral organs of each bird was carried out.

RESULTS

Kinetics of incorporation of ^{3}H-thymidine. Representative results obtained from 20 different experiments are given in Fig. 1. Leukocytes from uninoculated control birds exhibited little or no uptake of ^{3}H-thymidine during the entire course of labeling. Leukocytes from MDV-inoculated birds exhibited a remarkable increase in the rate of incorporation of ^{3}H-thymidine. After the addition of label the specific activity of DNA increased, reaching a maximum at 10 hr and declining thereafter. On the basis of many such experiments, the increase in specific activity found in leukocytes from MDV-infected birds ranged from 2.6- to 15-fold (average, 6.8) higher than in uninoculated birds. However, leukocytes from HVT-vaccinated birds were indistinguishable from the uninoculated controls in that they incorporated little or no ^{3}H-thymidine.

Time of maximal in vivo DNA synthesis in leukocytes from infected birds. Induction of DNA synthesis was monitored over the entire experimental period after inoculation of day-old chicks. Blood was drawn from uninoculated, HVT- and MDV-inoculated birds at weekly intervals. After each bleeding, leukocytes were separated and the rate of incorporation of ^{3}H-thymidine was studied. Representative results are given in Fig. 2. In Fig. 2A, each curve represents the kinetics of

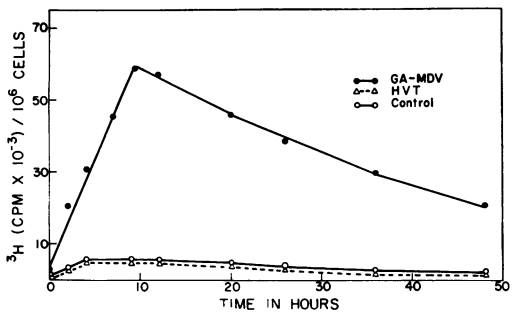

FIG. 1. *Incorporation of ^{3}H-thymidine into leukocytes. Uninoculated control* (○), *HVT-inoculated birds* (△), *and strain GA MDV-inoculated birds* (●).

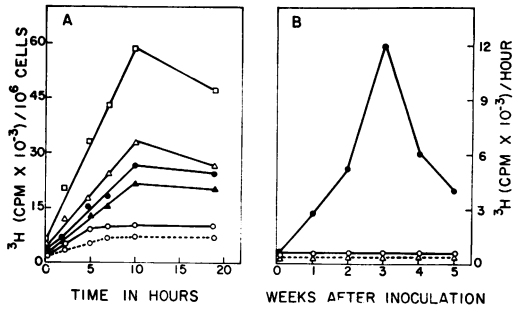

FIG. 2. *(A) Incorporation of ^{3}H-thymidine into leukocytes; leukocytes from uninoculated control and HVT-inoculated birds* (○——○). *Kinetics of incorporation remained at the same level for the entire 5-week period. Leukocytes from strain GA MDV-inoculated birds: first week* (○——○), *second week* (●), *third week* (□), *fourth week* (△), *fifth week* (▲). *(B) Rate of ^{3}H-thymidine incorporation calculated from the linear portion of (A). Uninoculated control birds* (○), *HVT-inoculated birds* (△), *GA MDV-inoculated* (●).

^{3}H-thymidine incorporation over a 16-hr assaying period for each successive week postinoculation. Figure 2B is a plot of incorporation rates calculated from the linear portion of the weekly incorporation curves represented in Fig. 2A. The time of maximal DNA synthesis in leukocytes from MDV-infected birds occurred between 3 to 4 weeks postinoculation. DNA synthesis in leukocytes from HVT-inoculated birds remained at a constant low rate throughout the entire experimental period of 5 weeks. As early as 1 to 2 weeks postinoculation, birds inoculated with MDV exhibited a 2- to 5-fold increase in the rate of induction of DNA synthesis over the uninoculated birds. When these inoculated birds were autopsied at 3 to 4 weeks after inoculation, i.e., at the time of maximal in vitro DNA synthesis, more than 60% were found to have developed tumors in the ovary, kidney, or spleen.

Isopycnic centrifugation of cell lysates in cesium chloride solution. The nature of the ^{14}C- and ^{3}H-thymidine-labeled products in leukocytes from normal and MDV (GA strain)-infected birds was studied by cocentrifugation of the mixed lysates in cesium chloride solution. The result is shown in Fig. 3A. The ^{3}H-thymidine-labeled macromolecules banded at the same density (1.698 g/cm^3, as determined by centrifugation in a model E analytical ultracentrifuge using SPO1 and *Micrococcus lysodeikticus* DNA as markers) as ^{14}C-thymidine-labeled material from uninfected controls. In another experiment, the lysate from ^{3}H-thymidine-labeled, MDV-infected leukocytes was centrifuged to equilibrium in CsCl. The result was similar to that shown in Fig. 3A. The radioactivity profile closely follows the absorbance at

260 nm. When the fraction from the heavy region of the density gradient was further analyzed in a model E analytical ultracentrifuge, only one DNA with a buoyant density of 1.698 g/cm³ (Fig. 3B) was detected. These experiments led to the conclusion that the labeled DNA in leukocytes from MDV-infected birds was mostly leukocyte DNA. No MDV-DNA was detected.

