Pathogenesis of Granulocytopenia and Bone Marrow Atrophy during Classical Swine Fever Involves Apoptosis and Necrosis of Uninfected Cells

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Granulocytopenia, a hematological hallmark of classical swine fever, is partially responsible for the suppression of innate immune defenses during classical swine fever. The present report demonstrates that this depletion was apparent as early as 3 days postinfection (p.i.). Both mature peripheral and bone marrow neutrophils were affected, whereas immature neutrophils increased absolutely in the periphery and coincidentally immature myeloid progenitors in the bone marrow. These data suggest that a pathogenic relationship exists between these compartments. The central event was not the arrest of hematopoietic cell proliferation or of the mobilization process, but instead apoptosis and possibly also necrosis were shown to play a role. This increase in apoptotic and dead cells was detected as early as 1–3 days p.i. In contrast, viral RNA in bone marrow hematopoietic cells (BMHC) was first detected 5 days p.i., and significant amounts of infected BMHC were detected only 7 days p.i., with the major target being the myeloid compartment. The increased caspase-3 activity observed supported a role for apoptotic cell death. Furthermore, the elevated caspase-9 activity indicated the involvement of the mitochondrial apoptotic pathway. Taken together, the results demonstrate that granulocytopenia and bone marrow atrophy are mediated by hematopoietic cell death and that indirect virus–host-mediated mechanisms are likely to be responsible.

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Key Words: granulocytopenia; bone marrow atrophy; apoptosis; necrosis; caspases; hematopoiesis; classical swine fever.

INTRODUCTION

For both the ontogeny and the maintenance of the immune system, the bone marrow is a crucial site due to its pivotal role therein. The development of an immune response against a given virus infection also relies on the correct functioning of the bone marrow environment. This is particularly evident with respect to the continued replacement of the polymorphonuclear leukocytes as they become depleted in the periphery (Cartwright et al., 1964; Dancey et al., 1976). Accordingly, an immunomodulatory virus infection could have substantial consequences on the efficiency of the bone marrow function and subsequently on peripheral immune defenses.

Classical swine fever virus (CSFV) offers much potential for studying the virus-induced immunomodulatory events. This is a small enveloped RNA virus of the Flaviviridae, genus Pestivirus, related to hepatitis C and Flaviviruses (Thiel et al., 1996). During classical swine fever (CSF), the immune system is severely compromised and is associated with hematological modulations, which include leukopenia, thrombocytopenia, and primary lymphoid organ perturbation (reviewed in Trautwein, 1988). The particular affinity that the virus possesses for myeloid and endothelial cells (Cheville and Mengeling, 1969; Ressang, 1973; Susa et al., 1992; Pauly et al., 1998; Summerfield et al., 1998a) early in infection would render these targets of particular importance for the immunomodulatory events. Leukopenia changes during CSF involve both lymphopenia and granulocytopenia (Susa et al., 1992; Summerfield et al., 1998a,b). While T lymphocyte depletion was shown to be associated with apoptosis probably mediated through Fas–Fas ligand interactions (Summerfield et al., 1998b), the mechanisms underlying the granulocytopenia remain unknown. Except for the thrombopoietic cells, which are depleted and infected (Hoffmann et al., 1971; Gomez-Villamandos et al., 1998), not much is known with regard to the pathological and pathogenic events in the bone marrow during CSF. In addition and unfortunately, most articles were not published in English (Pehl, 1959; Shara-brin, 1968; Manolescu and Popa, 1974).

The objective of the present study was to investigate the relationship between peripheral granulocyte depletion and changes in the bone marrow myeloid compartment following CSFV infection of pigs. Studies were performed in efforts to define the effects of CSFV infec-

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2 Abbreviations used: BMHC, bone marrow hematopoietic cells; CSF, classical swine fever; CSFV, classical swine fever virus; FCM, flow cytometry; PI, propidium iodide; SWC, Swine Workshop Cluster, for CD molecules identified on porcine leukocytes for which no equivalent human CD molecule is known.
tion on the bone marrow hematopoietic environment. One of the objectives of this study was to determine whether granulocytopenia was secondary to bone marrow atrophy and whether the latter was a consequence of bone marrow cell trafficking in response to the peripheral leukopenia, a consequence of suppressed hematopoiesis, or caused by virus-induced cell death. Furthermore, it was of interest to know which lineages of bone marrow hematopoietic cells (BMHC) were targets for CSFV infection and how infection of BMHC related to the detrimental effects of the virus in the bone marrow.

