Apoptosis is induced by infectious bursal disease virus replication in productively infected cells as well as in antigen-negative cells in their vicinity

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The kinetics of infectious bursal disease virus (IBDV) replication and induction of apoptosis were investigated in vitro and in vivo. After infection of chicken embryo (CE) cells with IBDV strain Cu-1, the proportion of apoptotic cells increased from 5.8% at 4 h post-infection (p.i.) to 64.5% at 48 h p.i. The proportion of apoptotic cells correlated with IBDV replication. UV-inactivated IBDV particles did not induce apoptosis. Double labelling revealed that, early after infection, the majority of antigen-expressing cells were not apoptotic; double-labelled cells appeared more frequently at later times. Remarkably, apoptotic cells were frequently located in the vicinity of antigen-expressing cells. This indicated that an apoptosis-inducing factor(s) might be released by cells that replicate IBDV. Since interferon (IFN) production has been demonstrated after IBDV infection, IFN was considered to be one of several factors. However, supernatants of infected CE cells in which virus infectivity had been neutralized were not sufficient to induce apoptosis. Similar results were observed in the infected bursae of Fabricius: early after infection, most of the cells either showed virus antigens or were apoptotic. Again, double-labelled cells appeared more frequently late after infection. This suggests that indirect mechanisms might also be involved in the induction of apoptosis in vivo, contributing to the rapid depletion of cells in the IBDV-infected bursa.

Introduction

Infectious bursal disease virus (IBDV) is a member of the genus Avibirnavirus in the family Birnaviridae. A non-enveloped, icosahedral capsid with a diameter of 60 nm contains two segments (A and B) of double-stranded (ds) RNA (Müller et al., 1979; Kibenge et al., 1988). Segment A encodes a polyprotein that is processed into the major structural proteins VP2 and VP3 (Hudson et al., 1986) and into VP4, a virus-encoded protease that shares a number of features with bacterial Lon proteases (Sanchez & Rodriguez, 1999; Birghan et al., 2000; Lejal et al., 2000). A second open reading frame encodes the small, non-structural protein VP5 of unknown function (Mundt et al., 1995). Segment B encodes the virus polymerase, VP1. Infection of 3- to 6-week-old chickens with IBDV causes an acute disease (Gumboro disease), characterized by high morbidity and mortality. Surviving chickens, as well as chickens infected immediately after hatching, develop an immunodeficiency resulting from the depletion of B lymphocytes. This immunosuppression results in increased susceptibility to opportunistic infections, impaired growth and failure of vaccination. Acute and immunosuppressive forms of the disease have a large economic impact on the poultry industry worldwide.

Lymphoid cells in the bursa of Fabricius (BF) are the target cells of IBDV. Infection results in lymphoid depletion and the final destruction of the BF as the predominant feature of the pathogenesis of infectious bursal disease (IBD) (Käuffer & Weiss, 1980; Becht & Müller, 1991; Burkhardt & Müller, 1987). Besides necrosis, marked atrophy of the infected BF without severe inflammatory response was also reported (Rosenberger, 1985). This suggests the involvement of apoptotic processes in the pathogenesis of the disease. The induction of apoptosis in IBDV-infected chicken peripheral blood lymphocytes has been reported (Vasconcelos & Lam, 1994). Apoptotic cell death was also observed in vitro in IBDV-infected Vero cells and chicken embryo (CE) cells (Tham & Moon, 1996). IBDV infection of susceptible chickens resulted in the induction of apoptosis of cells in the bursa (Vasconcelos...
Two viral proteins have been suspected to play a role in the induction of apoptosis. Fernandez-Arias et al. (1997) showed that apoptotic cell death was induced by the structural protein VP2 in mammalian cells, but not in CE cells. A VP5-deletion mutant IBDV strain induced apoptosis in a reduced number of infected CE cells compared with the parental strain; this mutant strain replicated more slowly than the parental strain (Yao et al., 1998). Results of previous studies indicated a correlation between virus replication and apoptosis in cells of the BF (Nieper et al., 1999). The involvement of indirect mechanisms was suggested by Inoue et al. (1994), since apoptosis was observed in T cells of the thymus of infected chickens, whereas IBDV antigens were found mainly in infiltrated B cells or in reticular cells. Furthermore, Tanimura & Sharma (1998) investigated serial sections of IBDV-infected BF and demonstrated apoptotic cells in not only antigen-positive but also antigen-negative bursal follicles.

