Marek’s disease virus EcoRI-Q gene (meq) and a small RNA antisense to ICP4 are abundantly expressed in CD4+ cells and cells carrying a novel lymphoid marker, AV37, in Marek’s disease lymphomas

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Mature lymphomas produced in Rhode Island Red (RIR) chickens infected with the RB1B strain of Marek’s disease virus (MDV) were examined for the presence of viral DNA and RNA and expression of viral antigens. In situ hybridization showed that all tumours examined contained viral DNA in areas of lymphoid infiltration. In 3/5 tumours, there was a correlation between the number and distribution of cells expressing the Marek’s disease EcoRI-Q gene (meq) and those that carried the lymphoid cell marker AV37. Expression of the MDV-specific phosphoprotein pp38 was infrequent in lymphomas but abundant in a splenic tumour which also expressed the viral glycoprotein gB. Northern blot analysis of lymphocyte fractions purified by immunoaffinity showed that CD4+ and AV37+ fractions from lymphomas expressed meq and the small RNA antisense to ICP4 (SAR). The results are consistent with the notion that transformed cells are CD4+ cells, carrying the AV37 marker and expressing meq and SAR.

Introduction

Marek’s disease (MD) is a lymphoproliferative disease of chickens caused by a herpesvirus. The virus infects both B and T cells but, following natural infection, mainly CD4+ lymphocytes are malignantly transformed (Schat et al., 1991). There is evidence that viral DNA is integrated as well as episomal in lymphoma cells and cell lines (Delecluse et al., 1993) but the precise role of the virus in transformation is not known. The fact that MD lymphomas develop rapidly compared to avian leucosis tumours suggests that Marek’s disease virus (MDV) may not act as an insertion mutagen, as in the case of avian leucosis virus (Anderson et al., 1992), but rather that it encodes proteins which, directly or indirectly, are responsible for transformation. Several experimental approaches have been used to identify MDV genes that are implicated in transformation. These have included comparison of the genomes of MDV and attenuated MDV (Silva & Witter, 1985; Maotani et al., 1986), characterization of cDNA libraries derived from cell lines (Kopacek et al., 1993; Li et al., 1994; Ohashi et al., 1994; Koptidesova et al., 1995; McKie et al., 1995; Peng & Shirazi, 1996) and investigation of the effect of antisense oligodeoxyribonucleotides and RNAs on the growth of cell lines (Kawamura et al., 1991; Xie et al., 1996). These experiments, particularly the work with antisense oligonucleotides and RNAs have suggested that meq, a homologue of the fos and jun oncogenes (Jones et al., 1992), ICP4, a homologue of herpes simplex virus immediate-early transactivator ICP4 (Anderson et al., 1992), the phosphoprotein complex pp38 (Cui et al., 1991) and sequences mapping in the BamHI-H region which encode a 7 kDa protein (Peng et al., 1993) might be involved in transformation. However, the significance of the results obtained with antisense oligonucleotides is not clear because evidence was not provided in every case that expression of the targeted genes had been reduced or inhibited in the treated cell lines. Moreover, viral gene expression in cell lines may not reflect expression in lymphomas since it is known that cell lines undergo selection or virus reactivation during passage in vitro. These observations strongly point to the need for analysis of viral expression in lymphomas to obtain meaningful results on the role of viral products in transformation.

Early studies using convalescent chicken antisera showed that only 2 to 3% of lymphoma cells expressed viral antigens
whereas cell lines varied considerably, ranging from 1 to 30% (Powell et al., 1974; Ross et al., 1977; Calnek et al., 1981). In contrast, 90% of the cells of cell lines expressed a tumour-associated antigen (MATSA) which was not expressed during cytolytic infection of fibroblasts (Powell et al., 1974; Witter et al., 1975). Up to 30% of lymphoma cells were reported to express the tumour antigen. Subsequently, the expression of the viral phosphoprotein pp38 in tumours was demonstrated using a monoclonal antibody (MAb) (Naito et al., 1986). Little is known, however, about the expression of other viral products in lymphomas. In this study, we have investigated the nature of the MDV-infected cells in mature lymphomas and the viral genes and proteins expressed in those cells. We have focussed particularly on expression of meq, pp38, a small RNA (SAR) antisense to ICP4 and a novel cell marker (AV37) associated with infected and transformed cells (Kaiser et al., 1996; Burgess et al., 1997).

