

IMMUNOHISTOCHEMICAL DETECTION OF B AND T LYMPHOCYTES IN MANDIBULAR LYMPH NODES OF EXPERIMENTALLY INFECTED PIGLETS WITH CLASSICAL SWINE FEVER VIRUS

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Classical swine fever (CSF) is a highly contagious viral disease of domestic pigs and wild boars with severe consequences on animal welfare, livestock production, trade and national economy. We investigated if colostral antibodies in piglets, originating from sows vaccinated with Chine strain (C-strain) and challenged with CSF virus, affected the distribution of the B and T lymphocytes in mandibular lymph nodes. Nineteen 45 days old cross breed pigs of both sexes were divided in three groups. All animals in the first group, originating from sows vaccinated with C-strain vaccine were serologically positive for the presence of specific colostral CSF virus antibodies. The second group consisted of pigs originating from unvaccinated sows serologically negative for CSF virus antibodies. Three healthy pigs serologically negative for the presence of CSF virus antibodies, originating from unvaccinated sows against CSF virus served as a control group. In the group of animals originating from unvaccinated sows against CSF, severe depletion of CD79 positive B lymphocytes was detected. In the group of pigs originating from sows vaccinated with C-strain (CSF virus antibody positive group of pigs), a reduced number of CD79 positive B lymphocytes was detected, but B cells were still present in the periphery of the germinative centres of secondary lymph follicles. There was an increase of the number of CD3 positive cells in the mandibular lymph nodes of pigs with or without maternal antibodies.

Key words: B lymphocytes; classical swine fever; lymphoid depletion; T lymphocytes

INTRODUCTION

Classical swine fever (CSF) is a highly contagious viral disease of domestic pigs and wild boars. The causative agent is the virus (CSFV) that belongs to the genus *Pestivirus* of the *Flaviviridae* family (van Oirschot, 1999, 2003; Paton and Greiser-Wilke, 2003). CSF has severe consequences on animal welfare, livestock production, trade and national economy (Moenning, 2000). During CSFV

infection lymphoid organs are depleted from lymphocytes while in the peripheral blood severe lymphopenia develops (Šuša *et al.*, 1992; Sato *et al.*, 2000; Summerfiled *et al.*, 2000; Sánchez-Cordón *et al.*, 2002, 2005). Such an event contributes to the transient immunosuppression and is most likely the cause of delayed immune response in infected animals (Sánchez-Cordón *et al.*, 2006). Monocytic and immature granulocytic cells have been recognized to be infected with the virus (Summerfiled *et al.*, 1998) as well as vascular endothelial cells, consequently leading to the damage of various tissues. Recently Carrasco *et al.*, provided evidence that dendritic cells are infected with CSFV. Tissue lesions and lymphocyte distribution depend on the virulence of the virus (Narita *et al.*, 2000). In countries where CSFV has not been eradicated, a vaccination program with the attenuated live vaccine (mostly with China strain) is established. In such circumstances colostral antibodies are expected to protect piglets up to a certain level. We wanted to know if colostral antibodies in piglets, originating from sows vaccinated with China strain (C-strain) and challenged with CSF virus, affected the distribution of B and T lymphocytes in mandibular lymph nodes.

MATERIAL AND METHODS

Animals, virus and experimental design

Nineteen 45 days old Landrace × Large White pigs of both sexes were divided in three groups. All eight animals in the first group originating from pigs vaccinated with C-strain vaccine were serologically positive for the presence of specific colostral antibodies on CSFV. The second group consisted of eight pigs originating from unvaccinated sows serologically negative for the presence of CSFV antibodies. Three healthy pigs unvaccinated against CSF, originating from unvaccinated sows were in the third, control group. All the animals were serologically negative for the presence of BVDV antibodies. The animals were housed in the Experimental-Diagnostic-Center of the Scientific Veterinary Institute "Novi Sad", Novi Sad.