The mechanisms of IBDV-induced apoptosis remain unknown. In this study, a double-labelling technique was used for the simultaneous detection of cells containing viral antigens and apoptotic cells. The IBDV serotype 1 strain Cu-1 used in the experiments is a tissue culture-adapted variant of the wild-type strain Cu-1wt (Nick et al., 1976; Zierenberg et al., 2000). Whereas infections of susceptible chickens with Cu-1wt are associated with high mortality rates (Nick et al., 1976; Cursiefen et al., 1979; Kaufer & Weiss, 1980; Zierenberg et al., 2000), no mortality has been observed following intrabursal infection of chickens with Cu-1 (Lange et al., 1987). In the latter case, however, replication of the virus results in destruction of lymphoid tissue in the BF, and clinical signs in infected chickens have been reported (Lange et al., 1987). Investigation of IBDV-infected CE cells and of cells in the BF of IBDV-infected chickens at the single-cell level should help to elucidate the significance of apoptosis in the pathogenesis of IBDV infection.

Methods

- **Virus and cells.** Primary and secondary cultures of CE cells prepared according to standard protocols were maintained at 38 °C and 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 5% foetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin. The tissue culture-adapted serotype 1 strain Cu-1 of IBDV (Nick et al., 1976; Nieper et al., 1999; Zierenberg et al., 2000) was propagated in CE cells as described previously (Cursiefen et al., 1979). CE cells were washed with PBS and infected with Cu-1 at an m.o.i. of 1. Mock-infected cells were used as controls. After adsorption of the virus for 60 min, the cells were rinsed and DMEM was added as above.

- **Inactivation of IBDV by UV light.** Virus particles purified by calcium chloride density centrifugation (Müller & Becht, 1982) were treated with UV light (wavelength 254 nm at a distance of 4 cm) for 30 min. Inactivation of virus infectivity was confirmed by plaque assay.

- **IFN assay.** The presence of IFN in IBDV-infected CE cells was demonstrated by the plaque-reduction method (Gelb et al., 1979a).

Briefly, IBDV-infected CE cells were frozen and thawed three times and then centrifuged for 20 min at 2000 g and the supernatants were collected. Virus infectivity was inactivated by heating the samples in a water bath at 70 °C for 30 min and exposing them to UV light for 15 min. Monolayers of CE cells were incubated overnight at 38 °C with serial twofold dilutions of these supernatants and infected with about 250 p.f.u./ml vesicular stomatitis virus (VSV). VSV replication was determined after 60 h by plaque assay. IFN titres were expressed as the reciprocal of the highest dilution that caused a 50% reduction in the number of plaques compared with the number in untreated cultures infected with VSV.

### Treatment of IBDV-infected cell supernatants

The supernatants of Cu-1-infected CE cells were harvested 24 h post-infection (p.i.) and cell debris was removed by centrifugation at 300 g for 10 min. CE cells were inoculated with these supernatants either untreated or after heat inactivation of IBDV infectivity by incubation with the neutralizing monoclonal antibody (MAb) B1 (Öppling, 1991) for 1 h at 38 °C. Neutralization was confirmed by plaque assay.

- **Chickens.** White Leghorn chickens were hatched from specific-pathogen-free eggs (Lohmann). Four chickens, at the age of 6 weeks, were inoculated intrabursally with 0.5 ml Cu-1-infected CE cell culture supernatant (2 × 107 p.f.u./ml). Four chickens were treated analogously with sterile PBS and served as uninfected controls. One infected chicken and one uninfected chicken were killed at 6, 12, 24 and 36 h after inoculation and the bursae were removed.