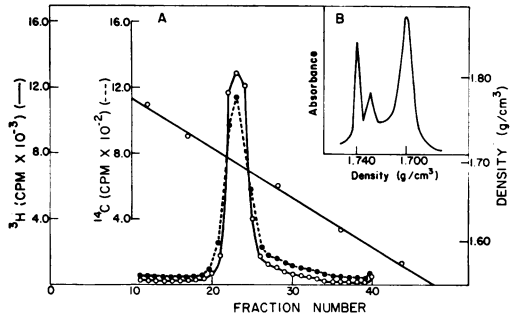

FIG. 3. *(A) Cocentrifugation of uninoculated leukocyte (●) with strain GA MDV-inoculated leukocyte lysates (○) in cesium chloride equilibrium density gradient. (B) Buoyant density determination of strain GA MDV-inoculated leukocyte DNA with Micrococcus lysodeikticus DNA and SPO1 DNA as markers in model E analytical ultracentrifuge. The ultraviolet absorption photograph was scanned with a Joyce Loebel microdensitometer.*

Correlations between DNA synthesis, isolation of virus from leukocytes, and tumor from inoculated birds. Table 1 shows a comparison of leukocytes from the blood of uninoculated, MDV-inoculated, and HVT-inoculated birds. The index of DNA synthesis is defined as the ratio in specific activity (counts per minute per microgram of DNA per hour) of DNA in inoculated birds to that of uninoculated birds. As shown in Table 1, leukocytes from HVT-inoculated birds showed an index of 1, similar to those of uninoculated birds. Of the 13 MDV-inoculated birds, three showed an index of 1. Upon necropsy, these birds revealed no gross lesions of any kind. The leukocytes from the remaining 10 birds exhibited an index ranging from 2.6 to 15. These birds developed tumors in gonad (ovary or testis), kidney, liver, or spleen. Infected leukocytes from HVT- and MDV-inoculated birds, including the three with DNA indexes of 1, gave characteristic plaques in

TABLE 1. *Correlation between DNA induction and gross tumor*

Bird no.	Virus inoculum	Dosage (plaque-forming units per chicken)	Post-inoculation time (weeks)	Index of DNA induction[a]	Gross lesions	Virus isolation
H 71	None		3	1.0	–	–
H 73	None		3	1.0	–	–
H 74	None		3	1.0	–	–
H 76	None		4	1.0	–	–
H 77	None		4	1.0	–	–
H 82	GA-MDV[b]	10^5	5	1.0	–	NT[c]
H 83	GA-MDV	10^5	3	14.0	K, G, S[d]	+
H 85	GA-MDV	10^5	4	1.0	–	+
H 90	GA-MDV	10^5	5	12.0	K, G, L, S	+
H 93	GA-MDV	10^5	3	9.0	K, G, S	+
H 94	GA-MDV	10^5	5	2.6	G	NT
H 96	GA-MDV	10^5	5	6.0	G, S	NT
H 102	GA-MDV	10^5	3	15.0	K, G, S	+
H 105	GA-MDV	10^5	3	11.5	G, S	+
H 106	GA-MDV	10^5	3	15.0	K, G, S	+
H 107	GA-MDV	10^5	5	3.9	K, G, L, S	+
H 108	GA-MDV	10^5	5	1.0	–	NT
H 110	GA-MDV	10^5	5	5.5	K, G, L	+
H 120	HVT[e]	10^5	3	1.2	–	+
H 121	HVT	10^5	3	1.0	–	+
H 123	HVT	10^5	5	1.0	–	+
H 124	HVT	10^5	4	1.0	–	NT
H 126	HVT	10^5	4	0.9	–	+

[a] Index of DNA induction = specific activity of DNA from inoculated bird/specific activity of DNA from control bird.
[b] GA strain of Marek's disease virus.
[c] Not tested.
[d] G, gonad; K, kidney; L, liver, S, spleen.
[e] Herpesvirus of turkey (FC126).

secondary duck embryo fibroblasts after 10 to 14 days.

DISCUSSION

This paper reports the first direct evidence that MDV possesses the potential to induce DNA synthesis in the host cells. The MDV genome was shown to induce in vitro host DNA synthesis in leukocytes from birds infected with MDV. This induction, in turn, correlated closely with in vivo tumor formation. Parenthetically, leukocytes infected with HVT do not show induction of DNA synthesis or in vivo tumor formation even though the HVT genome could be detected in the infected cells.

The kinetics of ^{3}H-thymidine incorporation in oncogenic MDV-infected leukocytes reached a maximum at about 10 hr postincubation and declined thereafter. This decline could be due partially to the release of nucleases in the medium as a result of cell degeneration. As cell degeneration continues, the increase of nucleotide pool size in the medium might also affect the incorporation of precursor molecules.

The product of in vitro incorporation of ^{3}H-thymidine into leukocyte culture was identified as DNA both by the diphenylamine test and by deoxyribonuclease digestion (98.5% rendered acid soluble). Equilibrium centrifugation in CsCl showed the density of this DNA to be 1.698 g/cm^3 compared to the bouyant density of MDV-DNA which is 1.706 g/cm^3 (7). Although the labeled DNA was predominently cellular and no viral particles were present in these cells, it is clear that they were infected because virus was isolated from such cells on secondary duck embryo fibroblasts after 10 to 14 days. The significant point of the data presented here is the presence in the infected leukocytes of the oncogenic MDV genome.

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