Induction of apoptosis in bone marrow neutrophil-lineage cells by classical swine fever virus

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The pathogenesis of bone marrow atrophy during classical swine fever (CSF) was investigated in vitro by using CSF virus (CSFV) infection of bone marrow haematopoietic cells (BMHC). The monocytic lineage had the highest susceptibility to CSFV infection, whereas the more mature SWC8+ granulocytic cells were not directly susceptible to infection. However, myelomonocytic precursors were targets for CSFV infection and continued to differentiate into SWC8+ granulocytic cells, which remained infected. This explains the occurrence of infected peripheral blood granulocytes during CSF. The infection of BMHC resulted in increased apoptosis and necrosis, mainly within the granulocytic lineage. Caspases 3 and 9 were particularly activated, relating to the mitochondrial pathway of apoptosis. Interestingly, the majority of infected cells were non-apoptotic, the apoptotic cells being primarily non-infected. This indicated an indirect mechanism for induction of apoptosis, but no role could be identified for bone marrow stroma cells such as macrophages or fibroblastoid cells. Furthermore, soluble factors including cytokines and reactive oxygen species were not primarily responsible. In contrast, contact between infected and non-infected BMHC was critical for increasing apoptosis in the latter. Taken together, these results in vitro relate to and help to explain further the apoptosis of BMHC that occurs in vivo during CSF. This experimental system will also be particularly useful for the study of CSFV gene products involved in leukocyte apoptosis.

Introduction

Classical swine fever virus (CSFV), a member of the genus Pestivirus in the family Flaviviridae, is known to cause severe leukopenia and atrophy of primary lymphoid tissue in infected swine (reviewed by Trautwein, 1988; Thiel et al., 1996). Peripheral lymphocyte depletion has been attributed to cell death and activation-induced apoptosis as the principal cause of T lymphopenia (Summerfield et al., 1998a). In addition to lymphocytes, mature granulocytes are depleted from the peripheral blood and are replaced by their immature precursors (Summerfield et al., 1998b). No decrease in the viability of peripheral granulocytes was detectable (Summerfield et al., 2000). However, when bone marrow haematopoietic cells (BMHC) isolated from infected pigs were analysed, an increased rate of cell death was observed, caused by apoptotic and possibly also necrotic processes (Summerfield et al., 2000). Considering the short half-life of granulocytes and the fact that no signs of suppressed haematopoiesis were observed in these previous studies, BMHC death appears to be a major cause of granulocytopenia during classical swine fever (CSF). The fact that BMHC death in vivo was not associated with virus infection and replication (Summerfield et al., 2000) was puzzling with respect to the mechanism of cell death. Consequently, the aim of the present in vitro study was to analyse in a controlled manner the influence of CSFV on BMHC with respect to infection, replication and cell death.

Methods

- BMHC isolation, preparation and culture. BMHC were isolated from the sternum of 3- to 6-month-old pigs as described previously (Summerfield & McCullough, 1997). Erythrocytes were lysed in NH4Cl buffer (0.15 M NH4Cl, 10 mM NaHCO3, pH 7.4). Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum (FCS, Sigma), 10 mM HEPES, 2 mM l-glutamine,
100 U/ml penicillin and 100 µg/ml streptomycin at 39 °C. Recombinant porcine granulocyte–macrophage colony-stimulating factor (GM-CSF, 5 ng/ml; Inamur & Takamatsu, 1995) or lipopolysaccharide (LPS, 1 µg/ml; Sigma) was added to stimulate BMHC proliferation.

Bone marrow stroma cells (BMSC) were generated by culture of adherent BMHC for 2–3 weeks in DMEM supplemented as described above. Non-adherent BMHC were removed after 3 and 7 days of incubation, in combination with a medium change. After a further 1–2 weeks, a monolayer of cells had developed with mainly fibroblast, endothelial and macrophage morphology.

Bone marrow-derived macrophages (BM-Mφ) were generated by culture of BMHC in DMEM supplemented with 20% (v/v) FCS and 20% (v/v) heparinized porcine plasma (all other components were as mentioned above) for 10 days at 39 °C. Non-adherent BMHC were removed after 3 and 7 days of incubation in combination with a medium change.

