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Short Communication

Dietary germanium biotite supplementation enhances the induction of antibody responses to foot-and-mouth disease virus vaccine in pigs

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We evaluated the potential ability of germanium biotite (GB) to stimulate the production of antibodies specific for foot-and-mouth disease virus (FMDV). To this aim, we measured the total FMDV-specific antibody responses and IgM production after vaccination against FMD both experimentally and in the field. GB supplementation with FMDV vaccination stimulated the production of anti-FMDV antibodies, and effectively increased IFN-γ and TNF-α levels. These results suggest that GB may be a novel alternative feed supplement that can serve as a boosting agent and an immunostimulator for increasing the efficacy of FMDV vaccination in pigs.

Keywords: adjuvant-like effect, foot-and-mouth disease virus, germanium biotite

Foot-and-mouth disease (FMD) is an acute and highly contagious viral disorder of cloven-hoofed animals such as cattle, sheep, and pigs. FMD causes extensive financial loss, and has a devastating effect on national and international live animal populations as well as the animal product trade [1]. A severe outbreak of FMD in Korea occurred during 2010～2011 [10]. Following this outbreak, emergency vaccination was carried out as a control measure and a routine vaccination program is still ongoing to further regulate FMD infection. Inactivated foot and mouth disease virus (FMDV) vaccine typically used in Korea contains intact FMDV delivered with incomplete oil-based adjuvants. This combination is effective for reducing disease incidence and has been successfully used for controlling FMD in Korea [10]. However, this vaccine has numerous disadvantages including a poor cell-mediated immune (CMI) response and a short duration of immunity compared to that conferred by natural FMDV infection [5]. Hao *et al*. [6] found that only 31.9% of the serum samples from 91 pigs vaccinated against FMDV had antibody titers required for protection against FMD infection. Therefore, a new approach for improving FMD vaccines that can generate a more robust and longer lasting immune response is needed.

Germanium biotite (GB) is an aluminosilicate mineral with numerous biological activities in animals. In particular, this compound has been found to improve host immune function. Our previous study showed that dietary supplementation with GB enhances the proliferative activity of lymphocytes and monocytes in pigs [3]. In addition, Jung *et al*. [8] demonstrated that continuous ingestion of GB promotes the expression of several cytokines such as interferon-gamma (IFN-γ), interleukin (IL)-4, and tumor necrosis factor-alpha (TNF-α). GB also increases the production of antibody against *Pasteurella multocida* in mice in a dose-dependent manner. Similarly, dietary supplementation with GB accelerates lymphocyte proliferation and $CD8^+$ T lymphocyte production induced by concanavalin A (Con A; a T cell mitogen) in mice [7]. Taken together, these findings suggest that humoral immune responses, particularly ones involving the production of anti-FMDV antibodies, could be stimulated by a feed supplementation with GB. However, little is known about the effect of GB on FMDV-specific antibody generation. Therefore, the purpose of the present investigation was to evaluate the impact of dietary GB administration on humoral immune responses in swine specifically focusing on the adjuvant-like effect of GB after FMD vaccination.

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This investigation included experimental and field studies that assessed the following: (i) FMDV-specific IgM and total antibody levels after FMDV vaccination, (ii) the effect of GB supplementation on the production of total anti-FMDV antibodies in a Korean commercial swine farm, and (iii) expression levels of IFN-γ, TNF-α, and IL-1β to confirm the non-specific immunostimulatory effect of GB. All procedures involving animals were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals by the Council for International Organizations of Medical Sciences (CIOMS; World Health Organization, Switzerland), and approved by the Institutional Animal Care and Use Committee of Chonnam National University (Korea; Approval No. CNU IACUC-YB-2010-1).

