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Latency and reactivation of Marek's disease virus in B lymphocytes transformed by avian leukosis virus

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The physical and biological state of the Marek's disease virus (MDV) genome in avian leukosis virus (ALV)-transformed cells is characterized using cell lines established from ALV tumours co-infected with the SB-1 strain of MDV. The MDV genome within the ALV-transformed cells was found to be methylated at 5′ CpG 3′ dinucleotides. Less than 2% of the tumour cells expressed MDV antigen and only one virus plaque that was characteristic of an MDV infection was noted when tumour cells were cocultured with fibroblasts permissive for a productive MDV infection. However, when methylation of the MDV genome was prevented by culturing the tumour cell lines in the presence of 5-azacytidine, both MDV antigen expression and viral replication increased. Based on these results, it appears that MDV resides within the ALV-transformed cells in a latent state and that MDV latency might be influenced, to some extent, by methylation of the MDV genome.

Introduction

Avian leukosis virus (ALV) and Marek's disease herpesvirus (MDV) are significant pathogens of poultry. It is known that infection with pathogenic strains of either of these viruses results in the formation of malignant lymphomas (Payne, 1982; Purchase, 1986). However, ALV and MDV malignantly transform different cell types of the lymphoid system. ALV transforms B cells, whereas MDV infection has been shown to cause the transformation of only T lymphocytes. However, it has been observed that chickens infected with ALV and a non-oncogenic strain of MDV (SB-1), used routinely as a vaccine (Schat & Calnek, 1978), experienced a higher incidence of lymphoid leukosis than those birds exposed to ALV alone (Bacon *et al.*, 1989). The mechanism of this enhancement is unknown. Our previous work showed that MDV was present in greater than 90% of the B cell tumours removed from birds receiving both viruses (Fynan *et al.*, 1992). These findings suggest that ALV and MDV may cooperate in the development of B cell tumours. Our previous studies did not, however, determine whether the entire infectious MDV genome was present in the ALV-transformed tumour cells. Moreover, the biological status of the MDV genome was not characterized.

In view of the effect of MDV infection upon enhancement of B lymphoid tumour development, we sought to examine the state of expression of the MDV genome in ALV-transformed cells. For convenience, cell lines were established from B cell lymphomas isolated from chickens infected with both ALV and MDV strain SB-1. In a separate report, we have shown that the cell lines resemble the primary tumours in that the ALV provirus is integrated at the same location in the *myc* proto-oncogene in the cell line and the tumour from which it was derived (Fynan *et al.*, 1992). In this report, we examine the expression of the MDV strain SB-1 using two of the ALV-transformed cell lines, and show, for the first time, that the MDV genome can persist in ALV-transformed B cells in a non-productive state that is capable of responding to exogenous stimuli by reactivating to produce infectious virus. The relevance of the presence of infectious MDV within an ALV tumour cell to MDV and ALV pathogenesis is also considered.

Methods

Plasmids. Plasmid pA5 was a gift from Dr Robert Silva, USDA Agricultural Station, East Lansing, Mich., U.S.A. This plasmid contains a 4·0 kb fragment of the repeat region of the MDV serotype 2 strain 281MI/1 (Carter & Silva, 1990). A plasmid (pBN22) containing exon III of the c-*myc* gene was obtained from W. Hayward, Sloan-Kettering Institute, New York, N.Y., U.S.A. A plasmid containing the gene for chicken β-actin (obtained from S. Hughes, NCI-Frederick

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Cancer Center, Frederick, Md., U.S.A.) was used to confirm equal loading of DNA samples.

Virus strains. MDV strain SB-1 was obtained from TriBio Laboratories, State College, Pa., U.S.A., and Intervet. ALV strain RAV-1 was used in these experiments.

Cell lines. Established ALV-transformed cell lines 229 and 740 were previously described (Fynan *et al.*, 1992).

Chick embryo fibroblasts. Primary and secondary chick embryo fibroblasts were prepared from 8- to 10-day-old embryonated eggs (Spafas) as described (Hsuing, 1982).

Preparation of radioactive DNA probes. DNA probes were labelled with [^{32}P]dCTP by the nick translation or random priming method according to the manufacturer's instructions (Gibco-Bethesda Research Laboratories).

