Equine respiratory disease is believed to be the second most important cause of poor performance, interruption of training, and premature retirement among performance horses (41, 50, 61, 62, 72). In Seoul, Korea, the Seoul Race Park experienced yearly epizootics of infectious upper respiratory diseases (IURD), with an estimated incidence of 29.6%, from 2001 to 2005. IURD affects the nasal passages and throat region and results in chronic coughing, exercise intolerance, weight loss, and nasal discharge (S. H. Ryu, H. C. Koo, Y. K. Park, J. M. Kim, W. K. Jung, Y. H. Park, W. C. Davis, and C. W. Lee, submitted for publication). As it is often debilitating and is recalcitrant to conventional therapy composed of antibiotics, anti-inflammatory drugs, bronchodilators, and expectorants (20), IURD usually requires an extended recovery period, even though it is not usually fatal. Reducing recovery time would be of great benefit to owners and trainers, as well as to the animal itself. Returning to full activity with a reduced hospital stay is an important financial and medical consideration, since an early discharge from a care facility reduces costs for owners and should also reduce the possibility of the horse being reexposed to IURD pathogens and, subsequently, suffering a relapse. To treat IURD, the owners of the Seoul Race Park paid, collectively, an average of $140,000 per year between 2001 and 2005, which is 11.8% of the total veterinary fees for that period (Ryu et al., submitted).

Compared with those from a healthy control group, blood samples collected in the spring and summer from an IURD patient group of horses at the Seoul Race Park had significantly lower proportions of cells expressing major histocompatibility complex class II (MHC-II) and CD2, CD4, CD4, CD25, CD8, and CD8 T lymphocytes, dendritic cells, and surface immunoglobulin M B lymphocytes in peripheral blood, as well as enhanced cell proliferative responses with phytohemagglutinin and increased phagocytic activity against Streptococcus equi and Staphylococcus aureus strains with high antibiotic resistance, the bacteria frequently identified as etiologic agents of equine respiratory diseases at the Seoul Race Park in Seoul, Korea. This study shows that BARODON may act as an immunostimulator and can be an effective alternative to antimicrobial feed additives for nonspecific improvements in equine immune responses, particularly against respiratory diseases.
is assumed to be related to the stimulation of membrane-associated lymphoid tissue by the mineral component. The immunostimulatory effect of BARODON in pigs has already been demonstrated through the proliferation and activation of porcine immune cells, particularly CD4+ CD8+ double-positive T lymphocytes in peripheral blood and in the secondary lymphoid organ (73, 74). Also, it was shown to have an adjuvant effect on hog cholera vaccine efficiency (45).

Based on the immunostimulatory effects of BARODON in animal husbandry and on the potential of equine lymphocyte subpopulation analysis to predict host responses against respiratory diseases, this study was designed to extend our previous study (Ryu et al., submitted) with more detailed immunologic characteristics and to evaluate BARODON as a nonspecific immunostimulating agent in Thoroughbred horses. A set of monoclonal antibodies (MAbs) specifically reactive with equine leukocyte differentiation antigens and a flow cytometric (FC) analysis were used to determine the proportion of leukocyte subpopulations. Lymphoproliferative responses, particularly the in vivo activation of T cells determined by examining the expression of the activation marker, the α chain of the interleukin 2 receptor (IL-2Rα) (CD25) (23, 25, 30, 31, 32, 49, 55, 57, 58), were analyzed before and after stimulation with mitogen. In addition, the phagocytic activities of immune cells from peripheral blood against S. equi subsp. equi, the most important etiologic bacterium in equine respiratory diseases, and S. aureus, which has been frequently identified in horses with IURD at the Seoul Race Park (Ryu et al., submitted), were examined in Thoroughbred horses treated with BARODON.

Increased proportions of CD4+, CD4+ CD25+, CD8+, CD8+ CD25+, and CD2+ T lymphocytes, dendritic cells, and surface immunoglobulin M (sIgM)-positive B lymphocytes in peripheral blood, as well as higher lymphoproliferative responses to mitogens and phagocytic activities against S. equi subsp. equi and S. aureus (P < 0.05), were observed in BARODON-treated horses compared with control group horses. These results imply that BARODON has immunoenhancing effects on the equine immune system, particularly in terms of enhanced resistance against IURD.

