

Ovine Fetal Immune Response to Cache Valley Virus Infection

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Cache Valley virus (CVV)-induced malformations have been previously reproduced in ovine fetuses. To evaluate the development of the antiviral response by the early, infected fetus, before the development of immunocompetency, ovine fetuses at 35 days of gestation were inoculated *in utero* with CVV and euthanized at 7, 10, 14, 21, and 28 days postinfection. The antiviral immune response in immature fetuses infected with CVV was evaluated. Gene expression associated with an innate, immune response was quantified by real-time quantitative PCR. The upregulated genes in infected fetuses included *ISG15*, *Mx1*, *Mx2*, *IL-1*, *IL-6*, *TNF- α* , *TLR-7*, and *TLR-8*. The amount of Mx1 protein, an interferon-stimulated GTPase capable of restricting growth of bunyaviruses, was elevated in the allantoic and amniotic fluid in infected fetuses. ISG15 protein expression was significantly increased in target tissues of infected animals. B lymphocytes and immunoglobulin-positive cells were detected in lymphoid tissues and in the meninges of infected animals. These results demonstrated that the infected ovine fetus is able to initiate an innate and adaptive immune response much earlier than previously known, which presumably contributes to viral clearance in infected animals.

Cache Valley virus (CVV) is a mosquito-borne bunyavirus of the family *Bunyaviridae*, genus *Orthobunyavirus*, Bunyamwera group (1), and endemic in North America (2). Serologic studies have shown that unlike most members of the family *Bunyaviridae*, the vectors of CVV also infect larger mammals, with virus being isolated from sheep, cattle, and horses and causing reproductive losses in small ruminants (2–8), similar to the disease caused by Akabane virus (9–11) and the newly identified Schmallenberg virus occurring in Europe (12). The virus has rarely been associated with meningoencephalitis in humans (13, 14). *In utero* ovine fetal infection causes abortion and fetal malformations, mainly affecting the musculoskeletal system (MSS) and the central nervous system (CNS) (2–5, 7, 15).

Previous studies describing experimental CVV-induced malformations in ovine fetuses showed that the development of fetal lesions depends on the fetal age when infected. If the virus is inoculated between 28 and 48 days of gestation (dg), fetal death and abortion or MSS and CNS malformations occur. No apparent malformations are observed if the virus is inoculated after 48 dg (2, 3). Virus isolation can only be made from infected fetuses in early development, and viral recovery from tissues of term abortions and malformed lambs is uniformly unsuccessful. The virus is cleared from infected tissues within a few weeks after infection (2, 15) and before the presumed age of fetal immunocompetency, at approximately 70 to 75 dg (16, 17).

The gestation period of the ewe is approximately 147 days. Ovine fetuses develop erythropoiesis, myelopoiesis, and megakaryopoiesis in the yolk sac and liver at approximately 17 dg (18). At approximately 20 to 25 dg, lymphocyte production begins in the thymus, and lymphocytes are in the bloodstream at 48 to 50 dg (18). At 45 to 50 dg, T and B lymphocytes and cells with surface immunoglobulins are present in the spleen and lymph nodes (19, 20). The lymph nodes become integrated into a lymphatic system around 65 dg (18). Establishing the time when fetuses are able to respond to antigens is difficult. Available data on adaptive immune response of ovine fetuses have been based on serum neu-

tralization assays, which were established for use in mature animals. In addition, gestation assay points used in experiments are somewhat arbitrary. Ovine fetal antibody response to viral infection at titers greater than 1:2 has been detected after 76 to 78 dg (11). Because the syndesmochorial placenta of ruminants prevents passage of immunoglobulins from the ewe to the fetus (21), antibodies in fetuses and precolostral newborns are those produced by the fetus. For this reason, *in utero* viral infections can be diagnosed in aborted fetuses and stillborn ruminants using serum neutralization tests. Such testing is necessary with CVV because the fetus clears the virus long before the end of gestation (2, 5).

