PBMCs are additional sites of productive infection of bovine papillomavirus type 2

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Bovine papillomavirus type 2 (BPV-2) is an oncogenic virus infecting both epithelial and mesenchymal cells. Its life cycle, similar to other papillomaviruses (PVs), appears to be linked to epithelial differentiation. Human and bovine PVs have been known to reside in a latent, episomal form in PBMCs; therefore, it is believed that blood cells, like all mesenchymal cells, function as non-permissive carriers. Here, for the first time in veterinary and comparative medicine, the BPV-2 E5 oncoprotein and the major structural L1 capsid protein, known to be expressed only in productive infections, were shown to occur in defined subsets of PBMCs. E5 oncoprotein was detected in sorted T- and B-cells as well as in monocytes by flow cytometry and Western blot analysis. However, CD4+ and CD8+ lymphocytes appeared to be the main circulating targets of the virus, thus possibly representing the most important reservoir of active BPV-2 in blood. L1 protein was identified by flow cytometry in a population of blood cells recognized as lymphocytes by morphological scatter properties. Western blot analysis was performed on lysates obtained from the sorted subpopulations of PBMCs and detected L1 protein in CD4+ and CD8+ cells only. Thus, this study showed that CD4+ and CD8+ lymphocytes are permissive for BPV-2 and are new, hitherto unknown sites of productive PV infection. In light of these observations, the life cycle of PVs needs to be revisited to gain novel insights into the epidemiology of BPV infection and the pathogenesis of related diseases.

INTRODUCTION

Bovine papillomaviruses (BPVs) are a heterogeneous group of viruses responsible for tumours of the skin, genital and paragenital area, eye, upper gastrointestinal tract and urinary bladder (IARC, 2007). BPVs, like all other papillomaviruses (PVs), are usually strictly species specific. However, cases of cross-species infection are known to occur. BPV type 1 (BPV-1) and BPV-2, belonging to the genus Deltapapillomavirus (de Villiers et al., 2004), are responsible for infections in equids resulting in sarcomas (Chambers et al., 2003; Trenfield et al., 1985), as well as in bison and water buffaloes leading to warts (Lerikat et al., 2006; Silvestre et al., 2009). In addition, a variant of BPV-8, classified in the genus Epsilonpapillomavirus, also causes papillomas of the skin in bison (Tomita et al., 2007). More recently, it has been suggested that BPVs could be responsible for cutaneous sarcomas in cats and captive African lions (Munday & Knight, 2010; Orbell et al., 2010).

BPV-1 and -2 are closely related serotypes (Shafti-Keramat et al., 2009) and can infect both epithelial and mesenchymal cells. Similar to other PVs, BPV-1/-2 replication and virion production are confined to the epithelial region of the lesions, whilst infection of mesenchymal cells appears to be non-productive (Campo, 2006; Shafti-Keramat et al., 2009).

BPV-2 is known to play a central role in bladder carcinogenesis of adult cattle reared on pasturelands rich in bracken fern. In these animals, tumours of the urinary bladder are common, and it is believed that BPV-2 and some immunosuppressive and/or carcinogenic chemicals of
bracken fern act synergistically (Borzacchiello & Roperto, 2008; Roperto et al., 2008). In >90% of cases, tumours of the urinary bladder are responsible for a severe clinical syndrome known as chronic enzootic haematuria (Maxie, 1993). The incidence of bladder tumours varies among cattle grazing on pastures contaminated with bracken fern but may be >90% in adult animals (Pamukcu et al., 1976; Roperto et al., 2010a, b).

BPV-2 DNA was amplified and sequenced in 78% of urinary bladder tumour samples of cattle and in 40–50% of normal samples as controls (Borzacchiello et al., 2003; Roperto et al., 2008). The high incidence of BPV-2 DNA in normal bladder possibly reflects the presence of a latent infection that may be activated by the immunosuppressive and/or carcinogenic chemicals in bracken fern (Borzacchiello et al., 2003). Recently, it has been shown that human PV (HPV) and BPV DNA is detectable in PBMCs (Bodaghi et al., 2005; Roperto et al., 2008; Wosiacki et al., 2005), plasma and serum (Widschwendter et al., 2003).

HPV DNA has been found as an episomal form in the unfraccionated PBMCs of blood donors. Transcripts from HPV DNA-positive PBMCs have not been found, nor has the blood cell (sub)population(s) preferentially harbouring HPV genomes been detected, which suggests that PBMCs probably function as non-permissive carriers (Bodaghi et al., 2005; Ho et al., 2005).

