Apoptosis in lymphoid tissues of calves inoculated with non-cytopathic bovine viral diarrhea virus genotype 1: activation of effector caspase-3 and role of macrophages

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INTRODUCTION

Apoptosis is a form of cell death recognized as an essential mechanism in morphogenesis and homeostasis of organs and tissues. The main morphological changes during apoptosis (cellular shrinkage, membrane blebbing, chromatin condensation at the nuclear periphery and nuclear fragmentation into apoptotic bodies) are the final result of a complex biochemical cascade of events (Huppertz et al., 1999; Rathmell & Thompson, 2002). The apoptosis cascade includes an initiation stage with induction of the cascade by external and internal stimuli, an execution stage with activation of effector proteases called caspases (cysteine-containing aspartic acid-specific proteases) and the apoptotic death stage including nuclear and cellular collapse (Huppertz et al., 1999; Hengartner, 2000). In vitro studies have elucidated two regulatory apoptosis pathways (intrinsic and extrinsic). Both pathways induce apoptosis via activation of the effector caspase-3, which, once activated, irreversibly executes cell death, so that activation of caspase-3 can be considered a hallmark of apoptosis (Stennicke et al., 1998; Huppertz et al., 1999).

Apoptosis may also be involved in the immunopathogenesis of some viral diseases. There are several potential mechanisms by which viruses activate the apoptotic pathway. Some viruses may do so through the direct action of a specific viral protein (Noteborn et al., 1994; Zhuang et al., 1995). Another mechanism is that viruses induce apoptosis indirectly through their effects on other cellular processes (Clem & Miller, 1994; Tolskaya et al., 1995). The mechanisms involved in the initial execution of the apoptotic death cascade as well as the precise sequence of these events in infection with bovine viral diarrhea virus (BVDV) remain unclear.

BVDV, classified within the family Flaviviridae along with other pestiviruses such as classical swine fever virus (CSFV) and border disease virus (Fauquet et al. 2005), has traditionally been divided, on the basis of genetic differences, into two different genotypes or species, known...
as types 1 and 2 (Ridpath et al., 1994; Fulton et al., 2003). According to their cytopathogenicity in cultured cells, BVDV strains fall into two biotypes: non-cytopathic (NCP) strains, which cause no cytopathic effect, and cytopathic (CP) strains, which induce apoptotic cell death (Zhang et al., 1996; Grummer et al., 1998; Brock, 2003). NCP strains are the most frequently isolated strains on farms; these strains give rise to mild infections with moderate symptoms, characterized by a short febrile period, transient leukopenia and lymphoid depletion caused by apoptosis, especially severe in lymphoid tissues of the intestinal tract, leading to a local immunosuppressive state (Wilhelmsen et al., 1990; Walz et al., 2001; Liebler-Tenorio et al., 2003a, b).

The mechanisms responsible for lymphocyte apoptosis in bovine viral diarrhoea have not yet been clarified. In vitro studies have shown that CP BVDV strains induce apoptosis (Zhang et al., 1996; Grummer et al., 1998; Yamane et al., 2005), whilst NCP strains interfere with the molecular mechanisms favouring this process (Schweizer & Peterhans, 2001; Bendfeldt et al., 2003).

A number of in vivo studies have suggested that lymphoid depletion may be caused by virus replication in lymphocyte populations, which – together with monocytes/macrophages and dendritic cells – are the main virus target cells in gut-associated lymphoid tissue (GALT) (Odeón et al., 1999; Stoffregen et al., 2000; Teichmann et al., 2000; Liebler-Tenorio et al., 2003a, b, 2004). On the other hand, monocytes/macrophages may be involved in lymphocyte destruction through indirect mechanisms linked to the biosynthetic activation of macrophages (Lambot et al., 1998), which is the principal cause of lymphocyte apoptosis in the course of other viral diseases caused by pestiviruses such as CSFV (Gómez-Villamandos et al., 2001; Sánchez-Cordón et al., 2002, 2003, 2005).