Each of the sixteen animals received intramuscular inoculation of 2×10^5 TCDI₅₀ of CSFV, Baker strain, while three control pigs received only 1 mL of phosphate buffered saline (PBS), pH 7.2, on the 55th day of life. After virus inoculation clinical signs and rectal temperature were monitored daily. All experimentally infected pigs died before the 22nd day post infection (pid). Euthanasia of pigs in the control group was performed with an intravenous injection of T61 (Intervet, International) at the end of the study.

Blood collection and specific antibody detection in serum

Pre-inoculated blood samples were taken from the jugular vein of all pigs. Specific CSFV antibodies in the sera were determined by the IDEXX® *Classical Swine Fever Virus Antibody Test Kit* according to the manufacturers instructions. Detection of specific antibodies to the Bovine Viral Diarrhea Virus (BVDV) was performed by VN test with NADL strain, according to the procedure described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Office International Des Epizooties (2004).

Histopathology and immunohistochemistry

Samples were taken from the mandibular lymph nodes after necropsy. Specimens were fixed for two days in 10% neutral formalin. The samples were dehydrated through graded series of alcohol and embedded in paraffin wax by routine techniques for light microscopy. Wax embedded sections (4µm) were cut and stained with haematoxylin and eosin (HE).

For the detection of CSFV glycoprotein E2(gp55) the monoclonal antibody WH303 (Veterinary Laboratories Agency, Addlestone, UK) was used. To demonstrate T and B lymphocytes, monoclonal mouse anti-human CD3 and CD79 antibodies (DAKO Citomation, Denmark) were used. Tissue sections were dewaxed and rehydrated in xylene and in a graded series of alcohol. Citric buffer was used for antigen retrieval by incubation in a microwave oven (560W) for 21 minutes. Endogenous peroxidase activity was abolished by incubation of tissue sections with 0.5% hydrogen peroxide in methanol for 15 minutes at room temperature. All tissue sections were incubated with 50% normal goat serum for 30 minutes at room temperature. Primary antibody WH303 was diluted 1:50 in PBS containing 10% normal goat serum and incubated for 18 hours at 4°C. Monoclonal antibodies for CD3 and CD79 were diluted 1:50 in PBS and incubated for 60 minutes at room temperature. Biotinylated goat anti-mouse immunoglobulin (DAKO, ChemMate, Denmark) was applied as the secondary antibody for 20 minutes at room temperature.

All tissue sections were incubated with streptavidin-peroxidase complex (DAKO, Denmark) for 20 minutes at room temperature and with chromogen 3-3' diaminobenzidine tetrahydrochloride (DAKO, Liquid DAB, Denmark) or 3-amino-9-ethylcarbamazole (DAKO, ChemMate, AEC, Denmark) and counterstained with Mayer's haematoxylin. Details of the primary antibodies, including dilutions and pre-treatments, are summarized in Table 1.

Table 1. Details of the immunolabeling reagents

<i>Primary antibody</i>	<i>Chemical fixation</i>	<i>Antigen or cell detected</i>	<i>Antibody dilution</i>	<i>Pre-treatment</i>
<i>Monoclonal WH303*</i>	<i>Neutral formalin solution 10%</i>	<i>CSFV protein E2(gp55)</i>	<i>1 in 50[†]</i>	<i>Citrate buffer</i>
<i>Monoclonal mouse anti-human CD3**</i>	<i>Neutral formalin solution 10%</i>	<i>CD3 (T lymphocytes)</i>	<i>1 in 50^{!!}</i>	<i>Citrate buffer</i>
<i>Monoclonal mouse anti-human CD79***</i>	<i>Neutral formalin solution 10%</i>	<i>CD79 (B lymphocytes)</i>	<i>1 in 50^{!!}</i>	<i>Citrate buffer</i>

