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Persistence of Marek's Disease Virus in a Subpopulation of B Cells That Is Transformed by Avian Leukosis Virus, but Not in Normal Bursal B Cells

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Previous studies have described an augmentation of avian leukosis virus (ALV)-induced lymphoid leukosis in chickens that were coinfected with a serotype 2 Marek's disease virus (MDV) strain, SB-1. As a first step toward understanding the mechanism of this augmentation, we have analyzed the tropism of the MDV for the ALV-transformed B cell. After hatching, chickens were coinfected with ALV and a nonpathogenic strain of MDV, SB-1. Seventy primary and metastatic ALV-induced lymphomas that developed in chickens between 14 and 20 weeks of age were found, with only one exception, to carry SB-1 DNA. The MDV genome was maintained in cell lines derived from the tumors. However, MDV DNA could not be detected in nontransformed bursal B cells from chickens carrying ALV lymphomas. Moreover, during and after the lytic phase of MDV infection, SB-1 DNA was near or below the level of detection in bursal cells, suggesting that MDV most likely infects only a small subpopulation of bursal cells. By contrast, ALV-transformed B cells from MDV-free chickens could be persistently infected with MDV in vitro. These findings indicate that ALV lymphoma cells, unlike nontransformed bursal B cells, are susceptible to persistent MDV infection and can serve as a reservoir of MDV that can potentially influence the physiology of the transformed cell.

Lymphoid leukosis (LL) and Marek's disease are two distinct diseases of poultry that are induced by different classes of viruses. LL, a neoplasia of B lymphocytes, is caused by a retrovirus, the avian leukosis virus (ALV) (5, 6, 31). Although ALV persists in most commercial flocks of chickens, the incidence of LL is low and control of the disease is primarily achieved by eradication of infected animals from breeding flocks. Marek's disease is characterized by peripheral nerve demyelination and the formation of T-cell lymphomas (28). The causative agent of Marek's disease is a DNA virus of the herpesvirus group, the Marek's disease virus (MDV) (10, 34), that is ubiquitous in the avian population and is currently controlled in commercial chicken flocks by vaccination.

There has been increasing evidence that coinfection of chickens with both ALV and MDV alters the disease processes associated with the individual viruses. The incidence of Marek's disease was increased when chickens infected with pathogenic strains of MDV were also infected with ALV (19, 20, 23, 29). Conversely, a vaccine strain of MDV, which is nononcogenic, enhanced the development of LL in chickens experimentally infected with ALV (4). In this latter case, an increase in the number of tumors per chicken and overall incidence of LL in experimental flocks, as well as an increase in the frequency of metastatic tumors, was observed. Because there was no apparent impairment of immunity to ALV caused by the vaccine strain of MDV, MDV was presumed to directly influence the process of development and progression of the ALV lymphoma.

The development of LL is marked by well-defined stages (2, 15, 30) that involve the immature B cells of the bursa of Fabricius. At 4 to 8 weeks after neonatal ALV infection, the appearance of 5 to 20 enlarged bursal follicles is the first evidence of transformation. Each hyperplastic follicle is filled with transformed B cells that are clonal and contain an integration of the provirus in the c-*myc* locus (15). The proviral insertion in the c-*myc* locus alters the sizes of the DNA fragments produced by restriction enzyme digestion, resulting in fragments that are diagnostic for individual transformed follicles. After an interval of several weeks, only one or two of these hyperplastic follicles progress to become a lymphoma; the remainder of the follicles regress with age. In the final stage of the disease, which may take from 14 to 25 weeks from the time of infection, cells from the bursal lymphoma metastasize to distal organs, and ultimately, death of the host ensues. One of the apparently essential events in the process of neoplastic transformation is the deregulation of the c-*myc* gene by insertion of a strong viral promoter in the *myc* locus (22). However, the fact that only a few of the hyperplastic follicles which have a deregulated c-*myc* gene progress to become malignant tumors suggests that other factors in addition to activation of c-*myc* are required to achieve neoplastic transformation (15). It is therefore possible that MDV influences the development and progression of LL tumors at any of several stages of lymphomagenesis.

As a first step in elucidating the mechanism of MDV augmentation of LL, we have analyzed the ability of the virus to infect and persist in the ALV-transformed lymphomas and the nontransformed B-cell populations of the bursa of Fabricius that are the targets of ALV transformation.

MATERIALS AND METHODS

Chickens and virus infection. The chickens used in this study were either an F_1 cross between two inbred lines of chickens, line 7_1 and line $15I_5$, that were maintained in

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pathogen-free conditions, free of ALV or MDV, at the USDA Avian Disease and Oncology Laboratory, East Lansing, Mich., or a commercial line, SC (HY-line International, Dallas Center, Iowa). Fertile eggs were incubated to hatching, and chickens were reared in the Wistar Institute Animal Facility under conditions that meet the standards of the National Institutes of Health and of the U.S. Department of Agriculture. On the second day after hatching, chickens were injected intraperitoneally with 2,000 PFU of a vaccine strain of MDV, SB-1 (Intervet, Millsboro, Del.). On the same day, the chickens were injected intravenously with culture supernatant containing 10^5 infectious units of the ALV RAV-1, which was prepared as previously described (13).

