Arch Virol (2016) 161:417–424
DOI 10.1007/s00705-015-2649-y

BRIEF REPORT

Experimental infection with cytopathic bovine viral diarrhea virus in mice induces megakaryopoiesis in the spleen and bone marrow

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Received: 8 April 2015 / Accepted: 13 October 2015 / Published online: 3 November 2015

Abstract Here, we infected mice with cytopathic bovine viral diarrhea virus 1 (cp BVDV1) by oral inoculation and investigated the effects of infection by histopathological, immunohistochemical (IHC), hematological methods. Twelve mice were infected, and samples were obtained at day 2, 5, and 9 postinfection (pi). Most of the infected mice exhibited clinical signs of illness such as reduced movement, crouching, loose feces, loss of appetite, and reduced water intake. Blood samples from six mice were positive for BVDV based on reverse transcription polymerase chain reaction (RT-PCR). Blood analysis also revealed thrombocytopenia and lymphopenia. Viral antigens were detected in the spleen (12/12), bone marrow (12/12), and/or mesenteric lymph nodes (4/12) of all infected mice by IHC analysis. The spleens showed significant histopathological changes including (i) substantially increased numbers of megakaryocytes, (ii) lymphocyte depletion, and (iii) hemorrhages. The bone marrow also had an increased number of megakaryocytes, although this increase was not as strong as it was in the spleen. Severe lymphoid depletion was observed in the mesenteric lymph nodes. Viral infections were present in the lymphocytes but not detected in megakaryocytes of the spleen, bone marrow, or mesenteric lymph nodes. These results suggest that the increased numbers of megakaryocytes may be a direct result of BVDV infection. BVDV infection in mice following oral inoculation of cp BVDV1 leads to megakaryopoiesis in the spleen and bone marrow to replenish the platelets.

Bovine viral diarrhea virus (BVDV) causes diseases in cattle worldwide, and produces significant economic losses for the cattle industry. BVDV is a member of the family *Flaviviridae,* genus *Pestivirus*, which also includes border disease virus and classical swine fever virus (CSFV) [1]. BVDV can be classified into two species, *bovine viral diarrhea virus 1* and *bovine viral diarrhea virus 2*, and two biotypes, cytopathic (cp) and non-cytopathic (ncp), which differ in their effects on cultured cells [2, 3]. BVDV infection can be associated with a wide range of clinical symptoms, including fever, diarrhea, persistent infection, immunosuppression, respiratory disease, reproductive dysfunctions, hemorrhagic syndrome characterized by thrombocytopenia and bleeding, and mucosal disease [4–6].

Acute BVDV infection is characterized by leukopenia and thrombocytopenia [7–9]. These hematological abnormalities have been associated with acute ncp BVDV2 infection [9, 10] and may be attributed to viral infection and replication. The pathogenesis of BVDV-induced thrombocytopenia remains unknown. However, onset of BVDV infection likely results in an accelerated rate of platelet destruction or a reduced rate of platelets production [11, 12]. In swine, CSFV also causes severe thrombocytopenia and megakaryocyte degeneration, suggesting that changes in platelets can be attributed to the rapid platelet depletion [13].

Previous research on BVDV has been impeded by the lack of suitable small animal models. Recently, our group assessed the potential of using mice as a model for BVDV

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infection, and we were able to successfully induce BVDV infection in mice by intraperitoneal injection [14]. However, the role of mice as a reservoir for BVDV infections has not been examined in detail. In this study, oral inoculation was selected to assess the infectious process, and to determine how differences in blood or tissue infection may influence transmission of BVDV infection compared with IP injections. The objective of the present study was to evaluate BVDV infection in mice after oral inoculation and to characterize disease progression through hematological, virological, immunohistochemical (IHC) and histopathological assessments.

Specific-pathogen-free BALB/c mice (6-8 weeks old) were purchased from Central Laboratory Animal Inc. (Seoul, Korea). All animals were maintained under pathogen-free conditions and handled in accordance with the guidelines and protocols approved for these experiments by the Kyungpook National University Animal Care and Use Committee. All experiments were repeated two times to confirm reproducibility. A total of 30 mice were inoculated orally with cp BVDV1 or were mock infected.

