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Bovine Herpesvirus-1-induced Pharyngeal Tonsil Lesions in Neonatal and Weanling Calves

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Summary

The potential involvement of the pharyngeal tonsil in the pathogenesis of bovine herpesvirus-1 (BHV-1) infection was examined in neonatal and weanling calves infected by intranasal aerosol. Calves were monitored from days 1 to 5, and on day 6 (neonates) or 8 (weanlings) and, in a second trial at day 4-5, by histology, electron microscopy, immunocytochemistry and virus isolation. Mucosal lesions of neonates were similar to, but less extensive than, those of weanling calves. Loss of microvilli and goblet cells, with minimal epithelial erosions as early as day 1, progressed to necrosis of epithelium and adjacent lymphoid tissue, and leucocyte exudation. Lesions and clinical disease were progressive up to and including day 6 in neonates, but resolving in weanlings on days 5 and 8. By transmission electron microscopy, the physical characteristics of the phagocytic cells appeared similar in both age groups, and viral replication was not identified in leucocytes. Virus was isolated from, or found by immunocytochemistry in, the pharyngeal tonsil of all calves examined, except for two weanlings on days 1 and 8. Virus as detected by immunocytochemistry was restricted to epithelium and superficial lymphoid tissue in neonates, but was found in deep lymphoid tissue around germinal centres in weanlings. The study showed that the pharyngeal tonsil is readily infected with BHV-1 and may be an important lymphoid tissue for early anti-viral responses. The delayed inflammatory response and reduced viral clearance may contribute to the increased susceptibility of neonatal calves to fatal BHV-1 infections.

Introduction

Typically, bovine herpesvirus-1 (BHV-1) produces a self-limiting infection localized to the respiratory and reproductive tracts. Abortion and secondary bacterial infections are the most important economic sequelae (Wyler, Engels and Schwyzer, 1989). In neonatal calves, BHV-1 infection often produces a fatal multisystemic disease characterized by infection of respiratory and gastro-intestinal mucosa and variable involvement of visceral organs (Baker,

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McEntee and Gillespie, 1960; Mechor, Rousseaux, Radostits, Babiuk and Petrie, 1987; Wyler *et al.*, 1989; Schuh and Walker, 1990). The high susceptibility of neonates to micro-organisms is usually attributed to defects in leucocytic function and inflammatory response (Sherman, Goldstein, Lippert and Wennberg, 1977; Castleman, Brundage-Anguish, Krietzner and Neuenchwander, 1987; Martin, Rubens and Wilson, 1988; Kohl, 1989; Wilson and Lewis, 1990). The portals of entry for many micro-organisms are the nasal and oral cavities, but most studies of lymphoid tissue have focused only on the palatine tonsil (Husband, 1987) or the mucosa-associated lymphoid tissues of the gut (Torres-Medina, 1984; Dharakul, Riepenhoff-Talty, Albini and Ogra, 1988; Momotani, Whipple, Thiermann and Cheville, 1988) or bronchus (Anderson, Moore, Hyde and Dungworth, 1986). The pharyngeal tonsil is a specialized mucosa-associated lymphoid tissue in the nasopharynx of most animals, including cattle (Nickel, Schummer, Sieferle and Sack, 1973; Anderson *et al.*, 1986; Schuh and Oliphant, 1992). In man, the pharyngeal tonsil is thought to be important for immunological responses to inhaled antigens and micro-organisms resident in the pharynx (Rowe, Heubner, Gilmore, Parrott and Ward, 1953; Brandtzaeg, 1984; Pagano, 1984; Richtsmeier and Shikhani, 1987; Scadding, 1990). Its role in animals has not been closely studied, but in sheep and cattle the pharyngeal tonsil may be a retention site for opportunistic bacteria such as *Pasteurella* spp. (Al-Sultan and Aitken, 1985; Yates, 1988).

