African swine fever virus: a B cell-mitogenic virus in vivo and in vitro

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The two major characteristics of pathogenesis in African swine fever virus (ASFV) infections of domestic pigs are massive B-cell apoptosis and haemorrhage. The effects of ASFV on porcine B cells have therefore been systematically examined in vivo, by using virus-infected pigs and SCID-Beige mice reconstituted with porcine bone marrow, and in vitro, by using porcine B-cell lines and B cells from normal and ASFV-infected pigs. Secretion of porcine Ig was stimulated by ASFV both in vivo and in bone marrow cultures in vitro, with the virulent Malawi isolate of ASFV being the most effective. Stimulation of Ig secretion in vitro depended on the presence of ASFV-infected macrophages and did not occur with supernatants from ASFV-infected macrophages. Although the virus alone did not stimulate proliferation of purified B cells in vitro, it was co-stimulatory with CD154 (CD40 ligand). The B cells recovered from ASFV-infected porcine lymphoid tissue were of activated surface marker phenotypes and, interestingly, expressed diminished levels of the B-cell co-stimulatory surface molecule CD21. In addition, they were highly sensitive to IL-4 and CD154. These results may be integrated into a model of pathogenesis in which those B cells activated indirectly as a result of virulent ASFV infection of macrophages are not rescued from apoptosis through interaction with CD154, due to the drastic depletion of T cells that occurs early in infection. The consequently diminished specific anti-ASFV antibody response would favour survival of the virus, with the non-specific hypergammaglobulinaemia being perhaps another example of pathogen-mediated immune deviation.

Introduction

African swine fever (ASF) is a highly contagious, haemorrhagic disease of pigs that was first described about 80 years ago (Montgomery, 1921) and which is caused by a large (~200 kbp), icosahedral DNA virus (ASFV). The virus replicates predominantly in cells of the monocyte/macrophage lineage and virulent isolates kill domestic pigs within 7–10 days of infection. With less-virulent virus strains, pigs may survive. Animals that survive are resistant to the same virus isolates but do not produce classical neutralizing antibody. Interestingly, in its natural hosts, the African bushpig and warthog, there is no disease but instead a persistent, non-pathogenic infection. Thus ASF in the domestic pig may be considered to be a veterinary model for the recently emerged haemorrhagic virus diseases of man.

As the virus results in hypergammaglobulinaemia in chronic infections (Pan et al., 1970), it has been suggested that ASFV may be a polyclonal B-cell stimulator (Wardley, 1982). Such polyclonal, non-specific B-cell activation has been observed in many chronic and acute infections by both DNA and RNA viruses (reviewed by Cash et al., 1996). In most cases, hypergammaglobulinaemia is not induced by viral antigens but by cytokines, especially IFN-γ (Coutelier et al., 1988; Huang et al., 1993). There are, however, cases where a direct mitogenic effect of isolated viral proteins can be demonstrated; for example, members of the Herpesviridae, Alphaviridae, Paramyxoviridae, Togaviridae and Retroviridae (Cash et al., 1996). In the case of ASFV, Wardley (1982) observed that virulent ASFV has a co-mitogenic effect on Ig secretion when total porcine peripheral blood lymphocytes were stimulated with pokeweed mitogen (PWM) in vitro. One protein, P36, produced by ASFV-infected macrophages (Arala-Chaves et al.,...
1988; Ribeiro et al., 1991), inhibited antigen-specific murine Ig secretion but enhanced non-specific Ig secretion in plaque-forming cell assays, once again indicating an indirect effect of ASFV on Ig secretion. In contrast to this apparent stimulation of B cells, extensive B-cell apoptosis has been observed during infection of pigs with virulent ASFV (Ramiro-Ibáñez et al., 1997; Oura et al., 1998). Thus, the balance between activation and apoptosis of B cells is likely to have a major impact on and to contribute to the pathogenesis of the disease. To date, however, there have been no studies of the mechanisms involved; for example, whether ASFV exerts a direct or an indirect effect on the fate of B cells (activation, Ig secretion or death).