The cp BVDV1 (NADL) strain used in this study was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), cultured in Madin-Darby bovine kidney (MDBK) cells, and subsequently used for BVDV inoculation. The virus was titrated in MDBK cells, and a multiplicity of infection of 0.01 was used for subsequent passages. Twelve mice were assigned to the cp BVDV1-infected group, and three mice were used for the mock-infected group. Fifteen mice were used for each experiment. The twelve mice in the infected group were given one of two BVDV doses (six mice per dose: 4×10^5 50 % tissue culture infective dose ($TCID_{50}$) (low dose), or 1.2×10^6 $TCID_{50}$ (high dose). Mock-infected mice were orally administered 0.4 mL of tissue culture medium (Minimum Essential Medium; Life Technologies Corp., Carlsbad, CA, USA).

At day 2, 5, and 9 postinfection (pi), one mock-infected and four cp-BVDV1-infected mice were euthanized with CO_2 gas to collect blood and tissue samples. Vacutainer tubes containing EDTA (Beckton Dickinson, Franklin Lakes, NJ, USA) were used for blood collection in order to prevent coagulation. Total and differentiated leukocytes (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), as well as thrombocytes, were counted using a VetScan HM5 Hematology System (Abaxis, Union, CA, USA). The spleen, mesenteric lymph nodes, and femur were removed at necropsy and fixed in 10 % buffered formalin. The femoral bone marrow, spleen, and mesenteric lymph nodes were routinely processed and embedded in paraffin for hematoxylin and eosin (H&E) staining. For the quantitative analysis of megakaryocytes in the spleen and bone marrow, the number of megakaryocytes was expressed as the mean of five sections per slide at 200-fold magnification. All evaluations were conducted separately by two pathologists.

RNA was extracted from blood using a PureLink® Total RNA Blood Kit (Invitrogen, Carlsbad, CA, USA). RT-PCR was performed in a one-tube system using the pan-pestivirus primer pair V324-326 (INtRON Biotechnology, Inc., Daejeon, Korea); this system amplifies a portion of the 5′-untranslated region of the pestivirus genome [14]. The predicted size of the amplified PCR product was 297 bp.

IHC staining was performed on formalin-fixed paraffin-embedded blocks as described previously [15] using the primary monoclonal anti-BVDV antibody (Ab) 15C5 (Syracuse Bioanalytical, Ithaca, NY, USA). Negative control slides were prepared by staining with isotype-matched immunoglobulin G at the same dilution as that used for the primary antibody.

Data are expressed as mean ± standard deviation (SD). Quantitative data with normal distribution were analyzed using two-tailed, two-sample unequal-variance Student's *t*-tests, where variation was presented as SD for small sample sizes. *P* values <0.05 were considered significant.

In this study, mice were infected with cp BVDV1 by oral inoculation. Most of the BVDV-infected mice exhibited clinical signs of illness such as reduced movement, crouching, loose feces, loss of appetite, and reduced water intake. Reduced movement and crouching behaviors were mainly monitored at early time points of infection, whereas loose feces, loss of appetite, and reduced water intake persisted for the duration of the experiment (Table 1). We observed overall body weight throughout the experimental period. As shown in Fig. 1A, body weight was slightly increased in high dose-infected mice until day 5; however, weight loss was observed only in high dose-infected mice ($p < 0.02$) when compared to mock- and low dose-infected mice at day 9 pi (Fig. 1A). Splenomegaly was not observed in any infected mice. Spleen weight was increased in low dose-infected animals (Fig. 1B), whereas spleen weight in high dose-infected mice showed a slight increase after 5 days, which remained unchanged until day 9 (Fig. 1B). In high dose-infected mice, spleen weight was less than that of mock-infected animals at day 9 pi ($p < 0.009$).

Platelet counts showed a significant reduction in all BVDV-infected mice during the course of the experiment. Platelet counts were significantly lower in mice infected with a low dose of virus compared with mock infection at day 9 pi ($p < 0.0008$) (Fig. 2A). In addition, lymphopenia was observed in low dose-infected animals at day 9 pi ($p < 0.02$) (Fig. 2B). Severe lymphopenia was also found in mice infected with a high dose at day 5 pi ($p < 0.01$) (Fig. 2B). Leukopenia was observed in high dose-infected mice at day 5 ($p < 0.00009$) and in low dose-infected mice

Table 1 Summary of clinical score and BVDV antigen detection in mice inoculated orally with low or high dose of cp BVDV1. Results are representative of two independent experiments