RESULTS

Peripheral granulocyte depletion and immature cell dominance during CSF

After infection of pigs with the highly virulent CSFV strain Brescia, elevated body temperatures (>39.5°C by 2 days postinfection (p.i.), >40°C by day 3 p.i.; data not shown) plus a rapid onset of leukopenia between 1 and 3 days p.i. (Figs. 1a–1c) were observed. By 3 days p.i., the number of leukocytes isolated from all infected animals analyzed had fallen to below 9000 cells/μl blood (Fig. 1a); the normal range for leukocyte counts in healthy pigs obtained from the specific pathogen-free (SPF) unit was 10,000–20,000 leukocytes/μl. The loss of leukocytes continued to develop, reaching the lowest levels by 5 days p.i. (Fig. 1a).

The differentiation stage of the neutrophils within the leukocyte preparations was analyzed by microscopy. Therein, a depletion—in terms of absolute counts—of mature granulocytes with segmented nucleus was noted to occur concomitantly with the general leukopenia (Fig. 1b). This was not simply a granulocytopenia, since as early as 3 days p.i. over 60% of the granulocytic cells were immature. When absolute numbers of immature granulocytes were calculated, an increase with time p.i. was observed (Fig. 1c). Mainly neutrophilic bands, but also some metamyelocytes and myelocytes, were present; the last type continued to increase, particularly on day 5 and 7 p.i. (data not shown).

Depletion of BMHC

Granulocytes have a particularly high turnover rate, with hemostasis depending on the continuous production of granulocytes in the bone marrow (Cartwright et al., 1964; Dancey et al., 1976). Consequently, it was decided to include this organ in the analyses. Although absolute quantification of bone marrow cellularity is difficult, when BMHC from infected pigs were analyzed, the cell harvest decreased with time p.i. (Fig. 2a). Figure 2 shows the BMHC harvest from the infected animals, as well as from six uninfected control pigs (x-axis, c) for the purpose of comparison. At 1 day p.i., the BMHC counts were at the lower end of the range for uninfected pigs. By 3 days p.i., the harvests were often less than 30% of those obtained from uninfected animals. Despite this loss, the remaining cells retained their proliferative capacity (Fig. 2b). Although there was variation between animals, the proliferative capacity of the BMHC remaining in CSFV-infected animals was close to that found with BMHC from uninfected pigs.

FIG. 1. CSFV-induced depletion of the more mature granulocytic cells and simultaneous relative increase of immature granulocytic cells in the peripheral blood. (a) Leukocyte counts, determined by microscopy of heparinized whole blood samples after lysis of erythrocytes with Türk’s solution. (b) Absolute numbers of polymorphonuclear segmented neutrophils (PMN). (c) Absolute numbers of immature peripheral granulocytes (IG; mainly neutrophilic bands, myelocytes, and metamyelocytes). The maturity stage of the cells was assessed by microscopic analysis of DiffQuik-stained leukocyte preparations. Peripheral leukocytes were obtained from uninfected control pigs (x-axis: c), and from CSFV-infected pigs at different days p.i. (x-axis: 1–7), from a total of 18 animals. Each symbol represents an individual animal for a particular day, which was kept identical throughout this and subsequent figures, in order to compare the individual animal results in terms of the different parameters analyzed in each figure. These symbols do not represent the same animals on different days, because each pig was slaughtered to obtain the leukocyte preparations.
Modulation of bone marrow granulocytic cell subpopulations

