Classical Swine Fever Virus in Plasma and Peripheral Blood Mononuclear Cells of Acutely Infected Swine

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With 2 figures and 2 tables

(Received for publication May 18, 1998)

Summary

The distribution of classical swine fever virus (CSFV) in plasma, monocytes, T and B lymphocytes in peripheral blood was monitored during experimentally induced acute classical swine fever infection in piglets. Six piglets were infected with 10^{13} TCID_{50} of virus and blood samples taken up to 18 days post-inoculation (p.i.). Infectious virus was detected in monocytes, T and B lymphocytes to similar titres in five of the six infected piglets. Infectious virus was detected earlier in plasma than in any of the mononuclear cell subpopulations. No significant difference was observed in the period of time in which virus could be isolated from the three cell subpopulations. While a progressive lymphopenia developed, a marked B cell depletion was observed. However, B cells were apparently replaced by non-IgM-bearing mononuclear cells, as the proportion ‘total lymphocyte/total leucocytes’ remained unaltered throughout the experiment. Virus titres in plasma and peripheral blood mononuclear cells showed a tendency to increase as the disease progressed to its outcome.

Introduction

Classical swine fever (CSF) is a highly infectious disease caused by the classical swine fever virus (CSFV), a member of the family Flaviviridae, genus Pestivirus (Francki et al., 1991). CSF has a large economic impact in the pig industry and can be responsible for severe losses, particularly when introduced into susceptible, non-immune herds. The tropism of the virus for cells of the immune system, especially peripheral blood mononuclear cells (PBMC), is a well-recognized characteristic (Van Oirschot et al., 1981). In acute disease, CSFV can be isolated from bone marrow stroma cells (Shimizu et al., 1995), blood, buffy coats and PBMC. The virus induces a severe lymphopenia characterized by a decrease in peripheral blood B cells (Van Oirschot, 1979a, b; Van Oirschot et al., 1981; Susa et al., 1992; Sarma and Sarma, 1996). In addition, alterations in the distribution of T lymphocytes of infected swine have been demonstrated, as well as the presence of viral antigens in these cells (Pauly et al., 1998). Despite that, the distribution of infectious virus between different major peripheral blood cell subpopulations (monocytes, T and B lymphocytes) during the course of infection is still unknown. The present study was carried out to determine CSFV distribution in plasma and PBMC during experimentally induced acute CSF.
Materials and Methods

Cells
Porcine kidney cells of the SK6 lineage were grown in Eagle’s minimal essential medium with Earle’s salts, supplemented with 0.05% yeast lactalbumin hydrolysate, 2 mM glutamine, 10% fetal calf serum and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 20 IU/ml mycostatin; E-MEM).

Virus
The strain Alfort 187 (Dahle et al., 1987) of CSFV was used for the experiments. The virus was multiplied in SK6 cells following standard procedures (Roche and Edwards, 1994) and stored at −70°C until use.

Animal inoculation and sampling
A group of six conventional 30 day old piglets was infected with $10^{3.8}$ tissue culture infectious doses (TCID50) of virus per piglet. Two piglets were kept as uninfected controls. All piglets were negative for the presence of CSFV or neutralizing antibodies against CSFV at the beginning of the experiment. Heparinized blood samples (10 mg of heparin/ml of blood) were collected from the vena cava of piglets on days 0, 2, 3, 5, 7, 9, 11, 14, 16 and 18 post-infection (p.i.).

Leucocyte counting
Blood smears were fixed in methanol and stained with Giemsa solution and absolute and relative cell counts were determined based on cell morphology as described by Jain (1986).

Preparation of polyclonal anti-swine IgM serum
Polyclonal anti-swine IgM serum was obtained from mice inoculated with three intraperitoneal immunizations with swine IgM (50 μg/animal/immunization) purified by gel filtration (Hudson and Hay, 1989). After the immunizations, blood was collected and the serum was separated and stored at −20°C.