Day of infection	Less movement	Loose feces	Anorexia (less drinking water)	Spleen	Bone marrow	Mesenteric lymph node	RT-PCR*
2	4/4	4/4	4/4	4/4	4/4	2***/4	4/4
5	0/4	4/4	4/4	4/4	4/4	0/4	0/4
9	2**/4	4/4	4/4	4/4	4/4	2***/4	2**/4

Four mice were euthanized at each time point. Two mice were inoculated with low dose and the other two mice were inoculated with high dose. Two independent experiments were performed and the results were the same

Low dose used was 4×10^5; high dose was 1.2×10^6

* RT-PCR for viral RNA detection was performed using the whole blood

** BVDV antigen was observed in only high dose-infected mice

*** BVDV antigen was detected in only low dose-infected mice

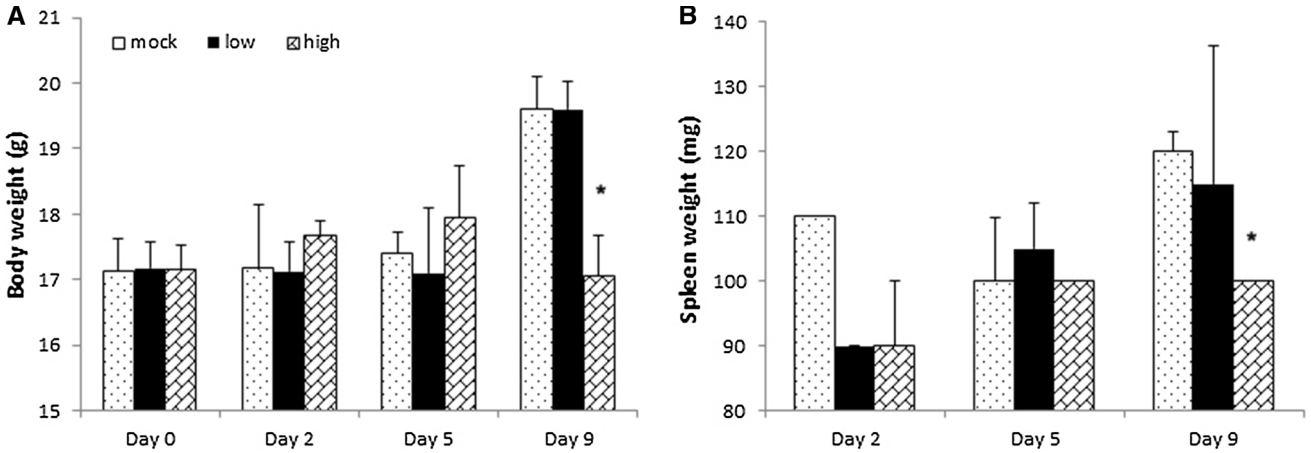

Fig. 1 Body weight (A) and spleen weight (b) of mock-infected mice and those infected with a low or high dose of virus. All animals exhibited same body weight at the start of the experiments. Standard error bars are shown. An asterisk indicates a significant difference ($P < 0.05$)

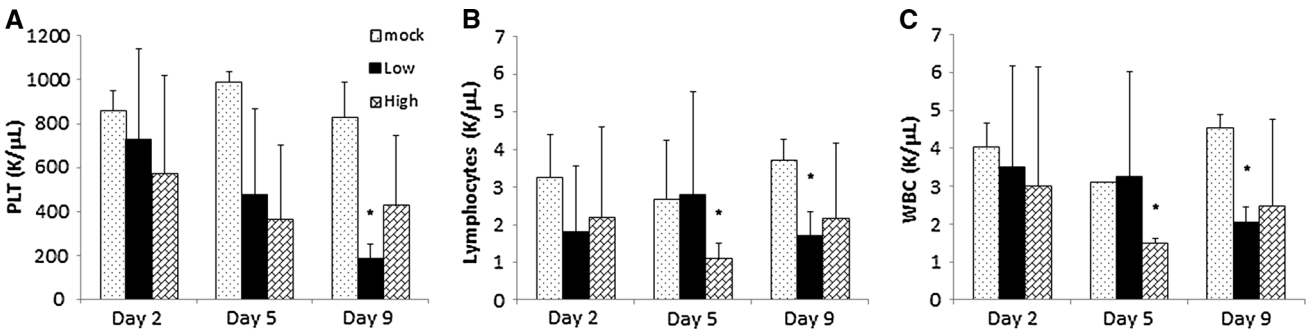

Fig. 2 Mean platelet (A), lymphocyte (B), and white blood cell (C) counts in mock-infected mice and those infected with a low or high dose of virus. A reduction was observed in both low and high dose-infected animals. Standard error bars are shown. An asterisk indicates a significant difference ($P < 0.05$)

at day 9 pi ($p < 0.001$) (Fig. 2C). Blood analysis in those animals showed that all other white blood cell types (neutrophils, monocytes, eosinophils, and basophils) were within normal limits.