- **DNA ladder analysis.** IBDV-infected and control cells grown in Petri dishes (80 mm diameter) were washed once with PBS and treated with proteinase K (1 mg/ml) at different times p.i. Cellular DNA was extracted with phenol–chloroform and precipitated overnight at —20 °C with ethanol–sodium acetate (Sambrook et al., 1989). Precipitated DNA was resuspended in 50 μl double-distilled water and treated with RNase A (0.5 μg/ml; Sigma) for 30 min. DNA fragmentation was visualized under UV light after electrophoresis in 2% agarose gel and ethidium bromide staining.

- **Annexin V staining.** CE cells grown on cover slips were washed once with cold PBS and incubated for 15 min at 37 °C with 50 μl FITC-conjugated annexin V (Annexin-V-FLUOS; Boehringer Mannheim) diluted 1:50 in incubation buffer (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2). In order to discriminate between apoptotic and necrotic cells, cells were additionally incubated with the DNA-binding dye propidium iodide (50 μg/ml; Sigma) in some experiments. After washing in the same buffer, the cells were allowed to dry and investigated by fluorescence microscopy.

- **DNA staining (sub-G0/G1, DNA peak technique).** Floating CE cells and attached CE cells, removed from Petri dishes by trypsin treatment, were collected in one tube by centrifugation and fixed at 4 °C in 1 ml ice-cold 70% ethanol. Ethanol was removed by centrifugation for 10 min at 300 g and 1 × 106 cells were incubated for 30 min at room temperature with 1 ml propidium iodide (50 μg/ml in PBS, supplemented with 1% BSA and 40 μg/ml RNase A). The DNA content of 1 × 106 cells was analysed by flow cytometry (FACS Calibur; Becton Dickinson) and the CellQuest research software. Compared with cells in the G0/G1 phase of the cell cycle, apoptotic cells appeared as a population of cells with a lower DNA content (sub-G0/G1 DNA peak technique; Telford et al., 1994). Cells to the left of the G0/G1 peak were therefore considered to be apoptotic. Each experiment was repeated at least three times.

- **Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay.** The BF was removed from IBDV-infected and uninfected chickens, fixed in 10% formalin and embedded in paraffin, sectioned and stained with haematoxylin and eosin. After deparaffinization, sections were dehydrated and permeabilized with Proteinase K (20 μg/ml) for 7 min at room temperature. Cells were incubated for 1 h at room temperature with 1 ml proteinase K (50 μg/ml) in PBS, supplemented with 1% BSA and 40 μg/ml RNase A. The DNA content of 1 × 106 cells was analysed by flow cytometry (FACS Calibur; Becton Dickinson) and the Cell Quest research software. Compared with cells in the G0/G1 phase of the cell cycle, apoptotic cells appeared as a population of cells with a lower DNA content (sub-G0/G1 DNA peak technique; Telford et al., 1994). Cells to the left of the G0/G1 peak were therefore considered to be apoptotic. Each experiment was repeated at least three times.

1108
paraffin according to standard protocols. Tissue sections were stained with Giemsa for histopathological investigations. For the detection of apoptotic cells, the in situ cell death detection kit AP (Boehringer Mannheim) was used according to the manufacturer’s instructions. Briefly, bursal sections were treated for 10 min at 37 °C with protease K (20 µg/ml). To minimize problems due to non-specific labelling, the TUNEL reaction mixture was diluted 1:2 with TdT buffer containing 1 mM cobalt chloride, 140 mM sodium cacodylate and 30 mM Tris–HCl, pH 7.2 (Nieper et al., 1999). The tissue sections were incubated with the reaction mixture for 60 min at 37 °C in a humidified chamber followed by incubation with an anti-fluorescein antibody conjugated with alkaline phosphatase. Apoptotic cells were visible by light microscopy after treatment with NBT/BCIP stock solution (Boehringer Mannheim).