Conventional 8-week-old pigs (with an average body weight of 22 kg) were obtained from a single healthy herd without any history of FMDV (Daehan Feed, Korea) and maintained in the animal facility of College of Veterinary Medicine, Chonnam National University (Korea) for the experimental study. Anti-FMDV antibody levels in all pigs were measured using a PrioCHECK FMDV type O kit (Prionics, Switzerland) to confirm that none of the experimental animals had prior exposure to FMDV. GB supplement was provided by Seobong BioBestech (Korea); components of the supplement were previously described [7]. The pigs were randomly divided into three groups of five. Pigs in group 1 were fed non-GB supplement as a negative control. Group 2 received pig feed supplemented with 1% (w/w) GB (1% GB group). Group 3 received pig feed supplemented with 3% (w/w) GB (3% GB group). All pigs were given the experimental diets for 2 weeks and then intramuscularly injected with an inactivated FMDV vaccine (Aftopor; Merial, UK). This vaccine contains a double-oil emulsion adjuvant with at least six 50% protective doses (PD50) of inactivated FMDV (O1 Manisa serotype). Five mL of blood were collected weekly from the jugular vein after FMDV vaccination until the end of the study. All pigs were euthanized for necropsy at 15 weeks of age.

The field study was conducted at a local farm with no history of FMDV infection located in Chonnam Province (Korea). The farm had a two-site production system with a nursery and finishing units with an all-in/all-out production system. All pigs were confirmed to be seronegative for FMDV. To minimize variability, 70 pigs 8 weeks old were randomly selected and divided into control and GB-fed groups. Pigs in the control group (n = 35) were fed non-GB supplement feed while animals in the GB-fed group (n = 35) received feed containing 3% (w/w) GB. After consuming the experimental diets for 2 weeks, the pigs were intramuscularly injected with an inactivated FMDV vaccine (Aftopor; Merial). Five mL of blood were collected from the jugular vein before vaccination and 4 weeks after FMDV vaccination. The blood samples were transported on ice to the laboratory to measure total FMDV-specific antibody production.

To measure total anti-FMDV antibody levels in the vaccinated pigs, a commercial PrioCHECK FMDV type O kit (Prionics) was used according to the manufacturer's instruction. Briefly, serum was collected by centrifugation at 2,000 × g for 10 min at 4°C. The serum samples and reference samples were added to each well, and incubated at 37°C for 60 min. Diluted anti-FMDV type O monoclonal antibody conjugate was dispensed to all wells and the samples were incubated for another 60 min. Next, 3,3′, 5,5′-tetramethylbenzidine (TMB) substrate was added and the reaction was inhibited with the addition of a stop solution. The optical density (OD) was measured at 450 nm and expressed as percent inhibition (PI) according to the formula stated in the manufacturer's protocol. PI values below 50% reflect an absence of anti-FMDV type O antibodies in the test serum while PIs over 50% indicate the presence of anti-FMDV type O antibodies.

The levels of FMDV-specific IgM in the FMDV vaccinated pigs were also measured using the PrioCHECK FMDV type O kit (Prionics) with some modifications. Briefly, the serum samples and reference sample were added to each well and incubated at 37°C for 60 min. Next, horseradish peroxidase-conjugated goat anti-pig IgM (AbD Serotec, USA) was added to all wells. After incubating for 60 min, TMB substrate was added and the absorbance was read at 450 nm. The OD value was corrected by subtracting the OD_{450} of the blank; the results are presented as the mean ± standard deviation (SD).