Methods

Source of tumours. Rhode Island Red chickens (HPRS RIR) were inoculated intramuscularly at 1 day of age with 1000 p.f.u. of RB1B (Schat et al., 1982). Mature lymphomas and splenic tumours were collected after 8 weeks. Spleens of chickens inoculated with HPRS16/att or CVI988 served as controls. Splenic tumours were also obtained from turkeys which had been infected with MDV-infected chickens. These turkeys were not infected with reticuloendotheliosis virus (REV) as shown by serology and PCR analysis of tumour DNA as described by Davidson et al. (1995).

Cell lines. RPL-1 (Naizerian et al., 1977), MSB-1 (Akiyama & Kato, 1974) and HP3 (Payne et al., 1981) cells were cultured as described previously (Ross et al., 1977).

MAbs and immunofluorescence tests. MAb BD1 (Li et al., 1994) is specific for the phosphoproteins pp38/24. MABS CT4 and CT8, both of IgG1 isotype, recognize the chicken homologues of the lymphocyte surface markers CD4 and CD8 respectively (Chan et al., 1988). The AV37 monoclonal (IgG2a) reacts with an antigen on the surface of transformed lymphocytes and circulating lymphocytes during the acute phase of infection (Burgess et al., 1997). The monoclonal mAb23B46, provided by Lucy Lee, is specific for meq. Spleen cells obtained from mice which had been immunized with recombinant fowlpox virus (rFPV-meq) overexpressing meq were fused with NS-1 myeloma cells. Hybridoma cultures positive for meq were selected, cloned by limiting dilution and the production of meq-specific antibodies was identified by ELISA, immunofluorescence and immunoprecipitation analysis of cells infected with rFPV-meq and wild-type FPV (Lucy Lee, unpublished). Immunofluorescence tests were carried out in this study using Cy2 goat anti-mouse IgG conjugate (BDS Inc., Pittsburgh, USA) at a final dilution of 1/1000. Cells were fixed in acetone for 10 min at room temperature.

Isolation of lymphocytes. Lymphoid cell suspensions were prepared by teasing tumours apart using forceps, suspending the cells in DMEM (Gibco) and layering over Ficoll–Hypaque (Pharmacia). Lymphocytes were isolated by centrifugation at 1350 ×g for 20 min and cells were collected from the interface, washed three times in cold PBA (PBS containing 2 mg/ml BSA and 0.1% azide) and resuspended in PBA at 10^8/ml.

Separation of cells using MiniMACS. Cells were separated using magnetic cell sorting (Miltenyi Biotec) and their purity determined by flow cytometry (Baigent et al., 1996). Briefly, 1 ml (10^6) cells were incubated for 30 min at 4 °C with 2.5 µl of 1:500 ascitic fluid and washed twice with PBA. The cells were incubated with 1 ml of rat anti-mouse IgG2a+ b or IgG1 MACS microbeads (Miltenyi Biotec) for 20 min at 4 °C and washed twice. Cells were resuspended in 1 ml and the prechilled MiniMACS column (Miltenyi Biotec) was primed with 0.5 ml PBA. Cells were added to the column which was then washed five times with 0.5 ml PBA. The retained cell fraction was recovered by forcing 1 ml PBA vigorously through the column.

Flow cytometric analysis of lymphocyte populations. For single staining, 10 µl of unsorted cells or 25 µl of sorted cells was added to a 96-well U-bottomed plate (Nunc). To unsorted cells 25 µl of primary MAb or appropriate control at 1:500 was added, incubated for 30 min at 4 °C and washed twice. Both sorted and unsorted cells were incubated for 20 min at 4 °C with 25 µl of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig and washed twice. The cell suspensions were examined on a FACS analyser (Becton-Dickinson).

For double staining, 25 µl containing 10^7 unsorted lymphoma cells was added to a 96-well U-bottomed plate followed by 25 µl of AV37 MAb (1:250 dilution) and 25 µl CT4 or CT8 at 1:100 dilution. After 30 min incubation at 4 °C and washing twice, 50 µl of goat anti-mouse IgG1 conjugated to FITC and 50 µl goat anti-mouse IgG2a conjugated to phycoerythrin (PE), both diluted 1:100, were added to the wells. After 30 min incubation at 4 °C, the cells were washed twice and examined on the FACS analyser.

In situ hybridization. MDV DNA fragments (37 kb spanning the internal repeats and part of U6, together with the 18 kb BamHI-B fragment mapping in U6) representative of approximately 45% of the genome were labelled with fluorescein-11-dUTP using random primers and the Klenow fragment of DNA polymerase I. Cells were fixed on silane-coated glass slides using buffered formal saline. Protocols for pretreating cells, for hybridization and for detection of bound fluorescein using alkaline phosphatase-conjugated anti-fluorescein antibody were as described in the DNA colour kit (Amersham) except that post-hybridization washing was carried out using 0.1 × SSC at 55 °C.