Previously, we have demonstrated that fetuses infected with CVV early in gestation (~35 dg) have low viral antigen and RNA signal in tissues around 56 dg and are able to clear the infection before development of an adaptive immune system at 75 dg (15). Similarly, age-based findings have been described in Akabane virus-infected fetuses (9, 11, 16). Because no effective serum neutralization antibody (an adaptive immune response) has been detected in ovine fetuses at the time when CVV is cleared from fetuses (18), it may be that the ovine fetus mounts an innate immune response for early viral clearance. To test this hypothesis, the expression of selected genes associated with the innate immune response was determined in tissues of CVV-infected and noninfected ovine fetuses, and CVV mRNA was quantified in selected tissues of these fetuses to correlate the measured innate response with viral clearance. In addition, fetal Mx protein, an interferon (IFN)-stimulated GTPase previously associated with

Received 20 July 2012 Accepted 28 February 2013

Published ahead of print 6 March 2013

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doi:10.1128/JVI.01821-12

TABLE 1 Primer sequences used for RT-qPCR

Gene	Accession no.	Primer sequence (5'–3')	
		Forward	Reverse
CVV	AF082576.1	CAC CAG CGA AAT CCC AAT CAC CA	CTC CAG ACA TAG CAC CCA CCA
TNF- α	X55152.1	CGG CGT GGA GCT GAA AGA CAA	CTG CGA GTA GAT GAG GTA AA
IL-1	NM_001009465.2	AGT GAT GGC TTG CTA CAG T	CCG AGG TCC AGG TGT T
IL-6	X68723.1	GAG GGA AAT CAG GAA ACT GT	CTC GTT TGA GGA CTG CAT CT
TLR7	HQ529279.1	ACT CCT TGG GGC TAG ATG GT	GCT GGA GAG ATC CCT GCT AT
TLR8	FJ905847.1	TCC ACA TCC CAG ACT TTC TA	GTT CTT GTC CTC ACT CTC TT
Mx1	JN377734.1	GTA CGA GCC GAG TTC TCC AA	ATG TCC ACA GCA GGC TCT TC
Mx2	NM_001078652.1	CAT CCA TAA ATC GCT CCC CTT GT	GCT CCT CTG TCG CCC TCT GGT
ISG15	FJ844480.1	TGA CGG TGA AGA TGC TAG GG	ACT GCT TCA GCT CGG ATA CC
IFN- α	X55152.1	ACC CAG CAC ACC TTC CAG CTC TT	CCT CGC AGC CCC TCC TC
IFN- β	EU276065.1	TGG TTC TCC TGC TGT GTT TCT C	CGT TGT TGG AAT CGA AGC AA

antiviral activity against bunyaviruses (22–26), was quantified in the fetal allantoic and amniotic fluids. Expression of the IFN-stimulated gene 15 (*ISG15*), which has been shown to be produced by fetuses in response to viral infection *in utero* (27–29), was evaluated in CVV-infected fetal tissues. Finally, the distribution of B and T lymphocytes and immunoglobulin-positive cells was evaluated in infected and noninfected, ovine fetal tissues in early gestation.

MATERIALS AND METHODS

Virus inoculation and sample harvesting. A group of 15 seronegative, pregnant Rambouillet ewes was housed in BSL2 confinement buildings according to protocols approved by the Institution Animal Care and Use and the Institutional Biosafety Committee. At 35 dg, ewes were inoculated *in utero* with a 1-ml inoculum containing 10^5 50% tissue culture infectious doses of CVV (infected group) or 1 ml of minimum essential medium (mock-infected/control group), as previously described (15). The viral inoculum was derived from the second passage of an isolate from allantoic membrane from an experimentally infected fetus (3). At 7, 10, 14, 21, and 28 days postinfection (dpi), three ewes (one mock infected and two virus infected) were humanely euthanized. Selected fetal tissues and their fluids were harvested and immediately frozen at -80°C or placed in RNA later (Ambion Life Technologies, Carlsbad, CA) and frozen at -80°C . The remaining fetal tissues and placenta were fixed in 10% buffered formalin diluted in deionized water or in Davidson's AFA fixative (glacial acetic acid, 37% formaldehyde, 95% ethanol).