It has been suggested that blood cells can be a site of latent virus, as episomal BPV DNA has been detected in circulating lymphocytes (Campo et al., 1994). Furthermore, both horizontal and vertical transmission of BPV-2 infection from peripheral blood of infected animals has been experimentally documented in healthy cattle (Stocco dos Santos et al., 1998). BPV-1/-2 E5 DNA has been detected in PBMCs of cattle suffering from urothelial tumours of the urinary bladder (Roperto et al., 2008) and horses affected by sarcoids (Brandt et al., 2008). Furthermore, the expression of BPV-2 E5 oncoprotein has been shown in PBMCs of haematuric cattle by both RT-PCR and Western blotting; immunocytochemical investigations on cytospin preparations from PBMCs showed that E5 was localized mainly in the cytoplasm of lymphocytes (Roperto et al., 2008).

Although further epidemiological studies are required to improve our understanding of PV transmission (IARC, 2007), it has been suggested that PBMCs may be PV carriers and blood may be a potential new route of transmission (Bodaghi et al., 2005; Chen et al., 2009; Roperto et al., 2008).

In this report, we have clearly documented expression of the BPV-2 E5 oncoprotein and L1 capsid protein in PBMC subsets derived from cows affected by urinary bladder tumours.

This study shows, for the first time, which blood cell subpopulations are involved in the potential spread of the virus by the bloodstream and that productive infections by BPVs can take place in PBMCs.

RESULTS

Immunoprecipitation for E5 and DNA detection and sequencing

In the four slaughtered cows tested in this study, E5 oncoprotein was detected in lymphocytes (Fig. 1). PCR analysis, validated by direct sequencing of the amplified product, confirmed the presence of BPV-2 sequences. The results are summarized in Table 1, and an example of PCR analysis of the blood cell subpopulations is shown (Fig. 2). BPV-2 DNA was amplified mostly in CD4+ and CD8+ cells. Polymorphonuclear cells (PMNs) were consistently negative.

Morphological patterns of the tumours

The histological patterns of the four examined tumours were diagnosed as papillary hyperplasia/papilloma (case 1), a low-grade papillary carcinoma co-existing with an epithelioid angiosarcoma (case 2), a low-grade papillary carcinoma (case 3) and a high-grade papillary carcinoma co-existing with a CIS (case 4). In all lesions, a diffuse chronic stromal inflammation was also observed.

Flow cytometry

Double-colour flow cytometry showed different expression levels of E5 oncoprotein in lymphocytes as well as in monocytes in the four cases examined. In particular, CD4+ T-cells were the only cells showing E5 oncoprotein in the cow suffering from papillary hyperplasia/papilloma (case 1). In the remaining cows with malignancies, >50% of CD4+ or CD8+ T-cells expressed E5 oncoprotein. In addition, >20% of circulating CD14+ monocytes showed the presence of E5 oncoprotein. Thus, monocytes were the second most important population of white blood cells carrying viral protein. However, CD4+ and CD8+ lymphocytes were more intensively stained than the CD14+ monocytes. Low levels of the oncogenic protein

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**Fig. 1.** E5 oncoprotein expression in lymphocytes of the peripheral blood. E5 oncoprotein was detected by immunoprecipitation. Lane 1, negative control (normal blood from healthy cattle); lanes 2–5, peripheral blood cells from the four haematuric cows tested.
were also shown to occur in CD21\(^+\) B-cells from one cow with high-grade bladder malignancy (case 4). PMNs were negative in all the cases (Fig. 3). The flow cytometry results are summarized in Table 1.

In the animal affected by a high-grade carcinoma coexisting with CIS (case 3), intracellular L1 protein was detected in a cell population identified as lymphocytes by morphological scatter properties (Fig. 4). Monocytes and PMNs were consistently negative.

Table 1. Data from flow cytometry (E5 oncoprotein) and PCR analysis (BPV-2 DNA)

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Method</th>
<th>CD4(^+)</th>
<th>CD8(^+)</th>
<th>CD14(^+)</th>
<th>CD21(^+)</th>
<th>PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Papillary hyperplasia/papilloma</td>
<td>FC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>2</td>
<td>Low-grade papillary carcinoma and angiosarcoma</td>
<td>FC</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
</tr>
<tr>
<td>3</td>
<td>Low-grade papillary carcinoma</td>
<td>FC</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
<td>NEG</td>
</tr>
<tr>
<td>4</td>
<td>High-grade papillary carcinoma and CIS</td>
<td>FC</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td>POS</td>
<td>NEG</td>
</tr>
</tbody>
</table>

Fig. 2. PCR amplification of BPV-2 DNA in PBMCs. (a) A DNA fragment of 125 bp was amplified in T-cells (CD4\(^+\) and CD8\(^+\)), B-cells (CD21\(^+\)) and monocytes (CD14\(^+\)), but not in PMNs. HAC, Healthy animal control; CTR\(^+\), positive control (DNA from a BPV-2-positive bladder tumour); CTR\(^-\), negative control (no DNA added); M8, molecular mass marker type 8 (Roche), with sizes given in bp.