Mucosa-associated lymphoid tissues are characterized by areas of lympho-epithelium containing microvillous (M) cells and intra-epithelial leucocytes. M cell areas lack cilia and goblet cells, creating a specialized, relatively unprotected focus for the selective sampling of antigens, and for the transportation of leucocytes and macromolecules across the mucosa. As a result of this normal activity, pathogens may be transported through the protective mucosal barrier to lymphoid tissue (Owen, 1983; Brandtzaeg, 1984; Wolf and Bye, 1984; Richtsmeier and Shikhani, 1987), with subsequent dissemination to distant body sites via lymphatics draining into the main vasculature. An early immunological response at the mucosal barrier provided by resident leucocytes, responding inflammatory cells and appropriate regulatory stimuli may affect the localization and modulation of infectious agents. The importance of this mucosal micro-environment in the pathogenesis of disease (Befus, Lee, Ernst, Egwang, McElroy, Gauldie and Bienenstock, 1986), particularly in the respiratory tract, is largely unexplored.

The objectives of this study were to compare the potential role of the pharyngeal tonsil in the pathogenesis of BHV-1 infection in neonatal and weanling calves by (1) characterizing the lesions histologically and electron-microscopically and (2) evaluating viral clearance by isolation of virus from tissue and by immunocytochemistry.

Materials and Methods

Ten Holstein and 11 Hereford-cross calves used in sequential and single time-point studies (see below), respectively, were obtained at birth, fed colostrum free from BHV-1 neutralizing antibody and infected when 36 to 50 h old (Mechor *et al.*, 1987). Twenty weanling Hereford-cross calves (sequential study) and 12 Hereford-cross calves (single time-point study) were obtained when 4 to 5 months old from two herds

free from BHV-1 infection. Serum neutralizing antibody to BHV-1 was measured by standard techniques (Rouse and Babiuk, 1974). All calves were subjected for 5 min to an intranasal aerosol (Bielefeldt Ohmann and Babiuk, 1985; Mechor *et al.*, 1987) of BHV-1 strain 108 (about 4×10^7 plaque forming units [PFU] per ml) or serum-supplemented Dulbecco's medium (controls). Clinical examinations were made daily. In the sequential studies, one to three calves chosen at random were killed on the days shown in Table 1. For the single time-point study, all calves were killed and examined 4-5 days after inoculation. Six calves were used in the control and infected groups, except for the infected neonatal group, which contained five animals. As previously described (Schuh and Oliphant, 1992), the pharyngeal tonsil was divided into three sections perpendicular to the rugae, and the cranial, middle and caudal portions were processed for histology, electron microscopy (scanning and transmission) and immunocytochemistry, respectively. Tissue from days 1, 3, 5 or 6 in the sequential studies, and all calves at 4-5 days were selected for electron microscopic examination. Equivalent mixtures of mouse monoclonal antibodies for BHV-1 (van Drunen Little-van den Hurk, van den Hurk, Gilchrist, Misra and Babiuk, 1984) and bovine coronavirus (Deregt and Babiuk, 1987) were used for the immunocytochemistry. The specificity, clone numbers and immunoglobulin isotype of both monoclonal antibody mixtures are given in Table 2. Viral antigen was detected on cryostat sections by means of an indirect immunocytochemical method with alkaline phosphatase-anti-alkaline phosphatase complexes (Bielefeldt Ohmann, 1987) or an immunoperoxidase method based on avidin-biotin complexes modified from Smith, Collins and Carman (1989) (Schuh and Oliphant, 1992). Viral antigen detection in formalin-fixed tissues by the immunoperoxidase method was modified by deparaffinization of the slides in xylene, hydration through graded alcohols and pretreatment for 10 min at 37°C with 0.1 per

Table 1
Number of neonatal or weanling calves sampled each day after intranasal aerosol infection with bovine herpesvirus-1

Age group	Experimental group	Number of calves sampled at the stated intervals (days) after inoculation								
		1	2	3	4	5	6	7	8	9
Neonates	Controls	0	1	0	0	0	1	0	0	0
	Infected	1	1	1	1	1	3	0	0	0
Weanlings	Controls	0	0	0	0	0	0	0	0	3
	Infected	3	3	3	2	3	0	0	3	0

Table 2
Components of bovine herpesvirus-1 and bovine coronavirus monoclonal antibody mixtures used for immunocytochemistry