Isolation and separation of total cellular DNA. DNA was isolated by standard methods (Ausubel *et al.*, 1987). Briefly, cells were washed twice in PBS and then resuspended in 100 mM-NaCl, 10 mM-Tris–HCl pH 8·0, 25 mM-EDTA, 0·5% SDS. Proteinase K was then added to a final concentration of 250 μg/ml. Samples were incubated at 50 °C overnight, phenol–chloroform-extracted and precipitated with 0·1 volume 2·5 M-sodium acetate and 2·5 volumes of ethanol.

Tumour DNA (5 μg) was digested with restriction enzymes and separated on an 0·8% agarose gel. Samples were transferred to nitrocellulose (Schleicher & Schuell) and hybridized with ^{32}P-labelled DNA probes as described (Sambrook *et al.*, 1989). Radioactive probe was removed from the membranes according to the manufacturer's instructions. Membranes were placed in a boiling solution of 0·1 × SSC [(20 × SSC is 3 M-NaCl, 0·3 M-sodium citrate, pH 7·0)/0·1% SDS] and rocked until cool. This procedure was repeated until no radioactive counts were detected.

Dot blot analysis. Cells (5×10^3 to 1×10^6 cells/well) were applied to nylon membranes (Schleicher & Schuell) and dried. Cells were lysed, denatured and neutralized *in situ* by wetting the membrane in 10% SDS for 3 min, denaturation solution (0·5 M-NaOH, 1·5 M-NaCl) for 5 min, neutralization solution (1·5 M-NaCl, 0·5 M-Tris–HCl pH 8·0) for 5 min, 2 × SSPE (20 × is 3 M-NaCl, 0·2 M-sodium phosphate, 0·02 M-EDTA, pH 7·4) for 5 min and dried. Prehybridization and hybridization were performed as described (Sambrook *et al.*, 1989). The radioactive DNA probe was removed from the membrane by the method described by the manufacturer. Nytran membranes were washed in 50% deioinized formamide/6 × SSPE at 68 °C for 30 min. Washes were repeated until blots were free of radioactive counts.

Fluorescent antibody staining. Cell-associated MDV antigen was detected by indirect immunofluorescence analysis. Fibroblasts grown on coverslips or cytocentrifuge preparations of lymphoid cell lines were fixed in 2% acetic acid in ethanol at −10 °C for 20 min. After rehydration, the cells were incubated with mouse monoclonal antibody Y5 which is specific for serotype II MDV antigens (Lee *et al.*, 1983). Cell-bound antibody was detected with fluorescein isothiocyanate-labelled goat anti-mouse immunoglobulin antibody using a Leitz epifluorescence microscope.

Results

Marek's disease virus DNA is present in ALV tumour-derived cell lines

We have previously shown that most ALV-transformed tumours isolated from MDV (strain SB-1)-vaccinated chickens contain MDV DNA (Fynan *et al.*, 1992). To analyse the persistence of the MDV genome in ALV-transformed cells, cell lines were established from a number of tumours. We have previously shown that ALV-transformed B cell lines contain MDV DNA sequences using hybridization with an MDV serotype 2-specific probe (Fynan *et al.*, 1992). The DNA fragment used as a probe in these experiments was derived from the short repeat region of the MDV genome (Fig. 1*a*).

The presence of MDV DNA in two ALV-transformed cell lines, 229 and 740, is shown in Fig. 2(*a*). Hybridization of the MDV strain SB-1-specific probe, pA5, to DNA digested with *Eco*RI and *Bam*HI isolated from cells productively infected with MDV strain SB-1 results in the detection of discrete bands of 3·2, 2·9, 1·1, 1·0 and 0·9 kb. The 1·1, 1·0 and 0·9 kb bands occur at twice the molar amount of the 3·2 and 2·9 kb fragments because they are derived from the internal repeats and occur at least twice per genome (see Fig. 1*a*). Hybridization to pA5 of *Bam*HI- and *Eco*RI-digested high M_r DNA derived from tumour lines 229 and 740 results in a pattern of identifiable bands that is similar to that seen in the lanes of MDV strain SB-1 productively infected cells. This shows that cell lines 229 and 740 contain SB-1 DNA