[†] In PBS (pH 7.2) containing normal goat serum 10%

^{!!} In PBS (pH 7.2) The Labeled streptavidin-biotin complex (LSAB) method (ChemMate, DAKO, Denmark, product No. K5003) was used with 3-3' diaminobenzidine tetrahydrochloride (DAKO, Liquid DAB, Denmark, product No. K3465) or 3-amino-9-ethylcarbamazole (AEC, ChemMate, DAKO Denmark, product No. K5003),

* Veterinary Laboratory Agency, Addlestone, UK, product No. PA0826

** DAKO, Denmark product No. A0452

*** DAKO, Denmark product No. M7051

Detection of viral RNA

RNA was extracted from tissues using Trizol reagent (GIBCO BRL) according to manufacturer's recommendation. Briefly, three volumes of Trizol reagent were added to 250 µL of sample material (supernatant from 10% tissue homogenate in PBS). This was then extracted sequentially with chloroform and isopropanol and then RNA was precipitated with 80% ethanol prior to resuspension in DEPC treated water.

The "one-tube" or "one-step RT-PCR" assay was performed by using reagents supplied in a commercial "Access RT-PCR system" (Promega, USA) according to the manufacturer's instruction. RT-PCR amplification was done using E2 gene specific primers described by Katz *et al.* (1993): gp55-U: 5'-ATA TAT GCT CAA GGG CGA GT-3' (sense, position in genome of the Alfort strain is 3378-3397); gp55-L: 5'-ACA GCA GTA GTA ATT TCT TTA-3' (antisense, position in genome of the Alfort strain is 3685-3662).

RNA sample (6 µl) was added to 44 µl reaction mixtures containing AMV/Tfl 1 x reaction buffer, dNTP mix (10mM), 25 pmol of sense and antisense primer, 1 mM of MgSO₄, 5 U of AMV RT and 5 U of *Tfl* DNA polymerase. The RT-PCR cycling conditions were as follows: 45 min at 48°C for RT, 94°C for 2 min for AMV RT inactivation and RNA/cDNA/ primer denaturation, 40 cycles of 30 s at 94°C, 1 min at 60°C and 2 min at 68°C, and a final extension step at 68°C for 7 min. Amplified products were analyzed by electrophoresis on a 1.5% agarose gel.

RESULTS AND DISCUSSION

Clinical signs and gross lesions

All pigs inoculated with CSFV became ill with characteristic clinical signs of CSF. High body temperature was detected 2 days post-inoculation (dpi). Rectal temperature was between 40.6°C and 41.4°C. Reduced appetite and apathy were noticed from 3 dpi. Conjunctivitis associated with eye discharge was recorded from 5 dpi. Yellowish gray diarrhea was noticed from 7-8 dpi. In few pigs aphonia developed. Neurological signs (convulsions, staggering gait, posterior paresis) were recorded from 7-14 dpi. Hyperemia, cyanosis and hemorrhages of skin were also noticed from 9 dpi. All experimentally infected pigs in both groups died until 22 dpi.

The mandibular lymph nodes were swollen and enlarged. Subcapsular to diffuse hemorrhages were found on necropsy which gave an almost dark appearance to the lymph nodes. Mostly, mandibular lymph nodes had a characteristic marbled appearance in cross-section.

Histopathology

In all experimentally infected pigs with CSFV microscopic changes corresponded with gross lesions. Huge hemorrhages were localized mostly subcapsularly, in the cortex and paracortex. All examined mandibular lymph nodes showed depletion of lymphocytes from follicles and a great number of

macrophages. Edema of endothelial cells with fibrinoid necrosis and thrombosis occurred in small blood vessels.

Detection of viral antigen

There was no immunolabelling for the glycoprotein E2 (gp55) in the control group of the pigs. Immunohistochemical examination of the mandibular lymph nodes of the experimentally inoculated pigs with CSFV demonstrated E2 (gp55) protein as brown granular intracytoplasmatic deposits of endothelial cells, macrophages (Fig. 1 and Fig. 2), and a large number of lymphocytes and plasma cells around trabeculae and subcapsular sinus (Fig. 2).