### Double labelling of cells for detection of apoptosis and viral antigens.

CE cells stained with FITC-labelled annexin V as described above were fixed with ice-cold acetone for 10 min. After drying, a polyclonal anti-IBDV rabbit serum (B22; Nieper et al., 1999) was added at a dilution of 1:100 and allowed to bind for 60 min at 37 °C in a humidified chamber. After washing three times with PBS, the cells were incubated for 30 min at 37 °C with a TRITC-conjugated anti-rabbit antibody (Sigma) diluted 1:200. The cells were washed three times with PBS and once with water and subsequently examined with a fluorescence microscope.

After performing the TUNEL assay as described above, sections of the BF were incubated overnight at 4 °C in a humidified chamber with the primary antibody B22, diluted 1:100. After washing, the sections were incubated for 30 min at 37 °C with a biotinylated anti-rabbit antibody (Sigma), diluted 1:100. This was followed by incubation for 30 min at 37 °C with streptavidin–peroxidase conjugate (Boehringer Mannheim), diluted 1:100. Staining was done with 3-amino-9-ethylcarbazole as substrate (Camon). Between the incubation steps, the sections were washed with TBS (100 mM Tris–HCl, pH 7.6, 0.9% NaCl).

### Results

**IBDV-infected CE cells undergo apoptosis**

In order to investigate the kinetics of apoptotic cell death after IBDV infection, cellular DNA of Cu-1-infected CE cells was extracted at 6, 8, 12, 24 and 36 h p.i. Fragmentation of the DNA, a characteristic feature of apoptosis, was first observed at 12 h p.i. as a faint, but clearly discernible DNA ladder. As shown in Fig. 1, the intensity of the DNA bands increased considerably thereafter. No DNA fragmentation was detected in mock-infected cells up to 36 h p.i.

Annexin V was used to monitor the onset of apoptosis. A few cells (<1%) in both the infected and the uninfected CE cell cultures showed green fluorescence of the cell membrane, indicating binding of annexin V, at 2 h p.i. At 4 h p.i., approximately 4% of cells were annexin V-positive in the infected monolayers, increasing to 10% at 14 h p.i. (Table 1). These cells were located close to each other. Again, in the uninfected cell cultures, only individual cells (<3%) distributed throughout the cultures were stained by annexin V (not shown). Since annexin V may also stain necrotic cells due to their disrupted cell membranes, double staining of infected and uninfected CE cell cultures with propidium iodide was also performed. In both cultures, no double-stained cells were observed. At 12 h p.i. and later, only a few cells in the infected cultures showed both green and red fluorescence, indicating negligible necrotic processes (data not shown).

### Table 1. Percentages of cells positive for viral antigens and annexin V in Cu-1-infected CE cell cultures

Viral antigens were detected by immunofluorescence assay. Numbers of stained cells were estimated by fluorescence microscopy.

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>Cells with viral antigens (%)</th>
<th>Annexin V-positive cells (%)</th>
<th>Double-labelled cells as a percentage of cells with viral antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4</td>
<td>7</td>
<td>5</td>
<td>7</td>
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<tr>
<td>6</td>
<td>7</td>
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<td>10</td>
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<td>8</td>
<td>7</td>
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</tr>
<tr>
<td>12</td>
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<td>8</td>
<td>80</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>10</td>
<td></td>
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</tbody>
</table>

--, None detected.
In order to quantify the number of apoptotic cells, IBDV-infected and control CE cells were incubated with propidium iodide and subjected to flow cytometry. In infected cell cultures, the proportion of cells with a lower DNA content increased considerably, indicating the induction of apoptosis (Fig. 2). The results of time-course experiments are shown in Table 2. In the infected CE cell cultures, 5–8% of cells were found to be apoptotic at 4 h p.i., which was comparable to the percentage of apoptotic cells in uninfected controls. At later stages of infection, the percentage of apoptotic cells in the controls remained approximately the same and did not increase to more than 21.0% apoptotic cells at 48 h p.i. However, at 12, 24, 36 and 48 h p.i., the proportion of apoptotic cells in the infected cell cultures increased to 18.5, 28.6, 45.2 and 64.5%. 