MATERIALS AND METHODS

Non specific immunostimulator BARODON. The composition of the anionic mineral complex BARODON is as follows: 600 g of sodium metasilicate (Na2SiO3) commercialized disodium trioxolate extracted from plants; Neder- land B.V., The Netherlands), 300 g of potassium carbonate (K2CO3), 9 g of sodium carbonate (Na2CO3), 9 g of sodium borate (Na2B4O7), 900 g of sucrose (C12H22O11), 10 mg of silver nitrate (AgNO3), 30 mg of sodium chloride (NaCl), and 120 mg of sodium thiosulfate (Na2S2O3) dissolved in a total of 1,000 mL of water. The product was patented in the United States (patent no. 6,447,810 B1) on April 26, 2019 by guesthttp://cvi.asm.org/Downloaded from

TABLE 1. MAbs specifically reactive with the equine leukocyte differentiation antigens used in this study

| MAb      | MAb isotype       | Moleculea                        | References |
|----------|--------------------|----------------------------------|------------|
| HB19A    | IgG2a              | EqCD5                            | 9, 35      |
| HB88A    | IgG1               | EqCD2                            | 6, 35      |
| B29A     | IgG2a              | sIgM                             | 35, 65     |
| E18A     | IgG2a              | sIgM                             | 21, 35     |
| DH59B    | IgG1               | CD172a                           | 15, 36, 55, 56 |
| H58A     | IgG2a              | MHC-I                            | 6, 15, 28, 54 |
| TH81A5   | IgG2a              | MHC-II                           | 1, 2, 15, 54 |
| HB61A    | IgG1               | EqCD4                            | 6, 35, 66  |
| HT14A    | IgG1               | EqCD8                            | 6, 21, 35, 66 |

a Equine (Eq) leukocyte differentiation molecules.

Mitogen-stimulated lymphoproliferative responses. A total of 107 peripheral blood mononuclear cells (PBMC) in a volume of 10 mL of medium were placed in a tissue culture flask and were incubated upright in the presence or absence of mitogen (one control flask with culture with only RPMI 1640 medium, the other flask for culture with 1 μg/mL of phytohemagglutinin [PHA] [Sigma-Aldrich]) for 72 h to stimulate equine leukocytes (31). Cells were then stained for IL-2Ra (CD25) expression on CD4+ or CD8+ T lymphocytes with biotinylated recom
FIG. 1. PCR products from Streptococcus equi subsp. equi and Staphylococcus aureus bacteria isolated from nasal swab specimens of horses with IURD, the phagocytic strains used in this study, amplified with corresponding species-specific primer pairs. Lanes: M, 100-bp DNA ladder (Takara Bio Inc., Otsu, Shiga, Japan); 1, S. equi subsp. equi isolate using sodA primer sets (230 bp of PCR product) and seeI primer sets (520 bp of PCR product); 2, S. equi subsp. equi ATCC 33398 using sodA primer sets and seeI primer sets; 3, S. equi subsp. zooepidemicus ATCC 43079 using sodA primer sets and seeI primer sets (notice the negative reactions of S. equi subsp. seeI with primer sets, which are specifically reactive to S. equi subsp. equi, S. equi subsp. zooepidemicus with seeI primer sets); 4 and 7, negative control (distilled water); 5, S. aureus isolate using nuc primer sets and Sa-442 primer sets; 6, S. aureus ATCC 25923 using nuc primer sets and Sa-442 primer sets.

TABLE 2. Antimicrobial susceptibilities of the Streptococcus equi subsp. equi and Staphylococcus aureus strains used for in vitro phagocytosis in this study

| Bacterium                  | Penicillins | Cephalosporins | Aminoglycosides | Tetracyclines (TE) | Macrolides (E) | Sulfonamides | Polypeptides | Quinolones (ENR) |
|----------------------------|-------------|----------------|-----------------|-------------------|----------------|--------------|--------------|-----------------|
|                            | AM | AMC | OX | P | CF | CEF | AN | GM | K | N | STR | SXT | TMP | B | PB | ENR |
| S. equi subsp. equi        | S  | R   | S  | R | S  |     | S  | R  | S  | S  | S  | S   | S  | S  | R  | S  | S  |
| S. aureus                  | R  | S   | S  | R | S  | S   | S  | S  | S  | S  | S  | S   | S  | S  | R  | S  | S  |