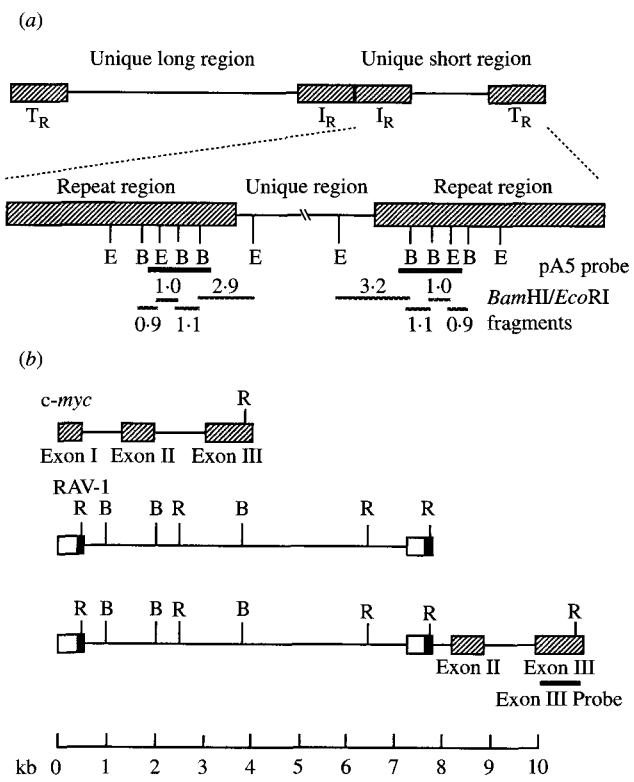

Fig. 1. (*a*) Schematic map of the MDV genome. The size and location of *Bam*HI and *Eco*RI restriction fragments detected by probe pA5 are shown. (*b*) Schematic drawing of ALV integration at c-*myc*. The region of hybridization to the c-*myc* exon III probe is shown.

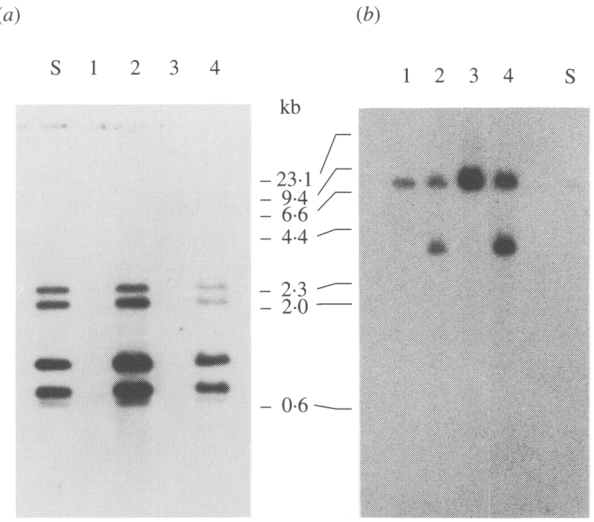

Fig. 2. Analysis of MDV strain SB-1 and ALV provirus integration in cell lines derived from ALV-transformed tumours. DNA (5 μg) samples isolated from cell lines 229 (lanes 2) and 740 (lanes 4), erythrocytes of the line of chicken [$15I_5$ (lanes 1) and SC (lanes 3), respectively] from which the original tumours were derived and chick embryo fibroblasts productively infected with MDV strain SB-1 (lanes S) were digested with the restriction enzymes *Bam*HI and *Eco*RI. DNA was transferred to a nitrocellulose membrane and probed with ^{32}P-labelled pA5 (*a*) or ^{32}P-labelled exon III of c-*myc* (*b*).

Table 1. *Expression of MDV in ALV tumour-derived cell lines*

	MDV antigen expression* (percentage of cells)		Number of MDV plaques†	
5-Azacytidine	−	+	−	+
Cell line 229	0·3	27	1	12
Cell line 740	1·3	21	0	25

* Cell lines were cultured in the absence or presence of 5-azacytidine (3 μM) for 72 h. MDV antigen was detected by indirect immunofluorescence analysis. A minimum of 1000 cells were counted.

† Cell lines were cultured in the absence or presence of 5-azacytidine (3 μM) for 72 h and then cocultured with secondary cultures of chick embryo fibroblasts at a ratio of one tumour cell/100 fibroblasts. Viral plaques were counted in cultures containing 4000 tumour cells.

and have not experienced any gross alterations of the genome within the region recognized by pA5. There is no hybridization of pA5 to DNA isolated from erythrocytes of uninfected chickens of the $15I_5$ and SC lines, showing the specificity of the pA5 probe for MDV DNA.

These tumour cell lines were established from B cell tumours from ALV-infected chickens. The B cell origin of the tumour cell lines was shown by expression of cell surface Ig M (data not shown), as in the tumours from which the cell lines were derived. Integration of the ALV provirus at the c-*myc* locus is typical of B cells that have become transformed by ALV (Ewert & DeBoer, 1988).