Real-time qPCR. To quantify relative numbers of CVV RNA copies, and abundance of genes of interest, real-time quantitative PCR (qPCR) was performed on samples of brain and muscle harvested from CVV-infected or mock-infected ovine fetuses according to a previously published protocol with minor modifications (30). Briefly, RNA was isolated from harvested samples and homogenized in TRIzol reagent (Gibco-BRL, Bethesda, MD) according to the manufacturer's recommendation. In order to eliminate contamination with genomic DNA, the extracted RNA was treated with the DNase I amplification reagent (Invitrogen, Carlsbad, CA), and the RNA concentration was quantified by spectrophotometry. RNA quality was determined by denaturing samples in a 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized on a UV transilluminator. The cDNA was synthesized from 500 ng of total RNA combined with primer mix containing oligo(dT) primer (0.2 $\mu\text{g}/\text{ml}$), random hexamer primer (300 $\mu\text{g}/\text{ml}$; Invitrogen), and deoxynucleoside triphosphate mix (10 mM each), followed by incubation at 65°C for 5 min. After incubation, SuperScript II reverse transcriptase (Invitrogen) was added to the reaction according to manufacturer's recommendations and reverse transcribed under the following conditions: 25°C for 10 min, 42°C for 60 min, and 70°C for 5 min. To test for genomic DNA contamination, control reactions were prepared without reverse transcriptase.

Specific oligonucleotide primers (Table 1) were generated in Oligo 5 program (Molecular Biology Insights, Inc.) for the following genes: the M segment of glycoprotein 1 of CVV, *alpha interferon* (*IFN- α*), *IFN- β* , *TNF- α* , *IL-1*, *IL-6*, *Mx1*, *Mx2*, *ISG15*, *TLR7*, and *TLR8*. The real-time qPCRs was performed using the ABI prism 7900HT system (Applied Biosystems, Foster, CA) with power SYBR green PCR Master Mix (Applied Biosystems). Primer specificity and efficiency ($-3.6 > \text{slope} > -3.1$) were confirmed by using a test amplification run. The data were normalized using cycle threshold (C_T) values for the ovine glyceraldehyde 3-phosphate dehydrogenase gene (*GADPH*) in each sample. Semiquantitative analysis was calculated with the $\Delta\Delta C_T$ method, and results are expressed as the relative fold changes compared to the lowest value for mock-infected samples. To calculate the relative amounts of viral mRNA, the relative fold changes were compared to the infected sample with the lowest $\Delta\Delta C_T$.

Protein slot blot. A protein slot blot assay was used to determine and compare relative concentrations of the Mx1 protein in the fetal fluids of infected versus noninfected fetuses. The starting concentration of protein in allantoic and amniotic fluids was determined by using the Bradford assay as recommended by the manufacturer (Bio-Rad Laboratories). According to the protein concentration, samples were prepared and diluted to 12 μg of protein for the amniotic fluid and 50 μg of protein for the allantoic fluid in a final volume of 200 μl of Tris-buffered saline (TBS). The volume was deposited onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) in a Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories) according to a modified protocol, as previously published (31). Briefly, nonspecific binding sites were blocked by immersing the membrane in 5% skim milk in TBS-Tween (TBST) for 1 h at room temperature on an orbital shaker. The membranes were incubated overnight at 4°C in 2.5% milk-TBST containing a rabbit anti-ovine Mx1 polyclonal antibody (a gift from Troy Ott, Penn State University) (32) at a concentration of 1:10,000. The membrane was washed three times and then incubated for 1 h with goat anti-rabbit IgG (1:10,000 dilution in 2.5% milk-TBST). After incubation with the secondary antibody, the membrane was washed three times and further incubated for 1 min with Bio-Rad chemiluminescence reagent. The relative concentration of the Mx1 protein in triplicate samples was determined quantitatively in relative light units using a Bio-Rad Chemidoc imager and assessed by using QuantityOne Image analysis software (31).

Immunohistochemistry. Immunohistochemistry was performed on 5- μm , deparaffinized sections of paraformaldehyde-fixed tissues mounted onto positively charged, silanized slides using an automated system for immunohistochemistry (DakoCytomation Autostainer; Dako, Carpinteria, CA) with minor modifications to a previously described immunohistochemistry protocol (15). Antigen retrieval was performed in a decloaker chamber (Biocare Medical, Concord, CA) with slides soaked in citrate buffer (pH 6.0). Sections were incubated with anti-ISG15 poly-