To date, there has been no detailed characterization of the cell populations undergoing apoptosis with a view to ascertaining their distribution, the extent of apoptosis or the mechanisms responsible for this process in lymphoid tissue from calves inoculated with BVDV. The intestinal mucosa is an ideal tissue for the study of virus–host interactions and pathogenic mechanisms, as it is the site of ileal Peyers patches composed of lymphoid cells, which are largely responsible for the generation of B lymphocytes in young ruminants.

**METHODS**

**Animals, virus and experimental design.** Ten male colostrum-deprived Friesian calves, 8–12 weeks old, were obtained from a herd free of tuberculosis, brucellosis, bovine leukaemia virus and infectious bovine rhinotracheitis, and were tested to confirm their BVDV-free status by antigen and antibody ELISA.

Calves were housed in the Animal Experimental Center of Córdoba University (Spain) under strict sterile conditions. Two animals were used as uninfected (UI) controls. These control calves received 10 ml tissue culture fluid free of virus, whilst the other eight calves each received a 10 ml intranasal inoculation of NCP BVDV genotype 1 strain 7443 (courtesy of the Institute fur Virology, TIIHO, Hannover, Germany) at a concentration of $10^5$ 50 % tissue culture infective doses ml$^{-1}$. This time point was defined as day 0. After virus inoculation, calves were examined twice daily for clinical signs throughout the study period. Before and after virus inoculation, blood tests were performed on all animals.

Animals were sedated and slaughtered in batches of two at 3, 6, 9, 14 days post-inoculation (p.i.). The two controls were killed at the end of the experiment, which was carried out in accordance with the Code of Practice for Housing and Care of Animals used in Scientific Procedures, approved by the European Economic Community Union in 1986 (86/609/EEC, amended by the directive 2003/65/EC).

**Processing of specimens for light and transmission electron microscopy (TEM).** A post-mortem examination was performed on all calves. During necropsy, tissue samples from the distal ileum (15 cm proximal to the ileocecal valve) of infected and uninfected calves were fixed in 10 % buffered formalin solution (pH 7.2) for histopathological and immunohistochemical studies. Samples of the distal ileum were also fixed in 2.5 % glutaraldehyde in 0.1 M PBS (pH 7.2) for ultrastructural analysis.

Samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax by routine techniques for light microscopy. Wax-embedded sections (3 μm) were cut and stained with haematoxylin and eosin for histopathological analysis or were processed for immunohistochemical studies.

For TEM, samples were post-fixed in 2 % osmium tetroxide, dehydrated in acetone and embedded in Epon 812 (Fluka Chemie AG). Sections (50 nm) for ultrastructural examination were counterstained with uranyl acetate and lead citrate, and viewed in a Philips CM-10 transmission electron microscope.

**Immunohistochemical techniques.** The avidin–biotin–peroxidase complex staining method was performed on serial sections (3 μm) of distal ileum. Identification of BVDV surface glycoprotein (Erns or gp48), monocytes/macrophages and cells expressing different chemokine mediators was carried out using different monoclonal (mAb) and polyclonal (pAb) antibodies. Details of the pAbs used in this study, including dilutions and pre-treatments, are summarized in Table 1.

For Erns detection, internal positive controls were used from tissues from control UI specific-pathogen-free calves that had not been exposed to BVDV. Mouse and rabbit non-immune sera were used in place of specific mAbs and pAbs as internal negative controls.

**Detection of apoptosis.** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) staining. For detection of DNA fragmentation, the paraffin wax sections of distal ileum, previously fixed in 10 % buffered formalin solution, were stained by a TUNEL method, using an In situ Cell Death Detection kit, POD (Roche Diagnostics), according to the manufacturer’s instructions. Internal positive controls consisted of distal ileum sections with apoptotic lymphocytes in GALT from a pig experimentally inoculated with CSFV (Sánchez-Cordón et al., 2003).

Cleaved caspase-3 (CCasp3) immunohistochemistry. Expression of CCasp3 in paraffin wax-embedded tissue sections of distal ileum, previously fixed in 10 % buffered formalin solution, was investigated using a pAb (anti-Asp175; Cell Signalling) according to the supplier’s instructions. Internal positive controls were the same as for TUNEL staining.