Preparation of cells, cell lines, and isolation of DNA. Cells were isolated from LL tumors and normal tissue of the bursa of Fabricius as previously reported (14). The lymphoid cells were freed from the tissues in a loose-fitting glass tissue homogenizer or by gentle grinding between frosted glass microscope slides. The cells were washed several times with Hanks balanced salt solution containing 5% fetal calf serum and run through a column of glass wool to remove any connective tissue or dead cells. Special precautions were taken to isolate normal lymphocytes from bursas which contained lymphomas to ensure that the samples were not contaminated with lymphoma cells. Individual bursal placea were scraped with a scalpel in Hanks balanced salt solution to release bursal follicles, and the follicles were washed by repeated $1 \times g$ sedimentation in Hanks balanced salt solution to remove adherent lymphocytes, including possible transformed B cells. Individual follicles were selected with a dissecting microscope to identify follicles that were approximately 0.5 mm in diameter, a typical size for nontransformed follicles. The cells were expressed from about 50 intact nonhyperplastic follicles by gentle grinding between frosted microscope slides.

Cell lines were established from primary bursal and metastatic LL tumors of ALV-infected chickens with culture conditions as previously described (16).

Total infected-cell DNA. Total cellular DNA was isolated from cells by standard methods (1). Infected cells were washed twice in phosphate-buffered saline and then resuspended in 150 mM NaCl–10 mM Tris (pH 8.0)–5 mM EDTA–0.5% sodium dodecyl sulfate (SDS). Proteinase K was then added to a final concentration of 250 μg/ml, and the samples were incubated at 50°C overnight and phenol-chloroform extracted before DNA was precipitated with 1/10 volume of 2.5 M sodium acetate and 2.5 volumes of ethanol.

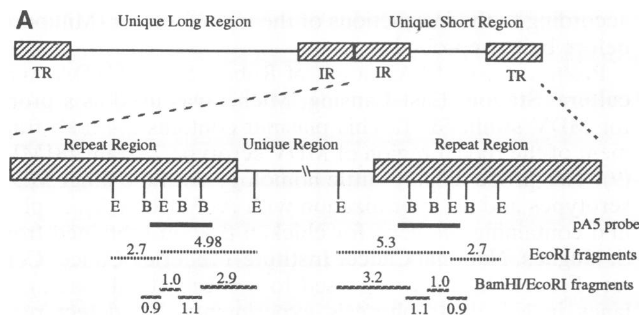
Gel electrophoresis and Southern blotting. For Southern blot analysis, 10 μg of tumor DNA was digested with restriction enzymes and separated on a 0.8% agarose gel. The samples were transferred to Immobilon-N (Millipore) and the membrane was treated according to the specifications of the manufacturer. The membrane was prehybridized for 3 to 4 h at 65°C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–0.5% SDS–5× Denhardt's solution–100 μg of salmon sperm DNA. The prehybridization solution was then removed, and hybridization solution (5× SSPE, 0.5% SDS, 5× Denhardt's solution, 100 μg of salmon sperm DNA) with ^{32}P-labeled probe was added. Hybridization was carried out at 65°C overnight. The following day, blots were washed three times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 5 min each at room temperature and twice in 0.1× SSC–0.1% SDS for 30 min at 50°C. The blots were then autoradiographed. The blots were washed to elute probes according to the instructions of the manufacturer (Millipore) before being reprobed.

Probes. Plasmid pA5, a gift of Robert Silva, USDA Agricultural Station, East Lansing, Mich., was used as a probe for MDV strain SB-1. This plasmid contains a 4.0-kb fragment of the repeat region of MDV serotype 2 strain 281MI/1 (9). This probe displays little homology with the other MDV serotypes and no hybridization with ALV (RAV-1). A plasmid containing the gene for chicken β-actin (obtained from S. Hughes, National Cancer Institute-Frederick Cancer Center, Frederick, Md.) was used to confirm equal loading of lanes in gel electrophoresis experiments. To detect rearrangements of the *c-myc* gene in LL tumors, Southern blots of *Eco*RI-digested DNA were hybridized with a probe (pBN22) encompassing exon III of *c-myc* (obtained from W. Hayward, Sloan-Kettering Institute, New York, N.Y.). Hybridization probes were radiolabeled by random priming with ^{32}P-labeled dCTP (17).

RESULTS

Primary and metastatic ALV-induced B-cell lymphomas contain MDV sequences. To assay for the presence of the MDV genome in LL tumors, a probe was obtained that was highly specific for MDV strain SB-1. The specificity of the pA5 probe for the MDV SB-1 sequences was demonstrated by its hybridization to DNA from SB-1-infected chicken embryo fibroblasts (CEF) but not to normal, uninfected CEF or ALV-infected CEF (Fig. 1B). Moreover, restriction fragments of 3.2, 2.9, 1.1, 1.0, and 0.9 kb generated following digestion with *Bam*HI and *Eco*RI are as predicted for the MDV serotype 2 (9). The 4-kb MDV sequence in the probe is derived from the viral short repeats. Therefore, the 0.9-, 1.0-, and 1.1-kb fragments, which are derived entirely from the repeat region, occur at twice the molar amount of the 2.9- and 3.2-kb fragments, which include repeat and unique sequences (Fig. 1A).