Fig. 3. E5 oncoprotein expression in fractionated PBMCs. E5 expression (blue shading) was detected by flow cytometry in the cytoplasm of CD4\(^+\) and CD8\(^+\) T-cells. Control cells are indicated by a green line. Although less intense, E5 expression was also present in CD14\(^+\) monocytes and CD21\(^+\) B-cells. PMNs were negative in all four cases.
Western blot analysis

To confirm the FACS results of flow cytometry, E5 Western blot analysis was performed on lysates obtained from the subpopulations of PBMC subsets, the purity of which ranged from 94 to 98%. E5 oncoprotein was detected in cells that were scored as E5-positive by FACS. CD4+ and CD8+ T-lymphocytes were the cell subpopulations showing the highest levels of E5 protein (Fig. 5). The lack of suitable antibodies hampered the FACS-mediated detection of L1-positive lymphocytes (see Methods). To overcome this difficulty and confirm the flow cytometric results, Western blot analysis of L1 was performed on lysates obtained from PBMC subsets. The L1 capsid protein was detected in CD4+ and CD8+ cells only (Fig. 6), and was not seen in CD14+ and CD21+ cells and PMNs.

DISCUSSION

This study focused on detection of the structural L1 capsid protein and E5 oncoprotein of BPV-2 in PBMCs of cattle suffering from PV-associated urinary bladder tumours. BPV-2 DNA was also amplified and sequenced from these cells. Our findings not only confirm and better define our previously obtained data on the specific detection of viral E5 mRNA and E5 oncoprotein in PBMCs (Roperto et al., 2008), but also have important implications regarding PV transmission and pathogenesis.

It has been suggested that productive BPV-2 infection is confined to epithelial cells, with the L1 capsid protein being expressed in the highly differentiated cells of the final epidermal layer (IARC, 2007; Moody & Laimins, 2010). Expression of the late L1 capsid protein in circulating T-cells suggested that the productive life cycle of PVs can also take place in PBMCs. This study showed that particular PBMC subsets, notably CD4+ and CD8+ lymphocytes, are permissive for BPV replication and therefore represent hitherto unknown sites of productive PV infections. Our findings support the concept of BPVs, like HPVs (Sarkola et al., 2008; You et al., 2008), not being strictly epithelial and are consistent with recurrent remarks suggesting the possible presence of the complete PV genome in non-epithelial cells (Füle et al., 2006). It is worth noting that HPV-16 L1 DNA has been detected in Schwann cells of peripheral nerves and endothelium of blood vessels. The same structures have shown an evident immunohistochemical reactivity for the E6 and E7 oncoproteins (Füle et al., 2006).

We were also able to identify the PBMC subsets harbouring BPV-2 E5. In veterinary medicine, previous studies have shown both the expression of E5 oncogene and the presence of E5 oncoprotein of BPV-2 in PBMCs (Brandt et al., 2008; Roperto et al., 2008). In human medicine, neither viral oncoproteins nor HPV-specific RNA are known to occur in PBMCs of PV-infected cancer patients (Pao et al., 1997). Although expression of oncogenes such as E6 and E7 mRNA of the high-risk HPV-16 and -18 has been documented in circulating cells by specific RT-PCR techniques, it was believed that a positive peripheral blood RT-PCR test indicated the presence of circulating tumour cells rather than infected blood cells (Tseng et al., 1999; Weismann et al., 2009). Very recently, it has been suggested that HPV in blood is attached to the outside of blood cells via a protein-containing moiety. HPV was detected on the
Our results suggest that CD4⁺ neutrophils but not on T-cells (Chen et al., 2009).

Our results suggest that CD4⁺ and CD8⁺ T-cells are the main circulating targets of the virus, indicating that they may represent the most important reservoir of active BPV-2 in blood. These BPV-infected T-cells appeared to be present in the initial stages of bladder tumours. In more advanced stages, monocytes and B-cells became infected, but >50% of positive cells were still composed of T-cell subpopulations. The weak fluorescence detected in PMNs suggested that these cells are not targeted by BPV-2. Although further studies are needed before drawing any definitive conclusions, the expression of E5 in monocytes and B-cells in animals with a longer history of urinary bladder tumours indicates that these cells might represent late targets of BPV-2. If so, the involvement of different subpopulations of PBMCs might reflect the clinical stage of the disease.