**Cell counting and statistical analysis.** In order to evaluate the number of immunolabelled cells that were present and to correlate the results obtained from the different antibodies used, two
RESULTS

Clinical and haematological findings

Moderate signs of disease (slight inactivity and apathy) were observed in the inoculated calves. There was a mild temperature increase at 2 days p.i., showing a biphasic behaviour that peaked at 7 days p.i. (39.5 °C). Between 2 and 6 days p.i., inoculated animals showed a marked leukopenia due to a drop in the number of neutrophils and lymphocytes as well as mononuclear cells, which were visible in the lamina propria. From 6 days p.i., these changes were more marked, appearing as isolated incidents of pyknosis and cellular fragmentation.

From 3 days p.i., Peyer’s patch lymphoid follicles displayed mild lymphoid depletion, as well as evidence of pyknosis and cell fragmentation characteristic of death by apoptosis. These changes coincided with the observation of an infiltrate comprising large mononuclear cells with abundant cytoplasm, identified as macrophages; some of these cells contained phagocytosed cell debris [tangible body macrophages (TBMs)]. Evidence of pyknosis and cell fragmentation within lymphoid follicles was more marked by 6 days p.i., and TBMs were more abundant. By 9 days p.i., the almost total loss of lymphocytes in the Peyer’s patch follicles was accompanied by a reduction in follicle size, together with reduced pyknosis and cellular fragmentation. However, a high level of macrophage infiltration remained until the end of the experiment. Although apoptosis was less evident in interfollicular lymphoid tissue, large macrophages were observed, becoming more abundant after 6 days p.i.

From 3 days p.i., ultrastructural examination showed changes consistent with lymphocyte apoptosis observed histologically, mainly in the lymphoid follicles. Apoptotic cells exhibited condensation and margination of chromatin, as well as fragmentation of lymphocyte nuclei and cytoplasm. These features were more widely spread in infected animals than in UI calves. As the experiment progressed, the signs of apoptosis increased, including the appearance of condensed nuclear chromatin and highly electron-dense membrane-bound cell fragments (apoptotic bodies) (Fig. 1a), either free or engulfed by macrophages. Similar alterations were also observed in some macrophages, as well as in some epithelial cells in both control and BVD-infected calves.

The presence of macrophages was increased in the areas of lymphoid follicles where lymphocyte apoptosis was

Table 1. Details of the immunolabelling reagents

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Antigen or cell detected</th>
<th>Antibody dilution</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb 15C5*</td>
<td>Viral glycoprotein Erns (gp48)</td>
<td>1:50†</td>
<td>Protease‡</td>
</tr>
<tr>
<td>Anti-human macrophage (MAC387) mAb§</td>
<td>Monocytes and macrophages</td>
<td>1:100†</td>
<td>Protease‡</td>
</tr>
<tr>
<td>Anti-human IL-1α pAb†</td>
<td>IL-1α</td>
<td>1:100§</td>
<td>TC/microwave#</td>
</tr>
<tr>
<td>Anti-bovine TNF-α pAb§</td>
<td>TNF-α</td>
<td>1:25§</td>
<td>TC/microwave#</td>
</tr>
<tr>
<td>Anti-ovine IL-6 pAb§</td>
<td>IL-6</td>
<td>1:50§</td>
<td>TC/microwave#</td>
</tr>
</tbody>
</table>

*Courtesy of Dr E. J. Dubovi, Cornell University, Ithaca, NY, USA.
†In Tris/HCl buffer, pH 7.6, containing 1 % normal horse serum.
§Courtesy of AbD Serotec.
||Courtesy of Endogen.
¶TC, 0.01 M tri-sodium citrate dehydrate (pH 3.2) (Merck); microwaved for 10 min at subboiling temperature.