Along with determining FMDV-specific IgM and total antibody levels, the expression of IFN-γ, TNF-α, and IL-1β mRNA in lymphocytes was measured to evaluate the effect of GB on the porcine immune systems. Lymphocytes were prepared as previously described [7] and total RNA was extracted from the cells using a PureLink RNA mini kit (Invitrogen, USA) according to manufacturer's protocol. An equal amount of targeted RNA was reverse transcribed using a QuantiTect reverse transcription kit (Qiagen, USA) according to manufacturer's protocol. In order to minimize variations in reverse transcriptase efficiency, all samples were transcribed simultaneously. Quantitative real-time PCR was then performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, USA) using a $MyiQ^2$ thermocycler and a SYBR green detection system (Bio-Rad Laboratories). The real-time PCR conditions were as follows according to manufacturer's protocol: 95°C for 10 min followed by 50 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec. The following oligonucleotide primer pairs were used to measure the expression of various cytokines: 5′-CAA AGC CAT CAG TGA ACT CAT CA-3′ and 5′-TCT CTG GCC TTG GAA CAT AGT CT-3′ (X53085) for IFN-γ, 5′-CGG GCT TAT CTG AGG TTT GA-3′ and 5′-CGG GCT TAT CTG AGG TTT GA-3′

(JF831365) for TNF-α, 5′-GGC CGC CAA GAT ATA ACT GA-3′ and 5′- GGA CCT CTG GGT ATG GCT TTC-3′ (NM_214055) for IL-1β, and 5′-CAG GTC ATC ACC ATC GGC AAC G-3′ and 5′-GAC AGC ACC GTG TTG GCG TAG AGG T-3′ (U07786) for β-actin as a reference control. The relative quantitation of IFN-γ, TNF-α, and IL-1β mRNA expression was performed using a comparative Ct method as previously described [9]. Antibody and cytokine levels are expressed as the mean ± SD. The mean values were compared between groups with a one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. All statistical analyses were performed using GraphPad InStat (ver. 3.0; GraphPad Software, USA). The p values < 0.05 were considered to indicate statistical significance.

IgM is the first immunoglobulin to be produced as part of the primary immune response. Levels rapidly increase in 3 ~ 5 days after vaccination and peak on days 7 ~ 14 following immunization. IgG responses are generally detected 9 days after vaccination followed by a 'class switch' from IgM to IgG [4]. IgG is the most common type of immunoglobulin in the blood and plays a major role in the antibody-mediated defense mechanism. Additionally, IgG provides particularly effective protection against FMDV through antibody-enhanced phagocytosis of the viral particles [2]. For this reason, we measured FMDV-specific IgM levels and total anti-FMDV antibody production.

Our data showed that the level of FMDV-specific IgM in the 3% GB-supplemented group was significantly increased 1 week after vaccination ($p < 0.05$) compared to the control group (Fig. 1A). This increase persisted for 2 weeks after vaccination. In addition, the level of total FMDV-specific antibody in the 3% GB-supplemented group was significantly elevated 2, 3, and 4 weeks post-vaccination ($p < 0.05$), and remained higher than that in the control group 5 weeks after vaccination (Fig. 1B). Similarly, total FMDV-specific antibody levels were markedly increased ($p < 0.001$) in the GB-supplemented group in the field study (Fig. 1C). These findings are similar to results from our previous investigation showing that administration of GB leads to a significant increase of both antibody levels and the B cell ($CD19^+$) ratio in mouse spleen [8]. A similar observation was also noted for a newly developed adenovirus vectored FMDV subunit vaccine that induced greater production of FMDV-specific IgM and IgG in pig serum [1]. Taken together, these data indicate that GB can enhance the antibody response to FMDV vaccination. However, further studies are required to elucidate the exact mechanism(s) underlying the effects of GB supplementation and the association of FMDV-specific humoral immune responses with GB.

The relative expression levels of IFN-γ, TNF-α, and IL-1β mRNA were measured along with the concentration