Therefore we examined systematically the effect of ASFV in vivo on porcine B cells, through characterization of B cells from ASFV-infected pigs and through reconstitution of SCID-Beige mice with porcine bone marrow (BM). In addition, we studied the interaction of ASFV with purified porcine B cells and B-cell lines in vitro.

**Methods**

**Virus.** The following ASFV isolates were used: the virulent African isolate Malawi Lilongwe 20/1 (Haresnape, 1984) and European isolate OUR T88/1 (Boinas, 1994), the less-virulent European isolate Malta/78 (Wilkinson et al., 1981), the non-pathogenic isolate OUR T88/3 (Boinas, 1994) and tissue culture-attenuated Ugandan (Wardley, 1982). Viruses were prepared from BM culture (Malawi, Malta, both OUR isolates), IBRS II cell culture (Uganda) or from infected spleen extract (Malawi) and virus titre was expressed either as haemadsorption units (HADₜₜ) (Wardley et al., 1977) or TCIDₚₚ (attenuated Uganda and OUR T88/3 isolates). Foot-and-mouth disease virus (FMDV; C Noville strain) and bluetongue virus (BTV) serotype 1 (Takamatsu & Jeggo, 1989) were also used as negative controls. Killed virus was prepared either by UV-irradiation or by heat treatment (95 °C, 5 min) and virus supernatant was prepared from infected BM cells by repeated filtration through 0.22 µm filters or 0.02 µm Anatop 10 filters (Anotec).

**Pigs and ASFV infection.** Twenty to twenty-five kilogram, large White/Landrace crossbred pigs of either sex were obtained from IAH Compton Laboratory. The virulent Malawi Lilongwe 20/1 isolate of ASFV (2 ml of 1 × 10⁹ HADₜₜ/ml; prepared from BM cultures) was inoculated intramuscularly in the rump. Blood, spleen and submandibular lymph nodes (SMLN) were collected from day 0 to day 6 post-infection. A total of three animals for day 0, one animal each for days 1, 3 and 5 post-infection and two animals each for days 2, 4 and 6 post-infection were sacrificed humanely in these experiments, in accordance with the requirement of the Animal (Scientific Procedures) Act 1986, under project licence number PPL 90/00866.

**Cells.** Pig BM cells were prepared from 3–4-week-old outbred pigs (6–8 kg) as described before (Malmquist & Hay, 1960). Mononuclear cells were isolated from total BM cells by gradient centrifugation as described below. Lymphocytes from mesenteric lymph nodes (MLN), spleen and SMLN were prepared by teasing the tissue in a Petri dish containing ice-cold Hanks’ balanced salt solution (HBSS). Cells were then passed through nylon mesh and lymphocytes were separated by gradient centrifugation (Nycocprep 1077 Animal, Nycomed) at 800 g for 20 min. B cells were negatively purified from MLN, spleen and SMLN lymphocyte cell suspensions by using a magnetic separation procedure. Lymphocytes were resuspended with a mixture of MAbs against CD3 (PT33) (Yang & Parkhouse, 1996), CD8 (11/295/33) (Saalmüller, 1996) and macrophage–granulocytes (w/C3, 74-22-15) (Saalmüller, 1996) and incubated for 30 min on ice. MAb-treated lymphocytes were washed three times in cold HBSS and then incubated with goat anti-mouse Ig-coated microbeads (Miltenyi Biotec) for 30 min on ice. After washing with cold MACS buffer (PBS containing 5 mM EDTA and 0.5% BSA), lymphocytes were resuspended in MACS buffer. T cells (CD3⁺), NK cells (CD8⁺) and monocyte/macrophages were depleted from lymphocyte preparations by using B5 depletion columns (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of B cells was more than 90% as analysed by staining with goat anti-pig Ig–FITC and FACS analysis.

A CD154 (CD40 ligand)-dependent porcine B-cell line was prepared by culturing purified MLN B cells (1.5 × 10⁶ cells/ml) with a γ-irradiated (5500 rad) monolayer of a fibroblast cell line expressing CD154 in the cell membrane (Wohlleben et al., 1996; obtained from A. Schimpl, Universität Würzburg, Germany) as described before (Takamatsu et al., 1999). The cells were cultured in 25 cm² flasks at 37 °C and viable B cells were collected every week by gradient centrifugation (Nycocprep, 800 g, 20 min) and transferred to new flasks containing the γ-irradiated, CD154-expressing cell monolayer.