**Table 2. Percentage of apoptotic cells as determined by flow cytometry in IBDV-infected and uninfected CE cell cultures**

Results represent means ± SD of three separate experiments.

<table>
<thead>
<tr>
<th>Time (h p.i.)</th>
<th>Uninfected cell cultures</th>
<th>Infected cell cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>6.0 ± 1.0</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td>8</td>
<td>11.0 ± 2.7</td>
<td>13.1 ± 4.1</td>
</tr>
<tr>
<td>12</td>
<td>13.8 ± 5.1</td>
<td>18.5 ± 4.6</td>
</tr>
<tr>
<td>24</td>
<td>18.6 ± 4.7</td>
<td>28.6 ± 4.3</td>
</tr>
<tr>
<td>36</td>
<td>21.3 ± 7.9</td>
<td>45.2 ± 3.9</td>
</tr>
<tr>
<td>48</td>
<td>21.0 ± 7.2</td>
<td>64.5 ± 4.9</td>
</tr>
</tbody>
</table>

**UV light-treated IBDV particles do not induce apoptosis**

Caesium chloride-purified IBDV particles, either untreated or treated with UV light, were added to CE cells at dilutions corresponding to an m.o.i. of 1. As monitored by flow cytometry 24 h later, untreated, replication-competent IBDV resulted in an increase in the proportion of apoptotic cells to 32.5% (Fig. 3a); UV light-treated virus particles did not result...
in an increase in the proportion of apoptotic cells compared to uninfected controls (Fig. 3 b).

**IBDV infection also induces apoptosis in antigen-negative cells**

In order to investigate the presence of apoptotic cells and cells containing IBDV antigens at the single-cell level, a double-labelling technique was applied. By using the polyclonal rabbit anti-IBDV antibody B22 and a TRITC-labelled secondary antibody on cells labelled earlier with FITC-conjugated annexin V, red intracytoplasmic fluorescence indicated the presence of viral antigens whereas green fluorescence indicated annexin V-positive cells. At 4 h p.i., only cells containing IBDV antigens and cells labelled by annexin V were observed, i.e. annexin V-positive cells did not contain viral antigens. At 6–8 h p.i., 7–10% of cells expressing viral antigens also showed green fluorescence; again, the majority of the cells expressing viral antigens were not annexin V-positive. However, annexin V-positive cells were located in close proximity to cells expressing viral antigens (Fig. 4a; 6 h p.i.). An increase in double-labelled cells was observed at later stages of the infection. At 12–14 h p.i., 60–80% of the cells containing IBDV antigens were also annexin V-positive. Most of these cells showed moderate expression of viral antigens, whereas labelling by annexin V was rarely detected in cells with large amounts of viral antigen (Fig. 4b; 14 h p.i.). However, a number of cells was still observed exhibiting either viral antigens or apoptosis. The results of these investigations are summarized in Table 1.

**IBDV-induced factors alone are not sufficient to induce apoptosis**

The presence of apoptotic cells in the vicinity of cells expressing viral antigens suggested the release of an apoptosis-inducing factor(s) into the supernatant by IBDV-infected cells. In order to test this hypothesis, supernatants of Cu-1-infected CE cells, either untreated or treated by incubation with a neutralizing anti-IBDV MAb, were added to uninfected CE cell cultures. At 24 h after the inoculation, the number of apoptotic cells was determined by flow cytometry. Incubation of caesium...
chloride gradient-purified IBDV particles with the neutralizing MAb prior to treatment of CE cells did not result in levels of apoptosis above background (Fig. 5a). Supernatants of IBDV-infected CE cells where the virus had been neutralized with the same MAb did not induce apoptosis in uninfected CE cell cultures. Numbers of apoptotic cells similar to those in the controls were observed (Fig. 5b). In cell cultures inoculated with untreated supernatants of infected CE cells, 37–9% of CE cells were apoptotic; in control cultures, inoculated with the supernatants of uninfected cultures, only 15–7% were apoptotic (Fig. 5c).