The above results would suggest that hematopoiesis was not arrested. Confirmation for this and further information on hematopoietic modulation during CSF came from analyses of myeloid BMHC populations by flow cytometry (FCM) using SWC3/SWC8 labelings: SWC3<sup>−</sup>SWC8<sup>−</sup> nonmyeloid cells (R1, Fig. 3a), SWC3<sup>+</sup>SWC8<sup>−</sup> more mature granulocytic cells (R2, Fig. 3a), SWC3<sup>low</sup>SWC8<sup>−</sup> myeloid progenitors (R3, Fig. 3a) and SWC3<sup>high</sup>SWC8<sup>−</sup> monocytic cells (R4, Fig. 3a). BMHC from infected pigs showed that there was an absolute decrease in all subpopulations. However, the relative representation of the more mature granulocytic cells (SWC3<sup>+</sup>SWC8<sup>−</sup>) was reduced (Fig. 3c). In contrast, the immature myeloid (SWC3<sup>high</sup>SWC8<sup>−</sup> myeloid progenitors; Fig. 3d) and monocytic cells (SWC3<sup>high</sup>SWC8<sup>−</sup>; Fig. 3e) increased, in a relative manner. These observations related to events in the periphery, wherein the immature cells came to dominate. Furthermore, both the peripheral depletion (Fig. 1) and the bone marrow depletion (Fig. 2) progressed in a kinetically similar manner.

Reduced viability of BMHC following CSFV infection

The maintained proliferative capacity and the increase of immature cells indicated that an arrest or a suppression of hematopoiesis was not responsible for granulocytopenia and bone marrow atrophy. Furthermore, previous work showed that peripheral lymphocytes died by apoptosis during acute classical swine fever (Summerfield et al., 1998b). Consequently, cell death was also considered as the responsible factor for depletion of granulocytes. However, using AnnexinV for external phosphatidyserine expression to quantify apoptotic cells and propidium iodide (PI) to quantify dead cells, no indication for reduced viability was observed (data not shown). This was in contrast to the results obtained with bone marrow samples. In comparison with control BMHC (Fig. 4a), both AnnexinV<sup>+</sup>PI<sup>−</sup> apoptotic cells (upper right quadrant) and AnnexinV<sup>−</sup>PI<sup>−</sup> apoptotic cells (lower right quadrant) had increased in BMHC obtained at 3 and 7 days p.i. (Figs. 4b and 4c). DNA content analysis was also made on these cells to detect very-late-stage apoptotic cells—with reduced DNA content, due to DNA fragmentation (Darzynkiewicz et al., 1992). Examples of results obtained from a control pig (Fig. 4d) and animals at 3 (Fig. 4e) and 7 days p.i. (Fig. 4f) identified an increased number of BMHC located in the sub-G1 region by 7 days p.i. (Fig. 4f).

Looking at all infected pigs, compared with uninfected control animals (defined as “c” on the x-axis of Fig. 5), an increase in AnnexinV<sup>−</sup>PI<sup>−</sup> apoptotic cells was evident as early as 1 day p.i. (Fig. 5a), peaking at 3 days p.i. The elevation of AnnexinV<sup>−</sup>PI<sup>−</sup> dead cells within the BMHC of infected pigs, also from day 1 p.i., was even clearer (Fig. 5b). In contrast to the AnnexinV<sup>−</sup>PI<sup>−</sup> cells, the AnnexinV<sup>−</sup>PI<sup>−</sup> population remained distinctly elevated throughout the course of the infection. As for BMHC located in the sub-G1 region, these were more difficult to identify. Only two animals tested at 7 days p.i. had particularly elevated levels of cells in the sub-G1 area (Fig. 5c). Interestingly, the pig with lower percentage of PI<sup>−</sup> BMHC at 7 days p.i. compared to the other animals (Fig. 5b) also had the lower level of sub-G1 cells (Fig. 5c). The occurrence of apoptosis in these PI-stained cells was confirmed by UV–light microscopy: the nuclei of the cells
contained condensed and fragmented DNA (data not shown).

Caspase activity

The above results indicated an increase in dead AnnexinV<sup>+</sup> and PI<sup>+</sup> cells at early times p.i. (1–5 days p.i.) without showing an increase in sub-G1 cells (Figs. 5b and 5c). This could be explained by a possible phagocytosis of late-stage apoptotic cells at these stages of the disease, making their detection difficult. Nevertheless, PI<sup>+</sup> cells with permeable membrane are usually also classified as late-stage apoptosis. Consequently, it was reasoned that a necrotic cell death pathway may also be involved in BMHC atrophy. To further evaluate this question, caspase-3, a central executing caspase in the cascade of enzyme activation typical of apoptosis,
was investigated. Interestingly, the results presented in Fig. 6a showed that an increase in caspase-3 activity was not detectable before day 3 p.i., but reached very high levels at 7 days p.i. These results would indicate that the early increase of PI<sup>1</sup> cells could relate more to a necrotic rather than an apoptotic mode of cell death. Nevertheless, particularly at 7 days p.i., the importance of caspase-3 and thereby also of apoptosis became evident, and even the BMHC from one animal with a small number of sub-G1 cells (Fig. 6c, triangle) had >20 times elevated caspase-3 activity similar to the two other animals in this group. When caspases that were upstream of caspase-3, such as caspase-8 and caspase-9, were analyzed, a different picture became apparent. Only caspase-9 levels were significantly elevated on days 5 and 7 p.i. (Figs. 6b and 6c).

