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INFECTIOUS DISEASE

Pathology and Viral Antigen Distribution following Experimental Infection of Sheep and Goats with Capripoxvirus

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Summary

Current understanding of capripoxvirus pathogenesis is limited since there have been no detailed studies examining cell tropism at well-defined intervals following infection. We undertook time-course studies in sheep and goats following inoculation of sheeppox or goatpox viruses in their respective homologous hosts, and examined tissues by light microscopy. A monoclonal antibody generated to a sheeppox virus core protein was used for immunohistochemical detection of viral antigen in tissue sections. Lesions and virus antigen were observed consistently in the skin, lung and lymph nodes. Antigen was detected at 6 and 8 days post inoculation for skin and lung, respectively, within cells which appeared to be of monocyte/macrophage lineage. In sheep skin capripoxvirus immunoreactivity was detected within previously unreported large multinucleated cells. In the lung, double immunolabelling detected the simultaneous expression of capripoxvirus antigen and cytokeratin indicating the presence of virus within pneumocytes. Lung double immunolabelling also detected the expression of capripoxvirus antigen in $CD68^{+}$ cells, confirming the presence of viral antigen within macrophages. Based on early detection of infected macrophages, dissemination of virus within the host and localization to tissues likely occurred through cells of the monocyte/macrophage lineage. Histological findings revealed similarities with both monkeypox and smallpox, thus capripoxvirus infection in sheep and goats may represent useful models with which to study strategies for poxvirus-specific virus vaccine concepts and therapeutics.

Keywords: capripoxvirus; goat; immunohistochemistry; sheep

Introduction

Sheeppox, goatpox and lumpy skin disease viruses are the three members of the genus *Capripoxvirus* subfamily Chordopoxvirinae, family Poxviridae (Buller *et al.*, 2005). These viruses are indistinguishable from each other using serology since there is at least 96% identity at the genetic level between different capripoxvirus isolates (Tulman *et al.*, 2001, 2002). Sheeppox and goatpox viruses are endemic in Asia, the Middle East and Africa north of the equator (Babiuk *et al.*, 2008a). Capripoxviruses cause severe disease in their host species, characterized by fever and skin lesions (Bowden *et al.*, 2008). In addition, with severe disease there are characteristic pox lesions in the internal organs including the lungs and digestive tract. The disease can result in high morbidity and mortality depending on the virus isolate and host species. Although sheeppox and goatpox viruses generally display a host preference, some isolates can infect both species and the disease caused by the same isolate can vary dramatically between sheep and goats (Babiuk *et al.*, 2009).

Previous studies investigating the pathology of sheep and goatpox have been limited and focused primarily on the development of skin lesions (Plowright

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et al., 1959; Krishnan, 1968; Murray *et al*., 1973). A few studies have used immunohistochemistry (IHC) for examining tissues from lambs infected with sheeppox virus in the field (Gulbahar *et al*., 2000, 2006) and recently for skin lesions from cattle infected with lumpy skin disease virus (Babiuk *et al*., 2008b). However, detailed studies investigating cell tropism at well-defined intervals following infection are lacking. Therefore, this study used histopathology and IHC to examine tissues from experimentally infected sheep and goats that had been characterized previously by real-time polymerase chain reaction (PCR) and virus isolation (Bowden *et al*., 2008). Both polyclonal anti-capripoxvirus sera from experimentally infected sheep and goats as well as monoclonal antibodies specific for capripoxvirus have been used in IHC to characterize the tissue and cell tropism of sheeppox and goatpox viruses. Capripoxvirus-infected cells have a characteristic morphology with abundant vacuolated cytoplasm, vacuolated nuclei with marginated chromatin and often multiple cytoplasmic inclusion bodies. These cells are termed 'sheeppox cells' (SPCs) or 'cellules claveleuses' (Borrel, 1903). Based on light microscopy and electron microscopy these cells have been suggested to be monocytes, macrophages or fibroblasts, but not endothelial cells (Murray *et al*., 1973). This study used a double immunolabelling method to identify infected cells.

Materials and Methods

Animals and Tissues

Ten adult Suffolk-cross sheep and 10 adult Saanen or Alpine goats were housed in microbiologically secure (biosecurity level 3) animal rooms at the National Centre for Foreign Animal Disease (NCFAD), Winnipeg, Canada. One sheep and one goat were killed for collection of uninfected tissue samples and the remaining animals were then housed in groups of three until such time as additional animals were killed for necropsy examination and sample collection. Animals were fed a complete balanced diet and water *ad libitum* and all experimental procedures complied with Canadian Council on Animal Care guidelines.

Animals were inoculated intradermally, in a shaved area on the right chest, with either $10^{4.9}$ 50% tissue culture infectious doses ($TCID_{50}$) of Nigerian sheeppox virus (sheep) or $10^{4.4}$ $TCID_{50}$ of Indian goatpox virus (goats) obtained from the Institute for Animal Health (Pirbright Laboratory, Pirbright, Surrey, UK), as previously described (Bowden *et al*., 2008). At 4, 6, 8 and 10 days post-inoculation (dpi), one sheep and one goat were killed by barbiturate overdose and subjected to necropsy examination. Thereafter one additional sheep (at 13 and 15 dpi), one additional goat (at 11 and 13 dpi) and two additional goats (at 15 dpi) were also killed for necropsy examination. The remaining four animals were kept for long-term serum collection (Bowden *et al*., 2008).