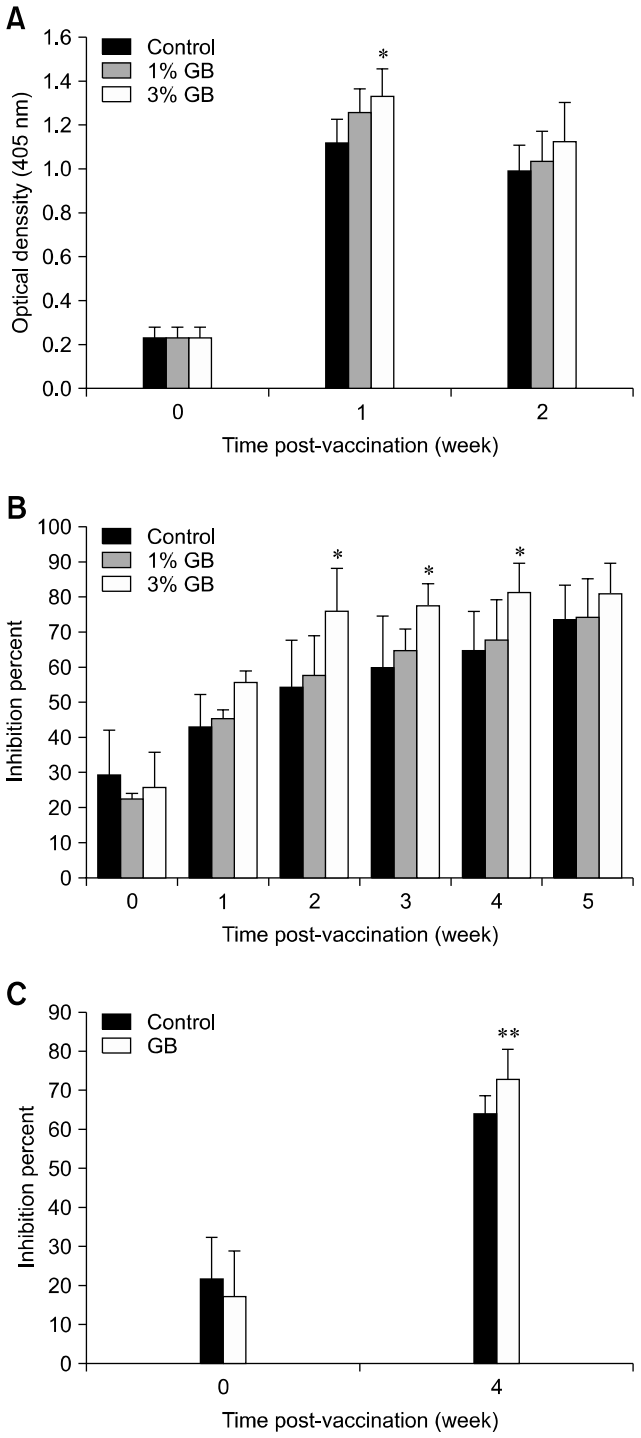

Fig. 1. Effects of GB supplementation on total FMDV-specific antibody and IgM production after vaccination against FMDV. Blood samples were collected from each pig in the experimental and field studies after FMDV vaccination. The levels of total FMDV-specific antibody (A) and IgM (B) were significantly increased in the 3% GB-supplemented group compared to the control group in the experimental study (*$p < 0.05$). (C) Total anti-FMDV antibody levels were also markedly increased (**$p < 0.01$) and maintained until 4 weeks post-vaccination in the field study.