paraffin-wax blocks from the distal ileum of each animal were selected. Sections were used for the detection of monocytes/macrophages (MAC 387), cells expressing different cytokines – tumour necrosis factor (TNF)-α, interleukin (IL)-1α and IL-6 – and the occurrence of apoptosis (TUNEL and CCasp3). Cell counts were carried out in 25 fields of 0.20 mm² (lymphoid follicles, interfollicular area, lamina propria and epithelium) chosen randomly in four diagonally positioned squares. The results were given as the number of positive cells per 0.20 mm². Cellular identification of monocytes/macrophages and cells expressing cytokines were based on morphological features, location and size of the cells. Evaluation of TUNEL labelling was carried out according to the criteria of Kerr et al. (1972): cell shrinkage, chromatin condensation, nuclear fragmentation, blebbing of the cell surface and phagocytosed apoptotic bodies, but only on cells with an approximate lymphocyte size, not labelled apoptotic bodies.

Data were analysed using the statistical analysis program GraphPad InStat version 3.0 (GraphPad Software). Data on immunopositive cells were assessed to calculate means ± SD, and differences between the values in UI and inoculated animals at each time point were analysed using the Mann–Whitney U-test for non-parametric distributions and an unpaired Welch-corrected t-test, with a significance value of P<0.05 for both.

Morphological studies of the ileum and detection of BVDV

From 3 days p.i., the intestinal mucosa of the distal ileum displayed a mild infiltrate comprising intraepithelial lymphocytes as well as mononuclear cells, which were visible in the lamina propria. From 6 days p.i., these changes were more marked, appearing as isolated incidents of pyknosis and cellular fragmentation.
observed. Therefore, the presence of phagocytosed cell debris (mainly apoptotic bodies) by macrophages (Fig. 1b) was accompanied, from 3 days p.i., by an enlarged cell size, loss of filopodia and an increase in lysosome numbers, changes that are indicative of phagocyte activation. Moreover, some macrophages displayed proliferation and dilation of the rough endoplasmic reticulum cisternae and Golgi complexes, changes indicative of secretory activation. As the infection progressed, increased evidence of apoptosis coincided with an increase in the number of macrophages showing clear signs of phagocyte activation; there was no correlative rise in the number of macrophages displaying changes indicative of secretory activity.

Macrophages, together with star-shaped cells identified as reticular or dendritic cells, appeared to be the main cells infected with BVDV by immunohistochemistry. Although from 3 days p.i. occasional antigen-positive macrophages were observed inside the slightly depleted lymphoid follicles and interfollicular areas, an increase in the number of infected macrophages was recorded at 6 days p.i. in these areas (Fig. 1c), together with abundant apoptotic bodies, both free and phagocytosed, staining positive for viral antigen. The number of infected macrophages peaked at 9 days p.i. in both lymphoid follicles and interfollicular areas (Fig. 1d).

Viral infection of macrophages was confirmed by ultrastructural examination. Subcellular structures related to viral infection were membrane-bound inclusions characterized by a moderate electron-dense granular content. These subcellular structures contained round or slightly oval particles of 45–55 nm in diameter, which were tentatively identified as mature BVD virions. Pleomorphism of virion-like particles was pronounced. The structures were not observed in samples from UI control calves.

**TUNEL- and CCasp3-positive cell distribution**

TUNEL staining identified mainly apoptotic bodies inside macrophages, but a signal was also seen in free apoptotic bodies and occasionally in the pyknotic nuclei of lymphocyte-like cells (Fig. 2a, c, e). CCasp3 was found mainly in lymphocyte-like cells displaying pyknotic nuclei, condensation and margination of chromatin and a diffuse cytoplasmic reaction. Occasionally, apoptotic bodies were labelled, but generally they were negative (Fig. 2b, d, f).
Fig. 2. (a, c, e) Follicles with free and phagocytosed (arrowheads) apoptotic bodies identified by TUNEL staining in UI animals (a) and in calves at 3 (c) and 6 (e) days p.i. (b, d, f) Pyknotic lymphocyte-like cells (arrows) immunostained with CCasp3 in UI animals (b) and in calves at 3 (d) and 6 (f) days p.i. Observe the scant presence of both lymphocyte-like cells positive by TUNEL staining and apoptotic bodies indicated by CCasp3, as well as the progressive increase in cellular structures reactive against both apoptotic markers. Bars, 80 μm (a, b); 125 μm (c, d); 100 μm (e, f). F, Lymphoid follicles; IA, interfollicular areas. (g, h) Mean (±sd) numbers of TUNEL-positive (g) and CCasp3-positive (h) cells in interfollicular areas (open bars), lamina propria (shaded bars) and epithelium (filled bars) (left axis) and in lymphoid follicles (△, right axis) in the distal ileum of UI animals (n=2) and calves inoculated with BVDV (n=2 calves per time point). The difference compared with controls was statistically significant (*P<0.05; Mann–Whitney U-test for non-parametric distributions).
number of CCasp3-positive cells was lower compared with
TUNEL-positive cells.