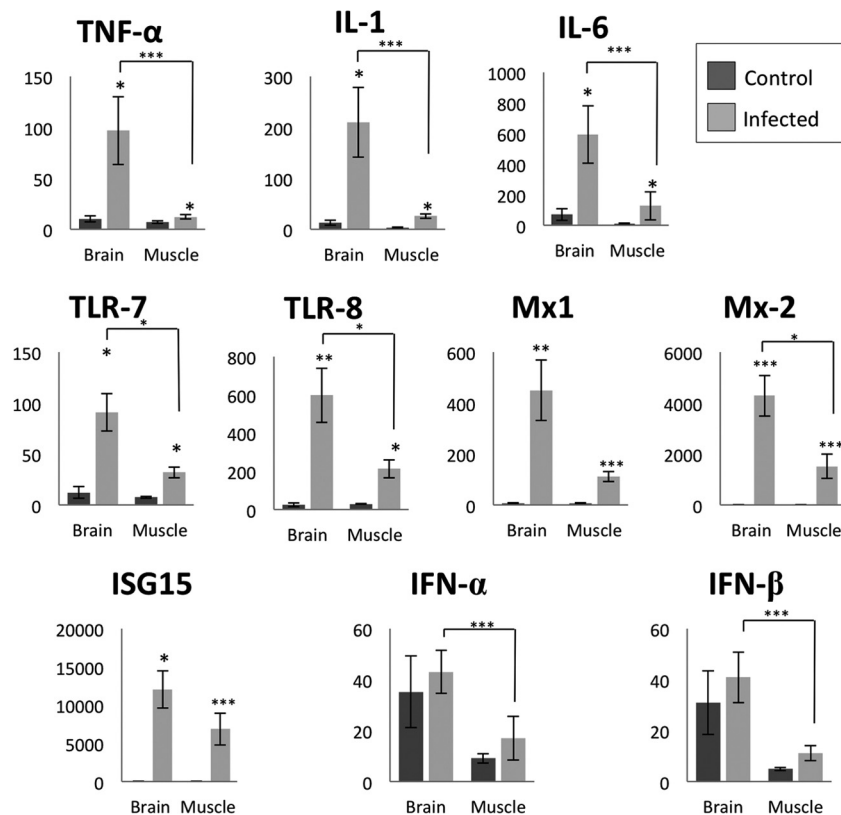


FIG 1 Relative levels of genes expression in brain and skeletal muscle of control and Cache Valley virus-infected animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

clonal antibody (diluted 1:500) for 30 min (the antibody was a gift from Thomas Hansen, Colorado State University) (33), CD3 (diluted 1:400) polyclonal antibody for 35 min (Dako), anti-CD79a polyclonal antibody (diluted 1:200) for 30 min (Dako), anti-ovine IgG (diluted 1:500) for 35 min, and anti-ovine IgM (diluted 1:2,000) for 30 min (KPL, Inc., Gaithersburg, MD). The primary antibody was followed by incubation with the secondary antibody (MACH 2 anti-rabbit polymer-HRP from Biocare Medical [CD3, IgG, and IgM] or ImmPress anti-mouse immunoglobulin (peroxidase) polymer from Vector Laboratories [ISG15, CD79a]) for 20 min. A chromogen complex, 3,3'-diaminobenzidine tetrachloride (DAB; Dako), was used to detect the targeted antigens, and sections were counterstained with hematoxylin. Slides were coverslipped with Permount mounting solution. Tissues were evaluated to determine percentage of infected cells in each examined organ and graded as follows: mild, $<3\%$ of cells positive; moderate, between 3 and 15% of cells positive; and marked, $>15\%$ of cells positive.

Statistical analysis. The quantitative data for mRNA expression and protein levels on fetal fluids were subjected to the Wilcoxon signed-rank test to determine differences between infected and noninfected groups. Differences between groups at different days postinfection were subjected to a one-pair Student t test. In order to reduce heterogeneity among samples, the data were log transformed if necessary, and P values below 0.05% were considered statistically significant. The data are presented as means and overall standard errors.

RESULTS

Real-time qPCR. No significant difference in gene expression was observed either between infected animals at each different days postinfection or between control animals at different days of gestation. Therefore, the data for each gene for each evaluated tissue

were pooled into two groups: control and infected animals (Fig. 1). qPCR analysis revealed that the *IL-1*, *IL-6*, *Mx2*, *ISG15*, *TLR7*, and *TLR8* genes were upregulated ($P < 0.05$) in the brain and skeletal muscle (SKM) of CVV-infected fetuses compared to noninfected fetuses. Only in the brain was the *TNF-α* gene significantly upregulated. All examined genes, but not *ISG15* and *Mx1*, had significantly higher upregulation in the brain compared to the skeletal muscle ($P < 0.05$). Similarly, the relative number of copies of CVV was significantly higher ($P < 0.05$) in the brain than in skeletal muscle (Fig. 2). Progressively lower means of relative number of copies of CVV were observed in both brain and SKM with the progression of the infection. A significant difference ($P < 0.05$) was observed in the brains of fetuses at between 14 and 28 dpi (CVV in the brains of 10-dpi fetuses was excluded from analysis due to high variability among these samples) and in SKM when comparing 10 to 14 dpi, 10 to 21 dpi, and 10 to 28 dpi.