Evaluation of death events in BMHC populations

To further evaluate the events occurring within the bone marrow following CSFV infection, the scatter characteristics of these BMHC samples were analyzed. Regions were created for setting electronic gates, in which Annexin<sup>V</sup><sup>2</sup> cells (Fig. 4: lower left quadrants), Annexin<sup>V</sup><sup>1</sup>PI<sup>2</sup> cells (Fig. 4: lower right quadrant), and Annexin<sup>V</sup><sup>1</sup>PI<sup>1</sup> cells (Fig. 4: upper right quadrant) could be visualized. In Fig. 7, a representative sample of BMHC from uninfected control pigs is shown in the left column; cells taken at 3 days p.i. are in the middle column, and those taken at 7 days p.i. are in the right column. A small population of cells with particularly low FSC and SSC was visible with all BMHC samples. These probably represented erythrocytes that had not been lysed, as well as contaminating platelets and cell debris that were likely to be present.

The FSC/SSC of the Annexin<sup>V</sup><sup>2</sup> viable BMHC (Fig. 7a) demonstrates that cells with high SSC were being depleted after infection, most notably by 7 days p.i. The SWC3/SWC8 labeling in Fig. 3 identified these as SWC3<sup>1</sup>SWC8<sup>1</sup> granulocytic cells. Annexin<sup>V</sup><sup>1</sup>PI<sup>2</sup> apoptotic cells tended to be FSC<sup>low</sup>SSC<sup>low</sup> for uninfected control BMHC (Fig. 7b, left), but an increase in FSC<sup>high</sup> was clear with BMHC from infected animals (Fig. 7b, middle and right). The SWC3/SWC8 labeling placed such cells within the myelomonocytic precursor and monocytic cell populations. Annexin<sup>V</sup><sup>1</sup>PI<sup>1</sup> dead cells in unin-
fected animal BMHC were also primarily FSC$^{\text{low}}$ SSC$^{\text{low}}$, with some SSC$^{\text{high}}$ (Fig. 7c, left). Following infection, the SSC$^{\text{high}}$ cells, with some increase in FSC, came to dominate (Fig. 7c, middle and right). SWC3/SWC8 labeling characteristics placed these BMHC within the granulocytic populations, although necrotic SWC3$^{\text{1}}$ SWC8$^{\text{2}}$ monocytic cells would also be present.

Infection of BMHC by CSFV in vivo

An important question concerning the understanding of the pathogenesis associated with the above hematological findings was the direct role for virus infection in the BMHC. The analyses of viral RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that the virus was present only at later times p.i. All BMHC samples from pigs slaughtered 1 day p.i. were negative (data not shown), as were five of six samples from 3 days p.i. One of the six animals analyzed 3 days p.i. gave a questionable result (Fig. 8a, lanes 1–6). It was not before 5 days p.i. that viral RNA was clearly detected in the BMHC (Fig. 8a, lanes 7–12). Figures 8b and 8c show the expression of CSFV glycoprotein gpE2 in BMHC collected from the virus-infected animals. Infected cells were first detected at 3 days p.i., in one of six animals (Fig. 8a). This was the pig for which the results of the RT-PCR are shown in lane 6 of Fig. 8a, which had <5% gpE2$^+$ BMHC. A similarly small number of infected cells was observed in BMHC at 5 days p.i., when all three pigs tested had gpE2$^+$ cells (Fig. 8a). By 7 days p.i., all three pigs had gpE2$^+$ cells, but two now possessed 20–25% gpE2$^+$ BMHC.