*Abbreviations for antimicrobials (Becton Dickinson and Company, Sparks, Md.): AM, ampicillin; AMC, amoxicillin; OX, oxacillin; P, penicillin; CF, cephalothin; CEF, cefotaxime; AN, amikacin; GM, gentamicin; K, kanamycin; N, neomycin; STR, streptomycin; TE, tetracycline; E, erythromycin; SXT, trimethoprim-sulfamethoxazole; TMP, trimethoprim; B, bacitracin; PB, polymyxin B; ENR, enrofloxacin. Abbreviations for susceptibilities: S, susceptible; R, resistant; I, intermediate.
group from 3 to 14 weeks of treatment. Among BARODON-treated groups, the proportions of MHC-II⁺ cells, dendritic cells, and sIgM⁺ B lymphocytes were significantly higher in the Tx-1 and Tx-2 groups than in the Tx-3 group (\( P < 0.05 \)), with no significant difference between the Tx-1 and Tx-2 groups (\( P > 0.05 \)) (Fig. 2 and 3F to H).

Before 3 days of culture with mitogen, significantly higher proportions of CD4⁺, CD4⁺ CD25⁺, CD8⁺, and CD8⁺ CD25⁺ T lymphocytes, and higher CD4/CD8 ratios in PBMC from BARODON-treated groups compared with the control group, were seen after 3 weeks of treatment (\( P < 0.05 \)), particularly in the Tx-1 and Tx-2 groups compared with the Tx-3 group (\( P < 0.05 \)). However, no significant difference was observed between the Tx-1 and Tx-2 groups (\( P > 0.05 \)) (Fig. 4A to D and Fig. 5).

Mitogen-stimulated lymphoproliferative activities of cells from peripheral blood. To examine the mitogen-stimulated lymphoproliferative responses, live PBMC were harvested from all groups following stimulation with PHA for 3 days and were analyzed by FC dual-color staining.

The proportions of large proliferating cells (R2)—CD4⁺, CD4⁺ CD25⁺, CD8⁺, and CD8⁺ CD25⁺ T lymphocytes—in BARODON-treated groups were significantly higher (\( P < 0.05 \)) than those in the control group after 3 weeks of treatment (\( P < 0.05 \)). Those significant differences were also found between the Tx-1 or Tx-2 and Tx-3 groups but not between the Tx-1 and Tx-2 groups (Fig. 4E to H and Fig. 6).

Phagocytosis. To examine in vitro phagocytic activity, PBMC were obtained from all groups and were compared following an experimental induction of phagocytosis with opsonized or nonopsonized S. equi subsp. equi and S. aureus bacteria isolated from the Korea Racing Association’s respiratory patient group and having high-level resistance to antibiotics (amikacin, penicillin, or trimethoprim-sulfamethoxazole) for respiratory disease (Ryu et al., submitted).

After trypan blue treatment, the PI (FL2) mean fluorescence for phagocytosis was reduced due to the quenching of PI signals by nonphagocytosed extracellular bacteria. However, there was no positive or negative effect from the addition of trypan blue on the FITC (FL1) signal for granulocytes and monocytes. The phagocytic activity in BARODON-treated groups was significantly higher (\( P < 0.05 \)) than that in the control group after 3 weeks of treatment (\( P < 0.05 \)). Among BARODON-treated groups, the phagocytic activity was significantly greater in the Tx-1 and Tx-2 groups than in Tx-3, and there was no significant difference between the Tx-1 and Tx-2 groups (\( P < 0.05 \)). The phagocytic activities against the opsonized S. equi subsp. equi and S. aureus were significantly greater than those against the nonopsonized S. equi subsp. equi and S. aureus (\( P < 0.05 \)) (Fig. 7).