Protein slot blot. A significant ($P < 0.01$) increase in the amount of Mx1 protein in the allantoic and amniotic fluid occurred in infected fetuses versus noninfected fetuses at each time point examined (Fig. 3). Similar to the quantitative data collected with the real-time qPCR, no effect of day or interaction of day versus infection was detected in evaluated samples.

Immunohistochemistry for ISG15. ISG15 was detected in several tissues of all infected fetuses, but no ISG15 immunolabeling was observed in control fetuses (Fig. 4). In infected fetuses, a strong signal was observed in numerous cells of the cerebral parenchyma and meninges, and fewer cells were positive for ISG15

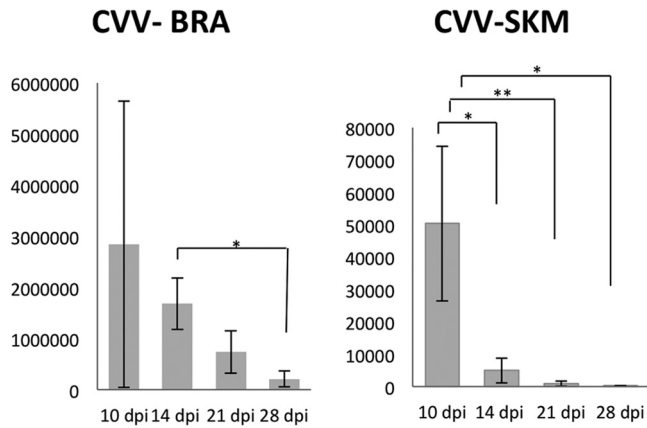


FIG 2 Relative number of Cache Valley virus RNA copies in main target tissues, brain (BRA) and skeletal muscle (SKM), at 10, 14, 21, and 28 dpi. *, $P < 0.05$; **, $P < 0.01$.

in the spinal cord. Most fetuses at 7 to 21 dpi had marked ISG15 antigen signal in the brain, with moderate signal observed at 28 dpi. The ISG15 antigen signal in the spinal cord of infected fetuses varied between mild to moderate at all test points. Multifocal clusters of cells with positive ISG15 immunolabeling were observed in the SKM of infected fetuses through 21 dpi with only rare cells having a positive signal at 28 dpi. Other tissues with clusters of cells with positive signal included: heart, smooth muscle of intestine, tongue, fibroblasts in the subcutaneous tissue, wall of large arteries, cells around small blood vessels, spleen (one fetus at 21 dpi), and rarely in the lungs, tonsils, and thymus of fetuses infected earlier.

Immunohistochemistry of B and T lymphocytes, IgG, and IgM. At 7 dpi/42 dg, scattered CD79a-positive cells were observed in the dorsal abdominal cellular lymphoid aggregates around the abdominal aorta, which correspond to sites of development of rudimentary renal or mesenteric lymph nodes, and rarely in the hepatic sinusoids in both control and infected animals. At 10 dpi/45 dg, slightly increased numbers of CD79a-, IgG-, and IgM-positive cells were observed in the same locations in infected and control animals (Fig. 5A to C). Rare IgM-positive cells were observed in the meninges of one infected animal. At 14 dpi/49 dg, slightly increased numbers of CD79a-, IgG-, and IgM-positive cells were observed in the sites described above, and at this time point, CD79a- and IgM-positive cells were in the spleen of one of the infected animals (Fig. 5D to F). Surprisingly, another infected animal also had a marked infiltration of CD79a-, IgG-, and IgM-positive cells in the meninges of the brain and spinal cord and in rare cells within the parenchyma (Fig. 5G to I). Increased numbers of CD79a-, IgG-, and IgM-positive cells were arranged around splenic arterioles in both infected and control animals at 21 dpi/56 dg and 28 dpi/63 dg, besides also being in the previously described sites. Numerous IgG- and IgM-positive cells were observed in the meninges of the spinal cord of two infected fetuses at 21 dpi, and rare immunoglobulin-positive cells were observed in one fetus at 28 dpi. Moderate to marked numbers of CD3-positive cells, evaluated only in sections of brain and transverse sections of the abdomen, including the lumbar spinal cord of control and infected fetuses at 21 and 28 dpi, were detected in the spleen and in renal and mesenteric lymph nodes in all fetuses. Scattered CD3-positive