Antibody mixture	Protein	Clone/subclone designations	Immunoglobulin isotype
Anti-bovine herpesvirus-1	gI	5G2, 1F10, 5G11, 1F8, 1E11, 3F3, 1B4	IgG2b, IgG2a, IgG1, IgG2a, IgG2a, IgG2b, IgG1
	gIII	1F11, 1D6-G11, 1E2, 3F12	IgG2a, IgG2a, IgG3, IgG2a
	gIV	3E7-50C1, PB136-2B2, 2C8-10B5, 3DP-2	all IgG1
	VP8	1G4	IgG1
Anti-bovine coronavirus	E2	HF8-8	IgG1
		BB7-14	IgG2b
	E3	BD9-8C	IgG2a

cent protease warmed to 37°C. Control procedures in each run included omission of the BHV-1 antibody, use of an irrelevant bovine coronavirus antibody and use of known BHV-1 positive and negative (bovine respiratory syncytial virus positive) formalin-fixed or cryostat lung sections. Virus was quantified in the pharyngeal tonsil by a microtitre assay (Rouse and Babiuk, 1974). Routine bacteriological examination of the pharyngeal tonsil was made by the Diagnostic Microbiological Laboratory, Western College of Veterinary Medicine, University of Saskatchewan.

Results

Clinical Response

All calves were serologically negative for BHV-1 at the start of the experiment. Calves infected with BHV-1 became pyrexial, with upper respiratory tract signs, within 1 or 2 days of infection, and control calves remained healthy. Weanling calves were recovering (normal body temperature and ameliorated clinical signs) 5 and 8 days after inoculation, but neonatal calves became moribund after 6 days.

Pathological Findings

Beginning at day 2, the mucus in infected neonatal and weanling calves increased and became progressively more opaque and tenacious. The epithelial surface of the pharyngeal tonsil and nasopharyngeal mucosa was hyperaemic and covered by multiple brownish foci, sometimes coalescing, slightly raised, and 1 to 3 mm in diameter. Histologically, the pharyngeal tonsil of neonatal calves, compared with that of weanlings, contained less dense accumulations of leucocytes in the lamina propria and epithelium, and there was an absence of germinal centres. In infected calves the earliest lesion, found at day 1 in weanlings and day 2 in neonates, consisted of multifocal epithelial erosions at the tips of the rugae and neutrophil accumulations in adjacent areas. There was also a loss of goblet cells and increased exocytosis of neutrophils across adjacent intact epithelium. Erosions progressed to ulceration with extension of necrosis into adjacent lymphoid tissue. The accompanying inflammatory response consisted of neutrophil, macrophage and lymphocyte infiltration with fibrin deposition. At the edge of the necrosis, intranuclear inclusion bodies were present in enlarged epithelial nuclei from days 2 to 5 after inoculation. Compared with control calves (Fig. 1), high endothelial venules in the parafollicular areas in all infected calves, particularly weanlings, were characterized by an increase in length and width, hypertrophy of endothelial cells, increased transendothelial migration of lymphocytes and neutrophils occluding the lumen (Fig. 2). In weanlings, increased lympholysis was present in germinal centres of secondary lymphoid follicles, a structure absent in neonatal calves. One weanling calf on day 5 and all three calves on day 8 showed attenuated epithelium covering denuded areas, organization of fibrin, and a reduction in neutrophil infiltration but an increase in mononuclear cells. In contrast, the lesions and inflammatory response in neonatal calves were progressive up to and including day 6. Direct comparison of the pharyngeal tonsil in the 4-5 day studies confirmed the overall uniformity of the necrotizing and inflammatory

response to BHV-1. Neonatal calves consistently had less necrosis and fewer inflammatory cells (Fig. 3), particularly mononuclear cells, than weanlings (Fig. 4).

Electron Microscopic Findings

By scanning electron microscopy, the earliest lesion seen was patchy loss of cilia, with separation and loss of cells. Epithelial erosions rapidly progressed to ulceration, with fibrin strands, cellular debris and leucocytes replacing the epithelium (Fig. 5) and increased leucocyte exudation over adjacent unaffected areas. At 4·5 days after inoculation, leucocyte exudation at the points of necrosis, and over remaining normal areas was more extensive in infected weanlings than in control weanlings or neonatal calves. Surface leucocyte exudation was not found in control neonatal calves.