The presence of viral RNA in whole blood samples was detected by RT-PCR in all low and high dose-infected mice at day 2, but it was absent at day 5. It was then detected only in high dose-infected mice at day 9 (Table 1). As a

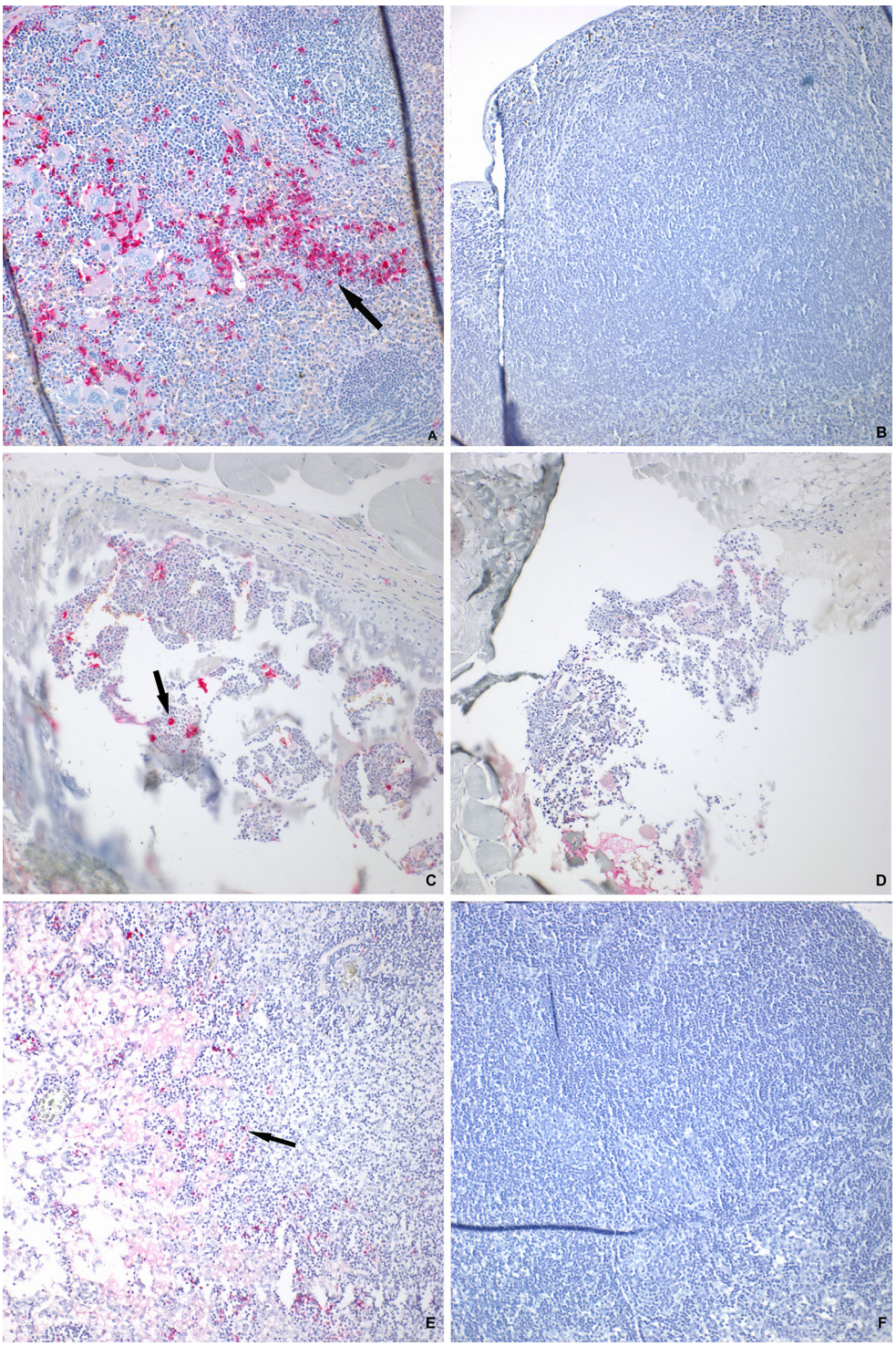

Fig. 3 Immunohistochemistry for BVDV antigen at day 9 pi after oral inoculation. Viral antigen was detected in the lymphocytes of the spleen (a), bone marrow (c), and mesenteric lymph nodes (e) (black arrows). Images of the spleen (b), bone marrow (d), and mesenteric lymph nodes (f) of a negative control mouse are also shown (original magnification, 200×)