To determine the relationship between MDV and the process of ALV-induced lymphoma development in chickens coinfected with both viruses, we first analyzed bursal and metastatic B-cell lymphomas for the presence of the MDV genome. Chickens were infected with RAV-1, a subgroup A strain of ALV, and SB-1, a serotype 2 vaccine strain of MDV, on the second day after hatching. Bursal and metastatic lymphomas which developed between 14 and 22 weeks of age were isolated for analysis. The tumor cells were analyzed for antigenic markers characteristic of the LL tumors by immunofluorescence and fluorescence-activated cell sorter analysis. All tumors analyzed in this study expressed cell surface immunoglobulin M and morphologically resembled immature bursal B cells, typical of LL lymphoma cells (11; data not shown). To verify further that the lymphomas were typical of ALV lymphomas, DNA extracted from the tumor cells was analyzed for rearrangement of the *c-myc* gene, which results from insertion of the ALV provirus in the *c-myc* locus. Southern blot and hybridization analyses of tumor sample DNA with a probe specific for exon III of *c-myc* detected bands between 2.5 and 4.0 kb, which are distinct from the 6.5-kb germ line *c-myc* restriction fragment (see Fig. 2). The ALV provirus was found integrated upstream from the *c-myc* locus in each of the lymphomas examined, as evidenced by hybridization of a probe for the U5 region of the ALV long terminal repeat to the rearranged *c-myc* fragment (data not shown). These results confirm that all of the tumors analyzed in this study were typical of the ALV-induced B-cell lymphomas (15). There



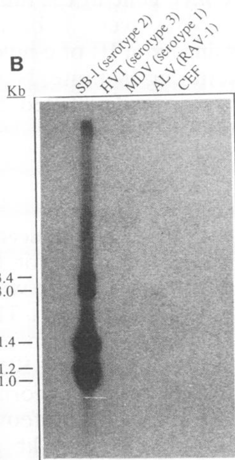


FIG. 1. Relationship of the pA5 probe to the SB-1 genome and its hybridization to chickens cells. (A) Schematic representation of the *Bam*HI (B) and *Eco*RI (E) restriction enzyme sites and fragments of the SB-1 genome which hybridize with the pA5 probe. IR, internal repeat region; TR, terminal short repeat region. (B) Specificity of the pA5 probe is indicated by hybridization to DNA from MDV SB-1-infected CEF but not to uninfected CEF or to CEF infected with serotype 1 or serotype 3 MDV or ALV RAV-1-infected CEF. Total cellular DNA was digested with *Bam*HI and *Eco*RI restriction enzymes. HVT, herpesvirus of turkeys.

was no evidence of T-cell lymphomas or neurological symptoms characteristic of Marek's disease in any of the chickens.

Both primary bursal lymphomas and metastatic lymphomas in chickens coinfected with ALV RAV-1 and MDV SB-1 were found to contain MDV DNA that gave a restriction pattern with the pA5 probe similar to that of the SB-1-infected CEF (Fig. 2). The specificity of the pA5 probe was demonstrated by its failure to hybridize with DNA from ALV lymphomas of non-SB-1-infected chickens (Fig. 2 [chickens 5 and 6]). Overall, more than 70 tumors that were obtained from birds receiving both viruses were analyzed, and of the primary bursal lymphomas examined, 100% were positive for MDV. With one exception, all of the metastatic tumors also contained MDV DNA. These results indicate that the SB-1 virus infects and persists in the ALV-transformed bursal cells when the host chickens are coinfected with both viruses at 1 day of age.

MDV is not present in nontransformed bursal lymphocytes of lymphoma-bearing chickens. The previous results raised the question of whether maintenance of a persistent SB-1 infection was restricted to the ALV-transformed bursal cells or if it was a general property of all bursal B cells, both transformed and nontransformed. To address this question, DNAs were isolated from normal, nontransformed B cells and transformed B cells from the bursas of the same chickens coinfected with ALV RAV-1 and MDV SB-1 and analyzed for the presence of MDV DNA. Special precautions were taken to ensure that the sample of normal bursal B cells was not contaminated with transformed cells that may have emigrated from the lymphoma. The intact bursal follicles were teased from the bursa and washed thoroughly to remove attached cells, and follicles of normal size were selected for isolation of total DNA. The analysis of four representative chickens is shown in Fig. 3. To verify that the normal bursal cells were free of transformed cells, each sample of DNA was analyzed by Southern transfer and hybridization with the exon III c-*myc* probe. As shown in Fig. 3, the exon III probe hybridizes to the cellular *myc*

