Distribution of viral antigen in tissues of new-born lambs infected with Rift Valley fever virus

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ABSTRACT

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The distribution of Rift Valley fever (RVF) viral antigen was studied by immunohistochemistry in the liver, spleen, prescapular lymph node, lungs and kidneys of eight experimentally infected new-born lambs and in four new-born lambs that died of RVF during the 1974–75 RVF epidemic. The eight experimentally infected lambs were euthanazed at 6, 12, 18, 24, 30, 33, 48 and 51 h post-infection (p.i.), respectively. Immunohistochemical staining utilized polyclonal hyperimmune mouse ascites fluid to RVF virus and peroxidase-diaminobenzidine as substrate.

Virus antigen was most prominent in the liver and was detected as early as 18 h p.i. in the cytoplasm of hepatocytes that were sparsely scattered throughout the lobules. At 24–33 h p.i., antigen was also present in or adjacent to small foci of hepatocellular necrosis. At 48–51 h p.i. and in one of the field cases, positive staining was widespread and most consistently present in the cytoplasm of large numbers of degenerated or necrotic hepatocytes and in a few acidophilic bodies. Immunohistochemical staining was rarely observed in hepatocyte nuclei. Almost diffuse histochemical staining was observed in disintegrated cells and in the cytoplasm of necrotic hepatocytes throughout the liver in the other three field cases with pannecrosis; only the primary foci of necrosis and a narrow periportal rim of intact hepatocytes did not stain. No staining was observed in bile duct epithelium, endothelial and Kupffer cells in the initial stages of infection, supporting the contention that hepatocytes constitute the primary site of RVF virus replication in new-born lambs. Few cells stained positively in the spleen, prescapular lymph node, lungs and kidneys.

Keywords: Immunohistochemistry, lambs, liver necrosis, pathogenesis, Rift Valley fever virus, sheep

INTRODUCTION

Rift Valley fever (RVF) is a peracute or acute disease mainly of domestic ruminants in Africa, caused by a mosquito-borne phlebovirus of the family Bunyaviridae. The disease is most severe in sheep, resulting in a 80–95% mortality rate in new-born lambs and a high percentage of abortions in pregnant ewes. Rift Valley fever usually follows above-average rainfall and the subsequent increases in the number of mosquito vectors. Major epidemics of RVF occurred in South Africa in 1950–1951 and 1974–1975, and limited outbreaks of the disease and sporadic isolation of RVF virus were recorded at irregular intervals during the intervening years and in 1981 (Meegan & Bailey 1989; Swanepoel & Coetzer 1994).

Detailed studies of the pathology of RVF in new-born lambs, including a description of the sequential development of the liver lesions, have been published

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(Coetzer & Ishak 1982; Coetzer, Ishak & Calvert 1982; Daubney, Hudson & Garnham 1931; Weiss 1957; Easterday 1965; Coetzer 1977). Hepatic necrosis is the most striking finding in animals that died of the disease, but lesions are also present in other organs, including the spleen, lymph nodes, kidneys, lungs, adrenal glands and digestive tract.

The immunohistochemical detection of viral antigens in formalin-fixed tissues is useful in retrospective and pathogenetic studies, and to confirm a diagnosis where isolation of the causative agent is not possible. This method was recently used to detect RVF virus antigens in calves with encephalomyelitis and hepatitis (Rippy, Topper, Mebus & Morrill 1992). This study documents the distribution of RVF virus antigen in various organs, of experimentally and naturally infected new-born lambs, with emphasis on the hepatic lesions.

MATERIALS AND METHODS

Experimental and field cases

Tissues of 12 Merino lambs, 1–4 d old, infected with RVF virus were studied: eight lambs (lambs 1–8; Table 1) were experimentally infected and four lambs (lambs 9–12; Table 1) were field cases randomly selected from the files of the Section of Pathology, Onderstepoort Veterinary Institute. The tissues of two uninfected, 3–4 d-old Merino lambs (lambs 13, 14; Table 1) served as controls.