result, six out of twelve animals were BVDV positive in blood samples. As expected, viral RNA was not detected in the mock-infected animals. Based on IHC analysis of BVDV-infected mice, the distribution of viral antigens in infected mice is summarized in Table 1. BVDV antigen was not detected in any tissue samples from mock-infected mice. On the other hand, viral antigen was detected in the spleen (12/12), bone marrow (12/12), and/or mesenteric

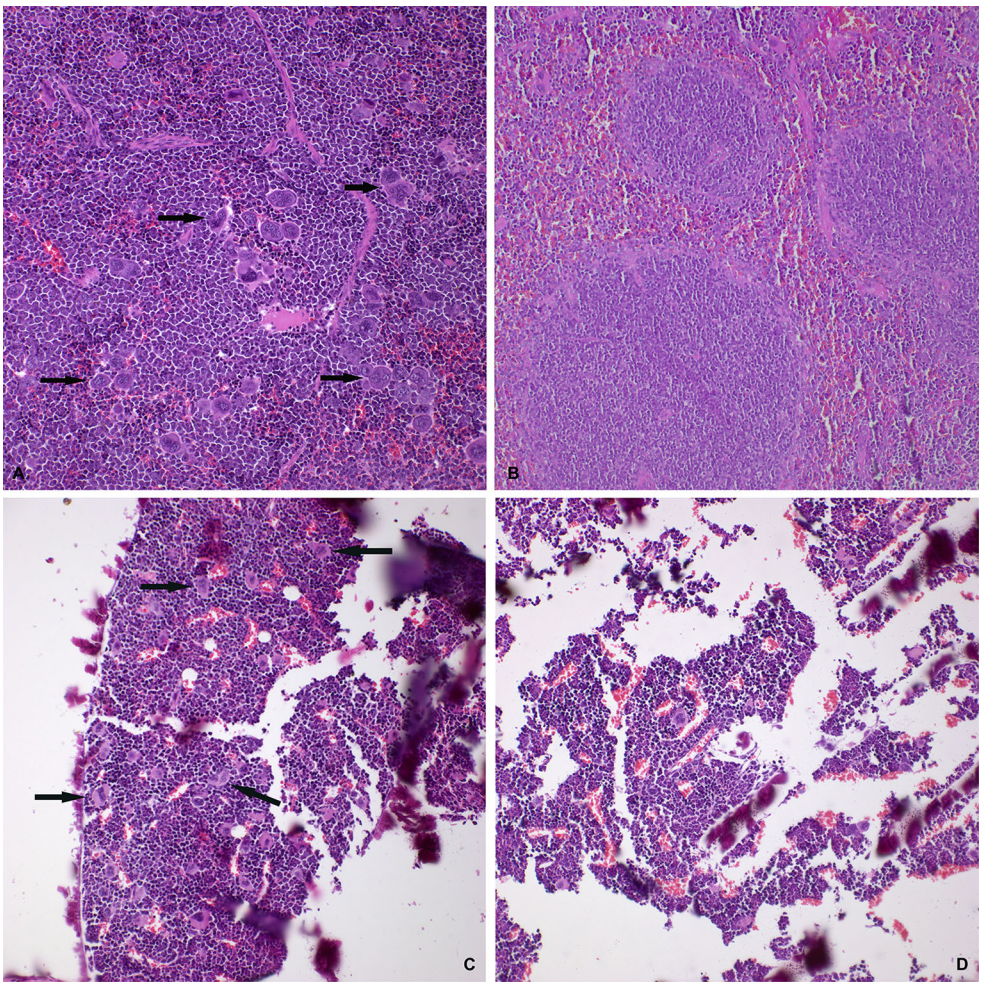

Fig. 4 Histopathology of the spleen and bone marrow at day 9 pi after oral inoculation. The cp BVDV1-infected mice showed increased infiltration of megakaryocytes in the spleen (a) and bone marrow (c) (black arrows). Images of the spleen (b) and bone marrow (d) of a negative control mouse are also shown (H&E; original magnification, 200×)