Using a triple SWC3/SWC8/gpE2 immunofluorescence analysis, with BMHC from an animal possessing 20–25% gpE2$^+$ cells, the BMHC targets for CSFV infection were identified (exemplified in Fig. 8c). By electronic gating on the gpE2$^+$ cells, a SWC3 versus SWC8 dot plot was obtained, wherein the gpE2$^+$ cells are depicted in black (Fig. 8d). Virus antigen was located mainly in the SWC3$^+$ myeloid BMHC. All myeloid cell populations—SWC3$^{\text{1}}$SWC8$^{\text{1}}$ immature myeloid precursors, SWC3$^{\text{2}}$SWC8$^{\text{2}}$ granulocytic cells, and SWC3$^{\text{1}}$SWC8$^{\text{2}}$ monocyotic cells—contained infected cells. Infection was not preferentially distributed within a particular myeloid BMHC population. Similar results were obtained when BMHC obtained from the other pig with over 20% infected BMHC 7 days p.i. were analyzed. These results also relate to those made with PBMC; target cells infected by CSFV at relatively early times p.i. were mainly within the myeloid compartment (Summerfield et al., 1998a).

Despite the association of virus infection with the myeloid compartment, this did not explain the death therein. The numbers of apoptotic and dead cells as well as the caspase-3 activity did not relate to the number of gpE2$^+$ cells.

DISCUSSION

During CSF, the immune system is severely compromised, yet the virus is classically noncytopathogenic (Thiel et al., 1996). Analyses of leukocyte apoptosis in the peripheral blood revealed an increase of apoptotic lymphocyte death following infection with the virulent CSFV strain Brescia (Summerfield et al., 1998b). However, when AnnexinV expression and PI incorporation in the granulocytic cells was determined after infection with the same strain, no increase of apoptotic or dead cells was found. This was surprising considering that mature neutrophils were severely depleted as early as 3 days p.i. In view of the short half-life (7–20 h) of mature neutrophils, one possible explanation for this depletion of mature neutrophils would be their reduced supply from the bone marrow. Theoretically, this could be caused by reduced hematopoiesis, a defect in the mobilization process, or cell death in the bone marrow. Considering that reports exist describing that CSFV induces regressive changes in bone marrow hematopoiesis, these results are consistent with the observations presented in this study.
in the bone marrow (Pehl, 1959; Sharabrin, 1968; Manolescu and Popa, 1974), one main objective of this study was to investigate the events in the bone marrow during the acute phase of the disease, in an attempt to elucidate its role in granulocytopenia.

Several lines of evidence currently presented suggested that hematopoiesis was not arrested. There was an absolute increase of immature granulocytes in the peripheral blood, the proliferative capacity of BMHC was maintained, and more immature myeloid progenitors increased in a relative manner in the bone marrow. As an alternative mechanism to explain the mature granulocyte depletion, there was the possibility that the virus infection was perturbing the bone marrow hematopoietic cell viability. An increase of both AnnexinV$^-$ PI$^+$ apoptotic and AnnexinV$^+$ PI$^+$ dead cells in the bone marrow was indeed noted. Most of these dying and dead cells were found to be granulocytic. Confirmation of the development of apoptotic events came from the detection of an increased number of sub-G1 cells (Darzynkiewicz et al., 1997). The increased caspase-3 and caspase-9 activities, which were detected, also pointed to the importance of apoptotic events, due to the association of the caspase cascade with programmed cell death (Porter and Janicke, 1999; Rathmell and Thompson, 1999). The late detection of sub-G1 cells and possibly also the failure to identify apoptotic peripheral granulocytic cells could be explained by phagocytes interacting with the external phosphatidylserine (PS) expressed on apoptotic cells. AnnexinV would identify this PS expression, but phagocytes in vivo could remove such cells before DNA fragmentation would occur (Fadok et al., 1992; Savill et al., 1993). The immunomodulatory changes associated with CSF would have resulted in increasing numbers of cells with external PS expression, placing an increasing burden on the phagocytes. Nevertheless, the AnnexinV$^+$ PI$^+$ cell numbers found 1–3 days p.i., combined with the low caspase-3 activity, would reflect cells undergoing also accidental or necrotic cell death. Such results do not necessarily indicate that two distinct mechanisms are involved in BMHC death. Apoptotic and necrotic modes of cell death do not exclude each other and can be caused by the same agents depending on the intensity of the death trigger or certain other conditions (Bonfoco et al., 1995; Leist et al., 1997; Zamzami et al., 1997; Plymale et al., 1999).