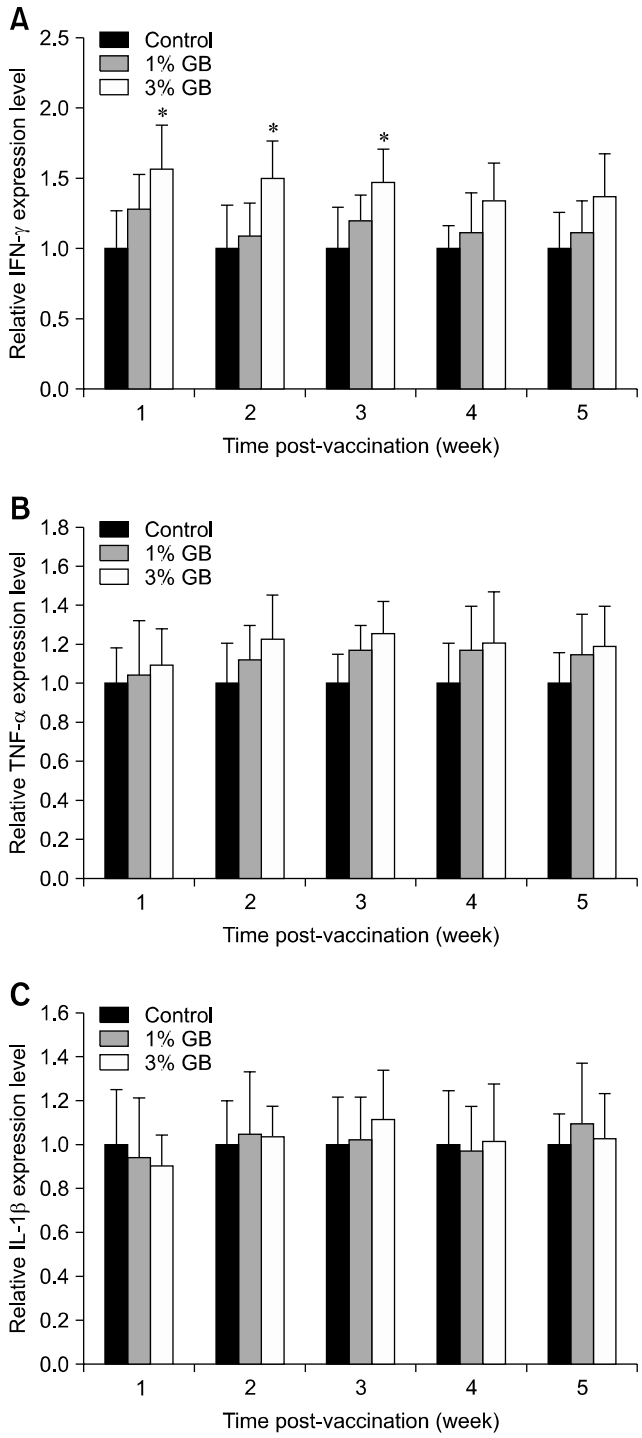

Fig. 2. Effects of GB supplementation on the expression of IFN-γ, TNF-α, and IL-1β mRNA in lymphocytes. The relative mRNA levels of (A) IFN-γ, (B) TNF-α, and (C) IL-1β in pig lymphocytes were measured using real-time PCR. IFN-γ mRNA expression was significantly increased in the 3% GB-supplemented group compared to the control group (*$p < 0.05$) after FMDV vaccination. The level of TNF-α mRNA was also increased but not significantly in the experimental animals.

of serum FMDV-specific IgG and IgM. It has been shown that GB acts as a non-specific immunostimulator by inducing the release of large quantities of TNF-α and subsequent activation of phagocytic cells such as macrophages [8]. In the present study, the relative expression of IFN-γ mRNA was significantly increased ($p < 0.05$) in the 3% GB-supplemented group (Fig. 2A) along with TNF-α mRNA expression (Fig. 2B). These results were similar to ones from our previous study indicating that administration of GB significantly increases the relative expression of both IFN-γ and TNF-α mRNA in mice [7].

In conclusion, data from the current investigation suggest that dietary GB supplementation can effectively stimulate the production of total FMDV-specific antibody and IgM while enhancing the expression of IFN-γ and TNF-α mRNA. Thus, GB acts as a non-specific immunostimulator. In our field study, we demonstrated for the first time the effect of GB on anti-FMDV antibody production in pigs. However, we did not evaluate the impact of GB on immune responses or directly address the mechanism(s) underlying the effect of GB supplementation on FMDV-specific antibody generation. Our results in combination with precise knowledge of the efficacy of GB supplementation as well as the associated mechanisms may be lead to the development of a new alternative feed supplement administered as a boosting agent and immunostimulator in pigs.

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Conflict of Interest

There is no conflict of interest.

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