Animals were anaesthetized by intramuscular administration of atropine sulphate (0.05 mg/kg; atropine injection; MTC Pharmaceuticals, Cambridge, Ontario, Canada) followed by intravenous administration of xylazine HCl (0.05–0.1 mg/kg; Rompun; Bayer, Toronto, Ontario, Canada) and ketamine HCl (5.0–10 mg/kg; Ketaset; Ayerst Veterinary Laboratories, Guelph, Ontario, Canada) and were then partially exsanguinated by jugular catheterization.

Histopathology and Immunohistochemistry

Tissues were fixed in 10% neutral phosphate buffered formalin and processed routinely. Sections were stained with haematoxylin and eosin (HE). For IHC, sections (5 μm) were air dried overnight and placed into a 60°C oven for 1 h. The dewaxed and rehydrated sections were quenched for 10 min in aqueous 3% H_2O_2 and rinsed in MilliQ water. For the monoclonal antibody, epitope retrieval was with high pH AR10 (Biogenex, San Ramon, California, USA) in a decloaking chamber (Biocare Medical, Walnut Creek, California, USA) and sections were then placed into Tris-buffered saline plus Tween (TBST) for 5 min. Sections were loaded from the TBST bath into a Dako autostainer (Dako, Carpinteria, California, USA) and incubated with F80G5 monoclonal antibody (NCFAD, Winnipeg), which was generated to *Escherichia coli*-expressed sheeppox virus ORF 057, a viral core protein, at a dilution of 1 in 1,500 for 1 h. The sections were then incubated for 30 min with Envision + anti-mouse (horseradish peroxidase (HRP) labelled) polymer kit (Dako) followed by a TBST rinse. 3,3′-diaminobenzidine (DAB; Dako) was used as the substrate chromogen and the slides were counterstained with Gill's haematoxylin.

For the polyclonal antibody, slides were pretreated with low pH Glyca (Biogenex) and incubated for 1 h with experimentally generated sera from sheep (diluted 1 in 1,000 in a 50/50 DaVinci Green/Background sniper solution; Biocare Medical) or goats (diluted 1 in 8,000 in TBST) followed by a TBST rinse. The sections overlaid with sheep antibody were incubated subsequently for 30 min with donkey anti-sheep IgG in 10% normal donkey serum (diluted 1 in 6,000; Jackson ImmunoResearch, West Grove, Pennsylvania, USA) followed by LSAB2 Streptavadin (Dako) for 10 min. The sections overlaid with

goat antibody were incubated for 30 min with goat HRP polymer kit (Biocare Medical) followed by a TBST rinse. DAB was used as the substrate chromogen and the slides were counterstained with Gill's haematoxylin. A negative isotype control (mouse IgG1; Dako) was run on a skin lesion from an infected goat.

For the poxvirus/cytokeratin double labelling, the slides were pre-treated with high pH AR10 (Biogenex). The monoclonal F80G5 was used as described above. After addition of the DAB, the sections were incubated with Denaturing Solution Kit (Biocare Medical) for 5 min to ensure that the second labelling procedure would not interfere with the first. An antibody specific for cytokeratins AE1/AE3 (Biogenex) was added for 10 min and the slides were rinsed with TBST and then incubated with Mach 2 (goat anti-mouse alkaline phosphatise conjugated) polymer kit (Biocare Medical) for 30 min. Development with Vulcan Fast Red substrate (Biocare Medical) and a Gill's haematoxylin counterstain completed the process.

For the CD68/poxvirus double labelling, slides were pre-treated with proteinase K (Dako) at room temperature for 15 min. Sections were incubated with monoclonal mouse anti-human CD68 (Clone EBM11; Dako), which reacts with the macrophage-specific marker CD68, at a dilution of 1 in 10 overnight at 4°C. Slides were then rinsed and incubated with Envision + anti-mouse (HRP labelled) polymer kit (Dako) for 30 min followed by a TBST rinse. DAB was used as the substrate chromogen. After the denaturing step, the F80G5 was run as previously described with a Mach 2 (alkaline phosphatase) polymer kit (Biocare) using Vulcan Fast Red (Biocare) as the substrate chromogen.

For caspase-3 immunolabelling, tissue sections were quenched for 10 min in aqueous 3% H_2O_2 and rinsed in MilliQ water. The slides were pre-treated with Dako Target Retrieval solution in a Biocare Medical decloaking chamber. The labelling was carried out using a Shandon sequenza unit (Thermo Scientific, Pittsburgh, Pennsylvania, USA). The primary antibody was a cleaved caspase-3 (Asp175) rabbit monoclonal antibody (Cell Signaling Technology Inc., Danvers, Massachusetts, USA) used at a dilution of 1 in 200 overnight at 4°C. The reaction was 'visualized' the following day using a HRP-labelled polymer, Envision® + system (anti-rabbit) (Dako) for 30 min and reacted with the chromogen (DAB). The sections were then counterstained with Gill's haematoxylin.

Results

Gross Pathology

Gross pathological changes in the animals in this study have been described previously (Bowden *et al.*, 2008) and consisted of typical pox lesions involving skin, mucosal surfaces, lungs, rumen and abomasum. Enlarged prescapular lymph nodes were observed as well as occasional small pale subcapsular lesions in the liver.