lymph nodes (4/12) of infected mice. In the spleen, bone marrow, and mesenteric lymph nodes, a positive antigen reaction was detected in lymphocytes (Fig. 3A, C, and E), but not in megakaryocytes. Other tissues showed negative results for the viral antigen as well.

Histopathological examinations were performed on the spleen, bone marrow, and mesenteric lymph nodes of infected mice at day 9 pi. All infected animals exhibited a high degree of histopathological changes compared to mock-infected mice, which showed no lesions. The most common characteristic observed in the spleen and bone marrow of all infected mice was the increased infiltration of megakaryocytes (Fig. 4A and C). In the bone marrow, aside from an increase in the number of megakaryocytes, no lesions were detected. Loss of lymphocytes and hemorrhages were observed in the spleen (Fig. 5A), and severe lymphoid depletion was found in the mesenteric lymph nodes (Fig. 5C) of all infected mice.

The number of megakaryocytes in the spleen and bone marrow after oral inoculation was significantly increased in mice compared to mock-infected mice. As shown in Fig. 6A, in low dose-infected mice, the number of megakaryocyte in the spleen increased until day 5 pi and decreased on day 9 pi. However, in high dose-infected mice, the number of megakaryocytes gradually increased until day 9. In both the low and high dose-infected groups, the number of megakaryocytes in the bone marrow increased until day 9 pi (Fig. 6B). Megakaryocyte counts in the bone marrow were lower than those found in the spleen.

The present study shows evidence of cp BVDV1 infection in mice after oral inoculation. Even though viral RNA was detected in whole blood samples of six out of twelve mice, most of the mice developed clinical signs of disease onset, and BVDV antigen was detected in the spleen and bone marrow of all mice. Histopathological