■ Virus infection. The moderately virulent CSFV strain Alfort/187 (kindly provided by K. Depner, Tierärztliche Hochschule Hannover, Germany; Summerfield et al., 1998b) and the virulent strain Brescia (kindly provided by H.-J. Thiel, University of Giessen, Germany; Summerfield et al., 1998a) were propagated in mycoplasma-free SK-6 cells as described previously (Knoetig et al., 1999). Cell lysates were prepared by sonication and were then clarified by centrifugation. Mock-treated controls were prepared in the same manner from non-infected SK-6 cells. UV-inactivated controls were prepared by exposing the cell lysate preparations to a 40 W UV lamp at a distance of 10 cm for 20 min (virus inactivation was controlled). BMHC were infected with CSFV, mock or UV-inactivated preparations on ice at an m.o.i. of 1 TCID₅₀ per cell unless otherwise specified. The inoculum was removed after 1 h by centrifugation and the cells were washed twice with PBS-A (350 g, 10 min, 4 °C). Virus titres were determined and calculated as described previously (Knoetig et al., 1999).

■ Monoclonal antibodies (MAbs) and flow cytometry (FCM). For definition of BMHC populations, anti-SWC3 (MAB 74-22-15, IgG1; Saalmüller, 1996) and anti-SWC8 (MAB MILA3, IgM, Serotec; Haveron et al., 1994; Saalmüller, 1996) MAbs were used. By an SWC3/SWC8 double immunoﬂuorescence analysis of BMHC, the granulocytic lineage was identiﬁed as SWC3⁻/⁺, SWC8⁻/⁺; the monocytic cells as SWC3⁻/⁺, SWC8⁻/⁺ and the early myeloid precursors as SWC3⁺/⁺, SWC8⁻/⁺ (Summerfield & McCullough, 1997). Fas expression was determined by using anti-Fas MAB CH-11 (Upstate Biotechnology). Indirect MAB labelling for triple immunofluorescence FCM was performed in a three-step procedure with isotype-speciﬁc conjugates [goat anti-mouse IgG, F(ab')₂ fragments, FITC- or PE-conjugated or biotinylated; Southern Biotechnology Associates] and streptavidin–SpectralRed conjugate (Southern Biotechnology Associates) as described previously (Summerfield & McCullough, 1997).

Interferon-α (IFN-α) was neutralized with a pool of MAbs (K9 and F17, each 1 µg/ml; Diaz de Arce et al., 1992). B. Charley and C. La Bonnardière (INRA, France) kindly contributed the interferon reagents. A MAB against GM-CSF (64-10) was generated in our laboratory by established methods (Butcher et al., 1991). Hybridoma supernatant from MAB 64-10 was used at 25% (v/v), a concentration found to block GM-CSF-induced BMHC proliferation (A. Summerfield, H. Gerber and K. C. McCullough, unpublished data).

■ Cell sorting. For immunomagnetic cell separation, BMHC were labelled using the following incubation steps: (i) MAB; (ii) biotinylated goat anti-mouse Ig (Jackson Immunoresearch Laboratories); and (iii) biotinylated paramagnetic microbeads (Miltenyi Biotec). Subsequently, the cells were sorted using the MACS magnetic cell separation system, following the manufacturer’s instructions (Miltenyi Biotec). After cell separation, the purity of the positive and negative fractions was controlled by FCM analysis (found to be over 95%).

■ CSFV detection. For detection of CSFV-infected cells, MAbs against the CSFV structural glycoprotein E2 (MAB HC/TC26, 10 µg/ml; kindly provided by Dr Bonnemeli AG, Switzerland; Greiser-Wilke et al., 1990) and the CSFV non-structural protein p125 (MAB C16, kindly provided by I. Greiser-Wilke, Hannover Veterinary School, Germany) were used. For this procedure, the cells were fixed and permeabilized (Cell permeabilization kit, Harlan Sera-Lab) before labelling with the MAb. This method, combined with the m.o.i. employed, permitted the detection of de novo E2 synthesis only, and not the input virus (Knoetig et al., 1999).

CSFV RNA was detected by RT–PCR after extraction of RNA with Trizol (Gibco BRL). The RT–PCR employed the Titan RT–PCR system (Boehringer Mannheim), according to the manufacturer’s instructions, with 1 µg RNA template plus 0.4 µM sense and anti-sense primers for CSFV (HCV-1, 5’ CCG TGA CCG TGG TAG GGG AAA 3’, and HCV-2, 5’ ATT TGG TCT TCG AGG CCC AGA 3’; Wirz et al., 1993) and porcine β-actin (5’ GGA CTT CGA GCA GAT GG 3’ and 5’ GCA CCG TGT CTT CAG AGA GG 3’). The latter were internal controls for the amount of input RNA. Reverse transcription was performed at 50 °C for 30 min followed by 25 cycles of PCR for DNA amplification (94 °C for 45 s; 25 cycles of 30 °C at 59 °C and 1 min at 68 °C).