Histopathology and Immunohistochemistry

Results of histopathology and IHC for infected goats and sheep (excluding the inoculation site) are summarized in Tables 1 and 2, respectively. In general, lesions were more widely distributed in goats. Lesions were observed most consistently in the skin, lung and lymph nodes and were similar between sheep and goats. Results obtained using the polyclonal antisera and the monoclonal antibody F80G5 were similar, and the results presented here

Table 1
Microscopical lesions and antigen distribution in goats inoculated with Indian goatpox virus

DPI	*Skin* HE/IHC*	*Lung* HE/IHC	*LNsub*† HE/IHC	*LNpre*‡ HE/IHC	*Liver* HE/IHC	*Kidney* HE/IHC	*Rumen* HE/IHC	*Abom*§ HE/IHC	*Bladder*¶ HE/IHC
4	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−
6	+/1+	+/−	+/−	+/−	−/−	−/−	−/−	−/−	−/−
8	+/1+	+/1+	+/−	+/1+	−/−	−/−	−/−	−/−	−/−
10	+/2+	+/1+	+/1+	+/1+	−/−	−/−	+/1+	−/−	−/−
11	+/3+	+/3+	+/1+	+/1+	+/1+	+/−	−/1+	+/1+	+/1+
13	+/4+	+/3+	+/−	+/2+	+/2+	−/−	+/3+	−/−	+/−
15	+/4+	+/4+	+/2+	+/2+	+/2+	+/−	+/4+	+/3+	+/1+

DPI, days post-infection; −, negative; +, histological lesion; 1+, weak immunolabelling (<20 cells); 2+, mild immunolabelling (<25% of the section); 3+, moderate immunolabelling (25–50% of the section); 4+, abundant immunolabelling (>50% of the section).
*Presence of histological lesions/positive immunolabelling of goatpox viral antigen.
†Submandibular lymph node.
‡Prescapular lymph node.
§Abomasum.
¶Urinary bladder.

Table 2
Microscopical lesions and antigen distribution in sheep inoculated with Nigerian sheeppox virus

DPI	*Skin* HE/IHC*	*Lung* HE/IHC	*LNsub*† HE/IHC	*LNpre*‡ HE/IHC	*Liver* HE/IHC	*Kidney* HE/IHC	*Rumen* HE/IHC	*Abom*§ HE/IHC	*Bladder*¶ HE/IHC
4	−/−	−/−	−/−	ns	−/−	−/−	−/−	−/−	−/−
6	+/−	+/−	−/−	ns	−/−	−/−	−/−	−/−	−/−
8	+/1+	+/1+	−/−	+/−	−/−	−/−	−/−	−/−	−/−
10	+/2+	+/1+	+/−	ns	−/−	−/−	−/−	−/−	−/−
13	+/2+	+/−	+/−	+/−	−/−	−/−	−/−	+/1+	−/−
15	+/4+	+/3+	+/1+	+/1+	−/−	+/−	+/2+	+/2+	+/1+

DPI, days post-infection; −, negative; +, histological lesion; 1+, weak immunolabelling (<20 cells); 2+, mild immunolabelling (<25% of the section); 3+, moderate immunolabelling (25–50% of the section); 4+, abundant immunolabelling (>50% of the section); ns, not submitted.
*Presence of histological lesions/positive immunolabelling of sheeppox viral antigen.
†Submandibular lymph node.
‡Prescapular lymph node.
§Abomasum.
¶Urinary bladder.

are based on the labelling with the monoclonal reagent. Capripoxviral antigen distribution was closely associated with histopathological lesions in both sheep and goats.

Skin: At 4 dpi, inoculation site lesions consisted of widespread dermal accumulation of large round (histiocytic) and/or spindle-shaped cells with vacuolated nuclei and chromatin margination consistent with cells previously described as SPCs. There was mild epidermal hyperplasia. At the inoculation site at 6 dpi, epithelial hyperplasia was more severe (25 layers thick) and epithelial necrosis was present. A large proportion of epithelial cells were swollen and vacuolated and numerous cells contained multiple clearly demarcated basophilic cytoplasmic inclusions. The dermis was severely expanded with oedema and haemorrhage, perivascular SPCs, neutrophils and a few lymphocytes. In the deep dermis there were numerous SPCs as well as severe endothelial hypertrophy and mild necrotizing vasculitis.

Microscopical changes associated with secondary skin lesions were first observed at 6 dpi. In the earliest lesions there was focal epidermal hyperplasia with scattered vacuolated, necrotic keratinocytes within affected areas as well as in hair follicle epithelium. There were moderate perivascular and periadnexal mononuclear cellular infiltrates within the dermis. At 8 dpi there was diffuse epidermal hyperplasia with scattered vacuolated keratinocytes containing basophilic cytoplasmic inclusion bodies. There was dermal expansion due to oedema, haemorrhage and infiltration of histiocytic and spindle-shaped SPCs containing basophilic cytoplasmic inclusions. By 10 dpi there was severe hyperplasia of the epidermis with necrosis of the upper epidermal layers and vesicle formation and dermal expansion due to a heavy infiltrate of SPCs (Fig. 1A). At 13 and 15 dpi there was extensive necrosis of both the epidermis and dermis with evidence of vasculitis and occasionally thrombosis.

In both sheep and goats, viral antigen was detected at the inoculation site at 4 dpi within large histiocytic or spindle-shaped SPCs distributed diffusely throughout the dermis and occasionally in pericytes and glandular epithelium. By 6 dpi antigen was widespread throughout the epidermis and dermis of the inoculation site. Numerous positive SPCs were observed infiltrating vessel walls in the hypodermis (Fig. 1B).