**Induction of IFN**

The presence of IFN in IBDV-infected CE cell cultures was demonstrated by VSV plaque-reduction assays. When CE cell cultures had been incubated with heat- and UV light-treated supernatants of IBDV-infected cells prior to VSV infection, the number of VSV-specific plaques was reduced and IFN titres of 32 were determined. No antiviral activity was present in the supernatants of control CE cell cultures that had not been infected previously with IBDV (data not shown).

**Distribution of apoptotic cells and cells containing IBDV antigens in the BF of infected chickens**

Chickens inoculated intrabursally with Cu-1 developed typical clinical signs of IBD by 30 h p.i. In a chicken killed at 24 h p.i., the BF was slightly enlarged, due to oedematous swelling. In those killed after 36 h, the bursae were enlarged considerably and had petechial haemorrhages. Histopathological changes such as interfollicular oedema, immigration of heterophilic cells and loss of lymphoid cells were consistent with earlier observations (Käfer & Weiss, 1976; Müller et al., 1979; data not shown).

Serial sections of bursae collected at 6, 12, 24 and 36 h p.i. were investigated consecutively for the presence of viral antigens by immunohistochemistry (IHC) and of apoptotic processes by the TUNEL assay. Brown staining of the cytoplasm, indicating viral antigens, was first observed in a small number of cells in the medulla of the follicles of infected BF at 12 h p.i. At 24 h p.i., the number of cells expressing viral antigens increased considerably and about 83% of the follicles were affected. At 36 h p.i., 90% of the follicles contained viral antigens, with antigen-positive cells located in the medulla and also in the cortex. IHC was negative in sections of uninfected BF. A few apoptotic cells, demonstrated by purple–black staining of the nuclei, were observed in both infected and uninfected bursae at 6 and 12 h p.i. These apoptotic cells were predominantly located in an area between the cortex and the medulla (data not shown). The number of apoptotic cells increased strongly up to 24 h p.i., especially in the cortex of the follicles, and almost all of the follicles showed an increase in apoptotic cells at 36 h p.i. compared with uninfected controls. Remarkably, some follicles were observed with small numbers of cells with viral antigens but large numbers of apoptotic cells.

**Fig. 5.** Apoptosis 24 h after treatment of IBDV-infected (shaded bars) or uninfected (open bars) CE cells with IBDV particles incubated with a neutralizing anti-IBDV MAb (a); with supernatants of infected cell cultures incubated with a neutralizing anti-IBDV MAb (b) or with supernatants of infected cell cultures without anti-IBDV MAb (c). The proportion of apoptotic cells was determined by the sub-G0/G1 DNA peak technique and flow cytometry analysis (means of three independent experiments, one sample each; error bars represent SD).

**Fig. 6.** Double labelling of the BF of infected chickens to demonstrate apoptosis and the presence of viral antigens. (a) Cells containing viral antigens (brown, long arrows) were observed mainly close to apoptotic cells (purple–black, arrowheads). Only a few cells were positive for both viral antigen and apoptosis (short arrows). Higher magnification (× 100) shows (b) a double-labelled cell (short arrow) located close to an apoptotic cell (arrowhead) and (c) cells positive for viral antigens (long arrows) located alongside an apoptotic cell (arrowhead).
Apoptosis is also induced in antigen-negative cells in vivo

The double-labelling method was applied to investigate the distribution of both apoptotic cells and cells containing viral antigens in the same section of the BF. At 24 h p.i., cells with brown cytoplasmic staining for viral antigens were observed. Remarkably, however, purple–black-stained nuclei, indicating apoptosis, were observed mainly in neighbouring cells. Most of the cells containing viral antigens were not apoptotic. Only moderate numbers of the cells were identified with both brown staining of the cytoplasm and purple–black-stained nuclei (Fig. 5a–c). These cells were located predominantly in the cortex of the follicles, whereas the majority of the cells positive only for viral antigens were found in the medulla. In sections prepared at 36 h p.i., a larger number of double-labelled cells was observed. However, numerous cells were also either positive by IHC or in the TUNEL reaction at this late time p.i.