Isolation of PBMC
PBMC were isolated by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as previously described (Mason et al., 1987). After centrifugation, the interface containing mononuclear cells was collected and several washings were performed to remove the Ficol-Paque and possible virus-containing plasma. The mononuclear cells were diluted in RPMI medium (Sigma, St Louis, MO, USA) supplemented with 1% fetal calf serum, at a final concentration of $2.5 \times 10^6$ cells/ml. The monocytes were isolated by the plate adherence method as previously described (Hunt, 1987). Briefly, PBMC were incubated for 90 min at 37°C in polystyrene plates. Non-adherent cells (i.e. lymphocytes) were removed by gentle washing with phosphate buffered saline, pH 7.4 (PBS) and separated. Adherent cells (monocytes) were detached from the dishes by incubation with PBS-glucose (PBS, pH 7.4; 5 mM glucose) for 15 min at 37°C. The purity of the cell populations was evaluated by analysis of cell morphology after Giemsa staining (Jain, 1986). The percentages of monocytes in the lymphocyte preparations ranged from 1 to 9% in infected animals and 1.7 to 6.67% in controls.

T and B lymphocytes were separated by panning as previously described (Wysocki and Sato, 1978; Mason et al., 1987). Briefly, polystyrene dishes were coated with 1 mg/ml mouse anti-swine-IgM serum prepared as described above and normal mouse serum (10 mg/ml) for 18 h at 4°C. Lymphocytes were adjusted to $2 \times 10^7$ cells/ml in PBS containing 5% fetal calf serum and distributed onto coated plates for 90 min at 4°C. Non-adherent cells (T lymphocytes) were removed by three gentle washings with PBS with 1% fetal calf serum. Adherent cells were subsequently removed by vigorous pipetting with the same buffer.

The two populations were submitted to differential counting for the detection of surface IgM by an immunoperoxidase assay using the polyclonal mouse anti-swine IgM serum followed by anti-mouse IgG/peroxidase conjugate (Dako, Glostrup, Denmark). The efficacy of lymphocyte separation was determined by calculating the percentage of B lymphocytes in adherent and non-adherent cell subpopulations. The percentages of Ig+ lymphocytes in adherent cells of infected animals ranged from 65 and 98.7% (mean 82.38, $\sigma = 9.1$). The percentage of Ig+ lymphocytes in non-adherent cells ranged from 0 to 21.7%.
In the control group, the percentages of Ig^+^ lymphocytes in adherent cells ranged from 71.7 to 98.7%. The percentages of Ig^+^ lymphocytes in non-adherent cells ranged from 0 to 10.5%.

**Percentages of B lymphocytes**

To determine the percentages of B lymphocytes in the inoculated animals, an immunoperoxidase test was performed to detect surface IgM in samples of PBMC isolated by density centrifugation on Ficoll-Paque. For this, 10^3^ PBMC were dispensed in 96 microwell plates, fixed, dried and stained as described below, except that polyclonal mouse anti-swine IgM serum was used as the detector antibody. The percentages of B lymphocytes were assessed by the surface IgM-expressing cells in PBMC samples.

**Virus isolation**

Samples of PBMC were tested for the presence of infectious virus by co-cultivation of PBMC as described previously (Ohmann et al., 1987), except that SK6 were used instead. Four ten-fold dilutions of PBMC (10^4^-10^1^ cells/well) were prepared in RPMI-1640 medium supplemented with 5% fetal calf serum, mixed with 3-4×10^4^ SK6 cells and the volume of medium was adjusted to 200 µl/well. The plates were incubated at 37°C in a 5% CO_2_ atmosphere for 4 days, fixed in 20% acetone in PBS for 10 min, dried for 4 h at 37°C and stained by immunoperoxidase as described below.

Virus isolation from plasma was performed by the inoculation of dilutions of 50 µl of plasma samples on to preformed monolayers of SK6 cells (final volume 100 µl/well). Sample dilutions (10^-5^-10^-1^) were distributed in 96 well microtitre plates, in quadruplicate. After an incubation period of 4 days at 37°C in a 5% CO_2_ atmosphere, the plates were fixed with 20% acetone in PBS and stained by immunoperoxidase as described below.

Titres were calculated by the method of Spearman-Kärber (Lorenz and Bögel, 1973). Titres in cells were determined by the same method, except that the results were adjusted to TCID_50_/10^2^ cells. Statistical analysis of infectious titres was performed by analysis of variance and the χ^2^ test.