DISCUSSION

Nonspecific immunostimulants are substances that induce an enhancement of the body’s native or acquired defense mechanisms regardless of the mode of antigen specificity (26, 51). Immunomodulators, also termed biologic response modifiers, may either enhance or suppress innate immune responses in a...
FIG. 3. Summary of FC analysis of R1 plus R2 (lymphocytes) (A), R2 (blasting and proliferating cells) (B), R3 (granulocytes) (C), CD2⁺ cells (all thymocytes, T lymphocytes, and NK cells) (D), MHC-I⁺ cells (E), MHC-II⁺ cells (F), dendritic cells (G), and sIgM⁺ B lymphocytes (H) in peripheral blood with gates placed only on 1, 2, and 3 at time zero. Horses were fed daily both with 6 to 7 kg of Omolene feed, which had already been mixed with 0.05% Barodon F. Gold, and with an additional 60 ml of Barodon-biogenic feed containing 10% Barodon F. Gold (Tx-1; \( n = 6 \)). Another group was fed only Omolene feed (Tx-2; \( n = 6 \)), while a third group was fed only Barodon-biogenic feed (Tx-3; \( n = 6 \)). The fourth group, an untreated control group, was fed general feed with no BARODON (Control; \( n = 6 \)). Significant differences between control animals and animals treated with BARODON are as indicated in the figure (a, \( P < 0.01 \); b, \( 0.01 < P < 0.05 \)). See Materials and Methods for details on the analysis of subsets by selective gating used to show the frequency of each cell population with gates placed on 1, 2, and 3.
non-antigen-specific way. The proposed action mechanism of nonspecific immunomodulatory preparations is macrophage activation and the subsequent release of cytokines that might enhance the immune response. Macrophages may recognize nonspecific immunomodulator particulate matter and become activated, resulting in the production of interferon (IFN), IL-1, tumor necrosis factor, or IL-6 (48, 59). These cytokines may affect humoral and cellular immune functions, including phagocytic activity, antibody production, and lymphocyte cytotoxicity. Mild fever, anorexia, and lethargy may be observed after the administration of immunostimulant preparations. This reaction likely reflects the increased circulating IL-1 and is not considered an adverse side effect. On the contrary, it may indicate recognition of the immunomodulator and activation of the immune system.

Regardless of the host species, immunostimulant preparations are used most often for treatment of chronic viral or bacterial infections with evidence of secondary immunosuppression (51). In equine medicine, nonspecific immunostimulatory preparations such as Baypamun P, Baypamun N, Lobelin, natural human IFN-α, and inactivated Propionibacterium acnes have been used to treat sarcoid skin tumors, equine respiratory disease complex, chronic respiratory disease, and inflammatory airway disease as a respiratory stimulant (27, 37, 42, 43, 47, 60, 64, 67, 75).

Recently, it has become possible to define the host immune system more specifically with MAbs against leukocyte differentiation antigens of various animals, including horses (5, 7, 69, 70). The efficacy of vaccines and new drugs can be evaluated in vivo by comparing the host response before and after application of those reagents (14, 17, 29). BARODON’s immunoenhancing effects on pigs as an adjuvant and as a nonspecific immunostimulant have been approved as follows (45, 73, 74): (i) increases in antibody titers and immune cell proportions in hog cholera- and Actinobacillus pleuropneumoniae-vaccinated pigs after BARODON treatment; (ii) improvements in average daily weight gain rates and feed conversion rates; (iii) increased proportions of CD4⁺ and CD8⁺ T lymphocytes, MHC-II⁺ lymphocytes, non-T/non-B (N) cells, and, particularly, CD4⁺ CD8⁺ double-positive T lymphocytes from peripheral blood and the mesenteric lymph nodes; and (iv) a higher stimulatory activity to mitogen (PHA, concanavalin A, and pokeweed mitogen). Likewise, the equine immune system was defined by using various MAbs specific to equine leukocyte differentiation antigens after application of the anionic alkali mineral complex BARODON to determine the horse’s ability to resist respiratory diseases as well as the immunostimulatory effects of BARODON and its potential as an immunostimulant and alternative to antimicrobial feed additives for improving host immune responses in Thoroughbred horses.

The increased proportions of cells expressing MHC-II, which play a major role in bacterial defense mechanisms, phagocytosis, and antigen presentation, as well as CD4⁺, CD4⁺ CD25⁺, CD8⁺, CD8⁺ CD25⁺, and CD2⁺ T lymphocytes, dendritic cells, and B lymphocytes in peripheral blood, from BARODON-treated horses indicate that BARODON has immunoenhancing effects on equine immune systems. The comparatively higher proportions of activated immune cells in the healthy control group than in the IURD patient group were also found to be associated with resistance to IURD, as noted in our previous study (Ryu et al., submitted).