Further investigation into cell death within the bone marrow demonstrated that it was most probably independent of the direct effects of virus infection. The first appearance of apoptotic and necrotic BMHC was clearly before the detection of viral RNA or protein. This relates to the noncytopathogenic nature of CSFV, also observed in BMHC cultures infected in vitro. In these cultures, CSFV infection induced apoptosis, but mainly in the un-
infected cells (Summerfield, unpublished results). From these results, a working hypothesis for future studies can be proposed wherein the supportive role of the bone marrow stromal system in hematopoiesis would be compromised by CSFV infection therein. Interestingly, Shimizu et al. (1995) did report a cytopathic effect in cultured bone marrow stromal cells. If this were definitively due to the virus itself, it would indicate a capacity of CSFV to disturb the bone marrow microenvironment. Nevertheless, the observed effect on the stromal cells in vitro appeared late (10 days p.i.), suggesting either an interaction of several factors or a required build-up of the infection before cytopathogenicity could be affected. Consequently, these observations are unlikely to be solely responsible for that observed in vivo. Furthermore, the virulent strain Brescia used in the present studies did not show any cytopathogenic effects in bone marrow stromal cells cultured in vitro (Summerfield et al., unpublished results).

Death receptors such as Fas have been reported to be upregulated on hematopoietic progenitor cells in vivo following infection with murine cytomegalovirus (Mori et al., 1997). However, no increase in Fas expression was found in the present study (data not shown). Moreover, the unchanged levels of caspase-8—usually active in death receptor induced apoptosis (reviewed in Rathmell and Thompson, 1999)—would point to a different apoptotic pathway. Attempts to identify serum factors with pro-apoptotic activity for BMHC in CSFV-infected pigs have been unsuccessful. Nevertheless, the increased levels of caspase-9 would relate to apoptosis such as that induced by stress following treatment with certain chemicals, irradiation, dexamethasone (Hakem et al., 1998; Kuida et al., 1998), reactive oxygen species, or nitrogen intermediates (Ushmorov et al., 1999; Stridh et al., 1998). The last two are particularly interesting as potential mediators of CSFV-induced BMHC death, because of their capacity to induce both apoptosis and necrosis under pathological conditions (Wood and Youle, 1994; McConkey, 1998; Leist et al., 1999).

Taken together, the results of present study describe a pathogenic model for peripheral granulocytopenia, which would relate to the susceptibility of CSFV-infected pigs to secondary bacterial infections (Trautwein, 1988). It appears that cell death events in bone marrow granulocytes are at least in part responsible for both the

FIG. 8. Infection of BMHC by CSFV. (a) Viral RNA was amplified by RT-PCR from total RNA isolated from BMHC samples at different days p.i. The electrophoresed products are shown (M, 100-bp marker; lane 1–6, 3 days p.i.; lanes 7–9, 5 days p.i.; and lanes 10–12, 7 days p.i.). The CSFV-specific band was found at 155 bp. (b) The expression of viral glycoprotein E2 in BMHC obtained from pigs at different time points p.i. was analyzed by FCM. Each symbol represents the relative proportion of E2⁺ BMHC from an individual animal for a particular day, but a different animal on different days, as for Figs. 1–3. (c) Histogram of E2 expression in BMHC from a pig with 24% E2⁺ BMHC at 7 days p.i.; the gray overlay represents the negative control obtained by labeling with an IgG2b-negative control mAb. (d) Triple immunofluorescence labeling of the glycoprotein E2 expression in the myeloid BMHC populations defined by SWC3 and SWC8 of the animal shown in (c); E2⁺ cells were gated and are depicted black.
peripheral granulocytopenia and the bone marrow atrophy. No direct involvement of the virus was detected, suggesting an indirect host-mediated effect. For the development of a working hypothesis for future investigations, the known inflammatory response of the monocytic cell system following CSFV infection (Knoetig et al., 1999) should be considered. Local and/or systemic inflammatory reactions and the subsequent local production of reactive oxygen or nitrogen species could mediate cellular stress processes known to result in apoptosis and necrosis. In this context, the granulocytic cells are likely to be particularly sensitive (Cotter et al., 1994).