What is the biological significance of the presence of E5 oncoprotein in PBMCs? The presence of E5 oncoprotein in lymphocytes of 13/15 cattle suffering from urothelial bladder tumours has been reported previously (Roperto et al., 2008). No metastases were found in any of the examined animals. Therefore, the possibility that E5-containing PBMCs may play an actual, direct role in neoplastic events in distant organs is unlikely, as urothelial tumours of the urinary bladder in cattle are known to be characterized by a relatively low metastatic potential as described above.

We believe that active PV-containing PBMCs may be responsible for spreading the infectious agent to numerous organs rather than playing a direct role in neoplastic events in distant sites, as urothelial tumours of the urinary bladder in cattle are known to be characterized by a relatively low metastatic potential as described above.

Although our data clearly argue in favour of the reproductive activity of BPV-2 in infected blood cells, further studies evaluating the haematogenous dissemination of PVs are urgently needed, as a viraemic phase of the infection has been suggested but not confirmed, thus remaining a controversial issue (Castellsagué et al., 2009; Syrjänen, 2010). In this context, cattle may serve as an animal model and may continue to lead the way in studying the comparative biology of PVs. Much of our understanding of PVs, including their life cycle and other relevant aspects of pathogenesis, has benefited and may continue to do so from investigations on animal models (Borzacchiello et al., 2009; Roperto et al., 2010a).

Finally, the presence of structural L1 capsid proteins in PBMCs should be regarded as a marker of productive BPV-2 infection, thus opening novel scenarios in the epidemiology and pathophysiology of these challenging virus infections.

**METHODS**

**Ethics statement.** We did not perform any animal experimentation, as we collected blood samples when the prophylaxis campaign to eradicate brucellosis from large animal breedings, put in place according to the Italian legislation, was ongoing. We obtained additional blood as well as tissue samples directly from public slaughterhouses, as the four animals tested were slaughtered after a mandatory clinical ante-mortem examination, as required by the European Union legislation.

**Blood samples, immunoprecipitation of BPV-2 E5 oncoprotein, BPV-2 DNA detection and sequencing.** The peripheral blood samples we examined were collected by veterinarian colleagues who harvested these samples to perform serum investigation to eradicate brucellosis from cattle that had been clinically suffering from chronic enzootic haematuria for several years. All animals had been raised in hilly/mountain cattle households in the South of Italy and were known to have grazed on pastures rich in bracken fern. The presence of E5 oncoprotein in the liver, placenta and fetal organs of haematuric cows (S. Roperto, unpublished data).

DNA was extracted from the subpopulations of white blood cells from the four cows using a QIAamp DNA Mini kit (Qiagen).
according to the manufacturer’s instructions, as reported elsewhere (Balcos et al., 2008). The amplified DNA was subjected to direct sequencing in an automated apparatus (Bio-Fab).

**Bladder samples.** Samples of bladder neoplastic urothelium were collected at public slaughterhouses from the four haematuric 4–8-year-old cows. Bladder samples were routinely divided into several parts. One part was fixed in 10% neutral buffered formalin. The remaining parts were immediately frozen in liquid nitrogen and stored at −80 °C until utilized for specific molecular procedures.

**Histopathology.** Tissues fixed in 10% neutral buffered formalin were routinely processed for paraffin embedding. Histological diagnosis was assessed on 5 μm haematoxylin and eosin-stained sections using the morphological criteria suggested in the recent report on the new histological classification of urothelial tumours of the urinary bladder of cattle (Roperto et al., 2010a).

**Cell isolation and sorting.** Peripheral blood (50 ml) was collected in EDTA-containing tubes and submitted to centrifugation for 40 min at 1000 g. PMNs were isolated according to the method of Carlson & Kaneko (1973) after removal of the buffy coat and erythrocyte lysis. The purity and yield of recovered cells were evaluated using an XT2000IV automated haematology analyser (Sysmex) and microscopically after cytocentrifugation and May–Grünwald Giemsa staining.