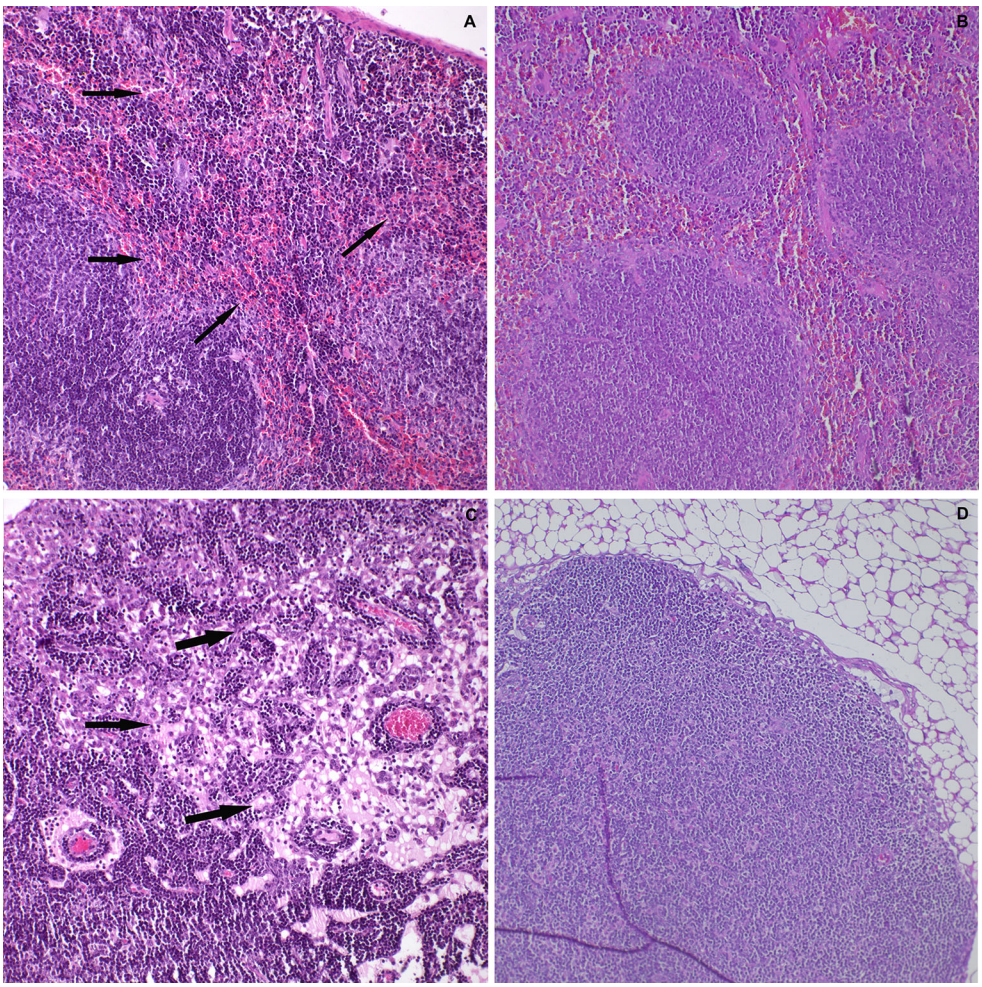

Fig. 5 Histopathology of the spleen and mesenteric lymph nodes at day 9 pi after oral inoculation. Lymphocyte counts were decreased in the spleen (a) and mesenteric lymph nodes (c). Images of the spleen (b) and mesenteric lymph nodes (d) of a negative control mouse are also shown (H&E; original magnification, 200×)

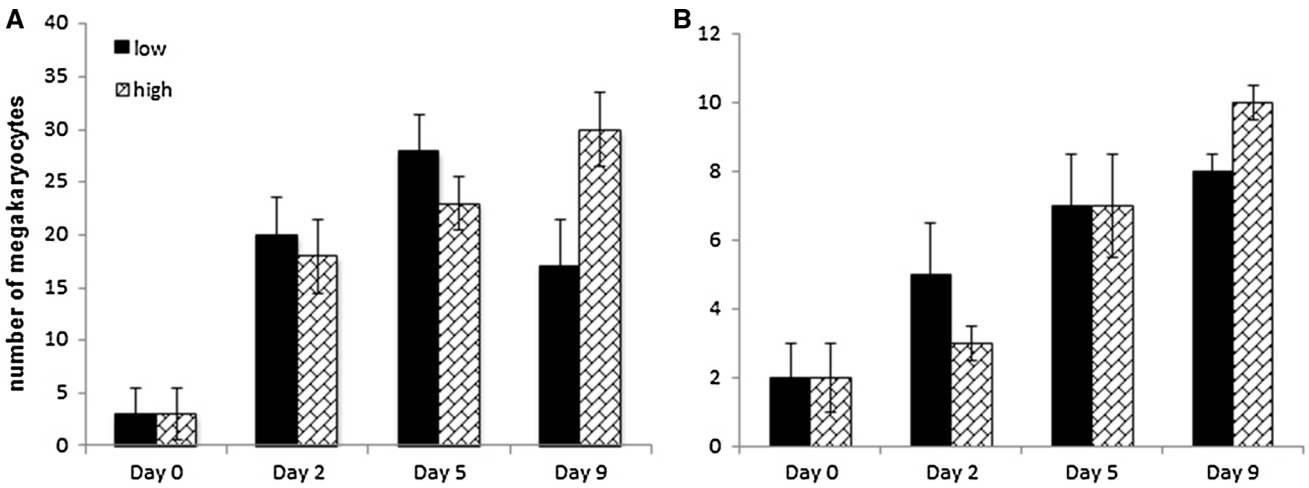

Fig. 6 Megakaryocyte counts in the spleen and bone marrow. Megakaryocytes in the spleen (A) and bone marrow (B) were consistently increased in high dose-infected mice until day 9 pi

changes were also observed in all BVDV-infected mice. Specifically, the following changes were observed: (i) the spleen and bone marrow showed a significant increase in the number of infiltrating megakaryocytes, (ii) the spleen showed loss of lymphocytes and hemorrhages, and (iii) mesenteric lymph nodes developed severe lymphoid depletion. Transient lymphopenia and thrombocytopenia also developed in virus-infected mice. These findings indicate that oral inoculation with cp BVDV1 exerts an influence on hematopoietic tissue, and as a result, thrombocytopoiesis is affected in the spleen and bone marrow in these mice. This is the first report, to the best of our knowledge, of experimental acute cp BVDV1 infection in mice.