Discussion

The rapid onset of severe destruction of the BF is the predominant feature of the pathogenesis of IBD. Since it has been observed that IBDV replicates in only about 20% of the lymphoid cells in the BF (Müller, 1986; Burkhardt & Müller, 1987), apoptosis induced by IBDV infection might be a significant feature of this process. In this communication, the kinetics of IBDV replication and induction of apoptosis in vivo and in vitro are reported. The IBDV strain Cu-1, which replicates in bursal cells as well as in tissue culture, was used. This procedure allowed the investigation of individual cells in culture and the determination of cell numbers, hardly possible in infected organs. The infection of susceptible chickens with the same virus strain revealed kinetics of virus replication and induction of apoptosis similar to those in cultured CE cells.

By application of the annexin V assay, used frequently to monitor early apoptosis (Martin et al., 1995), annexin V-positive cells were first observed in IBDV-infected CE cell cultures at 4 h p.i. The number of such cells increased with time. After 12 h p.i., however, with an increase in cytopathic changes in the monolayers, annexin V-positive cells were hardly detected, as only cells attached to the Petri dishes could be analysed by fluorescence microscopy. After this time, the onset of cellular DNA fragmentation was demonstrated by DNA laddering and flow cytometry. By applying these techniques, the cells in the culture medium, detached from the monolayers, could also be analysed; larger numbers of apoptotic cells were therefore detected (Tables 1 and 2). The small number of apoptotic cells present in uninfected controls may be attributed to physiological cell death of cultured cells.

Annexin V also binds necrotic cells, due to the disruption of their cell membranes. Necrotic cells are also stained by propidium iodide, however, whereas apoptotic cells are not. Double staining of infected CE cells with annexin V and propidium iodide showed annexin V-positive cells exclusively within 4–12 h p.i., indicating the induction of apoptosis rather than necrosis due to IBDV infection. The results of these time-course experiments contrast with recently published data. Tham & Moon (1996), who used IBDV strains 9147-P and 4365, reported DNA laddering immediately after infection of CE cell cultures and a decrease in intensity thereafter. These authors concluded that apoptosis and virus replication were independent. Our data, however, showed that apoptosis was associated with the replication process (Tables 1 and 2). Treatment of CE cell cultures with replication-incompetent, UV-inactivated IBDV failed to induce apoptosis. It can be speculated that UV light treatment does not impair binding of IBDV to cell surface receptors. Further experiments will have to be performed for a definite answer to the question of whether apoptosis is triggered via virus receptor activation.

In order to determine the significance of apoptosis in the pathogenesis of IBD, similar investigations were performed on the target cells in the BF of infected chickens. The intrabursal route of infection was chosen in order to determine the exact time of infection and to ensure a high concentration of virus at the preferred replication site. The small number of apoptotic cells in the uninfected BF represents the normal cellular turnover during maturation of B lymphocytes. After infection of chickens with Cu-1, the number of apoptotic cells in the BF increased considerably with time and correlated with the increase in cells expressing viral antigens. Again, these results indicated a correlation between virus replication and induction of apoptosis, in accordance with Nieper et al. (1999), who observed that most of the apoptotic cells in the BF contained viral antigens late (4 days) after infection.