In goats, positive immunolabelling for capripoxviral antigen was first observed in the secondary skin lesions at 6 dpi within the cytoplasm of perivascular macrophages and large spindle-shaped SPCs in the superficial dermis, as well as in a few epithelial cells within the stratum basale. For both sheep and goats at 11 dpi, viral antigen was detected extensively in the epidermis (primarily within the stratum basale and spinosum) as well as within the cytoplasm of histiocytic and spindle-shaped SPCs with a perivascular distribution in the dermis (Fig. 1C). No labelling was observed in the isotype negative control (Fig. 1D). At 13 and 15 dpi, a prominent feature was intense immunolabelling of SPCs within vessel walls. Virus antigen was also detected in pericytes and perineural cells as well as in SPCs throughout the dermis and extending between underlying muscle fibres. CD68 labelling was observed in numerous large cells throughout the dermis with SPC morphology, indicating that many of the SPCs were macrophages. However, in a significant number of SPCs with spindle morphology, CD68 labelling could not be detected, suggesting they were more likely to be fibroblasts.

One striking difference between the sheep and goats was the presence of large, spindle-shaped multinucleated syncytial cells with multiple basophilic intracytoplasmic inclusions throughout the dermis at 13 and

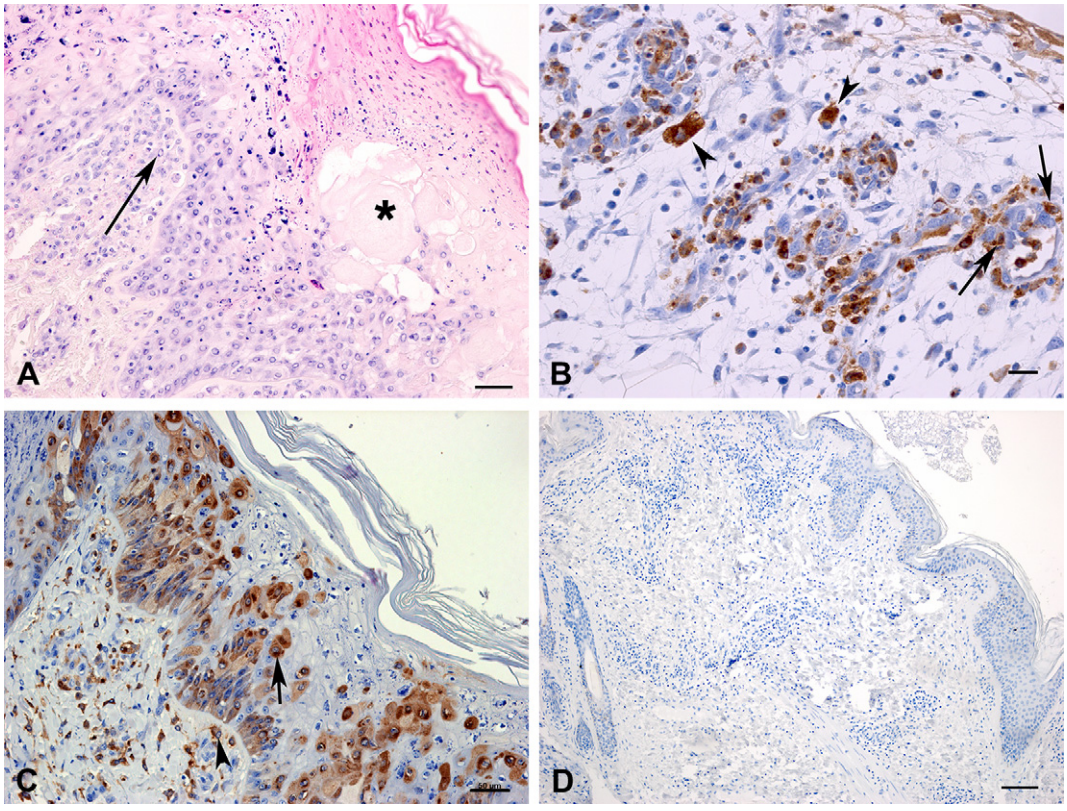

Fig. 1. Features of skin lesions in goats infected experimentally with capripoxvirus. (A) Severe epidermal hyperplasia with epidermal necrosis and vesicle formation (asterisk). The dermis is expanded by an infiltrate of mononuclear inflammatory cells and SPCs (arrow). HE. Bar, 50 μm. (B) Throughout the hypodermis, capripoxviral antigen is detected in numerous cells infiltrating vascular walls. Many of these appear to be macrophages (arrows). Note the large positively labelled SPCs (arrowheads). IHC. Bar, 20 μm. (C) Capripoxviral antigen in the cytoplasm of epidermal epithelial cells (arrow), as well as in SPCs throughout the dermis (arrowhead). IHC. Bar, 50 μm. (D) Skin lesion from an infected goat incubated with an isotype control antibody. IHC. Bar, 100 μm.

15 dpi in sheep skin, but not in goat skin (Fig. 2A). Positive immunolabelling for capripoxviral antigen was detected in the cytoplasm of these syncytial cells (Fig. 2B); however, these cells did not express CD68.

Lung: Lesions were first observed in the lungs at 6 dpi and consisted of mild, multifocal, lymphohistiocytic interstitial pneumonia. At 8, 10, 11 and 13 dpi there were multifocal well demarcated lesions