The experimental cases of RVF were those used for studies of sequential development of the liver lesions in RVF (Coetzer & Ishak 1982; Coetzer et al. 1982). The lambs were inoculated subcutaneously with 0,5 mℓ of an unpassaged field strain of RVF virus (titre $10^{7,5}$ MLD_{50}/0,25 mℓ) (Coetzer & Ishak 1982). The lambs were euthanazed by intravenous overdosages of barbiturates at predetermined times after inoculation (6, 12, 18, 24, 30, 33, 48 and 51 h; Table 1). The lamb killed at 51 h p.i. was in extremis. The field cases died during the 1974–1975 RVF epidemic. The diagnosis in these animals was confirmed by virus isolation and serum neutralization (Coetzer 1977).

Histopathology

At necropsy, tissues were collected in 10 % buffered formalin for histopathology. Tissues were routinely processed and 4–6 μm sections were stained with haematoxylin and eosin (HE).

Immunohistochemistry

An avidin-biotin complex (ABC) immunoperoxidase technique, similar to that previously described for the detection of Wesselsbron disease virus (Van der Lugt, Coetzer, Smit & Cilliers 1995), was used for immuno-histochemical staining of tissue sections. Briefly, 6 μm sections of the liver, spleen, prescapular lymph node, lungs and kidneys from each lamb were mounted on slides coated with 3-aminopropyltriethoxysilane, deparaffinized and hydrated. Endogenous peroxidase activity was quenched with 3 % H_2O_2 in methanol for 30 min, followed by a wash in phosphate-buffered saline (PBS) containing 0,1 % bovine serum albumin. The slides were then treated with 0,05 % protease XIV (37 °C for 15 min), and hyperimmune mouse ascitic fluid to RVF virus (1:500) was used as primary antibody. The ascitic fluid was prepared as described previously (Sartorelli, Fischer & Downs 1966; Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Shepherd, Hummitzsch, Erasmus & Barnard 1986). Biotinylated anti-mouse antibody (Dako), diluted to 1:500, and an avidin-biotin complex peroxidase solution, prepared according to the manufacturer's directions (Vector Laboratories), were then applied to the sections. Following this, sections were incubated with the chromagen 3,3'-diaminobenzidine 4HCl (DAB) (0,03 % in PBS with 0,006 % H_2O_2) for 8 min, and counter-stained with Mayer's haematoxylin.

To determine the specificity of immunohistochemical staining, liver sections were reacted with hyperimmune mouse ascitic fluid to Wesselsbron disease virus (Sartorelli et al. 1966; Swanepoel et al. 1986) and non-immune rabbit serum, and PBS was substituted for the primary antibody. The detection of RVF antigen by means of hyperimmune ascitic fluid was established initially with tissues from the lamb euthanazed at 48 h p.i. and one of the field cases. The ascitic fluid was used at dilutions of 1:10, 1:100, 1:500 and 1:1 000.

RESULTS

Histopathologic findings

Liver

A detailed description of the microscopic hepatic lesions of the new-born lambs used in this study has been reported previously (Coetzer 1977; Coetzer & Ishak 1982). The histopathology of lambs 1–12 is briefly reviewed and summarized in Table 1 to compare the localization of virus to the morphological lesions.