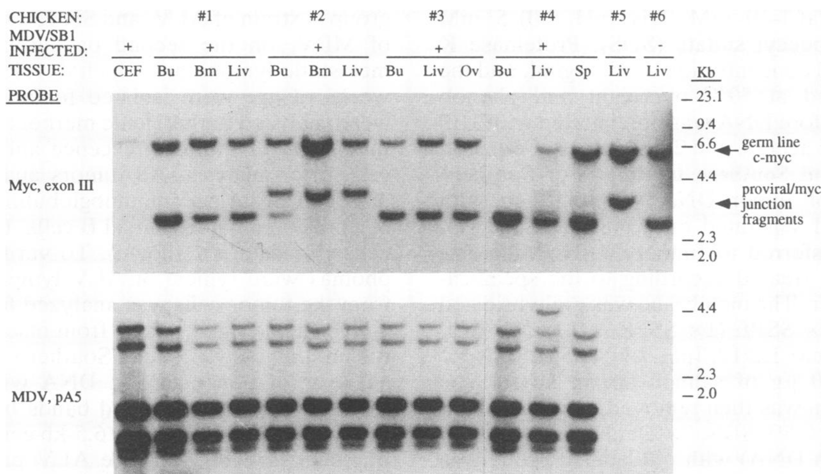


FIG. 2. MDV SB-1 DNA is detected in both primary bursal lymphomas and metastatic lymphomas of ALV RAV-1-infected chickens. Chickens no. 1 to 4 were coinfected at hatching with SB-1 and RAV-1; chickens no. 5 and 6 were infected only with RAV-1. Total cellular DNAs isolated from bursal lymphomas (Bu), metastatic lymphomas, liver (Liv), spleen (Sp), ovary (Ov), and bone marrow (Bm) were digested with *Bam*HI and *Eco*RI restriction enzymes. The samples of DNA were hybridized with the exon III probe of c-*myc* to identify the c-*myc*-proviral junction fragment which is present in the lymphomas but not nontransformed cells, which contain only the 6.5-kb germ line c-*myc* fragment. The exon III probe was eluted and the filter was rehybridized with the pA5 probe to detect the presence of the SB-1 genome.

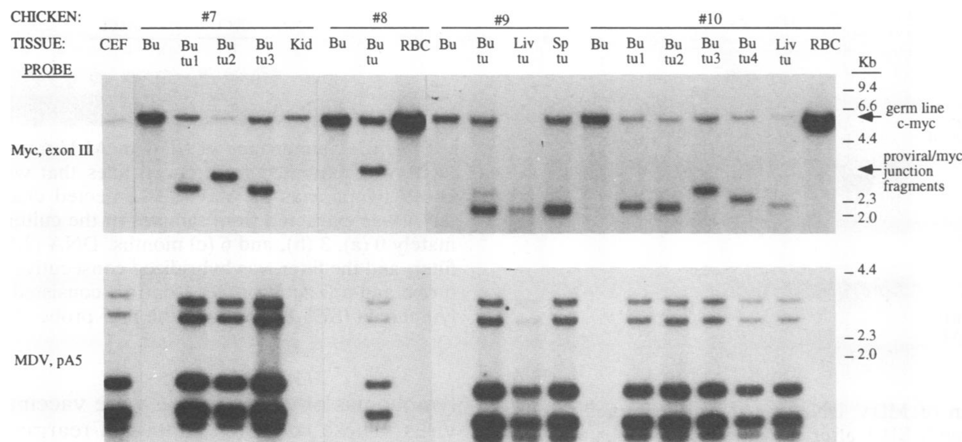


FIG. 3. Detection of MDV DNA in ALV RAV-1 lymphomas but not normal bursal lymphocytes of chickens coinfected with SB-1 and RAV-1. DNA was isolated and digested, and the filter was probed for the *c-myc*-proviral junction fragment and for SB-1 MDV probed as indicated in the legend to Fig. 2. Bu, nontransformed bursal lymphocytes; Bu tu, bursal lymphoma; Kid, nontransformed kidney cells; RBC, erythrocytes; Liv, liver; Sp, spleen.

gene, which is rearranged in the transformed (but not in the untransformed) bursal cells. Therefore, the presence of a rearranged *c-myc* gene marks the transformed cells. The same filters were washed and rehybridized with the pA5 probe to determine whether the MDV sequences were present in the cells. As shown in Fig. 3, the pA5 probe hybridized only to samples containing tumor DNA, as demonstrated by the presence of the rearranged *c-myc* gene. MDV sequences were not detected in samples containing only normal bursal cells, kidney cells, or erythrocytes from the same tumor-bearing chicken. The absence of a 2- to 4-kb *c-myc* fragment was indicative of the lack of a detectable number of tumor cells in these samples. Therefore, in contrast to the transformed B cells which carry MDV, the MDV was not present in the nontransformed bursal cells at the time at which the lymphomas were obtained.

Occurrence of MDV in bursal lymphocytes following SB-1 infection. Since ALV-transformed cells are derived from the bursal B-cell population, the question of when the cells that give rise to the LL tumor can be infected with MDV remains. Our objective was to analyze the susceptibility of bursal B cells to SB-1 infection both prior to and after transformation by ALV.

The appearance and duration of MDV DNA in the bursal lymphocyte population were monitored after neonatal infection with the SB-1 virus. Samples of bursal cells were taken every other day from 5 to 9 days after infection, a period which encompassed the duration of the initial cytolytic infection with MDV SB-1 (8, 33), and at 9 weeks of age. At each time, bursal B lymphocytes were purified, free of stromal cells, before analysis. In initial experiments, dot blots of 2.5×10^6 to 3×10^6 cells were made on nitrocellulose and the total cellular DNA blots were hybridized with the radiolabeled pA5 MDV probe. No specific hybridization was observed at any of the sampling times (data not shown). In two subsequent experiments, total cellular DNA was purified from bursal cells and 10 μg was analyzed by agar gel electrophoresis, Southern blotting, and hybridization with the pA5 probe. Most samples of bursal lymphocytes for each of the ages gave no specific hybridization. However, two of four chickens at 9 days postinfection showed weak but detectable bands (Fig. 4A [no. 3 and 4]). The sensitivity of this assay was analyzed by determining the highest dilution of an MDV^+ LL cell line in normal, uninfected bursal cells that could be detected by hybridization of Southern blot of total cell DNA with the pA5 probe. It was found that SB-1 DNA could be detected in samples containing as little as 1 MDV^+ cell, harboring 70 copies of MDV, when diluted in 10^4 uninfected bursal cells (Fig. 4B), suggesting that the number of MDV^+ bursal cells must be below that level of detection and therefore may represent a small subpopulation of bursal cells.