**Reconstitution of SCID-Beige mice with porcine BM cells.** Two-week-old SCID-Beige mice, of either sex, were obtained from a breeding colony at the Pabiright Laboratory Small Animal Unit and 2–3 × 10⁷ porcine BM cells were inoculated into the intraperitoneal cavity. Two weeks post-inoculation, a further 2–3 × 10⁷ porcine BM cells, with or without ASFV (10⁷–10⁸ HADₜₜ), were inoculated into the intraperitoneal cavity. Reconstitution of a porcine immune system was assessed by production of porcine Ig in serum from reconstituted mice by ELISA (see below).

**Detection of porcine Ig.** ELISA plates (Nunc-Immunoplate, Nalge) were coated with affinity-purified goat anti-porcine Ig (10 µg/ml, 50 µl per well, Southern Biotechnology Associates) in carbonate-bicarbonate buffer (50 mM, pH 9.6) for 1 h at 37 °C. The plates were washed with PBS/Tween (PBS containing 0.02% Tween 20). Samples or standard purified porcine Ig (Sigma) were diluted in PBS/Tween containing 3% ovalbumin (Sigma) and added to the plate (50 µl per well). After 2 h incubation at 37 °C, the plates were washed three times with PBS/Tween. HRP-conjugated goat anti-porcine Ig (Southern Biotechnology Associates) was then added to each well (50 µl per well) and incubated for a further 1 h at 37 °C. Plates were washed five times with PBS/Tween and then substrate (p-phenylenediamine, Sigma) was added and the absorbance was measured at 495 nm.

**Surface marker phenotype characterization by FACS analysis.** Negatively purified splenic B cells were characterized by flow cytometry (FACScan, Becton Dickinson) by using the lysis II program with the following MAbs: K52 1C3 (anti-porcine IgM), CC51 (anti-bovine CD21 which cross-reacts with pig CD21), 231 3B2 (anti-porcine CD21), K52 (anti-porcine IgM), MSA3 (anti-porcine MHC class II) and activated-cell marker Ki67 (Silvestrini et al., 1994). In each analysis, B cells were stained with all the MAbs for 20 min on ice, washed and then phycoerythrin-conjugated goat anti-mouse Ig (Southern Biotechnology Associates) was used to detect the bound MAbs. In the case of the intracellular activation marker Ki67, the cells were permeabilized with 0.02% Tween 20–PBS after being fixed with 0.01% paraformaldehyde on ice for 60 min and stained as above. The degree of cell membrane antigen expression was expressed as mean fluorescence intensity (MFI).

**Identification of proliferating cells by incorporation of 5-bromo-2′-deoxyuridine (BrdU).** BM cells from a normal pig were cultured for 4 days with or without ASFV (Malawi isolate) and cultured
cells were stained for the cell surface expression of light chain (MAb 139 3E1) or slgM (goat anti-pig Ig) as described above. For identification of proliferating cells, these cells (10⁶ cells in 0.5 ml) were incubated in culture medium containing BrdU (10 µM) for 30 min at 37 °C. After washing with PBS, cells were fixed with ice-cold ethanol for 30 min, washed in PBS and resuspended in 1% (w/v) paraformaldehyde solution (in 0.01%, v/v, Tween-20 in PBS) for 30 min at room temperature. Cells were then resuspended in DNase solution (50 000 U/ml in 4.2 M MgCl₂, 150 mM NaCl, pH 5) for 10 min at room temperature. After washing, the cells were incubated with 10 µl FITC–anti-BrdU (Becton Dickinson) for 30 min, washed and analysed by FACS.