At 6 h p.i., the hepatocellular changes were subtle and comprised increased cytoplasmic eosinophilia of a few scattered hepatocytes and the presence of a few neutrophils in the sinusoids. Slightly more advanced lesions, characterized by individual necrotic hepatocytes and occasional acidophilic bodies in the liver cell plates, spaces of Disse and sinusoids, were evident at 12 h and 18 h p.i. (Fig. 1).
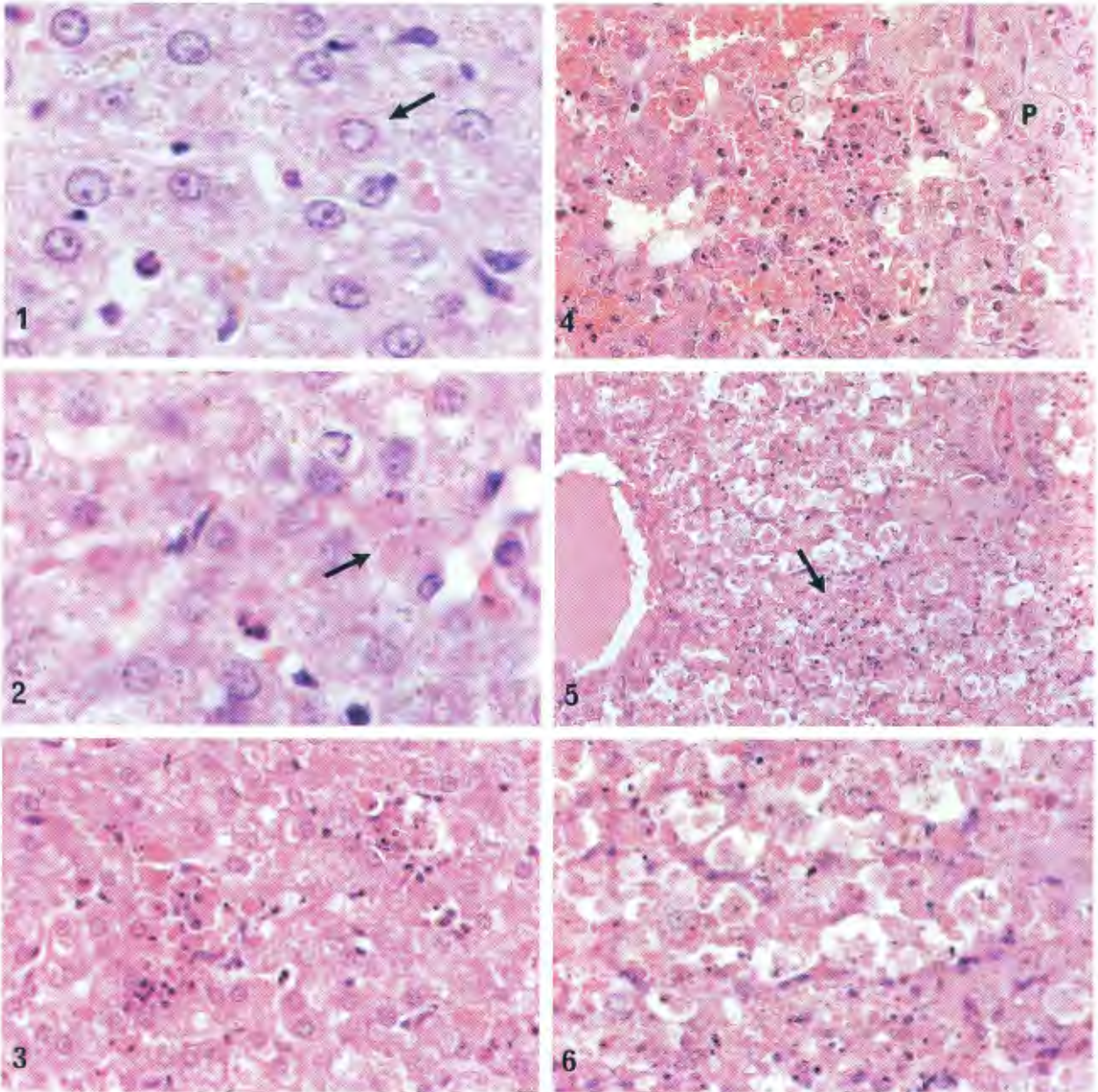
FIG. 1   Liver; lamb 3 (18 h post-inoculation). New-born lamb experimentally infected with a field strain of Rift Valley fever virus. Necrosis of a single hepatocyte (arrow) and a few neutrophils in sinusoids.
HE X 700

FIG. 2   Liver; lamb 4 (24 h post-inoculation). Slightly more advanced lesions characterized by a small focus of necrosis (arrow) as well as scattered necrotic hepatocytes in the remainder of the parenchyma.
HE X 700

FIG. 3   Liver; lamb 5 (30 h post-inoculation). Randomly distributed foci of necrosis infiltrated by neutrophils, and acidophilic bodies distributed in parenchyma.
HE X 350