The integration of ALV between exon II and exon III of c-*myc* in tumour cell lines 229 and 740 was demonstrated by examining the restriction fragment pattern around the c-*myc* gene. If integration of ALV has occurred, an *Eco*RI fragment of c-*myc*, detected by the exon III probe, will be a different size from that found in the normal allele (see Fig. 1*b*). As shown in Fig. 2(*b*), both cell lines 229 and 740 contain c-*myc* exon III on both the normal allele as well as an altered *Eco*RI restriction fragment, unlike the uninfected control cells. The presence of an additional restriction enzyme fragment of approximately 3·0 kb in both cell lines 229 and 740 compared to non-transformed chicken cell DNA from the same line of chicken indicates a disruption at the c-*myc* locus (Fig. 2*b*).

ALV tumour-derived cells maintained in culture produce very low to undetectable levels of MDV

Since the ALV-transformed cell lines contain MDV DNA, the biological activity of MDV within the ALV tumour-derived cell lines was studied. To determine whether infectious MDV was produced, tumour cell lines were cocultured with chick embryo fibroblasts which are permissive for an MDV productive infection. Even after 7 days of culture (typical for an MDV productive infection), no plaques characteristic of an MDV infection were observed in those cocultivations of cell line 740. Only one plaque was detected following culture of cell line 229 with chick embryo fibroblasts. Analysis of MDV viral antigen production by immunofluorescent staining revealed that only 1% of the cells expressed an MDV structural protein (Table 1). Note that the low level to undetectable amount of MDV antigen and virus production by these cells was not due to heterogeneity of tumour cells within the culture with respect to the presence of MDV genomes. That is, there is not a predominance of tumour cells that do not contain the MDV genome. We found that all of 20 clonal isolates of cell line 229 continue to harbour the MDV genome (data not shown). Therefore, the MDV genome appears to be maintained in most or all of the cells in the population. Thus, although MDV DNA was present and persisted in these tumour-derived cell lines, little evidence of viral replication was observed. Taken together, these results suggested that MDV persisted within the ALV-transformed cells in a latent or quiescent state.

MDV DNA is highly methylated in ALV tumour-derived cell lines but not in MDV-infected fibroblasts

Since latent herpesvirus genomes have been shown to possess methylation patterns distinct from genomes isolated from productively infected cells (Desrosiers *et*

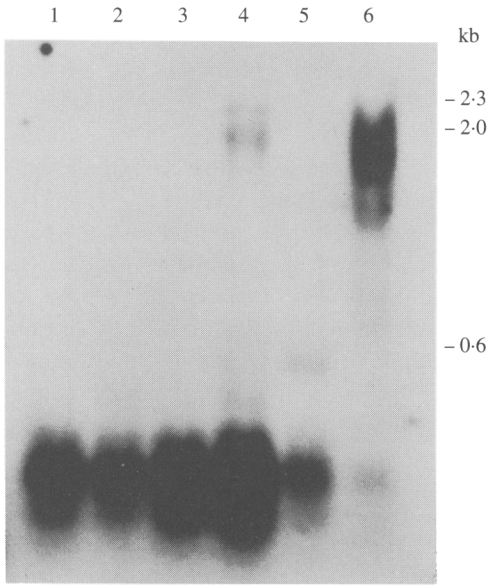

Fig. 3. Degree of methylation of MDV DNA in ALV-transformed tumour cell lines. DNA (5 μg) isolated from tumour cell lines 229 (lanes 3, 4) and 740 (lanes 5, 6) and DNA (0·5 μg) isolated from chick embryo fibroblasts productively infected with MDV strain SB-1 (lanes 1, 2) were digested with restriction enzymes *Msp*I (lanes 1, 3, 5) and *Hpa*II (lanes 2, 4, 6). The Southern blot was probed with ^{32}P-pA5.