The phagocytic activity against S. equi subsp. equi and S. aureus in the BARODON-treated group was significantly higher than that of the control group after 3 weeks of treat-
After mitogen (PHA) stimulation of PBMC for 3 days, the proportions of CD4^+ CD25^+, CD4^+ CD25^−, CD8^+ CD25^−, and CD8^+ CD25^+ T lymphocytes in the BARODON-treated group increased significantly compared with the control group. Significant differences were observed after 3 weeks of treatment. The cells expressing CD2^+ (all thymocytes, T lymphocytes, and NK cells) and MHC-I antigen were not significantly different within treatment groups (Tx-1, Tx-2, and Tx-3). However, when the immunological characteristics within the BARODON treatment groups were analyzed, the increased proportions of cells expressing MHC-II antigen, large blasting cells (R2), CD4^+, CD4^+ CD25^+, CD8^+, and CD8^+ CD25^+ T lymphocytes, dendritic cells, and B lymphocytes in peripheral blood, as well as the enhanced cell proliferative responses against PHA and phagocytic activity against *S. equi* subsp. *equi* and *S. aureus*, were all significantly greater in the Tx-1 and Tx-2 groups than in Tx-3. No significant difference was observed between the Tx-1 and Tx-2 groups. The increased proportions of these
immune cells should influence the activated lymphoproliferative responses by mitogen stimulus. Further studies using MAbs against other activation or regulatory molecules on equine immunomodulating cells and an analysis of their cytokine gene expression and protein secretion, including IL-10 or transforming growth factor beta, can more specifically elucidate the activity of BARODON.

Under stress, such as with strenuous exercise and long-distance transportation, alveolar macrophage activities and CD4+ T lymphocytes can be suppressed (71). According to clinical

FIG. 6. Summary of FC analysis of CD4+ (A), CD4+ CD25+ (B), CD8+ (C), and CD8+ CD25+ (D) T lymphocytes in peripheral blood with gates placed only on 1 and 2 and blast, proliferating lymphocytes (E) in peripheral blood with a gate placed only on 2 after 3 days of culture in RPMI alone or with PHA. Horses in one group were fed both Omolene feed and Barodon-biogenic feed (Tx-1; n = 6), while a second group was fed only Omolene feed (Tx-2; n = 6) and a third group was fed only Barodon-biogenic feed (Tx-3; n = 6). The untreated control group was fed general feed with no BARODON (Control; n = 6). Significant differences in the proportions of CD4+ (A), CD4+ CD25+ (B), CD8+ (C), and CD8+ CD25+ (D) T lymphocytes and cells in gate R2 (E) after culture in RPMI alone and with PHA, between control horses and BARODON-treated horses, are as indicated in the figure (*PHA = 3 days of PHA + 3 days of RPMI; a, P < 0.01; b, 0.01 < P < 0.05). See Materials and Methods for detailed information on the analysis of subsets by selective gating, used to show the frequency of each cell population.
experience and observations at the Seoul Race Park, the advance administration of BARODON in either an anionic feed additive form or a drinking water form reduced many clinical complications, including stress-induced respiratory disease, suggesting activation of immune cell populations, which is a result similar to that obtained after the treatment of horses with inactivated *P. acnes* (18, 43). Therefore, BARODON’s immunoenhancing effect in equine herds can improve the im-