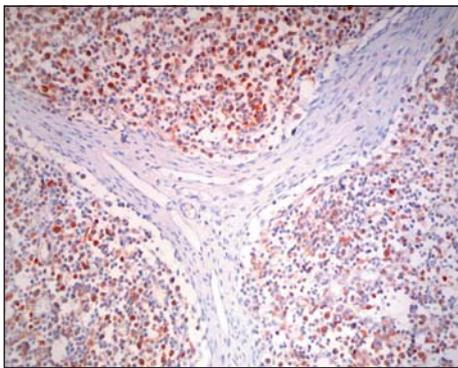


Figure 1. Mandibular lymph node, group with CSFV antibodies, WH303 (against E2 protein of the CSFV), LSAB, x400

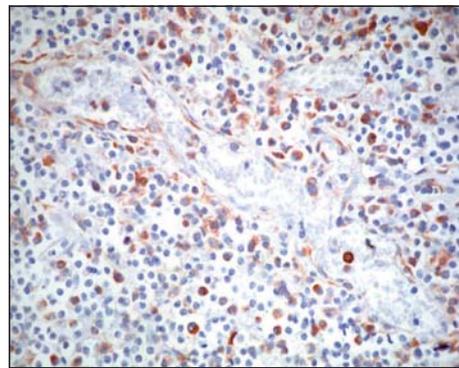


Figure 2. Mandibular lymph node, group without CSFV antibodies, WH303 (against E2 protein of the CSFV), LSAB, x200

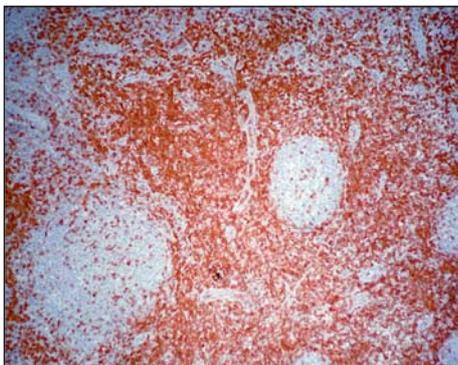


Figure 3. Mandibular lymph node, group without CSFV antibodies, 22 dpi, CD3 (against T lymphocytes), LSAB, x100

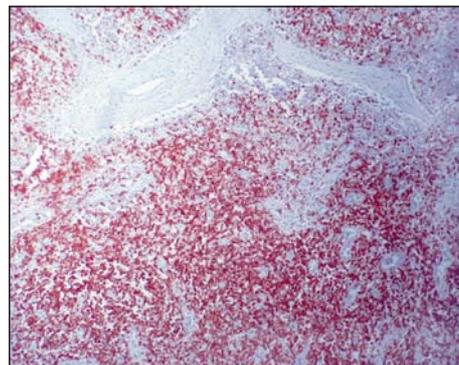


Figure 4. Mandibular lymph node, group with CSFV antibodies, 11 dpi, CD3 (against T lymphocytes), LSAB, x100

Detection of B and T lymphocytes in mandibular lymph nodes

The immunohistochemical detection of CD3 T lymphocytes in mandibular lymph nodes showed an increase in the number of CD3 T lymphocytes mostly around trabecules in both groups of experimental pigs (Fig. 3 and Fig. 4). Depletion of CD79 positive B lymphocytes was determined in both groups of pigs in the mandibular lymph nodes. In the group of animals originating from unvaccinated sows, (CSFV antibodies free group of experimental pigs), more severe depletion of CD79 positive B lymphocyte was detected (Fig. 6). On the other hand in the group of pigs originating from sows vaccinated with C-strain, (group of experimental pigs with the presence of the CSFV colostral antibodies), a reduced number of CD79 positive B lymphocytes was detected but CD79 positive B lymphocytes were still present in the periphery of germinative centres of the secondary lymph follicles of mandibular lymph nodes (Fig. 7).