■ Cell viability and apoptosis analysis. For quantification of apoptotic cells expressing phosphatidylserine (PS) on their surface and dead cells permeable to propidium iodide (PI, Sigma), dual-parameter analysis of annexin V–FITC (Bender Med Systems) and PI were performed (Vermes et al., 1995). To this end, 5 × 10⁶ cells were labelled with 2.5 µg/ml annexin V–FITC in 140 mM NaCl, 2.5 mM CaCl₂, 10 mM HEPES (pH 7.4) for 10 min. After FL1/FL2 compensation, PI (100 ng/ml) was added, in order to discriminate between apoptotic and dead cells, and the sample was acquired by FCM.

A second analysis of apoptosis used a method based on cellular DNA loss, typically found in late-stage apoptosis. Cells were fixed with 75% (v/v) ethanol (0 °C, 2 min) followed by washing and centrifugation. DNA was stained with 50 µg/ml PI plus 100 µg/ml RNase for 30 min at 37 °C and cells were then analysed by FCM. The DNA histograms obtained were used to quantify the apoptotic cells located in the sub-G₁ region (Darzynkiewicz et al., 1992).

The mitochondrial transmembrane potential (ΔΨₘ) of BMHC was measured by incubation with 40 nM 3,3´-diethyloxacarbocyanine iodide (DiOC₃(3)) (Molecular Probes) for 10 min at 37 °C. Formation of reactive oxygen species (ROS) was quantified by incubation of BMHC with 5,5´-dichloro-2′-fluorescein diacetate (DCFH-DA) (Molecular Probes) for 10 min at 37 °C (Rothe & Valet, 1990). During the reaction, HE is oxidized to ethidium, which emits a red fluorescence.

Formation of reactive oxygen species (ROS) was quantified by incubation of BMHC with hydroethidin (HE, 5 nM; Molecular Probes) for 10 min at 37 °C (Rothe & Valet, 1990). During the reaction, HE is oxidized to ethidium, which emits a red fluorescence.

Nuclear morphology was determined by fluorescence microscopy of cytospin preparations (100 µm, 5 min) of BMHC, which were stained with the DNA dye Sytox green (Molecular Probes).

■ Caspase activity. Caspase activity from cell lysates was tested with fluorescent substrates composed of peptides conjugated to 7-amino-4- (trifluoromethyl)coumarin (AFC) or 7-amino-4-methylcoumarin (AMC) as described previously (Summerfield et al., 2000). Substrates were Z-DEVD-AMC for caspase 3, Ac-LETD-AMC for caspase 8 and Ac-LEHD-AMC for caspase 9 (all from Calbiochem). Briefly, 50 µg protein obtained from cell lysates was mixed with 5 mM of the enzyme substrate in assay buffer [315 mM HEPES–NaOH, 31.25% (w/v) sucrose, 10 mM DTT, 5 mM HEPES–NaOH, 31.25% (w/v) sucrose, 10 mM DTT,
ROS scavengers. The potential role of ROS in the effect of CSFV on the viability of BMHC was investigated by using the ROS scavengers (all from Sigma) catalase (CAT, 1000 U/ml), superoxide dismutase (SOD, 2–200 U/ml), butylated hydroxyamisol (BHA, 100 µM) and N-acetylcysteine (NAC, 2 mM). CAT, BHA and NAC have been demonstrated to affect extracellular and intracellular ROS activity (Sundaresan et al., 1995; Katschinski et al., 2000; Schweizer & Peterhans, 1999; Zaragoza et al., 2000; Shimura et al., 2000).

PKH labelling for co-culture experiments. BMHC were labelled by using the red fluorescence cell linker kit PKH-26 GL (Sigma), following the manufacturer’s instructions. Labelled BMHC were detected in the fluorescence-3 channel of the FCM and thereby could be excluded when co-culture experiments with unlabelled cells were analysed.