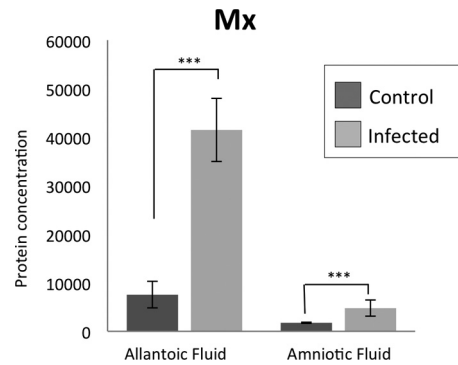


FIG 3 Relative amounts of ovine Mx1 protein in allantoic and amniotic fluid in control and infected animals. The presented values (y axis) are the relative light units using the Bio-Rad Chemidoc imager and assessed using QuantityOne Image analysis software. The starting protein concentration for the samples from the allantoic fluid was 50 μ g and for the amniotic fluid was 12 μ g. The data was log transformed for normalization. The infected fetuses had relatively higher amounts of Mx1 protein in the allantoic and amniotic fluid compared to the control fetuses. ***, $P < 0.005$.

cells were in the meninges of the brain and spinal cord of one infected fetus at 14 dpi and in the meninges of the spinal cord in one fetus at 21 dpi.

DISCUSSION

Ovine fetuses are able to mount an immune response associated with CVV infection earlier in gestation than previously described (10, 11, 16, 17). Although the fetal immune response was not able to completely clear virus by the end of the study at 63 dg (15), infected ovine fetuses were able to reduce the viral load within the CNS and MSS. Viral signal was also markedly reduced or absent in the ganglion, retina, kidney, and heart.

CVV targets mainly cells within the CNS and MSS, and viral signal was markedly reduced in the brain and almost completely cleared in the skeletal muscle after 21 to 28 dpi in fetuses infected at 35 dg (15). Similarly, a progressive reduction in viral load, as shown by mean relative amounts of CVV RNA copies, was observed in the brain and SKM in the present study, with significant differences observed between most days of infection in the SKM, and in the brain at between 14 and 28 dpi. In addition, in all groups, the relative numbers of CVV RNA copies in the brain were significantly higher than in the skeletal muscle.

The Mx protein, an IFN-stimulated GTPase involved in intracellular trafficking, membrane remodeling, and fusion processes, is capable of restricting growth of viruses, including influenza virus, measles virus, and bunyaviruses (including the *Orthobunyavirus*, *Phlebovirus*, and *Hantavirus* genera) (22–24, 26, 34–38). In viral infections, the Mx protein acts by interfering with transport of viral components in infected cells. In a model proposed by Haller and Kochs (35), MxA forms large, membrane-associated self-assemblies that store monomers of this protein. With infection, monomers bind to viral target structures, forming new assemblies involving viral intermediates that lead to mislocalization of viral components and consequent inhibition of viral replication (35). Human MxA has been shown to inhibit replication of La Crosse virus by binding to the viral nucleocapsid protein and forming large copolymers that accumulate in the perinuclear region (24). Previous studies have demonstrated antiviral activity of bovine Mx2 in cattle infected with bovine viral diarrhea virus