From 3 days p.i., there was a significant increase in the
number of TUNEL- and CCasp3-positive cells in both the
medullary region and the cortex of Peyer’s patch lymphoid
follicles. Positive cell counts peaked at 6 days p.i., declining
from 9 days p.i. due to atrophy of the Peyer’s patches. By
14 days p.i., counts were lower than those recorded in UI
animals (Fig. 2g, h).

In interfollicular areas, the number of TUNEL-positive
cells peaked at 3 days p.i., falling thereafter, whereas the
number of CCasp3-positive cells displayed a significant
increase from 6 days p.i. in interfollicular areas, lamina
propria and epithelium, thereafter remaining stable
(Fig. 2g, h).

**Quantitative and secretory changes in macrophages in the distal ileum**

Both BVDV-inoculated animals and UI controls displayed
numerous MAC387-positive myeloid cells, mostly mono-
cytes/macrophages and occasional neutrophils (Fig. 3a).
From 3 days p.i., there was a marked increase in the
number of macrophages in interfollicular areas. However,
in Peyer’s patch lymphoid follicles, in the lamina propria
and in the epithelium, this increase became statistically
significant at 6 days p.i., peaking at 9 days p.i. within
follicles and thereafter declining up to 14 days p.i., by
which time these structures were almost completely
destroyed; however, values remained significantly higher
in inoculated animals than in controls (Fig. 3b). Although
macrophages were mostly concentrated in peripheral
follicular areas, a large number were also scattered inside
follicles, where they were associated with pyknosis and cell
fragmentation characteristic of apoptosis.

The macrophage secretory activity observed ultrastructu-
 rally was confirmed by immunohistochemical techniques,
which enabled detection of various macrophage-secreted
cytokines. From 3 days p.i., there was a marked increase in
the number of TNF-α- and IL-1α-positive cells, mainly
macrophages, in the ileal lamina propria, where more
monocytes/macrophages stained positive for TNF-α than
for any other chemical mediator. An increase in the
number of monocytes/macrophages staining positive for
these cytokines was also observed in interfollicular areas;
the increase was significant from 3 days p.i. for TNF-α, and
from 6 days p.i. for IL-1α (Fig. 4a, b). However, within the
lymphoid follicles, despite the significant rise in macro-
phage numbers, there was no correlative increase in
secretory activity and few macrophages were immuno-
labelled for these cytokines (Fig. 4c, d), peaking in this area
at 9 days p.i. (Fig. 4a, b).

IL-6-reactive cells were relatively sparse; a few positive cells
were detected in the lamina propria, whilst some
immunostained epithelial cells were observed in the ileal
mucosa.

**DISCUSSION**

The inoculation of calves with the NCP BVDV genotype 1
strain 7443 resulted in an infection with moderate clinical
symptoms, which tend to go largely unnoticed (Liebler-
Tenorio et al., 2003a, b, 2004; Ridpath et al., 2007).
However, a marked lymphoid depletion of B-cell areas in
the intestinal mucosa, together with the presence of
pyknosis, cell fragmentation and TBMs, was observed.
Although similar findings have been reported following
infection by NCP strains of types 1 and 2 and in mucosal
disease (Wilhelmsen et al., 1990; Archambault et al.,
2000; Stoffregen et al., 2000; Teichmann et al., 2000;
Liebler-Tenorio et al., 2002, 2003a, b, 2004), the results obtained here demonstrate that GALT depletion was due to massive lymphocyte apoptosis, particularly in the B-cell compartments of Peyer’s patches. In vitro studies have sought to identify the mechanisms responsible for BVDV-induced apoptosis (Schweizer & Peterhans, 1999, 2001; Grummer et al., 2002a, b; Bendfeldt et al., 2003, 2007). To our knowledge, no detailed characterization and quantification of apoptosis has been described for lymphoid tissues of cows during acute infection of BVDV.