On the other hand, our results suggest that additional, indirect mechanisms are instrumental in the induction of apoptosis, since considerable numbers of apoptotic cells, but only small numbers of cells containing viral antigens, were observed in some follicles of the infected BF. Similar observations have been reported previously (Tanimura & Sharma, 1998). In the thymus of IBDV-infected chickens, apoptosis and histological lesions without obvious correlation to virus replication have also been reported (Sharma et al., 1993; Inoue et al., 1994). It was important, therefore, to investigate both apoptotic cells and cells containing viral antigens simultaneously and at the single-cell level. Early after infection, double labelling showed considerable numbers of apoptotic cells in which viral antigens could not be demonstrated, both in vitro and in vivo. One explanation might be the release of an apoptosis-inducing factor(s) by cells expressing viral antigens.

IFN, produced in vitro and in vivo after IBDV infection (Gelb et al., 1979a, b), was considered to be one of several possible apoptosis-inducing factors. Virus infections are known to be the most common cause of IFN production; above all, dsRNA is known to be a potent inducer of IFN (Jacobs & Langland, 1996), which might inhibit protein synthesis and also induce apoptosis (Lee & Esteban, 1994). Apoptosis has also been
described after infection with other dsRNA viruses, such as rotavirus (Superti et al., 1996) and infectious pancreatic necrosis virus (Hong et al., 1998), another member of the family Birnaviridae. The presence of IFN was demonstrated in supernatants of bovine viral diarrhoea virus-infected cell cultures, in which apoptosis had also been observed in uninfected cells (Adler et al., 1997; Perler et al., 2000). Interactions with IFN-induced apoptosis-inducing proteins are also known for numerous viruses, including vaccinia virus (Kibler et al., 1997), influenza virus (Bergmann et al., 2000) and reovirus, another dsRNA virus (Yue & Shatkin, 1997). Strategies used by other RNA and DNA viruses to control apoptotic host cell responses have been reviewed elsewhere (see for example Shen & Shenk, 1995; Teodor & Branton, 1997). In the case of IBDV infections, however, the involvement of other apoptosis-inducing factors, like TNF-α, has also to be considered.

Supernatants of IBDV-infected CE cells had been supposed to contain a factor(s) that might induce apoptosis in surrounding cells. However, treatment of CE cells with such supernatants, in which virus had been neutralized, had no relevant effect. One explanation might be that, in the absence of the viral genome, these conditions were not sufficient [IBDV neutralization strongly reduces binding of the virus to cellular receptors (Nieper & Müller, 1996), thus interrupting the replicative cycle, a prerequisite for this experiment].

The presence of apoptotic cells in IBDV-infected CE cells and bursal tissues that do not express viral antigens (Figs 4 and 6) might be explained by the observation that IBDV replication depends on the cell cycle (Müller, 1986); it might be speculated, therefore, that these cells are infected, but do not replicate the virus. Another explanation might be a factor that primed cells to be highly susceptible to apoptosis on infection, and apoptosis might be induced before viral antigens reach detectable levels.

Taken together, various mechanisms might be involved in the pathogenesis of IBD. (i) Early after infection, cells expressing viral antigens seem to be protected from apoptosis to ensure virus replication. This suggests inhibition of apoptotic cell death in productively infected cells and might be favourable for the virus. (ii) Induction of apoptosis in cells in the vicinity of productively infected cells might be attributed to antiviral mechanisms of the organism to prevent virus spread. (iii) Later in infection, productively infected cells also undergo apoptosis, induced by IBDV replication, to release the virus from infected cells. Knowledge of the pathways and virus components involved in the induction of apoptosis might help in the development of vaccines that elicit protective immunity without causing lesions in the BF.

The authors wish to thank Jörg Lehmann for help in flow cytometry analysis and Egbert Mundt for enabling the animal experiments. Thanks are due to Md. Rafiqul Islam and Peter Staeheli for critical reading of the manuscript. The technical assistance of Angelika Schneider is grateful acknowledged. These investigations were supported by the Deutsche Forschungsgemeinschaft.

This publication is dedicated to Professor Hermann Becht on the occasion of his 70th birthday on 8 July 2002.

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Received 3 August 2000; Accepted 16 January 2001