FIG. 4   Liver; lamb 7 (48 h post-inoculation). Multiple, coalescing foci of necrosis and haemorrhage. Periportal hepatocytes are degenerated (P).
HE X 350

FIG. 5   Liver; lamb 8 (51 h post-inoculation). More advanced lesions evidenced by widespread hepatocellular necrosis with sparing of few hepatocytes in periportal areas (on the right). Residual primary focus of necrosis is discernible (arrow).
HE X 300

FIG. 6   Liver; lamb 8 (51 h post-inoculation). Higher magnification to show severe necrosis and disintegration of hepatocytes in the lobules.
HE X 500

TABLE 1 Major histological lesions and immunohistochemical results in the liver of experimentally and naturally infected new-born lambs with Rift Valley fever virus

Lamb no.	Hours post-inoculation	Major histological lesion in the liver	Antigen staining in the liver*
1	6	Degeneration of isolated hepatocytes	0
2	12	Degeneration and necrosis of isolated hepatocytes	0
3	18	Degeneration and necrosis of isolated hepatocytes	+
4	24	Randomly distributed foci of necrosis	++
5	30	Randomly distributed foci of necrosis	++
6	33	Randomly distributed foci of necrosis	++
7	48	Extensive necrosis	+++
8	51	Extensive necrosis	+++
9	Natural case	Extensive necrosis	++++
10	Natural case	Pannecrosis	++++
11	Natural case	Pannecrosis	++++
12	Natural case	Pannecrosis	
13	Control	No lesions	0
14	Control	No lesions	0

*+ = positive staining of isolated hepatocytes
++ = positive staining of isolated hepatocytes and foci of necrosis
+++ = positive staining of large number of hepatocytes in lobules
++++ = positive staining of hepatocytes virtually throughout lobules
0 = no detectable virus-specific staining

At 24 h, 30 h and 33 h p.i., numerous single necrotic hepatocytes, some acidophilic bodies, as well as multiple, randomly distributed foci of necrosis (primary foci) involving groups of approximately 5–10 hepatocytes, were seen in the lobules (Fig. 2 and 3). Most of these foci were infiltrated by a few to moderate numbers of neutrophils and macrophages. Mild Kupffer cell proliferation was present throughout the lobules.

Widespread hepatocellular necrosis was present at 48 h p.i and in the lamb that was killed *in extremis* at 51 h p.i. Multiple, coalescing foci of necrosis, containing dense aggregates of cytoplasmic and nuclear debris, acidophilic bodies, fibrin, neutrophils, macrophages and red blood cells, were randomly distributed in the lobules (Fig. 4–6). The majority of hepatocytes in the remaining parenchyma were degenerated or necrotic with only narrow collars of viable hepatocytes remaining periportally. Eosinophilic, oval to slightly elongated, intranuclear inclusion bodies were occasionally observed in hepatocytes. Some central veins and portal vessels contained a few necrotic hepatocytes and cytoplasmic fragments.

In one field case (lamb 9), lesions were similar to those in the lambs killed at 48 h and 51 h p.i. In the three other field cases (lambs 10–12), the lesions were more severe and extensive with necrosis or disintegration of hepatocytes virtually throughout the entire liver (pannecrosis).

Other organs

Lymphoid necrosis of variable extent, characterized by nuclear pyknosis and karyorrhexis, occurred throughout the cortex and medulla of the lymph nodes of four animals (lambs 7, 10–12). The follicles were depleted of lymphocytes in lambs 10 and 11. Scattered lymphoid necrosis was seen in the white and red pulp of the spleen in six lambs (lambs 7, 8, 9–12) and neutrophils accumulated in the red pulp in five lambs (lambs 2, 4, 5, 7, 8). Five lambs (lambs 4, 6, 9, 11, 12) showed congestion and oedema of the lungs, and mild interstitial pneumonia accompanied by multifocal necrosis in alveolar walls and peribronchial lymphoid tissue. Scant necrosis of mesangial cells occurred in the renal glomeruli in lamb 10.