Results

Target cells for CSFV in the bone marrow

The susceptibility of BMHC to CSFV was determined by infection at different m.o.i. (0·1, 1 or 10 TCID₉₀ per cell) and monitoring of viral E2 expression at 24, 48 and 72 h post-infection (p.i.). Independently of the m.o.i., fewer than 5% of freshly isolated BMHC were E2⁺ at 24 h p.i. in the experiment shown in Fig. 1(a). Although the number of susceptible cells that could be identified at 24 h p.i. varied between experiments (3–25%), it was clear that only a minor subset of freshly isolated BMHC were susceptible to CSFV infection. As time progressed, the number of CSFV-infected BMHC increased up to 65%, again independently of the m.o.i. (Fig. 1(a)).

In order to identify the target cells in these cultures, triple immunofluorescence analyses were performed of SWC3 versus SWC8 and SWC8 versus E2, as well as cell-sorting experiments. For the latter, BMHC were separated into SWC8⁻ and SWC8⁺ fractions, whereby the non-granulocytic and granulocytic lineages could be separated. The unsorted cells and the SWC8⁻ and SWC8⁺ fractions were infected with CSFV (m.o.i. 1 TCID₉₀ per cell), cultured for 64 h and phenotyped in an SWC3/SWC8/E2 triple labelling. The SWC3/SWC8 labelling of all cells is shown in Fig. 1(b); plots on the left represent the unsorted cells; the middle plots represent the SWC8⁻ fraction and the plots on the right represent the SWC8⁺ fraction. The corresponding plots in Fig. 1(c) represent the phenotype of the gated E2⁺ infected cells alone. With the unseparated BMHC, similarly to freshly isolated BMHC, five populations were present: SWC3⁻ SWC8⁻ cells, composed mainly of stem cells and erythroid and lymphoid precursors (R1); more mature, SWC3⁻ SWC8⁺ cells of the B cell lineage (R2); SWC3 low SWC8⁺ myeloid progenitor cells (R3); SWC3⁺ SWC8⁻ monocyteic lineage cells (R4); and SWC3⁺ SWC8⁺ granulocytic lineage cells (R5) (Summerfield & McCullough, 1997). In this culture, wherein 21% of the total cells were E2⁺, the majority of SWC3⁺ SWC8⁻ monocyteic cells carried the virus antigen (58%; Fig. 1c, left plot), followed by the SWC3 low SWC8⁺ myeloid progenitor BMHC (31%), the SWC3⁺ SWC8⁺ granulocytic population (13%) and, last of all, the SWC3⁻ non-myeloid cells (8%). With the sorted SWC8⁻ BMHC, the SWC8⁻ populations dominated. However, 30% were SWC3⁺ SWC8⁺, representing cells that had differentiated during the time of culture after sorting (Fig. 1b, middle plot). In this culture, 43% became E2⁺ at 64 h after CSFV infection, showing a distribution of virus-infected cells similar to that seen with unsorted cells (Fig. 1c, middle plot). As for the sorted SWC8⁻ fraction, which remained predominantly SWC8⁺ after 64 h of culture (Fig. 1b, right plot), fewer than 1% were E2⁺ (Fig. 1c, right plot), primarily the few SWC8⁻ cells that contaminated this preparation.

This infection of BMHC cultures resulted in high titres of extracellular progeny virus. Titres of 10⁷–10⁸ TCID₉₀/ml were measured at 3 days p.i. after infection with both the Alfort/187 and Brescia strains.