Susceptibility of ALV-transformed B cells to MDV infection. Although it is clear that ALV-transformed B cells are harboring MDV, it was not known when, during lymphomagenesis, the B cells are susceptible to MDV infection. To determine whether the B cells could be infected by MDV after they had reached the preneoplastic stage of lymphoma development, chickens infected with ALV at hatching were kept in isolators free of MDV. At 9 weeks of age, these ALV-infected chickens were inoculated intraperitoneally

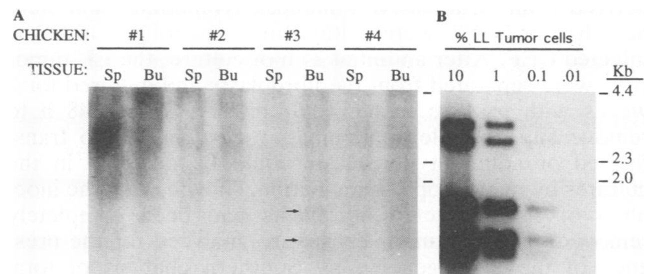


FIG. 4. (A) Detection of SB-1 DNA in samples of bursal lymphocytes from non-ALV-infected chickens. Chickens were infected at 1 day posthatching with the SB-1 strain of MDV. At 9 days after infection, total cellular DNA was obtained from samples of splenic (Sp) and bursal (Bu) lymphocytes and 10 μg digested by *Bam*HI-*Eco*RI restriction enzymes and Southern blots was probed with the pA5 SB-1 probe. Faint hybridization bands (indicated by arrows) were detected in two of four chickens after exposure of the autoradiograph for 3 days. (B) Sensitivity of assay to detect SB-1-infected lymphocytes. SB-1-infected transformed B cells from a cell line developed from an ALV-induced lymphoma were diluted with normal bursal cells from an SB-1-negative chicken. DNA was analyzed and the autoradiograph was exposed as for panel A.

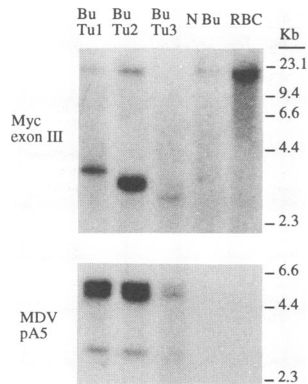


FIG. 5. Detection of MDV DNA in ALV-transformed B cells that were infected with SB-1 after transformation. DNA was isolated from bursal lymphomas (Bu tu 1 to 3), nontransformed bursal lymphocytes (N Bu), and erythrocytes (RBC) from chickens infected with ALV at hatching and with SB-1 at 9 weeks of age. DNA was digested with *Eco*RI, and Southern blots were hybridized with the exon III c-*myc* probe and then with the pA5 SB-1 probe.

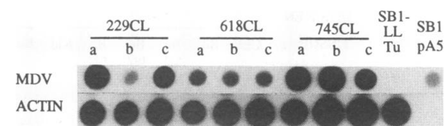


FIG. 6. Maintenance of SB-1 in ALV-transformed B-cell lines. DNA was extracted from B-cell lines that were established from B-cell lymphomas of ALV SB-1-infected chickens. Total cellular DNA was extracted from samples of the cultures taken at approximately 0 (a), 3 (b), and 6 (c) months. DNA (2.5 μg) was bound to a filter, and the filter was hybridized consecutively with the pA5 SB-1 probe and a β-actin probe. Controls consisted of an SB-1-negative lymphoma (SB1-LL Tu) and the pA5 probe (5 ng).

with SB-1 virus. At this age, ALV-transformed follicles are already present in the bursa. The chickens were sacrificed when metastatic lymphomas were evident, at between 14 and 20 weeks of age, and the lymphoma DNA was analyzed for characteristic provirus–c-*myc* junction fragments and for the presence of MDV DNA by Southern analysis. MDV infection was established in two of five chickens, as evidenced by the presence of SB-1 DNA in tissues. In both of the MDV^+ chickens, MDV DNA was detected in all of the bursal lymphomas but not in normal bursal B lymphocytes (Fig. 5). Therefore, postponing the timing of the MDV infection did not alter the tropism of the herpesvirus for the transformed cells. These results indicate that MDV can enter the ALV-transformed cells at or after the preneoplastic stage of transformation.

We also determined whether the SB-1 virus could infect and persist in neoplastically transformed B cells that were obtained from MDV-free chickens. Two ALV-transformed B-cell tumor lines, RP9 (27) and DT40 (3), which had been derived from terminal ALV-induced lymphomas and were negative for MDV, were cultured on a monolayer of SB-1-infected CEF. After an initial 24 h of culture, the LL tumor cells were separated from the fibroblasts and cultured for 2 weeks with passage to new culture flasks every 48 h to remove any possible contaminating fibroblasts. No transformed or adherent fibroblasts could be detected in the cultures by microscopic examination, showing that the inoculum of MDV-infected fibroblasts had been completely removed. The LL tumor cells were analyzed for the presence of the SB-1 genome by Southern analysis of total cellular DNA. Both cell lines were found to contain the MDV (data not shown), indicating that the ALV-transformed cells could be infected and that the virus persists in the tumor cell population without killing the cells. These results indicate that the ALV-transformed B cells are susceptible to MDV but does not rule out the possibility that infection of a small subpopulation of cells in the bursa occurs prior to transformation.