**Immunoperoxidase**

Immunoperoxidase tests were performed essentially as described by Saunders (1977), with minor modifications, as follows. Acetone-fixed plates were rehydrated with 100 µl of wash fluid (PBS with 0.5% Tween 80) for 5 min, the wash fluid removed and 50 µl of an appropriate dilution (1:300) of polyclonal swine anti-CSFV serum (kindly provided by Dr J. Bersano, Instituto Biologico, SP), prepared in dilution fluid (29.5 g NaCl, 1.55 g Na_2_ HPO_4_ 2H_2_ O, 0.23 g NaH_2_ PO_4_ H_2_ O, 5 ml Tween 80, H_2_ O to 1000 ml) was added to each well and incubated at 37°C for 15 min. Subsequently, the plates were washed three times with wash fluid and 50 µl of anti-swine IgG peroxidase conjugate (Dako), diluted at 1:150 in dilution fluid, was added to each well. After a further 15 min of incubation at 37°C, the conjugate was removed, the plates washed as described and the substrate 3-amino-9-ethyl-carbazole (Sigma), prepared as described (Harlow and Lane, 1988), was added to the wells in volumes of 50 µl/well. After 15–50 min of incubation at 37°C (depending on the intensity of the staining) the reaction was stopped by the addition of 50 µl/well of wash fluid. The presence of virus was indicated by the characteristic carmine-red colour of infected cells.

**Results**

**Clinical signs**

All infected animals developed clinical signs characteristic of CSF, which included depression, fever, anorexia, diarrhoea and conjunctivitis. Piglets 4 and 6 also showed neurological signs and locomotion disorders. The severity of the clinical signs was quite variable in the inoculated animals (Table 1). Five of the six infected piglets were culled in extremis between days 7 and 16 p.i. One piglet (no. 1) which displayed the mildest clinical signs, remained alive throughout the experiment and recovered from the infection, being culled at day 16 p.i.

**Leucocyte counts**

Figure 1 shows the results of the differential absolute leucocyte counts throughout the duration of the experiment. The results indicate a progressive decline in the absolute leucocyte
Table 1. Clinical signs of piglets infected with the Alfort 187 strain of classical swine fever virus (CSFV)

<table>
<thead>
<tr>
<th>Piglet number</th>
<th>Locomotion disorders</th>
<th>Depression</th>
<th>Fever</th>
<th>Conjunctivitis</th>
<th>Anorexia</th>
<th>Skin haemorrhages</th>
<th>Diarrhoea</th>
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<td>–</td>
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</tr>
</tbody>
</table>

As observed from day 3 post-inoculation. Symptoms were described as –, absent; +, present in low intensity; ++, present with moderate intensity; +++, present with high intensity.

subpopulation counts, for all infected swine. However, the decline was only statistically significant for lymphocytes, from piglets 2, 3, 4, 5 and 6. For piglet 1 (the one that recovered from the infection), a trend towards a slight increase in all cell subpopulation counts was observed from day 11 p.i. onwards.

Uninfected piglets showed variable leucocyte counts during the experiment. There was a tendency for increased total leucocyte counts in uninfected animals during the experiment (Fig. 1).

Percentage of B lymphocytes

The percentages of B lymphocytes in peripheral blood of infected animals showed a significant decrease as the disease progressed ($P = 0.0001$). Figure 2 shows that a severe and progressive B cell depletion occurred in all infected piglets. The mean percentage of B cells in infected animals on day 5 p.i. was 17% and on day 18 p.i. was 4.1%, whereas in uninfected animals, it was 20.1% on day 5 p.i. and 17.3% on day 18 after the beginning of the experiment.

Virus isolation

The results of virus isolation from plasma samples are shown in Table 2. Infectious virus was detected between days 5 and 14 p.i., from all infected animals. Virus titres varied from $10^{0.5}$ (piglet 5, day 5 p.i.) to $10^{4.75}$ TCID$_{50}$/50 μl (observed in piglet 4 on the last sample collected). The piglet that survived the infection (no. 1) had infectious virus in plasma samples from day 5 up to day 9 p.i. After day 9 p.i., no more infectious virus could be recovered.

Among the other piglets, there was an increase in titres of infectious virus in plasma as the disease progressed, except for piglet 2, where virus could not be recovered at the last sample collected before death (day 10 p.i.) The period of detection of infectious virus was significantly more prolonged in plasma than in PBMC ($P = 0.0001$). Virus isolation from plasma samples of control animals was negative throughout the experiment.