Induction of apoptosis in CSFV-infected BMHC cultures

Analysis of BMHC obtained from CSFV-infected pigs revealed an increase in both necrotic cells and cells undergoing apoptosis (Summerfield et al., 2000). Consequently, the influence of CSFV on the survival of BMHC in vitro was investigated. After infection of BMHC at an m.o.i. of 1 TCID₉₀ per cell, increases in the numbers of both dead and definable apoptotic cells were found at 48 h p.i., compared with mock-treated cultures (Fig. 2a). In fact, the elevated levels of apoptosis in CSFV-infected cultures were seen between days 2 and 4 of the culture, often with a maximum at 3 days p.i., when reduced cell counts (30–65%) were also found. The number of annexin V⁺ PI⁺ apoptotic cells following infection was four times that of control mock-treated cultures (lower-right quadrants) and the number of dead, PI⁺ cells was also higher in infected BMHC (upper-right quadrants). These results were confirmed by the observation that CSFV-infected BMHC cultures displayed an increased number of cells with reduced ΔΨₘ compared with mock-treated controls (Fig. 2b; lower left quadrants). In addition, over half of these definable apoptotic cells displayed increased ROS activity, as demonstrated by oxidation of HE to ethidine (Fig. 2b; upper quadrants). As further confirmation of a possible role for apoptosis during CSFV infection of BMHC, the DNA content of the above cells was analysed. Within the infected cultures, more cells were located in the sub-G₁ region (Fig. 2c).
Fig. 1. Kinetic analysis of CSFV infection in BMHC and identification of target cells. (a) Percentage of BMHC expressing viral glycoprotein E2 over a 4-day culture period, following infection with CSFV at an m.o.i. of 0–1 (■), 1 (□) or 10 (▲) TCID\textsubscript{50} per cell. The cells were stimulated with 1 µg/ml LPS. (b)–(c) SWC3/SWC8 expression of unsorted (left plots) and MACS-sorted SWC8\textsuperscript{−} (middle plots) and SWC8\textsuperscript{+} (right plots) BMHC. The BMHC were infected (m.o.i. 1 TCID\textsubscript{50} per cell), cultured for 64 h and then analysed in an SWC3/E2/SWC8 triple immunofluorescence labelling. In (b), the total BMHC are shown and the numbers in the regions defining the BMHC populations give the percentages of each population. In (c), only the E2\textsuperscript{+} cells are shown (determined by electronic gating), and the numbers in the regions give the percentage of E2\textsuperscript{+} BMHC relative to the total cells in that region. The results of a representative experiment are shown.

Caspases are proteases involved in the initiation and execution of the apoptotic process, although some of them have been associated with other cell processes. Addition of Z-VAD-FMK, a general caspase inhibitor, reduced virus-induced cell death by 70–85%, indicating the presence of apoptosis involving the caspase cascade. In order to confirm the role of caspases in the apoptotic events, caspase activity was quantified in cell lysates of BMHC that had been cultured for 72 h (Fig. 2d). Caspase 3 activity was clearly present in all cultures, but with 4–3-fold higher activity in CSFV-infected cells. For caspase 8, the difference between mock-treated and CSFV-infected cultures was not so great, although higher levels were seen in infected cells. The levels of caspase 9 activity were relatively low, but a clear 15-fold increase was discernible with CSFV infection (Fig. 2d).

No significant differences were found in the number of cells with reduced viability at the different m.o.i. employed, 0–1, 1 and 10 TCID\textsubscript{50} per cell (data not shown). Furthermore, no significant differences were observed when the degrees of virus-induced apoptosis with the moderately virulent Alfort/187 strain and the highly virulent Brescia strain were compared (data not shown).
Haematopoietic cell apoptosis induced by CSFV

**Fig. 2.** Influence of CSFV infection on the viability of BMHC. BMHC cultures stimulated with GM-CSF (10 U/ml) were either mock-treated or infected with CSFV and harvested after 72 h. (a) Dual-parameter analysis of PS-expressing cells (annexin V-labelled) versus dead cells (PI-labelled). (b) Dual-parameter analysis of mitochondrial transmembrane potential [DiOC\(_6\)(3)-labelled] versus ROS (ethidine-labelled). (c) Dual-parameter analysis of cell size (FSC) versus DNA content. The results of a representative experiment are shown. (d) Caspase 3 (C3), caspase 8 (C8) and caspase 9 (C9) activity detected in cell lysates of mock-treated (unfilled bars) and CSFV-infected BMHC (filled bars). Caspase activity from the virus-infected cultures was divided by that from the mock-treated cultures (fold-increase); these values are shown above the bars.

**Table 1.** Influence of BMHC stimulation on virus infection and viability

The percentages of E2\(^+\) and annexin V\(^+\) BMHC are shown, as well as the relative increase in annexin V\(^+\) BMHC in CSFV-infected compared with mock-treated cultures. A typical experiment is shown with BMHC cultured for 72 h prior to the analyses.

<table>
<thead>
<tr>
<th>Addition(s)</th>
<th>Annexin V(^+) (%)</th>
<th>Increase in annexin V(^+) (%)</th>
<th>E2(^+) (%)</th>
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<tbody>
<tr>
<td>No addition</td>
<td>18</td>
<td>36</td>
<td>100</td>
</tr>
<tr>
<td>LPS</td>
<td>7</td>
<td>27</td>
<td>285</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7</td>
<td>23</td>
<td>228</td>
</tr>
<tr>
<td>LPS + anti-GM-CSF</td>
<td>17</td>
<td>35</td>
<td>106</td>
</tr>
</tbody>
</table>
Influence of BMHC stimulation on culture characteristics following CSFV infection