By transmission electron microscopy, lesions were similar in both age groups. Necrosis, fibrin and cellular debris (Fig. 6) was associated with non-ciliated M cells (lympho-epithelium), ciliated epithelium and progression of necrosis into adjacent lymphoid tissue. Adjacent cells were often enlarged, with pale nuclei exhibiting peripheral clumping of chromatin and fewer than 50 viral particles

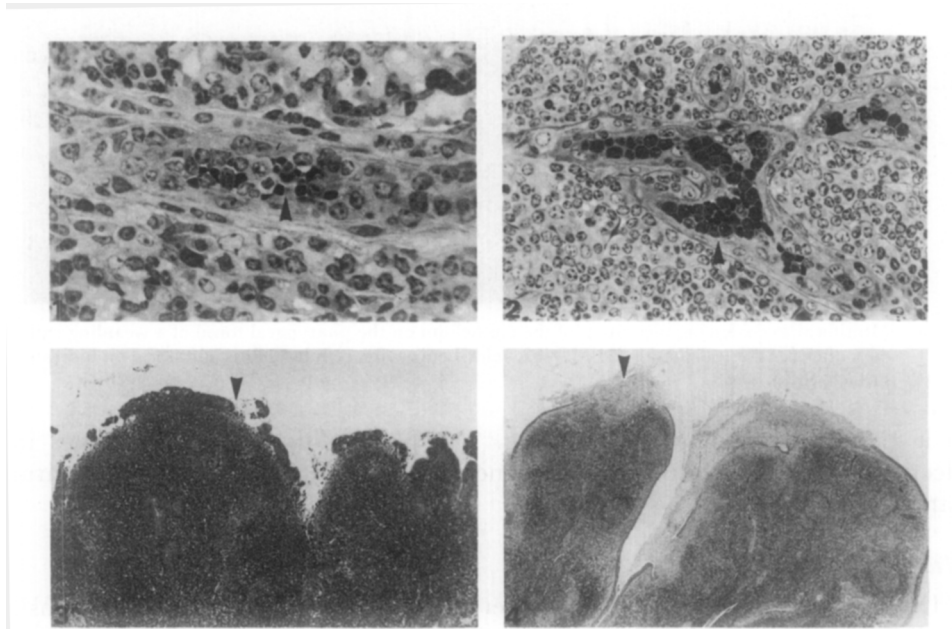


Fig. 1. Quiescent endothelium and lack of cellularity in the high endothelial venule (arrowhead) in the pharyngeal tonsil of a control weanling calf 4·5 days after inoculation with BHV-1. HE × 410.

Fig. 2. Enlargement of endothelial cells, increased cellularity of the endothelium and plugging of the venule lumen by neutrophils (arrowhead) in the pharyngeal tonsil of a weanling calf 4·5 days after inoculation with BHV-1. HE × 263.

Fig. 3. Ulceration of the epithelium (arrowhead) and minimal inflammatory exudate on the surface of the pharyngeal tonsil from a neonatal calf 4·5 days after BHV-1 inoculation. HE × 133.

Fig. 4. Ulceration and marked exudation of mucus, leucocytes and fibrin (arrowhead) in the pharyngeal tonsil from a weanling calf 4·5 days after BHV-1 inoculation. HE × 76.