**MATERIALS AND METHODS**

**Infection of pigs with CSFV**

Pigs (Swiss Landrace), 2–3 months old, were bred at the SPF unit of the Institute of Virology and Immunopathology (Mittelhäusern, Switzerland). A total of 18 animals were oro-nasally infected at a dose of $10^6$ TCID$_{50}$/animal with CSFV strain Brescia (provided by Dr. Thiel, University of Giessen, Germany), which has been shown in previous experiments to be highly virulent for pigs (Summerfield et al., 1998b). The animals were checked daily for clinical symptoms and temperature. Six animals were slaughtered at 1 and 3 days p.i., with three pigs slaughtered on days 5 and 7 p.i., in order to collect blood and bone marrow samples. For the purposes of obtaining control blood and bone marrow, six uninfected pigs of the same age, breed, and source were used.

**Preparation of cells**

Peripheral blood leukocytes were obtained by incubation of citrated blood in NH$_4$Cl buffer (0.15 M NH$_4$Cl, 10 mM NaHCO$_3$, pH 7.4) for 5 min at 4°C to lyse erythrocytes (Summerfield et al., 1998b). BMHC were isolated from the sternum and humerus by being flushed with PBS (Summerfield and McCullough, 1997). Erythrocytes in the BMHC preparations were lysed by incubation with the NH$_4$Cl buffer as described above.

**Hematopoietic cell growth**

The spontaneous proliferative capacity of BMHC was employed as a measure of the hematopoietic potential following CSFV infection. BMHC from infected pigs were compared with those from uninfected control animals, to determine whether the infection had an influence on this spontaneous proliferation. Freshly isolated BMHC were cultured at $3 \times 10^4$ cells/ml in microtiter plates. Cell proliferation was measured by culturing the cells for 24 h, followed by incubation with 1 $\mu$Ci $[^3H]$thymidine/well for an additional 18 h at 39°C. After harvesting of the radiolabeled cells onto glass fiber filters, counts per minute were read with a Trace 96 counter (Inotech, Switzerland).

**Monoclonal antibodies and flow cytometry**

For phenotyping, the following mAb were used: anti-SWC3 (mAb 74-22-15, IgG1, VMRD Inc., Pullman, WA; Saalmüller, 1996), anti-SWC8 (mAb MIL3, IgM, Serotec; Saalmüller, 1996; Haverson et al., 1994), and anti-CD95 (mAb CH-11, Upstate Biotechnology, Lucerne, Switzerland; Summerfield et al., 1998b). Using SWC3/SWC8 double immunofluorescence analysis of BMHC, granulocytic cells were identified as SWC3$^+$SWC8$^+$, monocytic cells as SWC3$^+$SWC8$^-$, immature common myeloid progenitors as SWC3$^+$SWC8$^-$, and nonmyeloid populations as SWC3$^-$SWC8$^-$. The last category included lymphocytes, stem cells, and nonleukocyte lineages (Summerfield and McCullough, 1997). Indirect mAb labeling for double and triple immunofluorescence FCM was performed in a two- or three-step procedure, respectively. Following mAb labeling, isotype-specific conjugates (goat anti-mouse IgG, F(ab')$_2$, fragments, FITC, PE, or biotin conjugated; Southern Biotechnology Associates) were used as third-step reagent. For triple immunofluorescence analyses, streptavidin-spectral red conjugate (Southern Biotechnology Associates) was used as third-step reagent for fluorescence 3 (Summerfield and McCullough, 1997).

Data were acquired with a FACScan flow cytometer, and the PC Lysis program (Becton Dickinson, Mountain View, CA) was used for analysis.

**Infection detection by immunofluorescence and RT-PCR**

For detection of CSFV-infected cells, the anti-CSFV E2 glycoprotein (gpE2) mAb HC26 was used (kindly provided by Dr. Bommeli AG, Bern, Switzerland; Greiser-Wilk et al., 1990). Due to the internal expression of gpE2 in infected cells, the cells were fixed and permeabilized (Cell Permeabilisation Kit, Harlan Sera-Lab, Crawley Down, UK) before being labeled with the mAb HC26. This method was shown to detect only de novo synthesized viral gpE2 and not phagocytosed virus (Knoetig et al., 1999). Detection of CSFV RNA was performed by RT-PCR and used 5' GTG GAC GAG GGC ATG CCC A 3' as sense primer and 5' TCA ACT CCA TGT GCC ATG TAC 3' as antisense primer as previously described (Hofmann et al., 1994; Wirz et al., 1993). Briefly, after reverse transcription using M-MuLV reverse transcriptase (Gibco BRL, Life Technologies, Basel, Switzerland) for 30 min at 37°C, amplification was performed utilizing Taq DNA polymerase (Promega, Catalys AG, Wallisellen, Switzerland) with 90 s of initial denaturation at 94°C followed by 30 cycles at 94°C for 30 s, 57°C for 60 s, 72°C for 30 s. The PCR products were then analyzed on a 2% agarose gel stained with ethidium bromide.