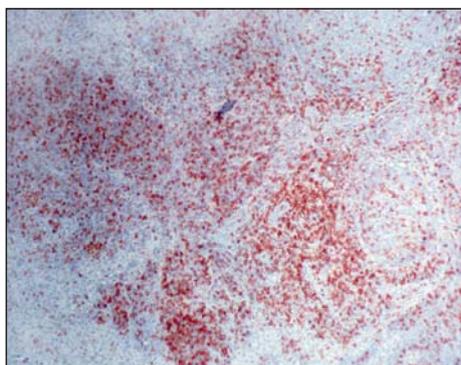


Figure 5. Mandibular lymph node, control group, CD3 (against T lymphocytes), LSAB, x100

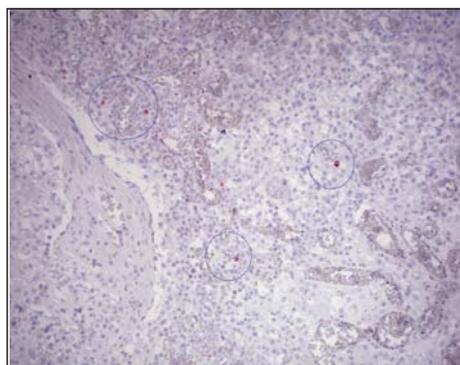


Figure 6. Mandibular lymph node, group without CSFV antibodies, 16 dpi, CD79 (against B lymphocytes), LSAB, x100. Note just a few positive B cells



Figure 7. Mandibular lymph node, group with CSFV antibodies, 11 dpi, CD79 (against B lymphocytes), LSAB, x100

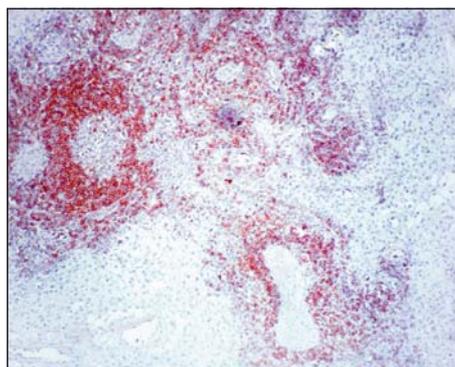


Figure 8. Mandibular lymph node, control group, CD79 (against B lymphocytes), LSAB, x100

Detection of viral RNA

In all the samples taken from pigs inoculated with CSFV, the RNA virus was found. The samples of mandibular lymph nodes and brain taken from pigs in the control group were negative for the presence of CSFV RNA.

Mandibular lymph nodes from both groups of piglets were depleted from B lymphocytes. More severe depletion of CD79 positive B lymphocytes was present in the second group of animals (free from CSFV antibodies) where only few immunolabeled cells for CD79 were determined. Our results are in correlation with results reported by Narita *et al.*, 2000. The authors discovered total depletion of B lymphocytes in lymph nodes of pigs infected with the highly virulent strain, and moderate depletion of B lymphocyte in the lymph nodes of pigs injected with the less virulent strain of CSFV. The depletion of CD79 positive B lymphocytes could have affected the delayed humoral response of pigs infected with CSFV (Sánchez-Cordón *et al.*, 2006). There is an initial depletion of T and B cells in the peripheral blood during CSF infection (Summerfield *et al.*, 2001). Here we provide the evidence that at the site of viral multiplication, i.e. lymph node, there is an increase of activated CD3 cells comparing to that in the controls. This is supported by the fact that CD3 positive cells practically replace B cells. We assume that as disease progresses, T cells activated at the site of virus multiplication and are most likely directly involved in the immune response. In the study of Sánchez-Cordón *et al.*, 2005, the role of monocyte/macrophage cells lineage and secretion of cytokines in the spleen from CSFV infected pigs was determined. The presence of viral antigens was necessary to induce TNF- α expression. TNF is cytokine involved in inflammatory response and its role is tightly connected with the induction of apoptosis. Since in our experiments maternally immune piglets had clinical evidence of the disease a few days later than the non-immune animals, we assume that passively transferred antibodies neutralized the virus to a certain level but could not prevent the fatal outcome of the disease. Also, the neutralizing activity of passively transferred antibodies prevented the total destruction of B lymphocytes but did not affect CD3 T lymphocytes presence in the lymph node. This result leads to the speculation that T cells *per se* are not the only cells responsible for B cell destruction. One possible explanation is that the signal for apoptosis comes from macrophages and that these cells probably produce a lower amount of TNF α in maternally immune piglets. The exact role of T cells in tissues during the course of CSFV infection needs to be evaluated in the future.