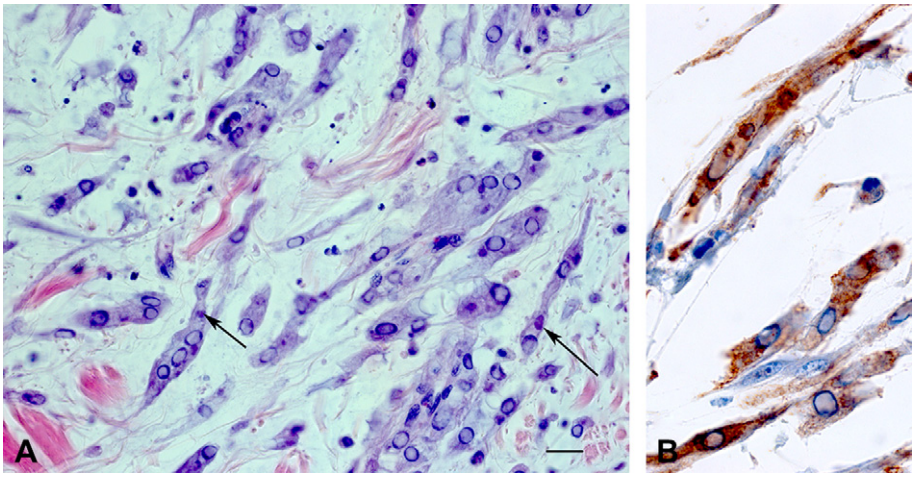

Fig. 2. Skin lesions at 15 dpi in sheep infected with capripoxvirus. (A) Within the dermis there are large spindle-shaped multinucleated cells which contain multiple basophilic intracytoplasmic inclusion bodies (arrows). HE. Bar, 20 μm. (B) Capripoxviral antigen (brown) within the cytoplasm of large multinucleated cells. IHC. Bar, 10 μm.

characterized by alveolar and septal oedema with infiltration by mixed mononuclear cells and SPCs (Fig. 3A). Many of these SPCs contained multiple basophilic intracytoplasmic inclusion bodies of varying sizes. Septal necrosis, type II pneumocyte hyperplasia, bronchiolar hyperplasia and vasculitis were also observed at later stages. By 15 dpi the lungs were diffusely affected with extensive necrosis, fibrin deposition, necrotizing bronchiolitis with epithelial intracytoplasmic inclusions and necrotizing vasculitis with thrombosis. In order to determine whether apoptosis played a role in the death of these cells, caspase-3 IHC was performed. Using this method, apoptotic cells in infected lung tissue were detected at the same level as uninfected control tissue (data not shown) and thus it is likely that the majority of the degenerating cells in infected lungs are undergoing necrosis.

Positive immunolabelling for capripoxviral antigen in both sheep and goats was first observed in the lungs at 8 dpi within the cytoplasm of a few perivascular and septal macrophages. At 11, 13 and 15 dpi, capripoxviral antigen was observed in numerous large spindle-shaped to histiocyte-like cells (SPCs) within the alveolar septae (Fig. 3B). Expression of CD68 was positive in numerous large SPCs within alveolar spaces and septa. Double immunolabelling with the anti-CD68 and F80G5 monoclonal antibodies confirmed the presence of virus within macrophages (Fig. 3C). Antigen was also detected within arteriolar smooth muscle cells (Fig. 3B) and bronchiolar epithelial cells. At 13 and 15 dpi, capripoxviral antigen was additionally detected in large cells lining the alveoli, which were identified as epithelial cells using a double labelling method and were interpreted to be hyperplastic pneumocytes (Fig. 3D).

Liver: Hepatic lesions were observed in goats, but not in sheep. These lesions were observed at 11, 13 and 15 dpi and consisted of a few scattered, well demarcated subcapsular and periportal lesions characterized by hepatocyte necrosis and loss with replacement by large spindle-shaped and round cells

