Comparison of Cell-Mediated Immunity Induced by Three Commercial Single-Dose *Mycoplasma hyopneumoniae* Bacterins in Pigs

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ABSTRACT. This study was to compare the degree of cell-mediated immunity induced by three commercial *Mycoplasma hyopneumoniae* bacterins using interferon- γ (IFN- γ) measurements, lymphocyte stimulation assays and delayed-type hypersensitivity tests. Serum IFN- γ levels were significantly elevated in all four vaccinated pig groups at 21 days post-vaccination ($P < 0.05$). Lymphocytes isolated 21 days post-vaccination exhibited significantly more proliferation in response to *M. hyopneumoniae* than lymphocytes isolated 0 day pre-vaccination ($P < 0.05$). Following intradermal injection of *M. hyopneumoniae* antigen at 14, 21 or 28 days post-vaccination, all pigs in the four vaccinated groups displayed skin reactions characterized by circumscribed, often erythematous nodules. Taken together, these demonstrate that all three commercial single-dose *M. hyopneumoniae* bacterins used in this study induce varying degrees of cell-mediated immunity.

KEY WORDS: cell-mediated immunity, *Mycoplasma hyopneumoniae*, vaccination.

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Mycoplasma hyopneumoniae is a causative agent of swine mycoplasmal pneumonia, a disease that is widespread and causes economic losses in the swine industry through stunted growth and susceptibility of the swine to infection by other pathogenic organisms [14]. *M. hyopneumoniae* is predominantly transmitted through nose-to-nose animal contact; less frequently, aerosolization of the agent maintains horizontal spread from infected to naive pigs of the same age, or vertical transmission from sows to their offspring [14]. To combat vertical and horizontal transmission, it has been suggested that vaccination of suckling piglets before *M. hyopneumoniae* infection during the first week of life may assist *M. hyopneumoniae* control. Since a correlation between cell-mediated immunity and protection from mycoplasmal disease has been reported [4, 16], cell-mediated immunity is important for control of mycoplasmal pneumonia in pigs. Single-dose *M. hyopneumoniae* bacterin was first introduced in 1998, and since then, several commercial single-dose *M. hyopneumoniae* bacterins have been routinely used in swine farms. Although one previous study reported the induction of cell-mediated immunity by two single-dose commercial *M. hyopneumoniae* bacterins [15], however, no one has compared induction of cell-mediated immunity by three commercial single-dose *M. hyopneumoniae* bacterins and by the same *M. hyopneumoniae* bacterin at different age. The purpose of the present study was to investigate the degree of cell-mediated immunity induced

by three commercial single-dose *M. hyopneumoniae* bacterins using interferon- γ (IFN- γ) measurements, lymphocyte stimulation assays and delayed-type hypersensitivity (DTH) tests in pigs at different ages.

Fifty colostrum-fed, cross-bred conventional pigs, farrowed from unvaccinated (for *M. hyopneumoniae*) and seronegative gilts, were purchased from a commercial farm. All pigs tested negative for porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus and *M. hyopneumoniae* by routine serological testing. Individual pigs were uniquely identified from seven days of age by single ear notches. These pigs were randomly sorted into five groups of ten pigs each.

Three commercial single-dose *M. hyopneumoniae* bacterins were investigated in this study. *M. hyopneumoniae* bacterin A (2.0 ml; Respire One, Pfizer Animal Health, New York, NY, U.S.A.) was administered to the pigs of groups T01 and T02 at 7 or 21 days of age, respectively. At 21 days of age, the pigs of groups T03 and T04 were vaccinated with *M. hyopneumoniae* bacterin B (2.0 ml; Mhyo, Boehringer Ingelheim Animal Health, St. Joseph, MO, U.S.A.) or bacterin C (1.0 ml; Mycoflex, Boehringer Ingelheim Animal Health), respectively. Pigs serving as the negative control (group T05) remained unvaccinated.

A minimum of 10 ml whole blood was collected for IFN- γ measurement and lymphocyte stimulation assay at 0 day pre-vaccination and 21 days post-vaccination. Porcine-specific IFN- γ levels in sera were measured with an enzyme-linked immunosorbent assay (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.) according to the manufacturer's instructions. Antigen for lymphocyte stimulation was prepared from *M. hyopneumoniae* (ATCC 25934), and concentration was adjusted to 4 mg/ml. The lymphocyte

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stimulation assay was performed as previously described [2].

DTH test was performed on 50 piglets from 5 groups at 14, 21 and 28 days post-vaccination as previously described [2]. Piglets were injected intradermally on the left inguinal area with 0.1 ml of a solution containing *M. hyopneumoniae* antigen (ATCC 25934; 300 µg/ml). Thirty-six hours after antigen injection, swollen erythema of the injection site was measured as previously described [12]. The longest and mid-point orthogonal diameters of the erythema were measured with engineering automated calipers and used to calculate the area of the erythema.

For single comparisons, ANOVA with post-hoc Tukey's test was applied to the primary variables (IFN-γ, lymphocyte stimulation assay and DTH test) among groups. Pearson correlation analysis was used to assess correlations of the primary variables among groups. For all statistical tests, $P < 0.05$ was considered statistically significant.