Infectious virus was isolated from PBMC of all infected animals with a tendency for increased titres as the disease progressed. Virus was isolated in B lymphocytes from all infected animals, except from piglet 1 (the piglet that survived the infection), whose B lymphocytes remained apparently free of infectious virus throughout this study.

In the group of infected animals, virus was isolated from day 7 p.i. up to death in all piglets, except from piglet 3, whose B lymphocytes showed no infections virus after the first sampling. Virus titres in B lymphocytes varied between $10^{0.025}$ TCID$_{50}$/10$^5$ cells (piglet 2 on day 7 p.i.) and $10^{3.5}$ TCID$_{50}$/10$^5$ cells (piglet 4 on day 11 p.i.).

In T lymphocytes, virus was isolated on days 5 p.i. (piglet 3) and 7 p.i. (piglets 4, 5 and 6) onwards, with the exception of piglets 1 and 2 (where the virus was isolated from T lymphocytes
only on days 5 and 7 p.i., respectively). Virus titres in T lymphocytes varied from $10^{0.075}$ TCID$_{50}$/10$^2$ cells (piglets 1, 2 and 3) to $10^{4.75}$ TCID$_{50}$/10$^2$ cells (piglet 6, last sample collected on day 7 p.i.), when the highest titre of infectious virus was detected.

Infectious virus was isolated from monocytes of all infected piglets. In these cells, virus isolation was positive in only 1 day of sampling. The exception was piglet 4, where virus was recovered from monocytes on days 9, 11 and 14 p.i. Titres varied from $10^{0.2}$ TCID$_{50}$/10$^2$ cells (piglet 4 on day 11 p.i.) to $10^{3.25}$ TCID$_{50}$/10$^2$ cells (piglets 5 and 6 on days 9 and 7 p.i., respectively). The presence of virus in monocytes of piglet 2 could not be investigated because cells were lost by contamination.

There were no statistically significant differences between infectious virus titres obtained from B, T lymphocytes and monocytes, from all infected animals ($P = 3.065$). Equally, the length of time in which virus could be isolated from the three cell populations was not
Fig. 2. Percentages of B and total lymphocytes (B plus T cells) in infected and uninfected animals. The lines represent the average of relative lymphocyte counts: ▲, B lymphocytes of infected animals; ■, total lymphocytes of infected animals; ▲, B lymphocytes of uninfected animals; △, total lymphocytes of uninfected animals.

significantly different ($P = 0.658$). Virus could not be recovered from plasma or PBMC of controls throughout the experiment.

**Discussion**

Despite the fact that the affinity of CSFV for cells of the immune system has long been recognized, little is known about the distribution of virus in PBMC subpopulations. Previous experiments had focused on the histological and functional alterations of lymphocytes during acute CSF (Van Oirschot, 1979a, b; Van Oirschot et al., 1981). Infectious virus was detected in lymphatic tissues, buffy coat cells (Ressang, 1973; Van Oirschot and Terpstra, 1977) and PBMC (Van Oirschot et al., 1981). Recently, viral antigens were detected in B lymphocyte follicles (Susa et al., 1992) and in peripheral and thymic T lymphocytes (Pauly et al., 1998). However, the concomitant evaluation of the presence of infectious virus in all PBMC subpopulations during acute CSF had not been addressed so far. In view of that, the aim of the present study was to determine the quantitative distribution of infectious virus in peripheral monocytes, T and B lymphocytes, in order to examine whether there would be any significant difference in virus affinity for any of these cell subpopulations. Blood samples of CSFV-infected piglets were periodically collected and examined for the presence of infectious virus during the course of experimental acute disease. It was observed that infectious virus was detected in similar titres in monocytes, B and T lymphocytes from peripheral blood during acute disease, as well as from plasma.

In previous in vitro experiments (Van Oirschot, 1980), infectious virus could first be detected in lymphoblasts stimulated with either T or B mitogens. It was then suggested that the virus could infect mature B and T cells. Later, the tropism of CSFV for B cell follicles during the course of disease was demonstrated (Susa et al., 1992).

Subsequently, CSFV-induced apoptosis on thymic T lymphocytes was evidenced (Bruschke et al., 1997) as well as the presence of viral antigens in these cells (Pauly et al., 1998). However, it remained unclear as to whether there would be any particular tropism of the virus for any PBMC subpopulations during the course of acute infection. Here, it was demonstrated that
Table 2. Titres of infectious virus isolated from peripheral blood mononuclear cells of classical swine fever virus-infected piglets

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Titres expressed as $\log_{10}$ of the 50 % tissue culture infective dose (TCID$_{50}$) per $10^2$ cells or per 50 µl (plasma).