Northern blot analysis. Total RNA was extracted from cell lines and lymphocyte fractions using Trizol (Gibco). Approximately 10 µg was added to each lane for electrophoresis. Hybridization with 32P-labelled double-stranded DNA probes was carried out according to established protocols as reported previously for meq and SAR RNAs (Jones et al., 1992; Li et al., 1994). Probes for detection of meq, pp38 and SAR were a BamHI-P fragment subfragment of meq, EcoRI fragment of BamHI-H and the 3 kb HindIII fragment of BamHI-A respectively. β-Actin RNA was detected using a dsDNA probe labelled with 32P by random priming. The template for probe synthesis was a 1 kb fragment obtained by PCR amplification of chicken DNA using the primer pair 5' AGCCAGCCATGGATGATGATATTG and 3' AGCCAGCCATGGATGATGATATTG. The conditions for hybridization were the same as for hybridization to meq RNA.

Results

Detection of viral DNA by in situ hybridization

A method for identifying infected cells in lymphomas by hybridization was developed. The method was validated using MDV-infected fibroblasts and cell lines as controls. The results
of in situ hybridization are shown in Fig. 1. Chick embryo fibroblasts (CEF) infected with MDV contained variable amounts of viral nucleic acid, as expected of a non-synchronous infection. Some cells contained much nucleic acid as shown by the presence of intense signal throughout the cell, while others contained smaller amounts of viral nucleic acid in the nucleus. The non-producer cell line RPL-1, on the other hand, contained fairly uniform but small amounts of viral nucleic acid which were intranuclear. Pretreatment of the cells with DNase prior to hybridization abolished the intranuclear signal whereas RNase had no effect (not shown) indicating that the probe is hybridizing to viral DNA. Hybridization was MDV-specific because the probe did not hybridize to T cells transformed by a retrovirus (Fig. 1c) while a probe prepared using lambda DNA or HVT DNA, of similar complexity as the MDV probe, failed to hybridize to the MDV cell line (not shown).

In sections of lymphomas, viral nucleic acid was invariably present in a proportion of infiltrating cells which comprised small, medium-sized and large lymphocytes (Fig. 2d). The signal was resistant to RNase but sensitive to DNase treatment indicating that, in latently infected cells, the test detected principally DNA as in the case of cell lines (Fig. 1). In some areas (Fig. 2d), the majority of the lymphocytes were infected but some apparently uninfected small and large lymphocytes were also present. Uninfected renal cells are also visible in Fig. 2d.

Expression of MDV antigens in lymphomas and cell lines
Serial sections from four kidney lymphomas and one splenic tumour were prepared for detection of viral antigens by immunofluorescence and viral DNA by in situ hybridization. The results are shown in Fig. 2 and are summarized in Table 1. The meq antigen was present in a diffuse form in the nucleus of infiltrating lymphocytes but was also associated with dense bodies in the nucleus giving rise to a stippled pattern of intranuclear fluorescence. pp38 was located in the cytoplasm whereas the AV37 antigen was present at the cell surface. In 3/4 lymphomas examined, there was a good correlation between the presence of viral DNA in infiltrating lymphocytes and expression of meq and AV37 antigens whereas expression of pp38 was infrequent. In two tumours (tumours 4 and 5, Table 1) expression of meq and AV37 antigens was weak or absent, and in one of them (tumour 5) expression of pp38 was
Table 1. Presence of MDV DNA and expression of viral antigens in tumours

Serial sections of kidney lymphomas (1–4) and of a splenic tumour (5) were examined for expression of viral antigens by immunofluorescence and viral DNA by hybridization. +, A few single positive cells; ++, wide spread positive cells; −, negative.

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Examination of three cell lines (RPL-1, MSB-1 and HP3) has shown that at least 90% of the cells of each cell line contain viral DNA and expressed meq whereas pp38 expression was variable (0-2 to 10%). Similarly, 90% of the cells of the RPL-1 and HP3 cell lines expressed AV37 antigen whereas MSB-1 cells did not express the antigen.

To determine whether meq is also expressed in MDV-induced tumours in a different avian species, serial sections of splenic tumours from turkeys which had been infected by contact with MDV-infected chickens were examined by immunofluorescence. The results showed that meq was expressed in a large proportion of the cells from the turkey tumours and that the areas which were positive for meq expression were also positive for expression of AV37 antigen (not shown). The MDV aetiology of these tumours was confirmed by PCR analysis of tumour DNA which showed the presence of MDV DNA and absence of REV sequences in the tumours (N. Ross, unpublished).