al., 1979; Kanamori *et al.*, 1987; Youssoufian *et al.*, 1982), we examined whether the MDV DNA in these tumour cell lines displayed this characteristic of latent viral genomes. Therefore, DNA was isolated and digested with the restriction enzymes *Msp*I and *Hpa*II (Fig. 3). Both enzymes cleave at the site 5′ CCGG 3′, but cutting by *Hpa*II does not occur when the internal cytosine residue is methylated (Waalwijk & Flavell, 1978). *Msp*I digestion of DNA isolated from fibroblasts productively infected with MDV strain SB-1 results in DNA fragments of equal to and less than 0·6 kb. This indicates that there are many *Msp*I sites within the region probed, as expected for an enzyme that recognizes only a four base pair sequence (Fig. 3). The digestion patterns of DNA from MDV strain SB-1-infected fibroblasts cleaved with *Msp*I or *Hpa*II are similar, indicating that viral DNA from productively infected cells possesses little methylation in the region probed (Fig. 3, lanes 1, 2). The *Msp*I digestion pattern of tumour cell DNA is similar to that seen from productively infected cells (see lanes 3 and 5). On the other hand, the *Hpa*II digestion pattern of tumour cell line DNA is quite different. When the same tumour cell DNA was digested with *Hpa*II, the restriction pattern is different from that seen after *Msp*I digestion. The higher M_r fragments present following *Hpa*II cutting, as compared to those seen following digestion with *Msp*I, presumably result from the inability of *Hpa*II to cut at methylated 5′ CCGG 3′ sequences. This difference in the digestion pattern of cell line DNA cut with *Msp*I and *Hpa*II is consistent with the hypothesis that there is methylation of 5′ CpG 3′ dinucleotides within the region studied.

These results indicate that the physical state of the MDV genome in the tumour cells is different from that present during an active infection and that the MDV genome is heterogeneous with respect to methylation. Furthermore, we have shown that the MDV genome in these ALV tumour cell lines is likely to be modified by methylation of 5′ CpG 3′ dinucleotides, a characteristic of inactive herpesvirus genomes (Desrosiers *et al.*, 1979; Kanamori *et al.*, 1987; Minarovits *et al.*, 1991; Youssoufian *et al.*, 1982).

Effect of 5-azacytidine on MDV DNA synthesis

Having shown that the methylation pattern of MDV DNA isolated from the tumour cell lines differed from that of productively infected cells, we examined whether a change in methylation would affect the biological state of MDV in these tumour cell lines. Therefore, to determine whether a decrease in the level of methylation would lead to an increase in viral gene expression, the established MDV–ALV tumour cell lines were treated with an inhibitor of methylation, 5-azacytidine (Taylor *et al.*, 1984). ALV tumour cell lines 229 and 740 were cultured for 3 days in the presence of 5-azacytidine. DNA was isolated from the cells and digested with *Msp*I or *Hpa*II as in Fig. 3. The inability of *Hpa*II to digest cell line DNA isolated from cultures that had not been exposed to 5-azacytidine indicated that the MDV DNA in the ALV tumour-derived cell lines was methylated at cytosine residues (Fig. 3, lanes 2, 4, 6). However, cell line DNA isolated from cultures that had been treated with 5-azacytidine showed a restriction enzyme pattern similar to that of DNA isolated from chick embryo fibroblasts productively infected with MDV (Fig. 4, lanes 2, 4, 6). These results suggested that the MDV genome in the ALV tumour cell lines was unmethylated at cytosine residues following exposure to 5-azacytidine.

Having shown that culture in the presence of 5-azacytidine inhibited cytosine methylation of MDV DNA, the effect of hypomethylation on DNA synthesis was examined. To quantify the degree of amplification of MDV DNA in 5-azacytidine-treated tumour cell lines, equal numbers of cells, 5-azacytidine-treated or untreated, were applied to nylon membranes by use of a 'dot blotter' (see Methods) and probed for MDV. Fig. 5(*a*) shows that a 20- to 30-fold increase in the amount of MDV DNA occurred in cells treated with 5-azacytidine as compared with untreated cells. Significantly, 5-azacytidine had little effect on the copy number