Cell cultures and proliferation assays. BM cells (2 × 10⁷ cells/ml) were cultured in 24-well plates for 2 days and then incubated with ASFV (10⁵ HAD₅₀) for a further 7 days. As negative controls, two other viruses (FMDV, 10⁴ TCID₅₀; BTV, 10⁴ TCID₅₀) were included. The positive control for Ig was provided by addition of PWM (10 µg/ml) to the cultures. Supernatants from each culture were assayed for porcine Ig concentration by ELISA (see above). Adherent cells from porcine BM cells were isolated by incubating cells at 37 °C for 1 h in tissue culture flasks and then washing out non-adherent cells. The adherent cell cultures were further incubated overnight and then washed and adherent cells were recovered by EDTA (0.02%) treatment. Adherent BM cells were cultured with or without ASFV for 5 days, washed and mixed with purified MLN B cells (1:1) that had been incubated for 5 days with or without ASFV. Mixed cells were further cultured for 7 days and cultured supernatants were examined for porcine Ig concentrations.

Purified splenic or SMLN B cells (2 × 10⁶ cells per well) from ASFV-infected pigs were cultured for 4 days, with or without γ-irradiated (5500 rad), membrane CD154-expressing cells (2 × 10⁶ cells per well) and with or without recombinant human IL-4 (5 ng/ml, Genzyme), and then [³H]TdR (0.2 µCi per well) was added to each well. Samples were harvested after a further 16 h and incorporation of [³H]TdR was determined by liquid scintillation counting (1205 Betaplate, Wallac).

Purified MLN B cells (2 × 10⁶ cells per well) from normal piglets or B cells (2 × 10⁶ cells per well) from CD154-dependent cultures were cultured with or without ASFV or ASFV-infected spleen extracts for 4 days. B-cell proliferation was measured by [³H]TdR uptake as described above. Cell membrane permeability, one of the characteristics of apoptosis, was examined by trypan blue exclusion test.

Analysis of apoptosis and cell cycle progression. Details of the methods employed have been published previously (Silvestrini et al., 1994; Takamatsu et al., 1997). Purified MLN B cells or CD154-dependent MLN B-cell cultures were incubated at 37 °C with or without ASFV for various periods. DNA synthesis of the B cells was measured by [³H]TdR uptake. Apoptosis was assessed by FACS through examination of cell size and granularity, quantification of hypodiploid DNA after ethanol fixation, digestion with RNase and propidium iodide staining, and agar gel electrophoresis of extracted DNA. Cell cycle analysis was done by FACS analysis of ethanol-fixed, RNase-digested, propidium iodide-stained cells.

Results

Viable pathogenic ASFV induces porcine Ig secretion in vivo and in vitro

The serum of SCID-Beige mice reconstituted with porcine BM cells contained 15–55 µg/ml porcine Ig 14 days after cell transfer. Levels of porcine Ig gradually declined subsequently, but detectable amounts were present more than 70 days after reconstitution. Injection of such reconstituted animals with the pathogenic Malawi isolate of ASFV (10⁶ HAD₅₀) was followed by a rapid increase in the level of pig Ig in the serum, reaching a peak of 1.44 ± 0.15 mg/ml 14 days after introduction of the virus; i.e. an enhancement of about 40 times the level of pig Ig observed in the uninfected, reconstituted control mice (Fig. 1). These high levels (1 mg/ml) of porcine Ig were maintained for more than 5 weeks after ASFV infection. After 8 weeks, the concentration of porcine Ig had decreased to approximately half (0.47 ± 0.15 mg/ml), but was still considerably higher than the value of 0.02 mg/ml in uninjected reconstituted mice.

The virus-induced enhancement of porcine Ig in reconstituted SCID-Beige mice was dose dependent (Fig. 2a) and porcine Ig was not induced by killed virus or virus culture supernatant (Fig. 2a). Interestingly, other isolates of ASFV, for example Malta/78 and OUR T88/1, failed to enhance porcine Ig secretion in the reconstituted mice. Although relatively less effective than in reconstituted mice, ASFV was also able to enhance porcine Ig secretion by BM cell suspensions in vitro, but in these experiments less-virulent strains of virus also induced Ig synthesis (Fig. 2b). Ig secretion was not induced by tissue culture-adapted ASFV (attenuated Uganda) in vivo (Fig. 2a) or in vitro (data not shown).