Fractionation of lymphoma cells

Lymphoma cells were separated into CD4+, CD8+, AV37+ and B cell fractions in order to identify those lymphocyte...
subsets which were infected and expressed viral transcripts and proteins. The results of flow cytometric analysis summarized in Table 2 show that the main lymphocyte population consisted of CD4+ cells (67 to 90%), while CD8+ cells constituted only 2 to 12% and B cells were negligible. The proportion of AV37+ cells ranged from 12 to 55%. The purity of the sorted CD4+ and CD8+ fractions determined by flow cytometry was 81 to 94% while the AV37+ fractions were 75 to 85% pure. In situ hybridization revealed that the majority of AV37+ cells were infected (60 to 80%) while 15 to 85% of CD4+ and 21 to 60% of CD8+ cells were infected.

To confirm that AV37+ tumour cells belonged predominantly to the CD4+ population, double-stainings were done with MAb AV37 and MAbs against CD4 and CD8. The results were analysed by FACS. Fig. 3 shows the results obtained using a lymphoma similar to tumour 4, Table 2. It is clear that the AV37+ cells are CD4+ and almost completely CD8−. Similar results have been obtained with a number of lymphomas.

Characterization of viral transcripts in cell fractions from lymphomas

Northern blot analysis was carried out mainly to examine transcription of the SAR in cell fractions because antibodies were not available to investigate expression of proteins that might be encoded by RNA antisense to ICP4. Moreover, it is not known whether SAR encodes a protein. The results (Fig. 4) indicate that SAR and meq transcripts were abundant in CD4+ and AV37+ fractions but not in CD8+ cells. Similar results were obtained using three individual tumours. The presence of multiple meq RNAs in the range 1.2 to 3.8 kb is consistent with previous reports of both spliced and unspliced sense transcripts as well as antisense transcripts in the region coding for meq (Peng & Shirazi, 1996a). Meq transcripts were not detected in the spleens of infected or uninfected chickens, but low levels of SAR (barely detectable in Fig. 4) were detected in all fractions, particularly CD4+ and AV37+ cells, from the spleen of the MDV-infected chicken that had a kidney lymphoma. pp38 and VP16 transcripts were not detected in any of the fractions (not shown). All fractions contained intact actin RNA in approximately similar amounts.

Discussion

In this study we have examined the nature of infected lymphocytes in lymphomas and some of the MDV antigens and transcripts expressed by those cells. We focussed our investigation on meq, pp38 and SAR since these have previously been associated with transformation of cell lines (Cantello et al., 1994; Li et al., 1994; Xie et al., 1996). Ideally, we would have wished to carry out all the tests on the same...
sections using both immunocytochemical and in situ hybridization techniques. However, this was not possible because immunocytochemistry and in situ hybridization were not compatible and antibodies were not available to detect all the viral products under investigation. SAR had to be examined by hybridization because it is not known if it encodes a protein. Moreover, the use of riboprobes for SAR detection in situ is prohibitive in view of non-specific binding to ribosomal RNAs (Cantello et al., 1994; Li et al., 1994). Consequently, the plan adopted had to be a compromise. Serial sections were processed for detection of meq, pp38 and AV37 by immunofluorescence while viral DNA was detected by in situ hybridization. Cell fractions were separated by immunoaffinity methods and examined for viral transcripts by Northern blotting.

The results of immunofluorescence and in situ hybridization tests on sections of lymphomas indicated that there is a good correlation between the number of infected lymphocytes (shown by the presence of viral DNA), expression of meq and AV37 in lymphomas. Expression of pp38 was less frequent than expected from the number of infected cells present in the sections. Northern blot analysis has shown further that meq transcripts were abundant in CD4+ cells, and cells bearing the AV37 marker. Since the majority of the cell lines cells expressed meq and contained viral DNA, we conclude that transformed cells in MD lymphomas are CD4+ AV37+ cells expressing meq.

The precise role of meq in oncogenicity is not known. Recent studies have shown that meq is a transactivator and that it may also have a role in transcriptional regulation by causing DNA bending (Qian et al., 1995, 1996). Interestingly, Peng & Shirazi (1996b) have reported that a truncated meq (meq-sp) which retains the capacity to bind to AP1 and to interact with fos and jun but lacks transactivating domains is produced by alternative splicing in chick fibroblasts infected with an attenuated strain of MDV. They postulated that meq-sp could compete with meq for heterodimer formation with c-jun and act as negative regulator of meq activity in vivo. Further work is required to determine whether meq-sp is produced in transformed cells or in cells infected with oncogenic MDV.