**Cell viability and apoptosis analysis**

For quantification of apoptotic and dead cells, dual-parameter analyses of AnnexinV-FITC and PI labeling
were performed (Vermes et al., 1995). To this end, 5 × 10^5 cells are labeled with 2 μg/ml AnnexinV-FITC (Bender Med Systems, Austria) in 140 mM NaCl, 2.5 mM CaCl_2, 10 mM HEPES (pH 7.4) buffer for 10 min at 4°C. After FL1/FL2 compensation, PI (1 μg/ml) was added, in order to discriminate between apoptotic and dead cells, and the sample was analyzed immediately by FCM. A second analysis for apoptotic cells was performed based on the method of Nicoletti et al. (1991), which identifies cells with reduced DNA content as a result of DNA fragmentation (Darzynkiewicz et al., 1992). Cells were fixed with 75% (v/v) ethanol (0°C, 2 min), followed by washing/centrifugation. PI (50 μg/ml plus 100 μg/ml RNase) was added to the cells for 30 min at 37°C, which were then analyzed by FCM. The DNA histograms obtained were used to quantify apoptosis in terms of the sub-G1 peak in a cell cycle profile. Morphological features of apoptotic cells were assessed by microscopic analysis of nuclear changes occurring during the late apoptotic stages. To this end, cytocentrifuged cells were stained using the DiffQuik staining kit (Baxter Diagnostics AG, Düdingen, Switzerland).

Caspase activity

Caspase activity from cell lysates was determined using fluorogenic substrates composed of peptides conjugated to 7-amino-4-(trifluoromethyl)coumarin (AFC) or 7-amino-4-methylcoumarin (AMC). For caspase-3 Z-DEVD-AMC was used, for caspase-8 Ac-IETD-AFC was used, and for caspase-9 Ac-LEHD-AFC (all Calbiochem, Juro Supply, Lucerne, Switzerland) was used. A total of 2 × 10^4 cells were centrifuged (500 g, 10 min, 4°C), resuspended in 100 μl hypotonic lysis buffer (25 mM HEPES, 5 mM MgCl_2), and subjected to four freezing/thawing cycles. The lysate was sonicated and then clarified by centrifugation (10,000 rpm, 10 min, 4°C). The protein concentration in the supernatant was determined using a NanoOrange protein quantitation kit (Molecular Probes, Leiden, The Netherlands) following the manufacturer's instructions. For the caspase assay, 25-μg aliquots of protein were mixed with a 5 mM concentration of the enzyme substrate in the assay buffer (312.5 mM HEPES, 31.25% sucrose, 10 mM DTT, 0.3125% CHAPS, pH 7.5) to obtain a final volume of 200 μl. As negative controls, samples were incubated for 1 h at 37°C with the specific caspase inhibitors (Z-VD-FMK as general caspase inhibitor, Z-DEVD-FMK as caspase-3 inhibitor, Z-IETD as caspase-8 inhibitor, and Z-LEHD-FMK as caspase-9 inhibitor), before the addition of the corresponding substrate. After incubation for 2–4 h at 30°C, fluorescence was read (for AFC conjugates: excitation 400 nm, emission 505 nm; for AMC conjugates: excitation 370 nm, emission 450 nm) in a Spectramax Gemini (Molecular Devices, Paul Bucher, Basel, Switzerland). The amount of AFC/AMC released was calculated using a standard curve prepared from a titration of AFC or AMC (Calbiochem). Enzyme activity was calculated by subtraction of the amount of AFC/AMC released in the negative controls from that obtained with samples and expressed as picomoles per liter of AFC/AMC released per minute and per microgram of protein.

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