Serum IFN-γ levels were not detected in all pigs from 5 groups at 0 day pre-vaccination but significantly elevated in all four vaccinated groups at 21 days post-vaccination, although pigs from group T01 exhibited significantly higher serum IFN-γ levels than pigs from other 4 groups (T02, T03, T04 and T5; $P < 0.05$; Fig. 1). Lymphocytes isolated from pigs at 21 days post-vaccination manifested significantly more proliferation in response to *M. hyopneumoniae* than lymphocytes isolated at 0 day pre-vaccination ($P < 0.05$; Fig. 2). Higher amounts of proliferation were observed in the lymphocytes of pigs from group T01 in response to *M. hyopneumoniae* than lymphocytes from the other three vaccinated groups (T02, T03 and T04; $P < 0.05$; Fig. 2). Additionally, *M. hyopneumoniae*-specific lymphocyte proliferation in pigs from group T01 strongly and significantly correlated with serum IFN-γ levels ($r^2 = 0.775$, $P < 0.008$). Following intradermal injection of *M. hyopneumoniae* antigen at 14, 21, or 28 days post-vaccination, all pigs in the four vaccinated groups (T01, T02, T03 and T04) displayed skin reactions characterized by circumscribed, often erythematous nodules 24 to 48 hr later. Pigs in group T01 (14.25 ± 3.12) had significantly greater DTH response size than pigs in other vaccinated groups (10.17 ± 2.38 for T02; 9.6 ± 2.52 for T03; 9.8 ± 3.18 for T04; $P < 0.05$). The nodules regressed slowly after 48 hr, leaving no scar or skin reaction. The scores from this DTH test in three vaccinated groups correlated strongly and significantly with serum IFN-γ levels: pigs in groups T01 ($r^2 = 0.757$, $P < 0.011$), T02 ($r^2 = 0.764$, $P < 0.010$) and T03 ($r^2 = 0.688$, $P < 0.028$).

The present study has demonstrated that three commercial single-dose *M. hyopneumoniae* bacterins induced various degrees of cellular immune responses in pigs, as assayed by IFN-γ measurements, lymphocyte stimulation assays and DTH testing. Interestingly, pigs vaccinated with *M. hyopneumoniae* bacterin A at 7 days of age displayed stronger cellular immune responses than the other pigs vaccinated with *M. hyopneumoniae* bacterin A, B or C at 21 days of age on the basis of IFN-γ measurements and lymphocyte stimulation assays. Although we have no clear explanation for this observation, we assume that strong immunity can be induced before the pigs become infected, when fewer

Fig. 1. Interferon-γ levels measured from blood samples collected from pigs at 0 day pre-vaccination (□) and 21 days post-vaccination (■). Variation is expressed as standard deviation; *Significant difference between 0 day pre-vaccination and 21 days post-vaccination ($P < 0.05$); †Significant difference among all groups ($P < 0.05$).

Fig. 2. *Mycoplasma hyopneumoniae*-specific lymphocyte stimulation index in million peripheral blood mononuclear cells (PBMCs)/ml from pigs at 0 day pre-vaccination (□) and 21 days post-vaccination (■). Variation is expressed as standard deviation; *Significant difference between 0 day pre-vaccination and 21 days post-vaccination ($P < 0.05$); †Significant difference among all five groups ($P < 0.05$).

pathogens were present that interfere with immune response.

Under field conditions, *M. hyopneumoniae* bacterin A was more effective in preventing and reducing the severity of lung lesions induced by *M. hyopneumoniae* than *M. hyopneumoniae* bacterin B [1]. However, this comparative study was not compared with cell-mediated immunity induced by commercial *M. hyopneumoniae* bacterins. In the present study, *M. hyopneumoniae* bacterin A induced higher cell-mediated immunity against *M. hyopneumoniae* than *M. hyopneumoniae* bacterin B under experimental conditions. These results suggested that the higher induction of cell-mediated immunity against *M. hyopneumoniae*, the lower the severity of lung lesions induced by *M. hyopneumoniae*.

Vaccination is most likely to be effective, if active immunity is established before *M. hyopneumoniae* infection. Pre-

viously, the prevalence of *M. hyopneumoniae* in lung tissue from 201 suckling piglets was 2.0%, significantly lower than 9.3% in a corresponding sample from 921 nursery pigs [8]. However, the percentage of nested PCR-positive pigs, as assayed from nasal swab taken at 6 weeks of age, was dramatically increased (16%) in the clinically infected herds [17]. Hence, the earlier the vaccination, the greater the chance of avoiding *M. hyopneumoniae* infection before vaccination.

The possible disadvantages of vaccinating piglets before weaning include the presence of maternal antibodies and an increased risk of more severe PCV2 infections after weaning [6]. *M. hyopneumoniae* bacterin administered in the presence of maternal antibodies exerted little to no effect on antibody response development [3, 7]. Moreover, the timing of *M. hyopneumoniae* vaccination may be critical for minimizing the effects of vaccine-induced potentiation of PCV2-associated disease [9]. Many pig producers consequently prefer to vaccinate with single-dose *M. hyopneumoniae* bacterin at 1 week of age to minimize immunostimulation to PCV2 replication. Finally, an early single-dose vaccination of one-week-old, seronegative or seropositive pigs led to significantly fewer lung lesions than unvaccinated pigs following experimental challenge at 14 and 25 weeks later [5, 13].

IFN- γ is a key immunoregulatory cytokine that controls the differentiation of naïve CD4⁺ T cells into CD4⁺ Th1 cells and mediates cell-mediated immunity [11]. Because CD4⁺ Th1 cells promote a DTH response [10], the DTH response observed in only vaccinated pigs also supports a protective cellular immune response that is induced by commercial *M. hyopneumoniae* bacterins. Induction of the cell-mediated immunity by single-dose *M. hyopneumoniae* bacterins may play an important protective role, since no correlation between serum antibody level induced by humoral immunity and protection against *M. hyopneumoniae* infection has been reported [4, 16]. On the basis of this study, the induction of *M. hyopneumoniae*-specific cell-mediated immunity by three commercial *M. hyopneumoniae* bacterins is the important protective cellular immune response leading to control of the *M. hyopneumoniae* infection in pigs.

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