In a recent study, we found that IP-injected or IN-inoculated mice did not exhibit any clinical signs of illness [15]. Contrary to our expectations, oral inoculation with cp BVDV1 led to clinical manifestations as a result of viral infection. All mice showed the same clinical manifestations with the exception of weight loss, which was observed in high dose-infected mice (Fig. 1A). The inference of our result is that animals acquiring virus by the oral route could develop clinical disease. From the findings reported here, we speculate that the propagation pathways are probably different between the oral and IP routes. The spleen is the primary replication site by the IP route, whereas after oral inoculation, the primary replication sites are in the vicinity of the entry site. Therefore, it appears that oral inoculation may provide an effective portal of entry for the spread of virus infectivity. The present results suggest that in order to induce clinical signs of BVDV infection in mice, the inoculation method and virus dosage used should be considered.

In this study, viremia was detected in six out of twelve mice, and viral antigen was detected in all of the spleens and bone marrow of infected mice (Table 1). These results are in disagreement with those obtained with IP-injected groups in a previous study [15]. In the IP-injected group, viremia was detected in all but one mouse, and viral antigen was detected in the spleens of all but one mouse, as well as in the mesenteric lymph nodes of four out of six infected mice [15]. However, we showed that in the orally inoculated group, viral antigen was detected in the mesenteric lymph nodes of low dose-inoculated mice on days 2 and 9 pi, but not on day 5 (Table 1). These differences can be assumed to reflect differences in the preferred location for antigen deposition (i.e., the injection method) in the mouse model of BVDV infection. Although viremia was not detected by RT-PCR in mice, our results demonstrated that all mice were infected with BVDV, as confirmed by IHC. Thus, this result suggests that IHC is an appropriate method for BVDV detection in mice and that the spleen is the most reliable tissue for IHC detection.

In IP-injected mice, histopathological changes were only observed in the lymphoid organs. We found lymphoid depletion in the mesenteric lymph nodes and the loss of lymphocytes in the spleen. The number of megakaryocytes was markedly increased in the spleen and bone marrow during the infection period. BVDV infection was likely established in mice following oral inoculation, causing these lesions. Despite the increases of megakaryocytes, we could not detect the viral antigen in the megakaryocytes of the spleen and bone marrow by IHC; however, other studies have shown the presence of viral antigens in megakaryocytes in other animals [9, 13]. The differences between these groups may be related to differences in the host used. Importantly, these results suggest that the increases of megakaryocytes in the spleen and bone marrow may be explained by hematopoiesis to replenish platelets.

We found that lymphopenia developed after cp BVDV1 inoculation and therefore was most likely due to acute viral infection. It has been suggested that lymphocytes migrate to lymphoid tissue, which is associated with lymphoid depletion in the mesenteric lymph nodes and loss of lymphocyte in the spleen. Lymphopenia in mice may be the direct result of acute BVDV infection. The decrease in cell counts was greater in high dose-infected mice at day 5 pi. The ability of BVDV to cause thrombocytopenia has been reported in several studies [9, 10, 12, 16]. Platelets are involved in the spread of BVDV throughout the body, indicating that thrombocytopenia can be attributed to BVDV infection in mice. Although the contribution of megakaryocytes to the development of thrombocytopenia in mice was not determined in this study, BVDV infection in mice is likely to cause decreased production and accelerated destruction of platelets. Thus, further studies are required to determine whether BVDV can infect megakaryocytes in mice.

In conclusion, oral inoculation of mice with cp BVDV1 resulted in changes consistent with BVDV infection such as development of viremia, appearance of viral antigens, and hematological and histopathological changes. The results of the present study indicate that thrombocytopenia and lymphopenia can result from BVDV infection, and significant increases in the number of megakaryocytes in the spleen and bone marrow of mice are due to decreased production and accelerated destruction of platelets, leading to replacement via hematopoiesis in the spleen and bone marrow. Our results provide new information regarding BVDV infection in mice. Additional studies are needed to understand the pathogenesis and the mechanism underlying BVDV infection in mice.

Acknowledgments This work was supported by the Basic Science Research Program of the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology (NRF 2012R1A1A3011238).

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