Immunohistochemical findings

Liver

The immunohistochemical findings in the livers of the experimentally infected and field cases of RVF are summarized in Table 1. Viral antigen was first detected at 18 h p.i. in individual viable hepatocytes (approximately five to eight positive cells per section) scattered throughout the parenchyma (Fig. 7). The staining pattern in the cytoplasm of these hepatocytes was diffuse and fine to coarse, granular brown.

At 24 h, 30 h and 33 h p.i., intense staining was consistently present in the cytoplasm, and occasionally in the nucleus of degenerated and necrotic hepatocytes and in cytoplasmic debris in or adjacent to necrotic foci. Viral antigen was also detected in the cytoplasm of single and small groups of degenerated hepatocytes in the remainder of the lobules, and in a few acidophilic bodies (Fig. 8 and 9).

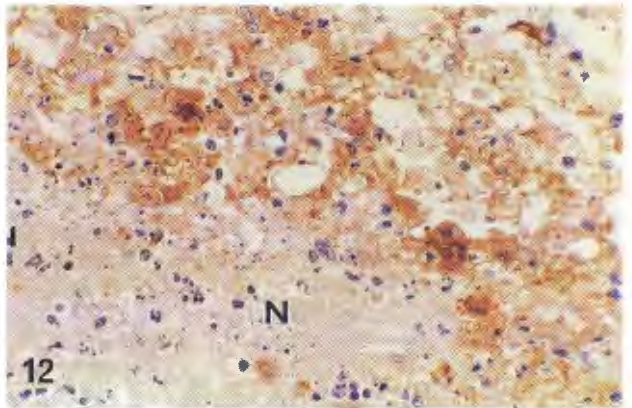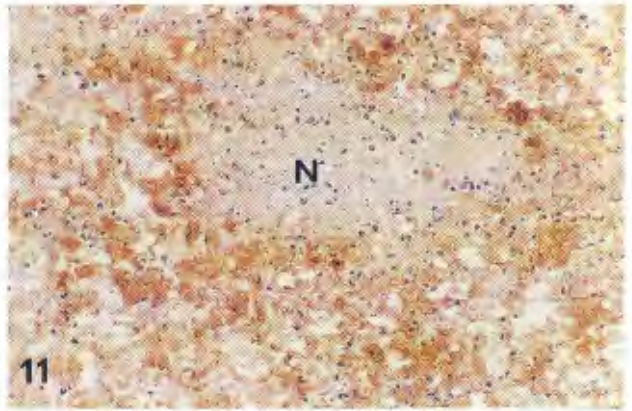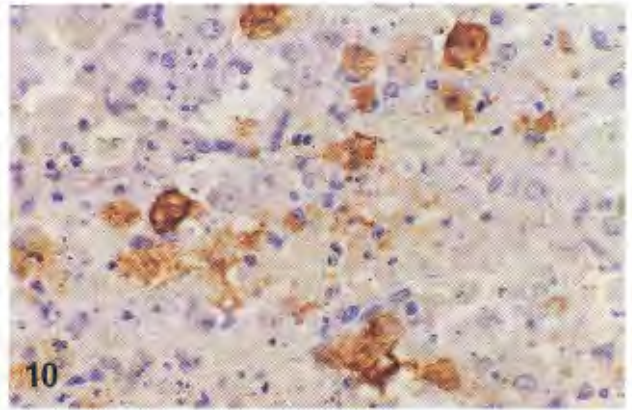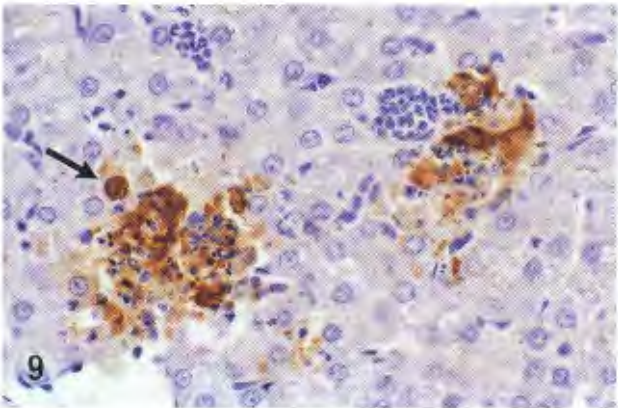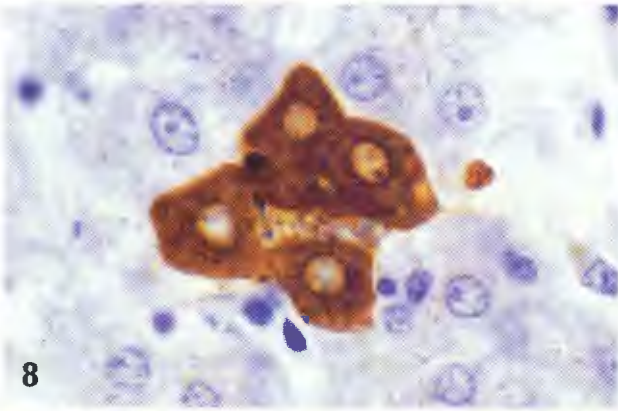At 48 h and 51 h p.i., distinct positive staining of degenerated and necrotic hepatocytes and some of
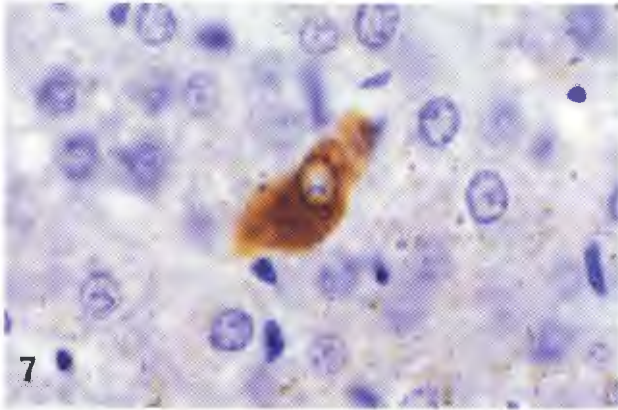
FIG. 7   Liver; lamb 3 (18 h post-inoculation). Rift Valley fever virus antigen is diffusely present in the cytoplasm of a viable, isolated hepatocyte. Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 700