BMHC cultures require certain haematopoietic growth factors for their survival, such as GM-CSF (Williams et al., 1990). Cultures without additional stimulation suffered from relatively high rates of ‘spontaneous’ apoptosis (10–30% annexin V+ cells in the mock-treated cultures, Table 1). Addition of either GM-CSF or LPS improved the viability of both mock-treated and virus-infected cultures (Table 1). However, the ratio of annexin V+ cells in infected BMHC increased compared with mock-treated BMHC. This related to an increase in the percentage of cells infected (Table 1). When GM-CSF bioactivity was blocked by an anti-GM-CSF neutralizing antibody, the level of apoptosis increased in both mock- and virus-infected cultures to that seen in the absence of stimuli, as did the number of virus-infected cells. It was also noted that the presence of both LPS and anti-GM-CSF antibodies reversed the influence of LPS, demonstrating the role of GM-CSF in the LPS-induced effect.

CSFV-induced apoptosis in granulocytic BMHC

Morphological analysis of infected BMHC stained with the DNA dye Sytox green revealed cells with condensed DNA, damaged cell membranes and DNA leakage, compared with mock-treated cultures (Fig. 3a). In order to identify which cells were subject to apoptosis and death, the forward-scatter (FSC)
Evidence that CSFV infection does not induce apoptosis directly in infected BMHC

UV-inactivation of CSFV or blocking virus infection with anti-E2 antibody abolished both E2 expression and CSFV-induced apoptosis (data not shown). This would imply that infection is an essential component in the observed induction of apoptosis within BMHC. However, a dual-parameter analysis of annexin V versus E2 expression showed otherwise (Fig. 4). The increase in annexin V+ cells following CSFV infection was not confined to the infected cells (Fig. 4a). In fact, the majority of apoptotic cells in the infected cultures were virus E2−. This divergence in the distribution of viral antigen and annexin V staining was also seen when the cells were labelled with annexin V together with MAb C16 against CSFV non-structural protein p125 (data not shown). An additional experiment confirmed that virus replication was associated more with the non-apoptotic cells. Infected and mock-treated BMHC, after 3 days of culture, were sorted into annexin V− and annexin V+ populations. Amplification of viral RNA by RT–PCR yielded a more intense CSFV-specific band from the annexin V− population than from the annexin V+ population (Fig. 4b, compare lanes 1 and 2). In contrast, the amounts of PCR product from the housekeeping gene β-actin were similar for both populations of virus and control cultures (Fig. 4b, lanes 3–6).

Role of soluble factors

Because of the above results, experiments were performed to investigate the role of soluble factors that are potentially involved in CSFV-induced apoptosis of BMHC. To this end, supernatants were harvested from mock-treated or CSFV-infected BMHC cultures (72 h p.i.), BM-Mφ (48 h p.i.) and BMSC (48 h p.i.). The capacity of these supernatants to induce apoptosis in freshly isolated BMHC was investigated. Any influence of the virus was controlled by adding anti-E2 MAb to particular cultures.

As shown in Table 2, when the BMHC were stained with annexin V after 24 h, the difference between the cultures treated with supernatant from mock- and virus-infected BMHC was not significant. There was a slight increase of annexin V+ BMHC cells when the supernatants were from CSFV-infected BMHC, and this increase was neutralized by addition of anti-E2 MAb. When the supernatants were from mock- or virus-infected BM-Mφ or BMSC, there was no difference in the number of annexin V+ cells in the treated BMHC at 24 h.

Viability analyses performed after 72 h now revealed increased rates of apoptosis when the supernatants were from the CSFV-infected cultures (BM-Mφ and BMSC are shown in Table 2). Again, blocking virus infectivity with anti-E2 MAb prevented this increase in apoptosis. In contrast, blocking IFN-α activity with neutralizing antibodies did not change the picture significantly.

The role of ROS, which are also potential soluble mediators of apoptosis, was investigated by using different ROS scavengers. These included CAT, SOD and BHA. None of these scavengers reduced the observed virus-induced cell death, as exemplified in Table 2 by the data obtained with BHA.