MDV persists in permanent cell lines derived from the ALV lymphomas. To extend our analysis of the relationship of MDV with the transformed B cell, permanent cell lines were established from both primary and metastatic ALV-induced lymphomas of chickens that were vaccinated with the SB-1 virus. These cell lines contained rearranged c-*myc* restriction fragments identical to that of the primary tumor from which they were derived and maintained the immature B-cell phenotype (data not shown). Several of these cultures were analyzed to determine whether the MDV genome was maintained in the cultured cells over an extended period of in vitro culture. As shown in Fig. 6, MDV DNA could be detected in cell lines that were derived from LL tumors of MDV-vaccinated chickens and maintained in culture for at least 9 months. Samples taken during these intervals indicate that although the relative number of MDV copies per milligram of DNA varied during culture, no consistent trend, i.e., loss or gain of copies of the MDV genome, was observed. These results show that MDV persists in the immortalized B cells with no apparent cytotoxic effect and suggest that the cell lines may be valuable tools for analyzing the physical and biological state of MDV in the transformed B cell.

DISCUSSION

Our aim is to understand the mechanism of MDV augmentation of ALV-induced B-cell neoplasia. As a first step toward that goal, we have analyzed the tropism of MDV for bursal B lymphocytes at different stages of lymphoma development. The SB-1 strain of MDV was found to be present in all but 1 of the 70 bursal and metastatic lymphomas examined. However, surprisingly, SB-1 DNA was not detected in nontransformed bursal lymphocytes of the chickens that had MDV^+ bursal tumors. These results indicate that the ability of MDV to infect and persist in the bursal B-cell population was restricted to the transformed B cell. This work extends that of Bacon et al. (4) showing enhancement of ALV-induced LL in the same $15I_5 \times 7_1$ line of chickens vaccinated with the SB-1 strain of MDV used in the present study. We have found no difference in the tropism of the SB-1 virus for the transformed B cells between the line $15I_5 \times 7_1$ or line SC chickens used in the present study. However, no attempt was made to determine whether the same degree of tumor augmentation occurs in line SC chickens as was reported for $15I_5 \times 7_1$ chickens. Our findings provide support for the hypothesis that MDV enhancement of ALV-induced B cell tumorigenesis may involve direct interaction of the MDV and the developing lymphoma. These findings raise questions concerning the basis of the selective persistence of MDV in the transformed B cells and the implications that this association has on the disease process associated with both viruses.

In SB1-infected chickens, most bursal cells are apparently not infected by MDV; if infected, they either quickly leave the bursal compartment or are killed by the virus or by

immune mechanisms, with the result that the viral DNA is not detectable in the general bursal B-cell population. Although the methods of total cell DNA dot blot analysis and Southern analysis are not sensitive enough to detect low levels of MDV infection, the results are consistent with previous studies of a low level of virus isolation following MDV infection during a short period (2 to 7 days) after neonatal infection (7, 8, 33). In situ analysis of MDV antigen (24) and viral particles (20) also indicates that very few cells in the bursal follicles are productively infected. Therefore, these findings suggest that the ability of MDV to persistently infect bursal cells is likely to be limited to a subpopulation of bursal B cells that could also be the target of ALV transformation.

It is notable that the target cells of ALV transformation appear to represent a small subpopulation of cells, the bursal stem cells, that are present in the bursa during late embryonic development until a few weeks after hatching (25, 26, 37). Therefore, selective infection of this minor cell population by MDV may go undetected by methods used in this study but would ensure that a high proportion of LL tumors would carry the MDV DNA. These bursal stem cells are normally not present in chickens after several weeks posthatching (37). Alternatively, ALV-induced transformation, which has been shown to block differentiation of the B cell (11), may arrest the bursal cells in a state in which they remain susceptible to MDV infection or able to resist cytolysis. This hypothesis is consistent with our observation that the ALV-transformed cells are susceptible to persistent MDV infection, unlike nontransformed bursal lymphocytes.

Furthermore, we cannot rule out the possibility that changes in the cell physiology of the bursal cell associated with ALV transformation and activation of c-*myc* render the cells susceptible to MDV infection or resistant to the cytolytic effects of MDV. It is clear from the present study that ALV infection alone, independently of cell transformation, does not render bursal B cells susceptible to persistent MDV infection, since most bursal B cells, although infected with the ALV, do not contain MDV DNA.

The findings of the present study may provide an explanation for the previous observations that the incidence of Marek's disease is increased by coinfection with ALV and MDV (18, 23). Although there is evidence that B cells in peripheral lymphoid tissues are the initial target cells of MDV during the early cytolytic phase of infection, virus-positive cells disappear within several days after infection (33). However, our experiments show that in contrast to normal B cells, MDV persists in the ALV-transformed B cells, which proliferate and metastasize within the host. Furthermore, we have recently demonstrated that the SB-1 virus, which is latent in the LL tumor cells, can be activated, resulting in a dramatic amplification of the MDV copy number and the elaboration of productive virus (21). Therefore, if pathogenic strains of MDV behave as the vaccine strains, we anticipate that the ALV-transformed cells can provide a reservoir of MDV, which is not present in ALV-free chickens, that enhances the risk of Marek's disease in chickens coinfected with the two viruses.