FIG. 8   Liver; lamb 4 (24 h post-inoculation). Note diffuse cytoplasmic Rift Valley fever viral antigen in a small group of hepatocytes not associated with necrosis. Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 700

FIG. 9   Liver; lamb 5 (30 h post-inoculation). Positive staining in the cytoplasm of degenerated or necrotic hepatocytes, an occasional acidophilic body (arrow) and cytoplasmic debris in and adjacent to necrotic foci. Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 350

FIG. 10   Liver; lamb 8 (51 h post-inoculation). Rift Valley fever viral antigen staining in large numbers of degenerated and necrotic hepatocytes and in cellular debris. Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 500

FIG. 11   Liver; lamb 11 (field case). Positive staining almost diffusely throughout the lobule. Note that staining is absent in a primary focus of necrosis (N). Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 350

FIG. 12   Liver; lamb 11 (field case). Higher magnification to illustrate positive staining of hepatocytes and cellular debris in a lobule sparing a primary focus of necrosis (N). Avidin-biotin-peroxidase complex method. Mayer's haematoxylin counter-stain X 500

the acidophilic bodies occurred throughout the lobules (Fig. 10). However, periportal hepatocytes, which were generally not necrotic, and primary foci of necrosis, did not show noteworthy positive staining. Rarely, indistinct, pale brown staining was present in the nuclei of viable or necrotic hepatocytes. Cytoplasmic fragments of necrotic hepatocytes, that were present in the lumen of a few central veins and portal blood vessels, also stained positive.