Mononuclear cells were isolated by stratifying the buffy coat layer onto 3 ml Ficol-Paque (Amersham Biosciences), followed by centrifugation for 30 min at 1700 g. The upper ring containing mononuclear cells was isolated and counted, and a minimum of 4 x 10^6 cells in 400 μl was put in flow cytometry tubes for labelling. The following primary antibodies were used: CD4+ (T-helper cell, clone YKIX302.9), CD8+ (T-cytotoxic cell, clone YCATE55.9), CD21+ (B-cells, clone CA21D6) and CD14+ (monocytes, clone VPM65) (all from Serotec). After 30 min incubation at 4 °C and two washes with PBS, secondary rabbit anti-mouse IgG (Fab-specific) FITC-conjugated antibody (Serotec) was added. All antibodies had been titrated previously to determine the appropriate dilution.

CD4+, CD8+, CD21+ and CD14+ cells were sorted by FACS (BD FACSVantage; BD Biosciences). After isolation, cells were immediately frozen at −30 °C. A minimum sorting yield of 1 x 10^6 cells with >90% purity was considered suitable.

**Flow cytometry for E5 and L1.** Flow cytometry for E5 was performed on the blood samples from the four animals after lysis of 6 ml blood in 50 ml erythrocyte lysis buffer containing 8% ammonium chloride. The cell concentration was adjusted to 2 x 10^6 cells in 200 μl, and labelling for membrane antigens was performed as described above except for the use of a secondary rabbit anti-mouse IgG (Fab-specific) phycoerythrin-conjugated antibody (Serotec). For detection of intracellular E5 oncoprotein, cells were permeabilized using Fix&Perm (BD Biosciences) for 20 min at 4 °C and 5 μl polyclonal goat anti-E5 serum (a kind gift from Dr M. S. Campo, University of Glasgow, UK) was then added. After 30 min incubation and two washes in PBS, secondary donkey anti-goat IgG (Fab-specific) FITC-conjugated antibody (Serotec) was added and incubated for 30 min. All samples were analysed using a FACSCalibur flow cytometer (BD Biosciences). At least 10,000 positive events for each membrane antigen were collected and, for each cell population, green fluorescence from tubes labelled for E5 was compared with that of control tubes in which the anti-E5 antibody was omitted. The data were analysed using CellQuest software.

Flow cytometry for L1 protein was performed on blood samples using a single-colour approach. This was due to the fact that the available antibody for L1 (an intracellular protein) was a purified mouse IgG, and thus a double-colour approach would have required a first step of labelling for surface markers followed by indirect labelling for L1 after permeabilization of the cells. However, as the antibodies for surface lineage-specific antigens were also mouse IgG, a double-labelling protocol was difficult, due to the non-specific binding of secondary anti-mouse antibodies to the surface markers. In a preliminary step, a surface labelling was also performed by omitting the permeabilization procedure in order to determine whether the antigen was also present on the cell membrane; this gave a negative result. Cells were permeabilized as described previously and intracellular labelling was performed using a purified mouse anti-L1 IgG (clone BPV1-I188; Abcam). After 30 min incubation at 4 °C and two washes, secondary rabbit anti-mouse IgG (Fab-specific), FITC-conjugated antibody (Serotec) was added. Green fluorescence from tubes labelled for L1 was compared with those with an appropriate isotype control. Lymphocytes, monocytes and PMNs were gated according to scatter properties alone.

**Western blot analysis for E5 and L1.** Leukocytes were solubilized at 4 °C in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl and 1% Triton X-100. Immediately prior to use, the following reagents were added: 1 mM DTT, 2 mM PMSF, 1.7 mg aprotinin ml⁻¹, 25 mM NaF and 1 mM Na₂VO₄ (Sigma-Aldrich). Lysates were clarified at 50,000 g for 20 min. The protein concentration was measured using a Bradford assay (Bio-Rad Laboratories). For Western blotting, 50 μg lysate proteins was heated at 100 °C in 2× pre-mixed Laemmli sample buffer (Bio-Rad Laboratories). Proteins were subjected to SDS-PAGE (15% polyacrylamide) under reducing conditions.

After electrophoresis, the proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences) for 1 h at 10 V in 192 mM glycine, 25 mM Tris/HCl (pH 7.5), 10% methanol using a Trans-Blot SD Semi-Dry Transfer cell (Bio-Rad Laboratories) according to the manufacturer’s instructions. The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS, pH 7.5) for 1 h at room temperature and washed with TBS plus 0.1% Tween 20. The filters were then probed with anti-E5 antibody or anti-L1 antibody by overnight incubation at 4 °C. After three washes in TBS, the membranes were incubated with HRP-conjugated anti-sheep IgG (Santa Cruz Biotechnology) or anti-mouse IgG (Bio-Rad Laboratories), respectively, for 1 h at room temperature. After appropriate washing steps, the bound antibody was visualized using an enhanced chemiluminescence system (Immun-Star WesternC kit; Bio-Rad Laboratories).

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