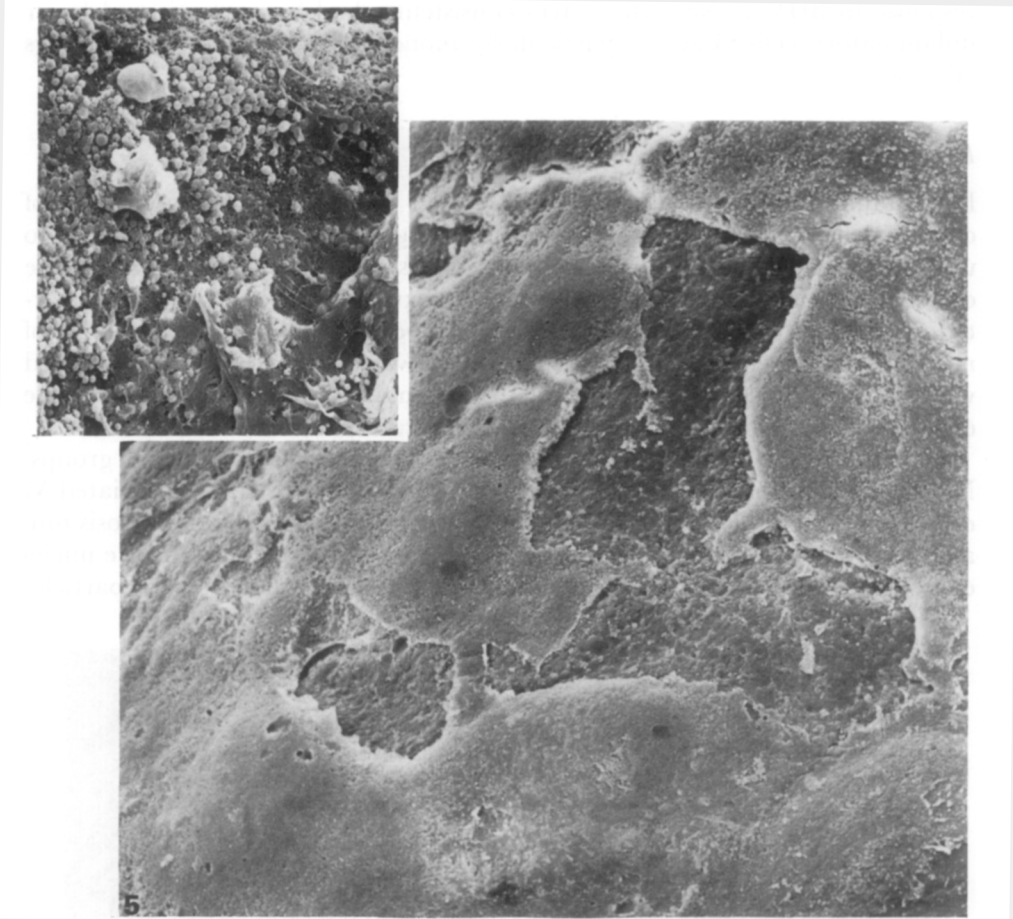


Fig. 5. Multifocal to coalescing ulceration of the epithelium on the pharyngeal tonsil of a weanling calf 3 days after BHV-1 inoculation. SEM $\times 62$. Inset: Leucocytes, cellular debris and fibrin on ulcerated surface. SEM $\times 198$.

in a given section of a nucleus (Fig. 7). Neutrophils and macrophages in ulcerated areas frequently contained necrotic debris (Fig. 8) but rarely viral particles.

Microbiology

BHV-1 was isolated (10^4 to 10^6 PFU per g of tissue) from the pharyngeal tonsil of all infected neonatal and weanling calves 4.5 days after inoculation. It was isolated from neonatal calves at 10^2 PFU per g (day 1), 10^3 PFU per g (day 2), 10^6 PFU per g (day 3), 10^3 PFU per g (day 4), 10^3 PFU per g (day 5), and 10^5 to 10^6 PFU per g (day 6). Pharyngeal tonsils from weanlings in the sequential study were evaluated in only one calf at 2 days after inoculation, and found to be positive (10^3 PFU per g). *Pasteurella haemolytica* was isolated in moderate numbers from the pharyngeal tonsil of one infected neonate (4.5 day study) and one weanling calf (2 days after inoculation), but not from any control calves. No other significant bacteria were isolated.