The consistent finding of MDV genomes in the ALV-transformed B cells supports a model of intracellular cooperation between these two viruses that may result in augmentation of lymphoma development. It is possible that portions of the SB-1 genome transactivate the retrovirus long terminal repeat in transformed bursal cells in a manner similar to that shown by Tieber et al. in CEF (36). Such a mechanism of transactivation may either enhance the ALV titer or could enhance transcription of the c-*myc* gene or cellular genes that are under the control of the proviral promoter in the hyperplastic cells and lymphomas. However, it remains to be seen whether MDV can transactivate the retroviral genes in B cells.

A scenario similar to that between MDV and the development of LL tumors is observed in the relationship between herpesviruses and the development of B-cell lymphomas in humans. Epstein-Barr virus has been found in a majority of Burkitt's lymphomas (38), a B-cell neoplasia of humans. In Burkitt's lymphoma, as in LL, the phenotype of the target cell of transformation is believed to represent a subpopulation of B cells (12). Similarly, another human herpesvirus, HHV-6, is found in a majority of the non-Hodgkin's B-cell lymphomas from AIDS patients (32). Moreover, in both human neoplasms, as in ALV-induced LL, the c-*myc* gene is deregulated in the lymphoma; this happens in Burkitt's and non-Hodgkin's lymphomas by translocation of the immunoglobulin gene to the chromosome carrying c-*myc* (32, 35) and in LL by proviral insertion in the c-*myc* locus (16). As is the case in LL, the herpesviruses in both of these human B-cell neoplasias appear to act as cofactors, since lymphoma development is not strictly dependent on the presence of the virus. These similarities between the human and avian systems suggest the possibility that similar mechanisms of tumor promotion may be operative. The avian system described here may therefore provide a useful model for the analysis of the mechanism of herpesvirus enhancement of B-cell lymphomagenesis and of herpesvirus-retrovirus interactions.

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REFERENCES

1. **Ausbel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1987. Current protocols in molecular biology. John Wiley and Sons, New York.
2. **Baba, T., and E. H. Humphries.** 1985. Formation of a transformed follicle is necessary but not sufficient for development of an avian leukosis virus-induced lymphoma. Proc. Natl. Acad. Sci. USA **82:**213–216.
3. **Baba, T. W., B. P. Giroir, and E. H. Humphries.** 1985. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology **144:**139–151.
4. **Bacon, L. D., R. L. Witter, and A. M. Fadly.** 1989. Augmentation of retrovirus-induced lymphoid leukosis by Marek's disease herpesviruses in white leghorn chickens. J. Virol. **63:**504–512.
5. **Burmester, B. R.** 1947. Studies on the transmission of avian visceral lymphomatosis. II. Propagation of lymphomatosis with cellular and cell-free preparations. Cancer Res. **7:**786–797.
6. **Burmester, B. R., A. K. Fontes, and W. G. Walter.** 1960. Pathogenicity of a viral strain (RPL 12) causing avian visceral lymphomatosis and related neoplasms. III. Influence of host age and route of inoculation. J. Natl. Cancer Inst. **24:**1423–1442.
7. **Calnek, B. W., J. C. Carlisle, J. Fabricant, K. K. Murthy, and K. A. Schat.** 1979. Comparative pathogenesis studies with oncogenic and nononcogenic Marek's disease viruses and turkey herpesvirus. Am. J. Vet. Res. **40:**541–548.
8. **Calnek, B. W., K. A. Schat, L. J. N. Ross, W. R. Shek, and C.-L. H. Chen.** 1984. Further characterization of Marek's disease virus-infected lymphocytes. I. In vivo infection. Int. J. Cancer **33:**389–398.
9. **Carter, J. K., and R. F. Silva.** 1990. Cell culture amplification of

a defective Marek's disease virus. Virus Genes **4**:225–237.