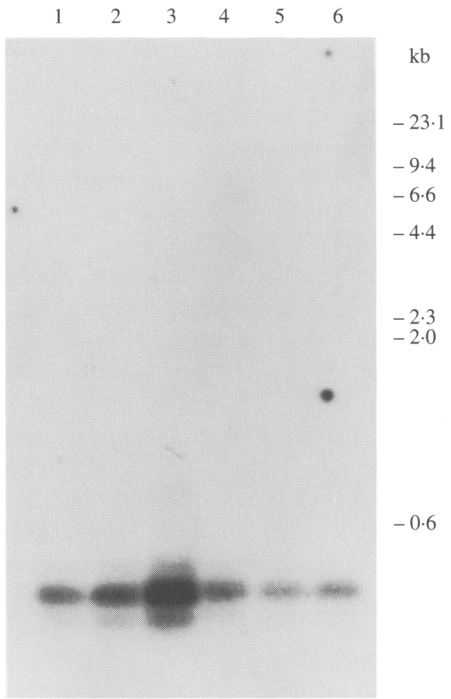

Fig. 4. Effect of 5-azacytidine on the degree of methylation of MDV DNA in ALV-transformed cell lines. DNA (5 μg) isolated from tumour cell lines 229 (lanes 3, 4) and 740 (lanes 5, 6) that had been cultured in the presence of 5-azacytidine (3 μM) for 72 h was digested with restriction enzymes *Msp*I (lanes 1, 3, 5) and *Hpa*II (lanes 2, 4, 6). DNA (0·5 μg) isolated from chick embryo fibroblasts productively infected with MDV strain SB-1 (lanes 1, 2) was digested with the restriction enzymes *Msp*I and *Hpa*II. The Southern blot was probed with ^{32}P-pA5.

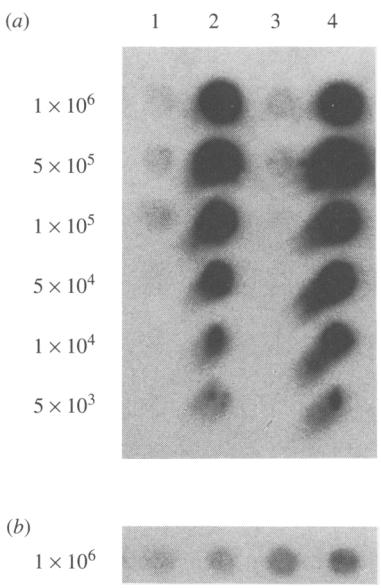

Fig. 5. Effect of DNA methylation inhibition on MDV DNA synthesis. Tumour cell lines 229 (lanes 1, 2) and 740 (lanes 3, 4) were cultured in the absence (lanes 1, 3) or presence (lanes 2, 4) of 5-azacytidine (3 μM) for 72 h. Cells were counted by trypan blue exclusion and applied to a nylon membrane using a dot blotting apparatus. The membrane was probed sequentially with ^{32}P-pA5 (*a*) and ^{32}P-*β*-actin (*b*). The numbers of cells/well are indicated on the left.

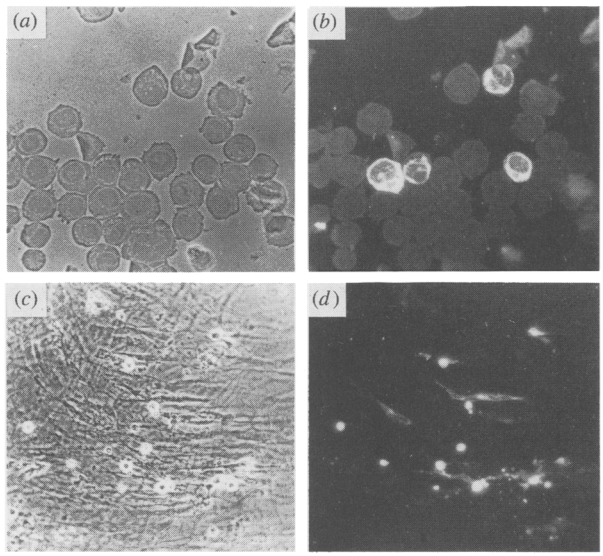

Fig. 6. MDV antigen expression in an ALV-transformed cell line. Cell line 740 was cultured in the presence of 5-azacytidine (3 μM) for 72 h. MDV antigen was detected by immunofluorescent staining with an MDV serotype 2-specific monoclonal antibody (*a*, phase contrast; *b*, immunofluorescent staining). Cell line 740 was cultured in the presence of 5-azacytidine (3 μM) for 72 h and then cultured with chick embryo fibroblasts for 24 h, after which lymphocytes were removed by washing (*c*, phase contrast; *d*, immunofluorescent staining). Immunofluorescent staining was done with mouse monoclonal antibody (Y5) specific for a late MDV protein, as described in Methods.

of the host cell *β*-actin gene (Fig. 5*b*). We conclude that undermethylation of the viral DNA is associated with a dramatic increase in MDV genome copy number per cell.