The results obtained in this experimental animal model suggested a similar pattern of distribution and numerical evolution of TUNEL-positive and caspase-3-positive cells in the distal ileum, although the number of TUNEL-positive cells was always greater. This may have been due to the lower specificity and sensitivity of the TUNEL method (Huppertz et al., 1999; Dukers et al., 2002), which can give rise to the staining of DNA chains broken due to processes other than apoptosis (false positives) (Labat-Moleur et al., 1998; Stahelin et al., 1998; Huppertz et al., 1999). However, the possible involvement of other effector caspases such as caspase-6 and -7 cannot be ruled out; their involvement might account for the greater number of TUNEL-positive cells.

The TUNEL technique mainly enabled identification of a large number of free and phagocytosed apoptotic bodies, whilst caspase-3 was observed chiefly in lymphocytes displaying pyknotic nuclei and margined chromatin. A large number of cells positive for TUNEL staining were negative for caspase-3. It is thus evident that joint use of both techniques enables in vivo assessment of two wholly distinct stages in the apoptosis cascade during BVDV infection. Thus, detection of caspase-3 in lymphoid tissues of calves inoculated with BVDV allowed us to identify, in vivo, the start of the execution stage – the ‘point of no return’ in the apoptosis cascade – as caspase-3 is one of the so-called effector caspases, a group of proteases that are activated after proteolytic conversion, giving rise to the irreversible execution of cell death via the degradation of vital cell proteins and the activation of endonucleases (Huppertz et al., 1999; Dukers et al., 2002). TUNEL staining, in contrast, enabled visualization of the final stage of death by apoptosis.

It has been shown in vitro that NCP strains such as the one tested here are able to induce an apoptosis-inhibiting effect in cell culture (Grummer et al., 2002b); this inhibition takes place at the mitochondrial level prior to activation of

**Fig. 4.** Mean ± sd of TNF-α-positive (a) and IL-1α-positive (b) cells in interfollicular areas (open bars), lamina propria (shaded bars) and epithelium (filled bars) (left axis) and lymphoid follicles (right axis) in the distal ileum of UI animals (n=2) and in calves inoculated with BVDV (n=2 calves per time point). The difference compared with controls was statistically significant (*P<0.05, Mann–Whitney U-test for non-parametric distributions; **P<0.05, unpaired Welch-corrected t-test). (c, d) Presence of numerous macrophages immunolabelled against TNF-α (c) and IL-1α (d) at 9 days p.i. in interfollicular areas (IA). Note the low number of cells releasing both cytokines inside follicles (F), where the lymphoid depletion and the reduction in size were manifest. Bars, 150 μm.
the caspase cascade. This mechanism may be linked to the lack of increase in effector caspase-3, which would presumably favour the establishment of persistent infection (Bendfeldt et al., 2003). However, the results obtained here indicated marked activation of caspase-3, which appears to be directly related to massive lymphocyte apoptosis, thus suggesting a mechanism other than that identified in in vitro studies. The regulation of apoptosis pathways (intrinsically and extrinsically) that induce cell death via activation of effector caspase-3 in GALT during BVDV infection needs to be studied in depth by in vivo models.

The question then arises of which stimuli are responsible for triggering the GALT lymphocyte apoptosis cascade during BVDV infection. Different studies have attributed lymphoid depletion to direct action of BVDV on lymphocytes (Wilhelmsen et al., 1990; Marshall et al., 1996; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003a), a view supported by in vitro studies suggesting that apoptosis is caused by the replication and accumulation of viral RNA (Zhang et al., 1996; Adler et al., 1997; Vassilev & Donis, 2000; Grummer et al., 2002b). Recently, in vivo studies showed that the direct action of BVDV is responsible only for the destruction of a small number of lymphocytes. Thus, although lymphocyte infection coincided with the start of cell death, the intensity of infection did not match the massive depletion of GALT structures, particularly during the early stages of the process (Pedera et al., 2009).