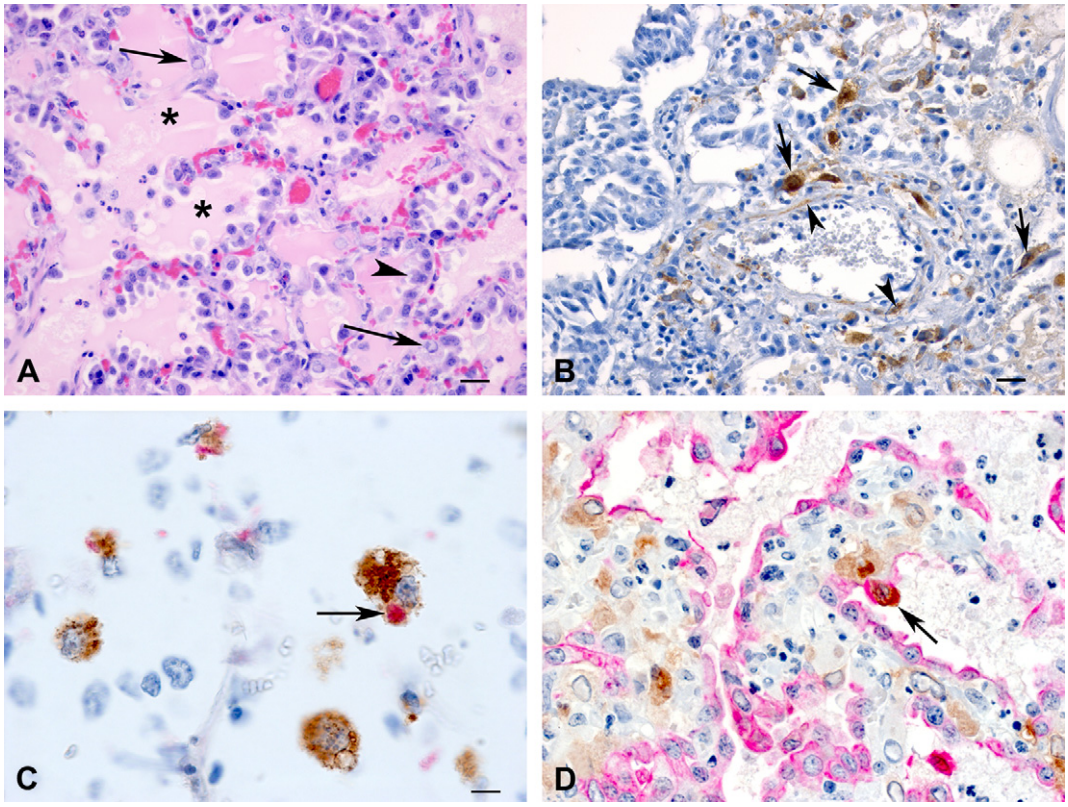

Fig. 3. Lungs from sheep infected with capripoxvirus. (A) SPCs were observed in alveolar walls and spaces (arrows). There is alveolar oedema (*) and type II pneumocyte hyperplasia (arrowhead) with infiltration by mononuclear inflammatory cells and scattered necrotic cells. HE. Bar, 20 μm. (B) Capripoxviral antigen in the cytoplasm of large spindle-shaped and histiocyte-like SPCs (arrows) as well as in pneumocytes and vascular smooth muscle cells (arrowheads). IHC. Bar, 20 μm. (C) Double immunolabelling showing simultaneous expression of CD68 (brown) and capripoxviral antigen (pink, arrow) within the same cell indicating the presence of capripoxviral antigen within macrophages. IHC. Bar, 10 μm. (D) Double immunolabelling showing simultaneous expression of cytokeratin (pink) and capripoxviral antigen (brown), within the same cell indicating the presence of capripoxviral antigen within hyperplastic pneumocytes (arrow). IHC. Bar, 10 μm.

consistent with SPCs as well as neutrophils (Fig. 4A). There was also evidence of biliary hyperplasia. In goat livers, antigen was detected at 11, 13 and 15 dpi and was limited to necrotic foci (Fig. 4B). Antigen was detected within SPCs as well as neutrophils based on morphology. It was difficult specifically to identify the SPCs in the liver; however, it appears that both macrophages and hepatocytes were involved based on morphology.

Lymph Node: Lymph node lesions most commonly affected the prescapular and submandibular nodes and occasionally the bronchial nodes. The earliest lesions (6, 8 and 10 dpi) included depletion of germinal centres and hyperplasia expressed as widening of the