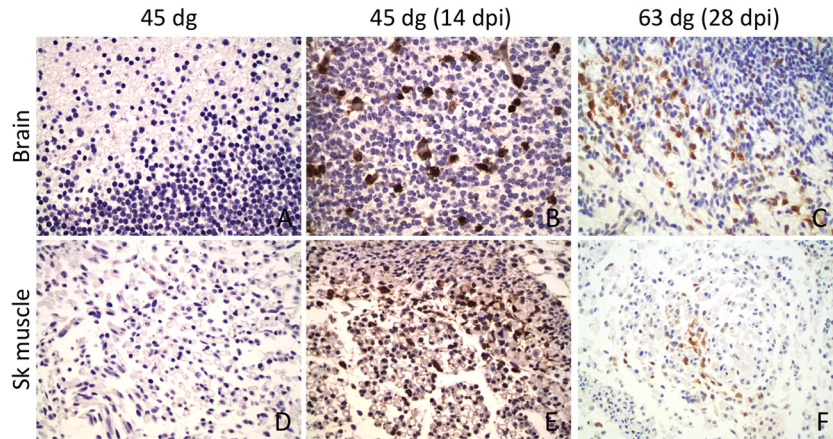


FIG 4 Immunohistochemistry for ISG15 in brain and skeletal muscle. A rabbit polyclonal antibody against ovine ISG15 protein was stained with DAB chromogen (brown) on the brains (A, B, and C) and skeletal muscle (D, E, and F) from 45-dg control fetuses (A and D) and from infected fetuses at 14 dpi (45 dg) (B and E) and 28 dpi (63 dg) (C and F). Numerous cells in the brains and skeletal muscle of infected fetuses are positive for ISG15. No positive cells were observed in the tissues of control fetuses.

(BVDV) (28, 39). Because Mx has a demonstrated antiviral effect against bunyaviruses, the present study quantified expression of the Mx molecule in infected tissues. A significant upregulation of ovine Mx1 and Mx2 was observed in evaluated tissues from infected fetuses compared to controls. Increased secretion of Mx1 was identified in fetal fluids in infected ovine fetuses. Thus, this protein likely contributes to the clearance of CVV infection.

The *ISG15* gene was markedly upregulated in the brain and in the skeletal muscle and highly expressed in the tissues from infected animals. Similar to the Mx protein, ISG15 is induced by type I IFN and has been associated with antiviral activity (29). In

recent studies, ISG15 has been shown to conjugate to proteins in a manner similar to ubiquitin (40, 41). Modifications and interference of antiviral signaling pathways involving ISG15 are mechanisms used by Crimean-Congo hemorrhagic fever virus, a bunyavirus, to evade the innate immune response (40). Upregulation of numerous ISG genes, including *ISG15*, and continuous stimulation of the innate antiviral response have been demonstrated in blood and tissues of bovine fetuses and cattle infected with BVDV (28, 39).

Although the *ISG15*, *Mx1*, and *Mx2* genes were abundantly expressed in all infected fetuses, the expression of other selected

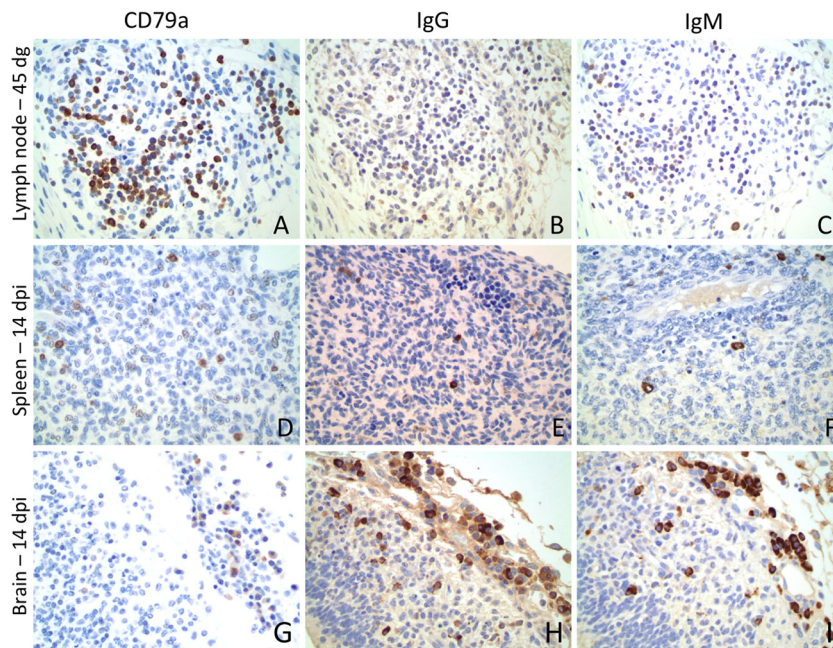


FIG 5 Immunohistochemistry for B cells, plasma cells, and ovine IgG and IgM in the lymph node, spleen, and brain. A polyclonal antibody against B cells and plasma cells (CD79a), ovine IgG, and ovine IgM were stained with DAB chromogen (brown). CD79a (A), IgG (B) and IgM (C) positive cells were observed in rudimentary lymph nodes at 42 to 45dg in control fetuses. CD79a (D)-, IgG (E)-, and IgM (F)-positive cells were observed in the spleens of infected fetuses. A remarkable number of CD79a (G)-, IgG (H)-, and IgM (I)-positive cells were seen in the leptomeninges of an infected fetus at 14 dpi.