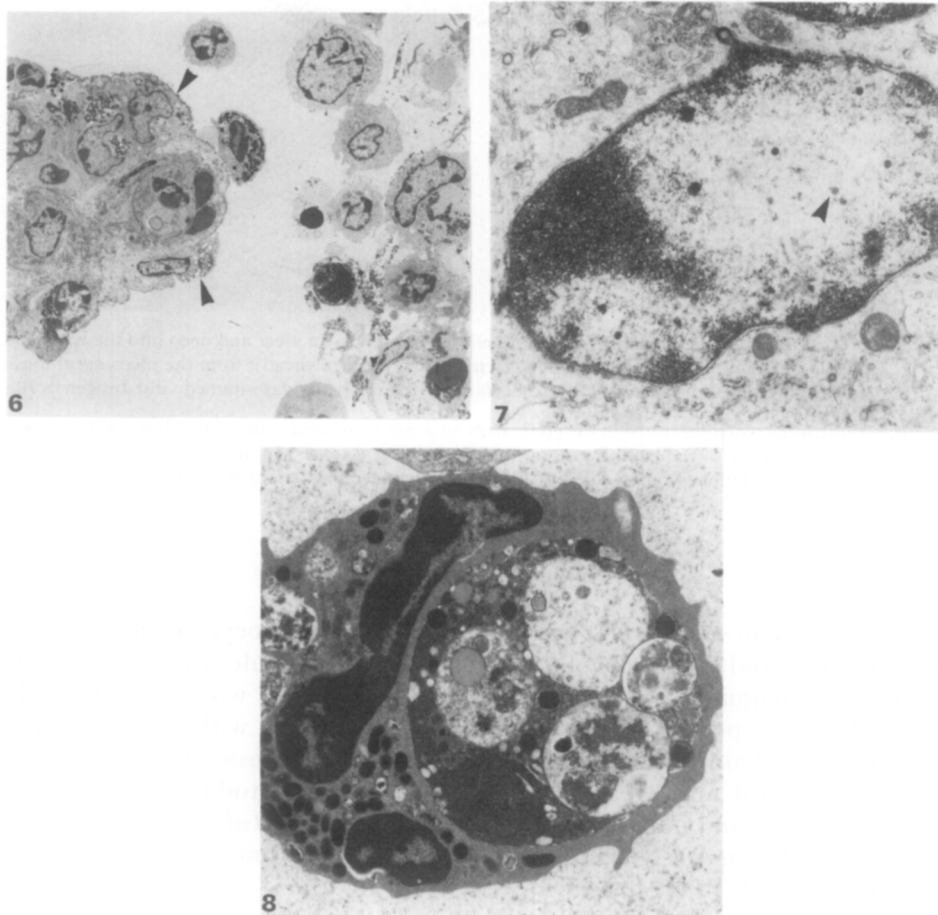


Fig. 6. Cellular exudation over the degenerating epithelium (arrowheads) of the pharyngeal tonsil from a neonatal calf 6 days after BHV-1 inoculation. Ciliated and M cells cannot be distinguished. TEM $\times 1287$.

Fig. 7. Epithelial cell with margination of nuclear chromatin and herpesvirus particles (arrowhead) from the pharyngeal tonsil of a weanling calf 4.5 days after BHV-1 inoculation. TEM $\times 909$.

Fig. 8. Neutrophil with phagocytosed cellular debris from the ulcerated surface of the pharyngeal tonsil of a neonatal calf 4.5 days after BHV-1 inoculation. TEM $\times 6123$.

Immunocytochemistry

Viral antigen was present in and around areas of epithelial necrosis in all calves in the 4.5 day studies. In the sequential study, all neonatal calves and all weanlings except two of three at days 1 and 8 after inoculation were positive. Weanlings at days 1 and 8 had only a few small areas containing viral antigen. Beginning 2 to 3 days after inoculation, virus was also found deep within the lymphoid mass in and around the germinal centres of the weanlings (Fig. 9), but remained superficial in the neonatal calves (Fig. 10).

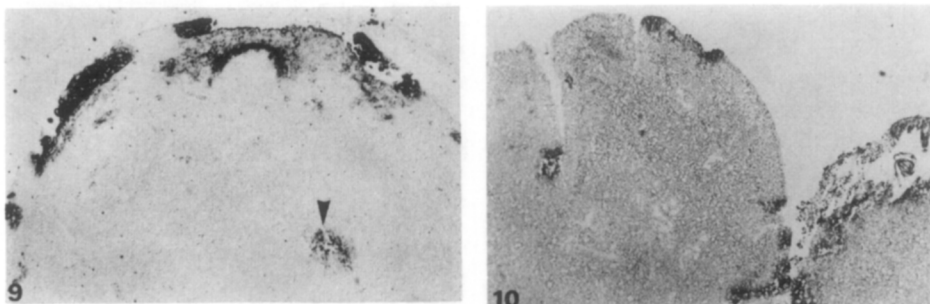


Fig. 9. Viral antigen staining intensely in the epithelium adjacent to an ulcer and deep into the lymphoid tissue of the lamina propria, including a germinal centre (arrowhead); from the pharyngeal tonsil of a weanling calf 3 days after BHV-1 inoculation. Immunoperoxidase-stained viral antigen $\times 70$.

Fig. 10. Viral antigen demonstrated in the epithelium adjacent to an area of ulceration but not deep in the lymphoid tissue of the lamina propria in the pharyngeal tonsil of a neonatal calf 4-5 days after BHV-1 inoculation. Immunoalkaline phosphatase-stained viral antigen $\times 39$.