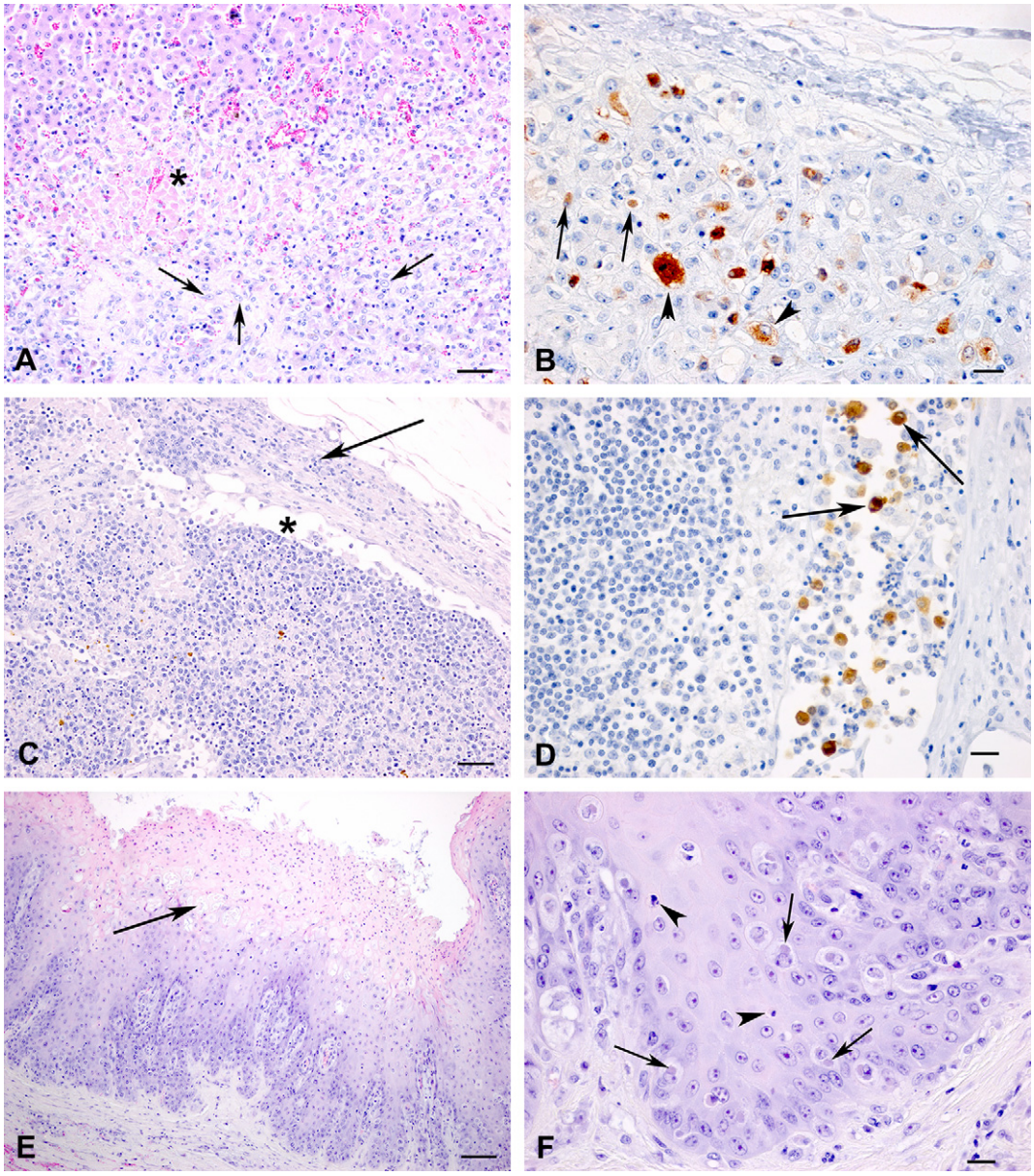

Fig. 4. Lesions at 15 dpi in goats infected with capripoxvirus. (A) Liver: there are multifocal areas of necrosis (*) with loss of hepatocytes in adjacent areas. Note the presence of cells with large vesicular nuclei consistent with SPCs (arrows). HE. Bar, 50 μm. (B) Liver: capripoxviral antigen in large SPCs (arrowheads) and neutrophils (arrows). IHC. Bar, 50 μm. (C) Lymph node showing extensive lymphocytolysis with subcapsular oedema (*) and capsulitis (arrow). HE. Bar, 50 μm. (D) Lymph node: viral antigen in macrophages (arrows) and neutrophils within the subcapsular sinus. IHC. Bar, 20 μm. (E) Rumen showing extensive epithelial hyperplasia with areas of ballooning degeneration of cells (arrow). The submucosa is expanded by oedema and inflammatory infiltration. HE. Bar, 100 μm. (F) Rumen. Higher magnification of (E) shows scattered individual cell necrosis (arrowheads) and cells with basophilic intracytoplasmic inclusion bodies (arrows). HE. Bar, 20 μm.

paracortex. At 11 dpi there was evidence of oedema and scattered lymphocytolysis. At 13 and 15 dpi, subcapsular sinuses contained moderate numbers of neutrophils and histiocytes and there were large multifocal areas of extensive lymphocytolysis affecting up to 50% of the node (Fig. 4C). Despite significant lesions observed within lymph nodes, positive immunolabelling was largely limited to clusters of macrophages within the subcapsular sinuses (Fig. 4D). At 15 dpi antigen was detected in macrophages and spindle cells within subcapsular proliferative inflammatory foci.

Rumen, Abomasum and Urinary Bladder: In the rumen, typical pox lesions were observed starting at 10 dpi as epithelial hyperplasia, spongiosis and necrosis, and progressed to severe oedema and cellular infiltration (SPCs and neutrophils) within the submucosa and underlying smooth muscle from then onwards (Fig. 4E). Numerous intracytoplasmic inclusion bodies were observed within epithelial cells (Fig. 4F). Antigen was first detected in the rumen at 10 dpi within small foci of degenerating epithelial cells. By 11 dpi there were clusters of positive SPCs within the lamina propria. At 15 dpi there was extensive immunoreactivity within the epithelium and the positive SPCs were abundant and widespread throughout the lamina propria, submucosa, within connective tissue separating smooth muscle bundles and extending to the serosal surface. Positive immunolabelling was also observed within the vascular smooth muscle and pericytes. Abomasal lesions consisted of severe expansion of the submucosa by oedema and a cellular infiltrate consisting of round and spindle-shaped SPCs as well as lymphocytes and plasma cells. Antigen-positive SPCs were observed in discrete inflammatory foci within the mucosa and submucosa of the abomasum at 11 and 15 dpi. Urinary bladder lesions were limited to serosal surfaces and underlying smooth muscle and consisted of oedema and infiltration of large SPCs. Within the urinary bladders of both sheep and goats, virus antigen was detected in SPCs distributed throughout the serosa and within connective tissue separating underlying smooth muscle bundles with primarily a perivascular distribution. Antigen was also detected within the smooth muscle fibres.

Other: Although a few small kidney lesions were observed in both sheep and goats, these consisted primarily of a non-specific mononuclear interstitial nephritis. These lesions were not consistent with poxvirus viral infection and no viral antigen was detected by IHC. No lesions or antigen were observed in the spleen, gallbladder, duodenum, jejunum, ileum, cecum, rectum, colon, oesophagus, nasal cavity, pancreas, salivary gland, tonsil or heart.

Discussion

The present study was conducted to examine the development and progression of microscopical lesions in association with the presence of capripoxviral antigen in multiple tissues at specific time points *post infection* in experimentally inoculated animals. For both sheep and goats, the most prominent histological lesions were observed in the skin, lung, lymph nodes and gastrointestinal tract. This is similar to observations made in previous studies involving natural (Gulbahar *et al*., 2006) and experimental (Plowright *et al*., 1959; Ramachandran, 1967; Krishnan, 1968; Murray *et al*., 1973) infections. However, studies using IHC to detect the presence of viral antigen have only examined field cases and thus information provided regarding early pathogenesis was limited (Gulbahar *et al*., 2000, 2006). In the present study, in both sheep and goats, abundant antigen was detected at the inoculation site as early as 4 dpi within dermal accumulations of SPCs, which were identified primarily as macrophages. At 6 dpi in the inoculation site, numerous positive SPCs were observed infiltrating vessel walls in the hypodermis and cytoplasmic viral inclusion bodies were observed. These data show that the viral antigen is found in macrophages at early time points and supports the suggestion by Bowden *et al*. (2008) that the viruses are disseminated through infected monocytes and macrophages to the systemic circulation with localization to skin and other tissues. The presence of infectious virus in whole blood from experimentally infected sheep and goats has been detected in previous experiments (Bowden *et al*., 2008; Babiuk *et al*., 2009) and for sheeppox, viraemia is known to be cell-associated (Kitching and Taylor, 1985).