* Death. –, no virus detected; BL, B lymphocytes; TL, T lymphocytes; MO, monocytes; PL, plasma; n.d., not determined due to cell contamination.

Virus distribution was similar in the three cell subpopulations examined, as statistically significant differences were not detected between infectious virus titres in monocytes, B and T lymphocytes.

Virus isolation from B lymphocytes was found to be similar to previous findings (Van Oirschot, 1980; Susa et al., 1992). However, we have been able to detect infectious virus within T lymphocytes as early as 5 days p.i., whilst Pauly et al. (1998) could detect virus in T lymphocytes only from 15 days p.i. onwards. These contrasting results could be due to the use of different cell separation and virus detection techniques employed in both studies. Pauly et al. (1998) separated B from T lymphocytes by passages over Nylon wool columns and viral antigen was detected by flow cytometry. Here, T lymphocytes were negatively selected by panning and the presence of infectious virus was determined by isolation in cell cultures. The mean cell separation efficiencies were 82.4 % (B cells) and 94.4 % (T cells). However, cell separation efficiencies were not shown in the previous study, so it was not possible to establish a comparison. Earlier detection of virus in T lymphocytes could also be due to the sensitivity of the techniques employed. It has recently been shown that the detection of viral antigen by flow cytometry is less sensitive than virus isolation on cell cultures (Porntrakulpipat et al., 1998). Moreover, the age at which experimental infection was performed (60–90 days of age in the previous experiment, as opposed to 30 days in this study), the virus strains used for infection and the amount of virus inoculated ($2 \times 10^7$ TCID$_{50}$ of Alfort Tübingen strain, as opposed to $10^{1.8}$ TCID$_{50}$ of the strain Alfort 187 used here) may also have contributed to such variation.
Infectious virus was also found earlier in plasma than in PBMC. However, as sampling was performed every other day during the infection, such an interval may have influenced the detection of the virus in plasma and cells. In addition, virus was also found in T lymphocytes and monocytes of two animals as early as it was found in plasma.

Perhaps the most striking finding of the present study was the replacement of the B cell subpopulation by some sort of as yet unidentified cell subset, as leucopenia progressed. Severe leucopenia is a well-established feature of acute CSFV infection (Van Oirschot et al., 1981). In this study, absolute leucocyte counts of infected piglets showed a progressive fall during the course of the disease. However, when the proportion of total lymphocytes in relation to total leucocytes (B plus T lymphocytes/total leucocytes) was determined, the proportion remained constant. As the percentages of B cells in relation to the total lymphocyte counts fell sharply during infection ($P=0.0001$), it seems that these cells were replaced by some sort of mononuclear IgM-negative cells. This substitution would keep relative total lymphocyte counts unaltered. Unfortunately, we could not determine the nature of the cells which apparently replaced B lymphocytes. It is possible that rapid depletion of B cells may have prompted immature (IgM$^-$) lymphocytes to circulate in peripheral blood, in an attempt to repopulate B cells destroyed in the course of infection. Alternatively, B cells may have been substituted by natural killer cells or T lymphocytes. However, due to the limitations of the present study, the nature of these ‘non-B cells’ remains undetermined, although such determination poses an interesting challenge for future studies.

The establishment of the identities of infected cell subpopulations in acutely CSFV-infected animals will contribute to further our knowledge on CSFV immunopathogenesis, especially the effects on the specialized functions of the immune cells. In this study, we have demonstrated that infectious CSFV was present to similar titres in the three major PBMC subsets. By decreasing the total leucocyte counts and, in addition, proportionally reducing the number of circulating B cells, the virus can seriously harm the humoral immune response and impair the development of an effective cellular immune response in acutely infected animals. Apart from decreasing the number of leucocytes, the virus is also able to multiply within the three major PBMC. Viral infection of leucocytes is a mechanism of preventing virus elimination. Infection and functional alterations of these cells may lead to an impaired immune response and persistence of virus in the host (Mims, 1974; Oldstone, 1991). These mechanisms seem to be at least part of the repertoire that CSFV may evoke to prevent its elimination from an infected host.

References


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