An additional experiment confirmed that soluble factors were not the elements mainly responsible for the observed CSFV-induced cell death in BMHC. In place of supernatant transfer, the infected BMHC were co-cultured with fresh indicator BMHC, but were separated from the latter by using a trans-well culture system. No difference was seen between co-cultures with mock-treated and virus-infected BMHC (Table 2). In fact, the slight increase in apoptosis observed when the supernatants from virus-infected BMHC were employed was no longer evident.
### Table 2. Role of soluble factors in CSFV-induced BMHC death

<table>
<thead>
<tr>
<th>Source of soluble factors</th>
<th>Infection</th>
<th>24 h</th>
<th>24 h + anti-E2</th>
<th>72 h</th>
<th>72 h + anti-E2</th>
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<tbody>
<tr>
<td>BMHC supernatant*</td>
<td>Mock</td>
<td>17</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>24</td>
<td>17</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BMHC trans-well†</td>
<td>Mock</td>
<td>22</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>19</td>
<td>18</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BM-Mφ supernatant‡</td>
<td>Mock</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>6</td>
<td>7</td>
<td>26</td>
<td>8</td>
</tr>
<tr>
<td>BMSC supernatant‡</td>
<td>Mock</td>
<td>16</td>
<td>15</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>14</td>
<td>15</td>
<td>18</td>
<td>6</td>
</tr>
<tr>
<td>Anti-IFN-α</td>
<td>Mock</td>
<td>8</td>
<td>ND</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>11</td>
<td>ND</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>BHA</td>
<td>Mock</td>
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<tr>
<td></td>
<td>CSFV</td>
<td>10</td>
<td>ND</td>
<td>23</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Supernatants (25%, v/v) from 72-h-old mock-treated or CSFV-infected cultures, added to freshly isolated indicator BMHC.
† 72-h-old BMHC were placed into trans-well inserts (pore size 0.8 µm), physically separated from the indicator BMHC.
‡ Supernatants (25%, v/v) from CSFV-infected BM-Mφ or BMSC (m.o.i. 5 TCID₅₀/ml, cultured 48 h p.i.), added to indicator BMHC.
ND, Not done.

### Table 3. Role of cognate interactions between infected cells and freshly isolated indicator BMHC in CSFV-induced apoptosis

<table>
<thead>
<tr>
<th>Infected cells</th>
<th>Infection</th>
<th>24 h</th>
<th>24 h + virus inhibition*</th>
<th>72 h</th>
<th>72 h + virus inhibition*</th>
</tr>
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<tbody>
<tr>
<td>BM-Mφ†</td>
<td>Mock</td>
<td>4</td>
<td>12</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>6</td>
<td>15</td>
<td>26</td>
<td>23</td>
</tr>
<tr>
<td>BMSC†</td>
<td>Mock</td>
<td>7</td>
<td>21</td>
<td>4</td>
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<tr>
<td></td>
<td>CSFV</td>
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<td>17</td>
<td>18</td>
<td>32</td>
</tr>
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<td>BMHC‡</td>
<td>Mock</td>
<td>7</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CSFV</td>
<td>16</td>
<td>15</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Virus inhibition was effected by adding 10 µg/ml anti-E2 MAb (BMHC–BMHC co-cultures) or by fixation of the cells with 1% (w/v) paraformaldehyde (BM-Mφ–BMHC and BMSC–BMHC co-cultures).
† BM-Mφ and BMSC were infected with CSFV (m.o.i. 5 TCID₅₀/ml) for 48 h and then added (2.5 × 10⁶ cells/ml) to freshly isolated indicator BMHC.
‡ BMHC from mock-treated or CSFV-infected (m.o.i. 5 TCID₅₀/ml) cultures were co-cultured at 72 h p.i. with freshly isolated indicator BMHC. Only the percentages of the freshly isolated BMHC are shown.
ND, Not done.

### Role of cognate interactions

Taken together, the above results demonstrated that CSFV-induced BMHC death was not a direct consequence of the presence of CSFV, but also was not induced by soluble factors released by infected cells. Consequently, the possible role of cognate interactions between CSFV-infected and non-infected BMHC in virus infection-induced apoptosis was analysed. To this end, BM-Mφ and fibroblastoid BMSC were employed. These two types of cell are known to be amongst the main regulatory elements in the bone marrow microenvironment (Dorshkind, 1990). After 48 h of culture, the cells were
harvested and co-cultured with freshly isolated BMHC. In order to inactivate the virus and inhibit its intercellular spread, infected cells in particular co-cultures were fixed with 1% (w/v) paraformaldehyde. The viability of the indicator BMHC at 24 h after co-culture revealed no significant differences between co-cultures with mock-treated or with CSFV-infected BM-Mφ and BMSC (Table 3). In contrast, by 72 h after co-culture, apoptosis was clearly enhanced when CSFV-infected cells were used. This was less evident when the BM-Mφ and BMSC were fixed before co-culture.