Induction of porcine Ig secretion requires ASFV-infected, adherent cells

To determine whether ASFV acts directly on B cells or indirectly through infection of macrophages present in BM cultures, monocyte/macrophage lineage cells were isolated from total BM cells by adherence to plastic, cultured with or without Malawi isolate ASFV for 5 days, washed and then cocultured for a further 7 days with purified MLN B cells; the
latter having been cultured for 5 days, with or without ASFV, prior to mixing with the adherent cells. As summarized in Table 1, stimulation of Ig secretion by either control or ASFV-

**Table 1. Stimulation of porcine Ig secretion requires ASFV infection of adherent cells**

Porcine adherent BM cells and purified MLN B cells were cultured separately for 5 days with or without Malawi isolate ASFV (10⁶ HAD₅₀/ml), mixed and cultured for a further 7 days. The concentration of porcine Ig in the cultures was determined by ELISA.

<table>
<thead>
<tr>
<th>Virus exposure</th>
<th>Adherent cells</th>
<th>B cells</th>
<th>Porcine Ig (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>ASFV</td>
<td>ASFV</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>ASFV</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>ASFV</td>
<td>None</td>
<td>1.43</td>
<td></td>
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</table>

**Fig. 2.** Induction of porcine Ig secretion by ASFV in vivo in SCID mice (a) and in vitro in BM culture (b). (a) Pig BM-reconstituted mice were infected 14 days later with the ASFV strain indicated and the amount of pig Ig in the mouse sera was estimated by ELISA after a further 2 weeks. Induction of Ig secretion in vivo was dependent on virus dose and was observed only with virulent Malawi isolate and not with killed virus or virus supernatant. sup, Supernatant. (b) Normal porcine BM cells were incubated in vitro with PWM or the viruses indicated and the amount of pig Ig in the culture supernatants was estimated by ELISA after 7 days incubation. A significant stimulation of Ig secretion in vitro was observed with all isolates of viruses tested, i.e. Malawi, Malta/78 and OUR isolates. FMDV and BTV were included as negative controls.

Table 1. Stimulation of porcine Ig secretion requires ASFV infection of adherent cells

Porcine adherent BM cells and purified MLN B cells were cultured separately for 5 days with or without Malawi isolate ASFV (10⁶ HAD₅₀/ml), mixed and cultured for a further 7 days. The concentration of porcine Ig in the cultures was determined by ELISA.

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<th>Porcine Ig (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>ASFV</td>
<td>ASFV</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>ASFV</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>ASFV</td>
<td>None</td>
<td>1.43</td>
<td></td>
</tr>
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</table>

**Fig. 3.** Induction of B-cell proliferation by ASFV in vitro. Lymphocyte proliferation was measured by [³H]Tdr uptake in cultures of total BM cells (a), total MLN cells (b), purified MLN B cells (c) and a CD154-dependent porcine B-cell line (d). Cultures were incubated for 4 days with (filled bars) or without (shaded bars) Malawi isolate ASFV (10⁶ HAD₅₀/ml, 100 µl per well) and for a further 16 h with [³H]Tdr. Some cultures, as indicated, were supplemented with either IL-4 (5 ng/ml), or γ-irradiated, cell membrane-expressed CD154 (CD40L). Significant induction is indicated by * (P < 0.001) or ** (P < 0.05).
ASFV is a B cell mitogen

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**Activation of porcine B cells by ASFV in vivo**

As the above experiments indicated a mitogenic effect of ASFV in vitro, we were interested to determine whether there was a corresponding activation of B cells in vivo as a result of infection with ASFV. Therefore, B cells recovered from pigs at various times after ASFV infection were examined for expression of activation antigens and for sensitivity to mitogenic stimuli. An increased fluorescence intensity of MHC II, sIgM, Ki67 and, marginally, IL-2R, peaking at day 4 post-infection (Fig. 4a), indicated B-cell activation in vivo. Interestingly, and in contrast, expression of the B-cell marker CD21 was reduced selectively in B cells recovered from infected animals (Fig. 4a). Similarly, activation in vivo was indicated by an increased sensitivity of B cells to stimulation with CD154 and IL-4 (Fig. 4b).