In one of the field cases (lamb 9), positive staining was similar in distribution and severity to that reported in lambs euthanazed at 48 h and 51 h p.i. In the other three field cases (lambs 10–12), positive staining was less intense than that in lambs 7–9, but occurred almost diffusely throughout the lobules in the cytoplasm of necrotic and disintegrated hepatocytes. Hepatocytes in the periportal areas and the primary foci of necrosis (Fig. 11 and 12) remained unstained. A small number of macrophages in portal areas contained intracytoplasmic antigen.

In all the cases, the bile duct epithelium did not contain viral antigen. Endothelial cells and Kupffer cells in animals in the early stages of infection (6–33 h p.i.; lambs 1–6) were also negative, while localization of viral antigen in cells other than hepatocytes in lambs 7–12 was difficult to determine owing to extensive cellular necrosis in the lobules.

Other organs

Scattered individual cells, probably lymphoid cells, stained weakly positive in the splenic red pulp in three animals (lambs 8, 10 and 12) and in the cortex, paracortex and medullary cords of the lymph nodes in lamb 10 and 12. Some of these antigen-containing cells were necrotic. Small numbers of unidentified cells in alveolar walls and alveolar macrophages in the lungs of lambs 11–13 contained clumps of virus-specific, coarsely granular material. Viral antigen was also found in a few intravascular monocytic cells in the lungs of these three field cases. In the renal glomeruli of lambs 7 and 8, coarse antigen-specific staining was evident in a small number of mesangial cells.

Positive staining was not detected in sections from the control animals or in the control sections.

DISCUSSION

New-born lambs are highly susceptible to RVF (Swanepoel and Coetzer 1994), with infection resulting in very high mortalities and severe but characteristic liver lesions (Coetzer 1977; Coetzer & Ishak 1982). The cytopathic changes in target cells are considered to be directly virus-induced (Peters, Liu, Anderson, Morrill & Jahrling 1989). Hepatic lesions progress from degeneration and necrosis of individual hepatocytes with the formation of acidophilic bodies (6–18 h p.i.) to coagulative necrosis of small groups of hepatocytes (primary foci) throughout the lobules at 24–33 h p.i. Lambs that are in extremis or die of the disease, generally show extensive necrosis or pannecrosis with residual primary foci still discernible throughout the liver.

The present findings indicate that RVF virus antigen occurs primarily in the liver, and that the amount of antigen increases progressively, reaching its maximum at the time of death. Immunohistochemical staining of varying intensity of individual or small groups of degenerated or necrotic hepatocytes was first detected 18–33 h p.i. In two lambs euthanazed later or in extremis, and in one field case, virus antigen was widely present in affected hepatocytes throughout the lobules. In three other field cases that died of the disease, staining occurred diffusely throughout the liver. The fact that staining of primary foci of necrosis became less intense during late and terminal stages of the disease, may be explained by the release of antigen from completely destroyed cells.

Staining occurred diffusely in the cytoplasm of affected hepatocytes, and was rarely observed in nuclei, where the antigen probably corresponds to a non-structural protein (NSs) which is synthesized during RVF virus replication, and which is presumed to form the intranuclear inclusions seen histologically (Coetzer 1977; Swanepoel & Blackburn 1977; Struthers & Swanepoel 1982; Struthers, Swanepoel & Shepherd 1984).

The lack of immunohistochemical staining observed in bile duct epithelium, endothelial and Kupffer cells in the initial stages of infection, supports the contention that hepatocytes constitute the primary site of RVF virus replication in new-born lambs (Coetzer et al. 1982); which corresponds with the conclusion reached in experimentally infected rats (Peters & Anderson 1981; Anderson, Slone & Peters 1987). Few cells were observed to stain immunohistochemically in the spleens, lymph nodes, lungs and kidneys in some of the lambs. Although the precise identity of the cells involved in the spleen and lymph nodes could not be determined, they resembled lymphoid cells. Staining in lungs and kidneys appeared to be associated with alveolar lining cells and mesangial cells, respectively.

The findings confirm the value of immunohistochemical detection of virus antigen in the diagnosis of RVF, and in distinguishing the disease from Wesselsbron virus infection in new-born lambs with severe hepatic disease (Van der Lugt et al. 1995).

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