It was possible that the important cognate interactions were with infected BMHC, and not the BM-Mφ or BMSC. In order to test this hypothesis, infected or mock-treated BMHC were cultured for 3 days and then labelled using the fluorescent cell-membrane dye PKH-26 (to identify and separate them from the freshly isolated indicator BMHC in the co-cultures). Co-culture was then performed with freshly isolated BMHC in the order to inactivate the virus and inhibit its intercellular spread. The number of apoptotic cells induced in the indicator BMHC was approximately twofold greater when infected cells were used, compared with the mock-treated cells (Table 3). Released virus was not the cause of this increased apoptosis, because addition of anti-E2 MAb did not alter the results.

Discussion

Increased numbers of necrotic and apoptotic uninfected cells have been identified in the bone marrow of CSFV-infected pigs (Summerfield et al., 2000). The present work employed in vitro BMHC cultures to establish, in a controlled manner, the role of the CSFV infection therein. The target cells for CSFV within the BMHC population were mainly within the myeloid lineages, and the monocytic lineage was the most susceptible. This contrasted with the more mature, SWC8(high) granulocytic cells, which were not susceptible to CSFV infection. However, the present work demonstrated that CSFV could infect the immature, less-differentiated cells, such as the SWC3(low) SWC8(low) myeloid progenitors. Their differentiation along the granulocytic lineage was apparently not impaired by this infection. It was through such infection of the myeloid progenitors that infected SWC8(high) granulocytic cells were observed. This characterization is particularly pertinent in that it demonstrates how and why infected SWC8(high) granulocytic cells appear in the peripheral blood of CSFV-infected pigs (Summerfield et al., 1998c).

Concerning the influence of CSFV infection on BMHC, several features of apoptotic cell death were identified: PS expression on the cell surface, reduced ΔΨm, increased ROS generation, loss of DNA and increased caspase activity. The particularly strong increase in caspase 9 in CSFV-infected cultures would point to a role for the mitochondrion-initiated pathway of apoptosis (Budihardjo et al., 1999; Rathmell & Thompson, 1999). It is clear that CSFV-induced BMHC death does not relate to the apoptosis observed with another pestivirus, the cytopathic form of bovine viral diarrhoea virus (Zhang et al., 1996; Hoff & Donis, 1997; Schweizer & Peterhans, 1999; Jungi et al., 1999). This may be expected, considering that CSFV strains, such as those employed in the present study, are primarily non-cytopathogenic for kidney cell lines, Mφ and fibroblastoid BMSC. Although Shimizu et al. (1995) reported a cytopathic effect of CSFV in cultured BMSC, we have never observed this phenomenon. Of course, the effect on the stromal cells in vitro reported by Shimizu et al. (1995) was only detectable late (10 days) p.i., a time-point well beyond our observations on the induction of apoptosis.

The majority of apoptotic BMHC in CSFV-infected cultures were uninfected, indicating that this effect of CSFV on BMHC was not a direct consequence of virus infection and replication. A possible explanation would be that infected cells were modulated in their haematoregulatory activity. Important therein are the cellular components of the bone marrow stroma. However, no role could be identified for factors or cognate interaction between BMHC and infected Mφ or other stromal cells, at least in the context of induction of apoptosis in the BMHC. Furthermore, none of our investigations pointed to a role for soluble factors released from infected cells in CSFV-induced apoptosis of BMHC.

Interestingly, an increased rate of apoptosis was observed when uninfected BMHC were co-cultured with virus-infected BMHC. This effect was dependent on cell contact between infected and uninfected cells. Such an observation would implicate death receptors on BMHC interacting with their ligands on CSFV-infected BMHC, but not stromal cells.

Taken together, the present results describe an in vitro model for the investigation of the characteristic bone marrow atrophy and peripheral loss of mature granulocytes found in CSF. The observed CSFV-dependent induction of apoptosis in granulocytic cells appears to relate to observations made with BMHC isolated from CSFV-infected pigs (Summerfield et al., 2000). This cell death could be central to the observed loss of mature granulocytes in the blood during CSF, being replaced by immature granulocytes, and in the development of bone marrow atrophy (Summerfield et al., 2000). The experimental system employed will prove particularly useful for further analyses of CSFV pathogenesis and for the study of CSFV gene products involved in leukocyte modulation and apoptosis.

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