**CD154 rescues B cells from ASFV-infected pigs from apoptosis**

Because B cells from ASFV-infected pig spleen showed an activated phenotype and increased response to CD154, we examined whether B cells from ASFV-infected pig spleen could be rescued from apoptosis by CD154 in vitro. Purified B cells or total lymphocytes from ASFV-infected pig spleen (Malawi isolate, at day 4 post-infection) were incubated at 37 °C with or without cell membrane-expressed CD154 and the cell membrane permeability was examined by trypan blue exclusion test (for purified B cells) and cell cycle/FACS analysis of sIgM+ cells (for total lymphocytes). B cells from ASFV-infected pigs died more quickly than did similar cells from normal pigs (Fig. 5a) and cell cycle/FACS analysis demonstrated an increase in hypodiploid DNA, indicating that these cells had entered apoptosis (Fig. 5b). On the other hand, when B cells were incubated with CD154, the number of membrane-intact B cells (non-apoptotic cells) from ASFV-infected pig remained similar to normal pig B cells (Fig. 5a) and only 18% of B cells showed hypodiploid DNA (Fig. 5b) compared with 64% without CD154.

**ASFV-infected macrophages and ASFV-infected pig spleen extracts do not induce apoptosis of B cells in vitro**

Finally, we looked for a direct effect of ASFV, ASFV-infected macrophages or soluble factors released into the spleen of ASFV-infected animals on B-cell apoptosis. A CD154-dependent porcine B-cell line, which is sensitive to apoptosis,
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Figure 5. B cells from ASFV-infected pigs enter apoptosis in vitro and can be rescued by CD154. (a) Purified splenic B cells from normal (■) or ASFV-infected (Malawi isolate, at day 4 of infection) (○) pigs were incubated in vitro at 37 °C with (○) or without (■) cell membrane-expressed CD154. Cell viability was examined daily by the trypan blue exclusion test. (b) Total lymphocytes from normal or ASFV-infected pig spleen were incubated at 37 °C with or without cell membrane-expressed CD154. Apoptosis of sIgM+ cells in the cultures was examined by staining the cells with anti-porcine Ig–FITC and then propidium iodide after ethanol fixation and RNase treatment. The samples were analysed by FACS. M1, hypodiploid DNA; M2, intact DNA.

Table 2. Total lymphocyte and B-cell recovery from ASFV-infected pigs

Pigs were infected with Malawi isolate ASFV and sacrificed daily from 2 days post-infection. The number of animals analysed at each time-point is shown. Spleens were weighed and cell suspensions were prepared for identification and quantification of B cells by staining with goat anti-pig Ig–FITC and FACS analysis. B-cell recovery is shown as a percentage of total lymphocytes.

<table>
<thead>
<tr>
<th>Time post-infection (days)</th>
<th>Total lymphocyte recovery (cells/g × 10^6)</th>
<th>B-cell recovery (%)</th>
</tr>
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<tbody>
<tr>
<td>0 (n = 3)</td>
<td>38.0 ± 7.0</td>
<td>10.7 ± 2.1</td>
</tr>
<tr>
<td>2 (n = 2)</td>
<td>40.4 ± 10.7</td>
<td>12.1 ± 2.9</td>
</tr>
<tr>
<td>3 (n = 1)</td>
<td>29.0</td>
<td>19.8</td>
</tr>
<tr>
<td>4 (n = 2)</td>
<td>3.2 ± 1.5</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>5 (n = 1)</td>
<td>2.3</td>
<td>10.6</td>
</tr>
<tr>
<td>6 (n = 2)</td>
<td>0.7 ± 0.4</td>
<td>5.5 ± 0.8</td>
</tr>
</tbody>
</table>

was co-cultured with Malawi isolate ASFV-infected or uninfected macrophages for 4 days and the cell viability was examined. There was no difference in B-cell viability between B cells co-cultured with ASFV-infected macrophages (B-cell viability 94.0%) and uninfected macrophages (B-cell viability 93.6%).

Spleen tissue (4 g) from normal and day 4 ASFV-infected pigs was extracted in 10 ml of HBSS and serial dilutions of spleen extracts, either untreated or UV-treated (to prevent virus replication), were added to CD154-dependent B-cell cultures. With or without UV treatment, the ASFV-infected pig spleen extracts (cell-free) induced strong B-cell proliferation in a dose-dependent manner. A normal spleen extract also induced B-cell proliferation but to a considerably lesser extent than ASFV-infected spleen extract and not in a dose-dependent manner (optimum at 1:64 dilution) (data not shown). There was no effect on numbers of viable cells in the cultures (data not shown).