Other authors have suggested that indirect mechanisms may be involved in lymphocyte destruction in the course of BVDV infection (Ellis et al., 1998; Liebler-Tenorio et al., 2002, 2003a, 2004). Whilst few studies have identified monocytes/macrophages as the potential cause of this process through soluble factor release (Lambot et al., 1998), in vitro research has shown that cytokine release appears to be inhibited in the case of NCP strains (Bielefeldt-Ohmann & Biabiuk, 1988; Schweizer & Peterhans, 2001; Baigent et al., 2002; Yamane et al., 2005).

In the present in vivo experiment, the progressive depletion of Peyer’s patches and increased evidence of apoptosis were accompanied, from 3 days p.i., by a significant rise in macrophage numbers, both in lymphoid follicles and in interfollicular areas. Some macrophages showed signs of viral infection (Odeón et al., 1999; Kelling et al., 2002), together with subcellular changes indicative of phagocytic activation and, in some cases, of secretory activity. However, the number of macrophages that immunostained positively for TNF-α and IL-1α, cytokines with proven ability to induce apoptosis (Zheng et al., 1995; Saldeen, 2000), remained low throughout the experiment in lymphoid follicles, where most apoptotic cells were found. These results would thus appear to rule out a major involvement of macrophages and macrophage-secreted chemical mediators in the apoptosis of follicular B lymphocytes during BVDV infection. However, these cytokines may still play a minor role, as in vitro studies have shown that TNF-α is responsible for 15 % of the total apoptotic effect induced by CP strains, and cells infected with NCP strains may also induce elevated TNF-α receptor levels (Yamane et al., 2005). Moreover, the low levels of IL-1 secretion by macrophages in lymphoid follicles, also observed in vitro, suggest that monocyte infection by both CP and NCP strains of BVDV inhibits IL-1 production (Jensen & Schultz, 1991). Therefore, the synergistic action of TNF-α and IL-1 reported as enhancing apoptosis in other immunosuppressive viral processes (Le & Vilcek, 1987; Gómez del Moral et al., 1999; Sánchez-Cordón et al., 2002, 2005) would appear not to take place in follicles during acute infection with the NCP 7443 strain.

The appearance of TBMs and the progressive increase in TBM numbers from the onset of infection, particularly within lymphoid follicles, were associated with an increase in cell debris due to lymphocyte apoptosis, a mechanism regulated by the presence of apoptotic cell-surface molecules recognized by phagocyte cell receptors (Savill et al., 1993). The increase in macrophage numbers was therefore assumed to be a consequence, rather than a cause, of apoptosis.

Nevertheless, the steady increase in apoptosis in interfollicular areas in which T lymphocytes predominated was matched by an increase in the number of macrophages, many of which displayed signs of viral infection and cytokine secretion (TNF-α and IL-1α). Thus, apoptosis among T-cell subpopulations, although less marked than in B-cell areas, may involve an indirect mechanism mediated by pro-apoptotic cytokines released by macrophages, as reported in other pestivirus infections (Gómez-Villamandos et al., 2001; Sánchez-Cordón et al., 2002, 2003, 2005).

It would thus appear that the indirect mechanisms regulating lymphocyte apoptosis within follicles (B-cell area) and in interfollicular areas (T-cell area) are different; the main cause of apoptosis within follicles and the possible involvement of other mechanisms in interfollicular areas still need to be clarified. In this respect, further research is required into the possible involvement of different T-lymphocyte subpopulations with cytotoxic capacity, and into the possible changes induced by virus infection in follicular stromal cells and their effect on the maintenance both of lymphocyte homeostasis and of the follicular microenvironment.

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Apoptosis in BVD: role of macrophages and caspase-3