Expression of MDV in ALV tumour-derived cell lines following treatment with 5-azacytidine

Since the level of MDV DNA synthesis was increased by exposure of the tumour cell lines to 5-azacytidine, experiments were performed to determine whether this treatment would disrupt the quiescent state and lead to the expression of MDV antigens.

The tumour cell lines were cultured in the presence of 5-azacytidine and examined at 72 h for MDV antigen expression by immunofluorescence analysis. MDV strain SB-1 protein expression was detected using an MDV serotype 2-specific monoclonal antibody to an MDV structural protein (Lee *et al.*, 1983). After culture of the tumour cell lines in medium containing 5-azacytidine for 72 h, an increase in the number of cells expressing MDV antigen was observed. The level of antigen expression in cell lines 229 and 740 increased from 0·3% and 1·3% of the untreated cells to 27% and 21% of cells of lines 229 and 740, respectively (Table 1). As an example, Fig. 6(*a*) shows phase contrast microscopy of tumour cells of line 740 following treatment for 72 h with 5-azacytidine.

Immunofluorescent staining for an MDV antigen shows cells expressing MDV protein (Fig. 6*b*). As shown in Fig. 6, a clear distinction could be made between MDV antigen-positive and -negative cells. These experiments show that MDV protein expression is enhanced following exposure to 5-azacytidine, at least for proteins detected by the Y5 antibody.

The increase in MDV DNA synthesis and protein expression following exposure to the methylation inhibitor, 5-azacytidine, suggested that the ALV B cell tumour lines might be capable of producing infectious MDV. Therefore, these tumour cell lines were cocultured with chicken embryo fibroblasts, which are permissive for MDV infection, and observed for c.p.e. typical of MDV infection. Since treatment with 5-azacytidine was shown to increase the level of MDV DNA synthesis and antigen detection, untreated tumour cell lines as well as those exposed to 5-azacytidine for 72 h were cultured with chick embryo fibroblasts. Tumour cell lines were cultured with secondary cultures of chicken embryo fibroblasts at a ratio of one tumour cell for every 100 chick embryo fibroblasts. The ALV tumour cells were washed off after 24 h of culture and the fibroblast cultures were allowed to continue. After 4 days of culture, plaques typical of an MDV strain SB-1 infection were observed in wells that had received cell lines 740 or 229 pretreated with 5-azacytidine (Table 1). Only one plaque was noted in a well containing cell line 229 which had not been exposed to azacytidine. Fig. 6(*c*) shows a representative MDV-type plaque in chick embryo fibroblasts cultured with cell line 740. Immunofluorescence analysis of this culture (*d*) shows that the MDV-producing cells are chick embryo fibroblasts, based on their distinctive spindle-shaped morphology, and not residual B cells. Therefore, infectious virions had been released from the original 5-azacytidine-treated B cells and passed on to permissive chick embryo fibroblasts.

The presence of fibroblasts infected with MDV strain SB-1 demonstrates that these cell lines are capable of producing infectious MDV virions and is evidence that an intact, non-defective MDV genome is present in these cell lines. Given these findings, we conclude that MDV strain SB-1 resides for the most part in these ALV tumours, in an inactive or latent state, and can be reactivated by treatment with the cytosine methylation inhibitor, 5-azacytidine.

Discussion

Continuous cell cultures have been established from ALV-transformed B cells derived from chickens that had also been infected with the vaccine strain of MDV, SB-1. In a previous report, we have shown that, unexpectedly, almost all ALV tumours occurring in chickens that had also been infected with the non-oncogenic MDV vaccine strain SB-1 contained MDV DNA (Fynan *et al.*, 1992). However, the functional potential of the MDV genome in those cells was not studied.

This report describes the functional potential of the MDV genomes contained in two continuous cell cultures of ALV B cell tumours isolated from chickens co-infected with MDV strain SB-1 and ALV (RAV-1). These cell cultures, called 229 and 740, were of B cell origin and contained ALV proviral insertions in the c-*myc* locus, which is a characteristic of ALV-transformed B cells (Ewert & DeBoer, 1988). Cell lines 229 and 740 were examined for the presence of SB-1 DNA using a probe specific for the MDV short repeat region (pA5). Based upon this analysis, both lines contained the MDV genome. Preliminary analysis by pulsed field gel electrophoresis showed that the majority of the MDV homologous DNA does not exist in a linear or episomal form but probably is present as a high M_r form, such as concatemers or integrated into the host cell DNA. However, this method of separation does not allow us to distinguish between these high M_r physical forms (data not shown). Although little or no infectious MDV or MDV antigens were detected in either cell line kept in maintenance culture, viral DNA synthesis, gene expression and viral plaque formation were observed in cultures incubated with 5-azacytidine. This shows that an infectious MDV genome is present in 229 and 740 cell lines in a quiescent state. Moreover, the MDV genome could be activated by cultivation with a drug that inhibits DNA methylation.