All infected piglets (with or without passive antibodies) during this experiment succumbed to the disease. The only difference was found during the pathohistological examination in CD79 depletion in the lymph nodes. We did not measure the titer of CSFV antibodies before challenge since ELISA kit detected only the presence or absence of antibodies. In the experiment of Terpsta and Wensvoort (1988) CSFV antibody titer of 1/32 could prevent both clinical signs of diseases and the horizontal spread of the virus. Obviously, the amount of colostrum CSFV antibodies in the blood of inoculated pigs originating from vaccinated sows with C-strain was not sufficient to prevent clinical signs of CSF.

In conclusion we found that depletion of B lymphocytes (CD79) was severe in the second group of piglets (without maternally derived antibodies), while in the group of piglets with maternal antibodies the depletion of B lymphocytes was less severe. A significant amount of CD3 lymphocytes was detected in all infected piglets in lymph nodes where a high expression of E2(gp55) was determined. The role of activated CD3 T cells in the lymph node should be elucidated in the future.

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IMUNOHISTOHEMIJSKO DOKAZIVANJE B I T LIMFOCITA U MANDIBULARNOM LIMFNOM ČVORU PRASADI EKSPERIMENTALNO INFICIRANIH VIRUSOM KLASIČNE KUGE SVINJA

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SADRŽAJ

Klasična kuga svinja (KKS) je infektivno virusno oboljenje domaćih i divljih svinja, koja ima značajan uticaj kako sa stanovišta dobrobiti životinja, tako i sa stanovišta svinjarske proizvodnje, trgovine i nacionalne ekonomije. Želeli smo da ustanovimo da li specifična kolostralna antitela prasadi poreklom od krmača

vakcinisanih sa Kina sojem (K soj) vakcinom protiv KKS, utiču na distribuciju B i T limfocita u mandibularnom limfnom čvoru, nakon veštačke infekcije sa virusom KKS. Devetnaest prasadi starosti 45 dana, podeljeni su u tri grupe. Jedinke iz prve grupe poticala su od krmača vakcinisanih K sojem protiv KKS i bila su serološki pozitivna na prisustvo specifičnih kolostralnih antitela protiv virusa KKS. Drugu grupu sačinjavala su prasadi poreklom od nevakcinisanih krmača protiv KKS, koja su bila serološki negativna na prisustvo specifičnih antitela protiv virusa KKS. Tri zdrave jedinke poreklom od nevakcinisanih krmača, koja su bila serološki negativna na prisustvo antitela protiv virusa KKS predstavljale su kontrolnu grupu. U grupi životinja poreklom od nevakcinisanih krmača protiv KKS, utvrđena je deplecija B limfocita najvećeg stepena. U grupi životinja poreklom od krmača vakcinisanih sa K sojem, utvrđeno je izražano smanjenje broja CD79 pozitivnih limfocita, ali su B limfociti i dalje ostali prisutni na periferiji germinativnih centara sekundarnih limfnih folikula. Broj CD3 pozitivnih T limfocita u mandibularnim čvorovima bio je uvećan u obe grupe eksperimentalno inficiranih životinja sa virusom KKS u odnosu na kontrolnu grupu.