Discussion

The development of tonsillar necrosis, superficial leucocyte exudation and inflammation, and a response of the high endothelial venules were delayed and reduced in magnitude in neonatal calves compared with weanlings. In neonatal calves potentially fatal infection, associated with the continued presence of viral antigen and active inflammation, contrasted with repair and clearance of viral antigen in weanling calves. Other studies also indicated delayed immune and inflammatory response (Sherman *et al.*, 1977; Castleman *et al.*, 1987; Martin *et al.*, 1988) and reduced viral clearance (Castleman *et al.*, 1987; Kohl, 1989) in neonates.

Immunocytochemical identification of viral antigen was superior to virus isolation for demonstrating virus early in infection and differences in localization of BHV-1. Primary follicles and germinal centres in secondary lymphoid organs usually form several weeks after birth, as a result of activation and clonal expansion during postnatal antigen priming (Osmond, 1985; Neinen, Cormann and Kinet-Denoel, 1988). In the neonatal pharyngeal tonsil, secondary follicles are absent and BHV-1 antigen was restricted to areas of superficial necrosis. In weanling calves, antigen extended into deep lymphoid tissue and germinal centres, in a pattern reminiscent of antigen presentation (Owen, 1983). Schuh and Oliphant (1992) showed that the normal pharyngeal tonsil of neonatal calves differed from that of older animals in having relatively fewer leucocytes, fewer CD8⁺ T cells (cytotoxic/suppressor phenotype) and less MHC class II expression. The absence of prior antigen priming and lack of resident leucocytes may contribute to the inefficiency of viral clearance and to the delayed immune response in the neonatal calf.

Selected samples examined by electron microscopy appeared to indicate that BHV-1 rapidly affected ciliated respiratory epithelium and specialized M cell areas (lympho-epithelium), but loss of cilia made classification of the infected cells difficult. Preferential entry of BHV-1 through lympho-epithelium, as

reported for some viral (Owen, 1983; Wolf and Bye, 1984; Dharakul *et al.*, 1988) and bacterial infections (Owen, 1983; Momotani *et al.*, 1988) in mucosa-associated lymphoid tissues, could not be demonstrated. Traditionally, respiratory viruses are thought initially to affect ciliated respiratory epithelium. Early uncomplicated BHV-1 infection is characterized by discrete multifocal necrosis of respiratory mucosa, a pattern that would be compatible with entry through specialized areas such as lympho-epithelium.

Functional differences in the ability of neonatal leucocytes to migrate towards, phagocytose and kill micro-organisms have been found in several species by *in vitro* assays (Martin *et al.*, 1988; Kohl, 1989). Compared with older calves and adult cattle, normal neonatal and young calves have reduced reactive oxygen generation in neutrophils but increased bacterial ingestion capabilities (Hauser, Koob and Roth, 1986), greater directed migration of neutrophils (Zwahlen and Roth, 1990) and monocytes with reduced chemotactic but similar phagocytic ability (Kohl, 1989). Despite these reports of age-related differences, a clear indication that phagocytic dysfunction occurs *in vivo* has not been demonstrated. Electron microscopic examination of necrotic areas in the pharyngeal tonsil of BHV-1-infected calves indicated that there were no obvious differences in the response or physical characteristics of phagocytes in neonates and weanlings. Furthermore, virus particles were rarely found in phagolysosomes and virus replication was not evident in any leucocytes, suggesting that direct effects of virus on leucocytes are minimal.

Pathogenic micro-organisms, particularly *Pasteurella haemolytica*, were cultured from only one infected neonate and one infected weanling, despite the suggestion (Al-Sultan and Aitken, 1985; Yates, 1988) that *Pasteurella* spp. are resident in the palatine or pharyngeal tonsils of ruminants.

This study has confirmed that BHV-1 is pathogenic in the pharyngeal tonsil of cattle. In neonatal calves, the number of resident and responding leucocytes may be inadequate and the anti-viral inflammatory response is delayed. Differences in viral distribution and reduced viral clearance from the pharyngeal tonsil indicates a deficiency in modulation of the virus in neonates compared with weanling calves. The pharyngeal tonsil is probably the first major lymphoid structure of the respiratory tract that encounters BHV-1 under natural circumstances. An inadequate and delayed immunological response by the pharyngeal tonsil of neonates may permit viral dissemination.

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