10. **Churchill, A. E., and P. M. Biggs.** 1967. Agent of Marek's disease in tissue culture. Nature (London) **215**:528–530.
11. **Cooper, M. D., H. G. Purchase, D. E. Bookman, and W. E. Gathings.** 1974. Studies on the nature of the abnormality of B cell differentiation in avian lymphoid leukosis: production of heterogeneous IgM by tumor cells. J. Immunol. **113**:1210–1222.
12. **Dalla-Favera, R., M. Bregni, J. Erikson, D. Patterson, R. C. Gallo, and C. M. Croce.** 1982. Human c-*myc* onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc. Natl. Acad. Sci. USA **79**:7824–7827.
13. **Ewert, D. L., N. Avdalovic, and C. Goldstein.** 1989. Follicular exclusion of retroviruses in the bursa of Fabricius. Virology **170**:433–441.
14. **Ewert, D. L., and M. D. Cooper.** 1978. Ia-like alloantigens in the chicken: serologic characterization and ontogeny of cellular expression. Immunogenetics **7**:521–538.
15. **Ewert, D. L., and G. F. DeBoer.** 1988. Avian lymphoid leukosis: mechanisms of lymphomagenesis, p. 37–53. *In* K. Perk (ed.), Immunodeficiency disorders and retroviruses. Academic Press, Inc., Boston.
16. **Ewert, D. L., and W. T. Weber.** 1987. Effect of phorbol myristate acetate on in vitro growth of lymphocytes from the chicken thymus and bursa of Fabricius, p. 67–78. *In* W. T. Weber and D. L. Ewert (ed.), Avian immunology. Alan R. Liss, Inc., New York.
17. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
18. **Frankel, J. W., W. F. Farrow, C. O. Prickett, M. E. Smith, W. F. Campbell, and V. Groupe.** 1974. Responses of isolator-derived and conventional chickens to Marek's disease herpesvirus and avian leukosis virus. J. Natl. Cancer Inst. **52**:1491–1497.
19. **Frankel, J. W., and V. Groupe.** 1971. Interactions between Marek's disease herpesvirus and avian leucosis virus in tissue culture. Nature (London) New Biol. **234**:125–126.
20. **Frazier, J. A.** 1974. Ultrastructure of lymphoid tissue from chicks infected with Marek's disease virus. J. Natl. Cancer Inst. **52**:829–837.
21. **Fynan, E., D. L. Ewert, and T. M. Block.** Latency and reactivation of a Marek's disease virus in B lymphocytes transformed by avian leukosis virus. Submitted for publication.
22. **Hayward, W. S., B. G. Neel, and S. M. Astrin.** 1981. Activation of a cellular oncogene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
23. **Jakovleva, L. S., and N. P. Mazurenko.** 1979. Increased susceptibility of leukemia-infected chickens to Marek's disease. Neoplasma **26**:393–396.
24. **Jeurissen, S. H. M., R. Scholten, L. A. T. Hilgers, J. M. A. Pol, and G. F. De Boer.** 1989. In situ detection by monoclonal antibody D-35.1 of cells infected with Marek's disease virus that interact with splenic ellipsoid-associated reticulum cells. Avian Dis. **33**:657–663.
25. **Neiman, P., C. Wolf, P. J. Enrietto, and G. M. Cooper.** 1985. A retroviral *myc* gene induces preneoplastic transformation of lymphocytes in a bursa transplatation assay. Proc. Natl. Acad. Sci. USA **82**:222–226.
26. **Neiman, P. E., E. B. Gehly, L. M. Carlson, R. C. Cotter, and C. B. Thompson.** 1988. Bursal stem cells as targest for *myc*-induced preneoplastic proliferation and maturation arrest. Curr. Top. Microbiol. Immunol. **141**:67–74.
27. **Okazaki, W., R. L. Witter, C. Romero, K. Nazerion, J. M. N. Sharma, A. Fadly, and D. Ewert.** 1980. Induction of lymphoid leukosis transplantable tumors and the establishment of lymphoblastoid cell lines. Avian Pathol. **9**:311–329.
28. **Payne, L. N.** 1982. Biology of Marek's disease virus and the herpesvirus of turkeys, p. 347–431. *In* B. Roizman (ed.) The herpesviruses, vol 1. Plenum Publishing Corp., New York.
29. **Peters, W. P., D. Kufe, J. Schlom, J. W. Frankel, C. O. Prickett, V. Groupe, and S. Spiegelman.** 1973. Biological and biochemical evidence for an interaction between Marek's disease herpesvirus and avian leukosis virus in vivo. Proc. Natl. Acad. Sci. USA **70**:3175–3178.
30. **Purchase, H. G.** 1986. The pathogenesis and pathology of neoplasms caused by avian leukosis viruses, p. 171–196. *In* G. F. DeBoer (ed.), Avian leukosis. Martinus Nijhoff Publishing, Boston.
31. **Purchase, H. G., and L. N. Payne.** 1984. Leukosis-sarcoma group, p. 360–405. *In* M. F. Hofstad, H. J. Barns, B. W. Calnek, W. M. Reid, and H. W. Yoder, Jr. (ed.), Diseases of poultry, 8th ed. Iowa State University Press, Ames.
32. **Salahuddin, S. Z., D. V. Ablashi, P. D. Markham, S. F. Josephs, S. Sturzenegger, M. Kaplan, G. Halligan, P. Biberfeld, F. Wong-Staal, B. Kramarsky, and R. Gallo.** 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science **234**:596–601.
33. **Shek, W. R., B. W. Calnek, K. A. Schat, and C.-L. H. Chen.** 1983. Characterization of Marek's disease virus-infected lymphocytes: discrimination between cytolytically and latently infected cells. JNCI **70**:485–491.
34. **Solomon, J. J., R. L. Witter, K. Nazerian, and B. R. Burmester.** 1968. Studies on the etiology of Marek's disease. I. Propagation of the agent in cell culture. Proc. Soc. Exp. Biol. Med. **127**:173–177.
35. **Taub, R., I. Kirsch, C. Morton, G. Lenoir, D. Swan, S. Tronick, S. Aaronson, and P. Leder.** 1982. Translocation of the c-*myc* gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. USA **79**:7837–7841.
36. **Tieber, V. L., L. L. Zalinski, R. Silva, A. Finkelstein, and P. M. Coussens.** 1990. Transactivation of the Rous sarcoma virus long terminal repeat promoter by Marek's disease virus. Virology **179**:719–727.
37. **Toivanen, P., and A. Toivanen.** 1973. Bursal and post-bursal stem cells in chickens: functional characteristics. Eur. J. Immunol. **3**:585–595.
38. **zur Hausen, H., H. Schulte-Holthausen, G. Klein, W. Henle, G. Henle, P. Clifford, and L. Santesson.** 1970. EBV DNA in biopsies of Burkitt tumours and anaplastic carcinomas of the nasopharynx. Nature (London) **228**:1056–1058.