These results are significant because they show that the B cell compartment is capable of harbouring the MDV genome in a stable state. Furthermore, the MDV genome within the transformed B cells is able to be reactivated. Therefore, B cells which have been shown to be a site of productive MDV infection *in vivo* may also be a reservoir for latent virus.

Our previous work showed the coincidence of MDV in ALV-derived B cell tumours (Fynan *et al.*, 1992). Since chickens co-infected with the non-oncogenic MDV strain SB-1 and ALV experience a greater frequency of ALV-induced B cell tumours than those receiving ALV alone, it is tempting to speculate that the reported MDV strain SB-1-mediated enhancement of ALV tumorigenesis is, in part, a consequence of an intracellular effect of MDV upon the ALV genome, or upon the host cell that harbours the two viruses. Alternatively, MDV may be an innocent bystander coincidentally resident in the same cells that are transformed by ALV. More work is needed to distinguish between these possibilities.

In quiescent ALV-transformed cell lines 229 and 740, the region of the MDV genome recognized by the pA5 probe showed resistance to digestion with *Hpa*II but not

with *Msp*I. Since these two restriction enzymes both cleave at 5′ CCGG 3′ sequences, but *Hpa*II is unable to cleave when the internal cytosine is methylated, it is likely that this region of the MDV genome is heavily methylated. The DNA of mammalian cells is modified by methylation at 2 to 7% of the cytosine residues (Ehrlich & Wang, 1981). This modification has been associated with a transcriptionally inactive state (Doerfler, 1982). The pA5 region of the MDV strain SB-1 genome may contain DNA sequences that must be repressed to maintain the virus in a latent state. For example, based upon genomic location and homology compared to other herpesviruses, the pA5 region may contain an origin of replication and a cleavage/packaging site used during productive infection (Carter & Silva, 1990). This suggests that methylation of this region of the SB-1 genome within the pA5 region may be important in the maintenance of the latent state.

The quiescent genomes of retroviruses, adenoviruses and herpesviruses in their respective latently infected cells have been shown to be heavily methylated at 5′ CpG 3′ residues (Desrosiers *et al.*, 1979; Groudine *et al.*, 1981; Hoffman *et al.*, 1982; Kanamori *et al.*, 1987; Minarovits *et al.*, 1991). Moreover, inhibition of 5′ CpG 3′ methylation with 5-azacytidine has been associated with the reactivation of such latent genomes (Ben-Sasson & Klein, 1981; Groudine *et al.*, 1981; Hoffman *et al.*, 1982; Niwa & Sugahara, 1981). Therefore, cultures of cell lines 229 and 740 were incubated with 5-azacytidine and tested for evidence of MDV genome reactivation. Significantly, although the copy number of the host cell gene (actin) remained relatively stable, 5-azacytidine-treated cultures experienced a 20- to 30-fold increase in the copy number of MDV DNA sequences. In addition, a viral late antigen was detectable. Most significantly, unlike untreated cells, cultures incubated with 5-azacytidine released infectious MDV, as assayed in cocultivation experiments.

The pA5 region of the MDV genome isolated from 5-azacytidine-treated 229 and 740 cell cultures was shown to have gained sensitivity to the restriction enzyme *Hpa*II. This shows that this region of the genome had become hypomethylated as a result of 5-azacytidine treatment. Although 5-azacytidine may have many effects upon target cells (Taylor *et al.*, 1984), we feel it is possible that the reactivation of the latent MDV genomes in 5-azacytidine-treated 229 and 740 cultures was the result of hypomethylation. However, the ALV-transformed cell lines may contain MDV DNA in a physical state such that it is unable to undergo replication in response to treatment with 5-azacytidine.

This report shows, for the first time, the presence of latent MDV in a B cell population. The identity of any host, ALV or MDV genes that regulate the MDV genome in latently infected B cells remains to be determined. Similarly, the pathological significance of infectious MDV in B cells, in general, and ALV-transformed B cells, in particular, is unclear. Both of these aspects require further investigation.

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