IFN-stimulated genes was not altered. Previous studies have demonstrated that ovine fetuses within the second and third trimester of gestation are able to produce levels of interferon similar or greater to those produced by adult animals in response to a viral infection (42). Since type I IFN-induced genes were upregulated, it is presumed that the expression of type I IFN genes were also upregulated at the same or similar times and that, due to the short half-life of type I IFNs, the timing of upregulation of the type I IFN(s) was not detected at the time points evaluated. Even though less likely, one must consider the possibility of the upregulation of both ISG15 and ovine Mx by an IFN-independent pathway.

CD79a-positive cells (B lymphocytes and plasma cells) were observed in the spleens of CVV-infected fetuses at 49 dg, a finding which is in agreement with previous studies that identified B lymphocytes in ovine fetal lymph nodes and the spleen as early as 47 to 48 dg (19, 20, 43). Both infected and noninfected fetuses had scattered CD79a-, IgM-, and IgG-positive cells in lymphoid aggregates in the dorsal aspect of the abdominal cavity, sites of development of rudimentary renal and/or mesenteric lymph nodes, as early as 42 and 45 dg, respectively. The identification of CD79a-positive cells in regional lymph nodes earlier than observed in the fetal spleen supports the previously proposed idea that extrasplenic sites have the potential of producing B lymphocytes (43, 44) and potentially explains the fact that splenectomized fetuses are also capable of colonizing Peyer's patches with B lymphocytes (43, 45).

A marked infiltration of CD79a-, IgM-, and IgG-positive cells was observed within the meninges with fewer cells within the parenchyma of the brains and spinal cords of infected fetuses at 49 dg. It appears that the ovine fetus not only mounts an innate immune response to a viral infection but is also able to stimulate an adaptive immune response. Unfortunately, blood samples were not available from the fetuses during these experiments; therefore, fetal neutralizing antibodies could not be assayed. In ovine fetuses infected with Akabane virus, the earliest day of gestation where IgM- and IgG-positive cells were observed in tissues was at 59 to 60 dg. Titers of >1:4 were detected only after 100 dg (10, 46). Two other studies detected Akabane virus neutralizing antibodies after 76 to 78 dg, with titers ranging from 8 to 64 in one of these studies (11, 16).

CVV is a viral pathogen with a tropism for the ovine fetal CNS, skeletal muscle, and fetal membranes. The study of early infection demonstrated that a tropism correlated well with the CNS and musculoskeletal malformations observed in spontaneous CVV disease. With CVV, the development of arthrogryposis probably has a multifactorial pathogenesis involving effects on developing neurons, myocytes, and fetal membranes. CVV is the only source of viral infection shown to cause oligohydramnios. The virus is partially cleared from most fetal tissues by ~65 dg (28 dpi), before the presently accepted onset of ovine immunocompetence.

The ovine fetus is able to mount an immune response associated with CVV infection earlier in gestation by upregulation of genes that participate in the innate immune response. The infected fetus upregulates Mx genes, which have been shown to restrict growth of bunyaviruses. In addition, cells of the adaptive immune response—CD79a-, IgM-, and IgG-positive cells—are found in large numbers in infected tissues. Whether the immunoglobulin in these cells is actually CVV specific is still unclear. Further studies should be conducted for better understanding of the

immune system development in the ovine fetuses exposed to CVV and other viral infections.

ACKNOWLEDGMENTS

This study was supported by the USDA Animal Formula Health Research grant AH-9249.

We thank Andy Ambrus, the staff of the histology laboratory, Hoai Jaclyn Li from the Department of Veterinary Pathobiology, Andrew Steelman from the Department of Veterinary Integrative Biosciences at Texas A&M University, and Pamela J. Ferro from the Texas Veterinary Medical Diagnostic Laboratory for their excellent technical support.

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