Discussion

The principal pathogenic features of highly virulent ASF in domestic pigs are haemorrhage and destruction of lymphoid tissues, including intense B-cell apoptosis (Ramiro-Ibáñez et al., 1997; Oura et al., 1998), and finally death of pigs within 10 days without specific immune responses against the virus (Pastor & Escribano, 1990; Norley & Wardley, 1984). Both haemorrhage and destruction of lymphoid tissues appear to result from an indirect effect, perhaps through an altered...
cytokine profile, as the only infected cells observed when these processes are initiated are macrophages (Ramiro-Ibáñez et al., 1997; Oura et al., 1998). Paradoxically, in spite of the evident and extensive destruction of B lymphocytes in acute infection, pigs with a chronic infection with a sub-lethal virus isolate are hypergammaglobulinaemic (Pan et al., 1970). Thus, an assessment of the impact of ASFV on the B-cell component of the immune system is clearly relevant to the pathogenesis of the disease. In this work, we have demonstrated that ASFV, directly and/or indirectly, stimulates B-cell activation. These observations may help to explain the hypergammaglobulinaemia observed in ASF and may also be relevant to the massive B-cell apoptosis that is also characteristic of the disease.

The stimulation of porcine Ig synthesis occurring in ASFV-infected pigs was also seen in SCID mice reconstituted with pig BM cells and subsequently infected with the virus. The stimulation of Ig synthesis was very marked, with reconstituted BM cells and subsequently infected with the virus. The infected pigs was also seen in SCID mice reconstituted with pig BM cells were infected during the acute stage of the infection approximately 40 times the average concentration of pig Ig in control, uninfected pig BM-reconstituted animals. Interestingly, only the viable virulent Malawi isolate of ASFV stimulated pig Ig synthesis effectively in the reconstituted animals. In pigs, many more cells were infected during the acute stage of the infection with Malawi isolate of ASFV and a higher level of virus replication was observed in comparison with infection with the less-virulent Malta/78 isolate (Oura et al., 1998). The fact that pig Ig synthesis in reconstituted SCID mice was dependent on the dose of ASFV (Fig. 2a) means that it is likely that rapid and aggressive replication by highly virulent ASFV is required for effective porcine Ig secretion in vivo. At the same time, however, the mice survived virus challenge, and so in this respect the pig–SCID model provides a manipulable experimental in vivo model. On the other hand, systematic investigation of the virus-mediated mechanisms responsible for the stimulation of Ig synthesis observed is difficult to conduct in vivo. For this reason, it was necessary to study the effect of ASFV on pig Ig synthesis in vitro. With cultures of porcine BM, the stimulatory effect of ASFV on B cells was confirmed by increased Ig secretion, B-cell proliferation and FACS analysis of BrdU+ cells and shown to depend on the presence of adherent cells. As ASFV does not infect B cells, we therefore assume that the virus infected adherent cells (i.e. macrophages) and provided the stimulus for the B cells indirectly. Unlike the situation in SCID mice, however, less-virulent ASFV was also able to induce pig Ig secretion in vitro, and this may be due to the presence of all the necessary B-cell stimulation/differentiation factors, such as cell–cell interaction or cell-soluble factors, in an enclosed microenvironment such as that which exists in vitro.

In addition to the indirect effect of ASFV on porcine B cells, ASFV was co-mitogenic for purified B cells and a B-cell line, provided that CD154 or IL-4 was present. Although it is possible that macrophage-derived factors contaminating the virus preparation may have contributed to the stimulation observed, an alternative explanation is that ASFV may bind to B cells without causing a productive infection. The nature of such an interaction is unknown, but the immunosuppressive protein P36, which is produced by ASFV-infected cells (Arala-Chaves et al., 1988; Ribeiro et al., 1991), is an attractive candidate worthy of study.