**FIG. 7.** Representative dot plot profiles of PBMC with gates placed only on 1, 2, and 3 at time zero from a control horse (A) and a BARODON-treated horse (B) labeled with one MAb (Table 1) for granulocytes and monocytes (G/M) following phagocytosis with opsonized *Streptococcus equi* subsp. *equi*, which had been previously stained overnight with PI, and a summary of FC analysis of PBMC following phagocytosis with nonopsonized (C) or opsonized (D) *S. equi* subsp. *equi* and with nonopsonized (E) or opsonized (F) *Staphylococcus aureus*. Profiles A and B show the frequency of *S. equi*-negative G/M<sup>−</sup> cells (lower left quadrant), *S. equi*-negative G/M<sup>+</sup> cells (upper left quadrant), *S. equi*-positive G/M<sup>−</sup> cells (upper right quadrant), and *S. equi*-positive G/M<sup>+</sup> cells (lower right quadrant). Horses in one group were fed both Omolene feed and Barodon-biogenic feed (Tx-1; *n* = 6), while horses in the second group were fed only Omolene feed (Tx-2; *n* = 6) and horses in the third group were fed only Barodon-biogenic feed (Tx-3; *n* = 6). The untreated control group was fed general feed with no BARODON (Control; *n* = 6). Significant differences between the phagocytic capabilities of control animals and animals treated with BARODON are as indicated in the figure (a, *P* < 0.01; b, 0.01 < *P* < 0.05). Detailed information on the concentration of BARODON in each feed and instructions on how to perform phagocytosis and opsonization, stain *S. equi* subsp. *equi* and *S. aureus* with PI, and analyze subsets by selective gating to determine the frequency of each cell population are provided in Materials and Methods.
mune responses of horses to equine respiratory bacterial infection, as shown by the increased phagocytosis and, moreover, possibly treatment against S. equi subsp. equi and S. aureus strains with high-level resistance to common antibiotics for IURD. Considering that (i) the doping control system has prohibited the preventive use of antimicrobial feed additives and (ii) veterinarians have been very much concerned about this when selecting antibiotics in a clinical situation, BARODON’s immunoenhancing effect, with its mineral composition, in equine herds can be useful when aimed at equine respiratory bacterial infections under racetrack conditions. Clinical experience with Omolene feed containing 0.05% BARODON at selected stables in the Seoul Race Park showed that the feeding of more than 150 g of BARODON per year reduced significantly the prevalence of IURD compared with containing BARODON or general feed not during this study. Further controlled field studies will elucidate the potential effects of BARODON against equine respiratory disease.

The major ingredient in BARODON is a mineral that may affect vital biological processes, including immune responses. Silica, among several candidate substances in BARODON, may be responsible for these immunoenhancing effects. Subchronic and short-term exposure to silica was shown to enhance respiratory defense mechanisms by increasing the proportions of neutrophils, T lymphocytes, and NK cells and by activating phagocytes to release more reactive oxygen species, which have been known to help in the pulmonary clearance of infectious agents in lungs (3, 4, 33). More specifically, in the thoracic lymph nodes of rats with subchronic silicosis, a significantly higher percentage of CD8+ T cells and, to a lesser degree, CD4+ T cells was proliferated and activated, with the expression of IL-2R and intercellular adhesion molecule 1 and enhanced expression of IFN-γ mRNA, which may be an important priming cytokine for a systemic preactivation not only of alveolar macrophages but also of peritoneal macrophages that have experienced no direct contact with silica particles (8, 23, 34, 40).

The immunostimulant’s function was found to be mediated predominantly by macrophage activation, with stimulus-induced gene expression, and finally by increased functional competence (63). Therefore, multiple doses are expected to give pulses of immune stimulation, such as cytokine release, due to the increased persistence of the product within macrophages. In this study, 6 to 7 kg of daily feed intake of Omolene feed included 3 to 3.5 g of Barodon F. Gold. However, a daily intake of 60 ml of Barodon-biogenic feed can provide horses with 8.58 g of Barodon F. Gold, given its concentration and specific gravity. Nevertheless, BARODON’s effect on the enhancement and activation of immune responses without clinical side effects in horses was higher in the anionic feed additive form of BARODON, Omolene feed, than in the drinking water form of BARODON, Barodon-biogenic feed. This difference cannot be fully explained in this study.

Further studies are needed to determine the optimal amounts for daily intake and the best method of ingestion of BARODON for the efficient stimulation of equine mucosal immunity without possible adverse side effects. In a study with the non-specific immunostimulant OM-85 BV in human patients, stimulatory effects on T-lymphocyte subpopulations persisted during treatment but decreased to baseline values within 3 months after discontinuation of immunotherapy (16). However, the duration of immunomodulatory effects after discontinuation of BARODON was not investigated in this study. Although more studies are needed to elucidate the exact mechanism of action of BARODON and its enhancing effect on the equine immune system, this study suggests that BARODON is a potential immunostimulant and an alternative to antimicrobial feed additives for improving equine immune responses and that its use results in the improved capability of horses to endure an attack of infectious respiratory diseases.

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