A similar pathogenesis, where virus is disseminated by means of a monocyte-associated viraemia, is observed in experimental infection of monkeys with either variola virus (Jahrling *et al*., 2004) or monkeypox virus (Zaucha *et al*., 2001). In the present study, the earliest secondary lesions appeared in the skin at 6 dpi and in the lung at 8 dpi, and antigen was detected primarily in perivascular macrophages. It seems likely based on these data, as well as viral infectivity data (Bowden *et al*., 2008), that there was inoculation site seeding of the skin and lung, followed by later involvement of other organs. Extensive lesions and large amounts of viral antigen detected in skin and lungs suggest that these organs served as targets for viral replication.

In a previous immunohistochemical study (Gulbahar *et al*., 2006), positive labelling of poxvirus antigen was only observed in SPCs and degenerate

epithelial cells in the skin and the respiratory and gastrointestinal tracts. In the present study, antigen was additionally detected in pericytes, perineural cells, fibroblasts and vascular smooth muscle cells within the skin and within the respiratory and digestive tracts. This wide demonstration of viral antigen within mesenchymal cell types is similar to what was observed with experimental variola virus infection of monkeys, which suggested widespread permissive infection and cell-to-cell spread through connective tissues of different organs (Chapman *et al*., 2010). Of interest was the absence of lesions and viral antigen in both the liver and spleen in the early stages of infection in both sheep and goats. This differs from what is described with ectromelia virus in mice (mousepox), in which there is extensive replication and necrosis in liver and spleen followed by seeding of other tissues (Fenner and Buller, 1997). At later stages in goats (11–13 dpi), there were scattered areas of hepatocyte necrosis, but these lesions were mild. This pattern is more similar to what has been observed in human smallpox, where although there is seeding of other tissues such as the liver, spleen and lymphoid tissues, variola virus does not appear to replicate extensively enough to cause significant organ damage (Fenner *et al*., 1988). Similarly, in a study examining the pathology of experimental aerosolized monkeypox in cynomolgus monkeys, there was no evidence of monkeypox-associated hepatic changes before 13 dpi (Zaucha *et al*., 2001).

In lymph nodes, antigen detection was limited to a small number of macrophages within the subcapsular sinuses in both sheep and goats infected experimentally with capripoxvirus, despite extensive necrosis in later stages. This is in contrast to what was observed with experimental infection of monkeys with monkeypox virus, in which lymph nodes with diffuse necrosis showed antigen distribution throughout all regions of the node (Zaucha *et al*., 2001). One possible explanation for this is that the lesions in the lymph nodes are immunological or viral mediator-induced rather than them representing a site of virus replication.

The animals examined in this study showed extended virus shedding in nasal secretions up to 64 dpi and 41 dpi for sheep and goats, respectively (Bowden *et al*., 2008), and it was hypothesized that replication and shedding might occur at specific mucosal sites without lesion development. We examined multiple sections of nasal mucosa that did not contain lesions and were unable to detect antigen in these tissues; however, this could be related to the sampling of very small areas.

Immunolabelling with the macrophage marker CD68 revealed many of the large SPCs throughout the skin and lung to be of monocyte/macrophage lineage. In a previous study SPCs identified as macrophages were suspected to be infected. However, attempts to use double immunolabelling to phenotype the infected cells were unsuccessful (Gulbahar *et al*., 2006). In the present study, SPCs were confirmed to be primarily macrophages in the dermis and macrophages and epithelial cells in the lung, using a double immunolabelling method. There were also many other large cells in the dermis, similar to SPCs, which were more spindle-shaped in morphology. These were suspected to be fibroblasts based on their morphology; however, attempts to identify these cells immunohistochemically were unsuccessful due to lack of available antibodies.

The large syncytial cells that were observed in the sheep skin have not been reported previously and immunohistochemical analysis revealed that these cells contained viral antigen. The formation of syncytial cells occurs with certain poxvirus infections as a result of intercellular fusion (Buller and Palumbo, 1991). A recent review of poxvirus entry and membrane fusion concluded that fusion is mediated by a newly recognized group of viral protein components of the mature virion membrane and that the same group of entry/fusion proteins appear to be required for vaccinia virus-induced cell–cell fusion (Moss, 2006). With some viruses such as measles, inducing the formation of syncytia is a mechanism of viral cell-to-cell spread (Flint *et al*., 2009). Although vaccinia virus induces syncytia, the role that they play in the spread of the virus is unclear. Since the syncytia are thought to form between cells that are each already infected rather than between infected and uninfected cells, this suggests they do not contribute significantly to viral spread (Norkin, 2010). The mechanism of viral entry and spread has not been specifically investigated for capripoxviruses. The finding of syncytial cells within skin lesions suggests that it is likely that capripoxviruses employ fusion as a mechanism for viral entry, similar to what is seen for vaccinia virus, and could also indicate a mechanism of viral spread.

This description of the distribution of histological lesions and viral antigen in sheep and goats infected experimentally with Nigerian sheeppox and Indian goatpox, respectively, showed the range of affected organs and cells to be more widespread than previously reported. Early detection of infected macrophages indicates they are likely to play a role in the dissemination of the virus within the host. Infected SPCs were identified as macrophages and epithelial cells. Histological and immunohistochemical findings revealed similarities in monkeypox and smallpox pathogenesis. With regards to human orthopoxvirus infection, there is no single animal model that summarizes all known

aspects of the disease (Chapman *et al*., 2010) and, although capripoxviruses are not closely related to orthopoxviruses, capripoxvirus infection in sheep and goats may represent a useful model for the study of strategies of smallpox virus vaccine concepts and therapeutics.

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