All of the above leads to the conclusion that ASFV may activate B cells through direct binding and through indirect effects mediated via the infected macrophage. If so, infection with this virus should result in B-cell activation in vivo, and indeed this proved to be the case. B cells recovered from infected animals had enhanced expression of surface (MHC class II and mIg) and intracellular (Ki67) activation markers (Silvestrini et al., 1994). Interestingly, expression of the pan B-cell marker CD21 was considerably reduced on these B cells, an observation which is not entirely a feature of ASF, as a reduction of surface CD21 has also been observed with activated porcine B cells in general (Takamatsu et al., 1999). In the specific case of ASF, however, this reduction in CD21 expression might be expected to diminish specific antibody responses, as CD21 is involved in the regulation of B-cell activation and proliferation (Tedder et al., 1994). This would certainly enhance virus survival, as serum transferred passively from recovered pigs can delay the onset of ASF symptoms in pigs infected with a virulent strain of ASFV (Wardley et al., 1985), suggesting that in certain circumstances antibodies may play a role in protection against ASF.

Recent observations demonstrate that highly virulent ASFV infections cause systematic apoptosis of B cells in lymphoid tissues (Ramiro-Ibáñez et al., 1997; Oura et al., 1998). Apoptosis of B cells occurs in several of the stages of B-cell development, in particular in germinal centres, where low-affinity B cells are eliminated by programmed cell death. Conversely, naive B cells and unstimulated memory B cells, with their high expression of bcl-2 and absence of CD95 (Fas) expression (Baixeras et al., 1994; Koopman et al., 1997), are usually resistant to apoptosis and their entry into apoptosis is generally associated with activation. The interaction of CD154 with B cells via CD40 is a critical signal that influences B-cell differentiation, proliferation, rescue from apoptosis (Van Kooten & Banchereau, 1996) and induction of memory B cells (Foy et al., 1994). Thus, if ASFV stimulates B-cell activation, a feasible hypothesis is that the resulting activated B cells may enter the programmed cell death pathway by default unless rescued by, for example, the transiently expressed CD154 (CD40 ligand) of activated T cells. Consistent with this hypothesis, apoptosis of B cells from ASFV-infected pigs was prevented by CD154 in vitro. Moreover, the early and extensive T-cell apoptosis observed in lymphoid tissues infected with the highly virulent Malawi isolate of ASFV (Oura et al., 1998) could deprive the virus-activated B cells of the necessary CD154 rescue signal. Finally, splenic T cells recovered from animals infected with the Malawi isolate of
as ASFV failed to proliferate in response to mitogens (Childerstone et al., 1998), perhaps indicating a defect in CD154 expression and/or function on T cells during ASFV infection. Although the activated CD4+ T cell is the major cell type expressing CD154, a recent study showed expression of CD154 on a wide variety of cells including monocytes and dendritic cells (Grewal & Flavell, 1998). Dendritic cells are another important cell type controlling the fate of B cells. Thus, the effect of the expression of CD154 on various cell types other than T cells and of dendritic cells on ASFV-induced, activated B-cell apoptosis requires study in the future.

Finally, extracts of grossly infected spleens prepared 4 days after infection with the pathogenic Malawi isolate were tested for the presence of factors that might induce apoptosis of porcine B cells. Both untreated and UV-irradiated, ASFV-infected, day-4 spleen extract induced strong B-cell proliferation on CD154-dependent porcine B-cell cultures and no induction of cell death was observed. This result also supports the hypothesis that apoptosis of B cells in ASFV-infected pigs is due to the absence of rescue signals for activated B cells rather than to the production of factors that induce apoptosis.

In conclusion, infection of pigs with ASFV results in B-cell activation through both direct and indirect effects. Some of these activated B cells would be expected to differentiate into typical Ig-secreting cells. In highly virulent ASFV infection in pigs, however, the majority of activated B cells would enter the programmed cell death pathway unless rescued. We postulate that the extensive T-cell loss in highly virulent ASFV infection in domestic pigs plays an important role in B-cell apoptosis, depriving the cells of rescue signals such as CD154 and IL-4. This scenario may explain the extensive B-cell loss and the paradoxical non-specific hypergammaglobulinaemia seen in ASF. Both of these pathogen-mediated effects on the humoral immune response would favour survival of the virus.

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References


ASFV is a B cell mitogen


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