The role of pp38 in transformation is not clear because its expression was not consistent with meq expression either in lymphoma cells or in cell lines. The fact that pp38 expression was abundant in a splenic tumour, which also expressed gB, suggests that pp38 expression probably reflects virus reactivation in the splenic tumour. However, previous studies have reported extensive pp38 expression in lymphomas of the bursa and proventriculus (Naito et al., 1986). It has also been suggested that pp38 might have a role in the maintenance of the transformed state, since an oligonucleotide antisense to pp38 RNA inhibited the growth of cell lines (Xie et al., 1996). Unfortunately, the effect of the oligonucleotide on pp38 expression could not be demonstrated, because the cell line
used for the experiment did not express detectable amounts of pp38. It is possible therefore that growth inhibition could have occurred by some other mechanism. Consequently, the role of pp38 in oncogenicity remains uncertain. Further work involving mutagenesis of pp38 in the virus is required to evaluate its role in oncogenicity.

The Northern blot analysis showed that SAR transcripts were abundant in CD4+ and AV37+ fractions derived from lymphomas. Lower levels of SAR were also present in cell fractions from the spleen of a chicken bearing a lymphoma but were not present in cell fractions from spleens infected with attenuated MDV or from uninfected chickens. The precise role of SAR in latency and transformation is not known. Recent studies (Li et al., 1994, and unpublished observations) have shown that SAR is highly spliced, non-polyadenylated and has the potential to encode a polypeptide of 150 amino acids. Our results suggest that SAR may have a role in latency and transformation. It is possible that SAR could function by reducing or inhibiting expression of ICP4 thereby preventing cytolytic replication. It is also possible that SAR might combine with sense ICP4 RNA to form a double-stranded RNA (dsRNA) complex which could induce type 1 interferon production and hence block virus replication in lymphocytes. The induction of interferon by dsRNA is well documented (Sekellick & Marcus, 1986; Daly & Reich, 1995) and there is evidence that interferon can modulate expression of MDV antigens in splenocytes (Volpini et al., 1995).

Our work has shown a correlation between neo expression, viral DNA and AV37 antigen expression in lymphomas. Almost all MDV-transformed cell lines so far examined express the AV37 antigen, except for MSB-1. The lack of expression of the AV37 antigen on MSB-1 cells may be a feature of the particular clone available and further clones are being examined.

Taken together the results of double stainings and Northern blotting suggest that MDV-transformed cells are almost entirely CD4+ cells carrying the surface marker AV37. It is not known at present if the AV37 antigen is synthesized de novo as a result of infection and transformation or whether it is a marker for lymphoid cells that are susceptible to infection and transformation. Certainly the AV37 antigen appears to be unique in that it is present on MD lymphoma cells but is not specific to T cells, since it has also been detected on B cells, which do not become transformed by MDV (S. Burgess, unpublished results). The AV37 antigen has not been detected on normal embryonic or adult tissues (P. Kaiser, IAH Compton, personal communication). The detection of the AV37 antigen in lymphomas induced by MDV in turkeys (this study) suggests that it is not restricted to the chicken. In this respect, the AV37 antigen is similar to MATSA, which is also expressed on MDV-transformed lymphocytes and cell lines from turkeys (Powell et al., 1984). However, there are a number of important differences. AV37 antigen is not specific for MDV infection, since it is also found on a number of cell lines produced by other oncogenic viruses (Kaiser et al., 1996). It has a molecular mass of 75 kDa (Kaiser et al., 1996) and it does not appear to be an activation antigen as reported for MATSA (McCull et al., 1987).

Finally, our results have shown that the composition of lymphomas varies considerably. Tumour 4 (Table 2) is an extreme case where 95% of the lymphocytes were CD4+ but only 12% expressed the AV37 antigen and 15% were infected as shown by in situ hybridization. Lymphomas also contained a proportion of uninfected CD8+ cells. It is possible that some of these, apparently uninfected cells, could be part of an immune response which varies in intensity between individual tumours. However, the assumption that these cells were not infected with MDV needs to be confirmed. In situ hybridization may not have been sensitive enough to detect a single copy of latent MDV DNA. Further work using in situ PCR should be able to resolve this point.

References


Marek's disease virus encodes a basic leucine zipper gene resembling the fos/jun oncoproteins that are highly expressed in lymphoblastoid tumours. *Proceedings of the National Academy of Sciences, USA* 89, 4042–4046.


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