Research in Veterinary Science 91 (2011) e68–e72

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Evaluation of peripheral lymphocytes after weaning and vaccination for *Mycoplasma hyopneumoniae*

Andrew R. Kick [a,1], Mary B. Tompkins [a], J. Mark Hammer [b], Patricia A. Routh [a], Glen W. Almond [a,*]

[a] Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606, USA
[b] Novartis Animal Health, Greensboro, NC 24708, USA

ARTICLE INFO

Article history:
Received 26 August 2010
Accepted 11 November 2010

Keywords:
Pig
Weaning
Mycoplasma hyopneumoniae
Immune cells

ABSTRACT

This study evaluated immune cell populations in pigs following weaning and vaccination for *Mycoplasma hyopneumoniae*. Piglets ($n = 24$) were weaned (day 0) at 16 (±1) days of age, and randomly assigned to the vaccination group ($n = 16$) or control group ($n = 8$). Complete blood cell counts, flow cytometry and serology were completed for blood samples collected on days 0 (within hours of weaning), 3, 7, 14, 30 and 60. The *M. hyopneumoniae* S:P ratios (sample optical density: positive control optical density) were negative in the vaccination group until days 30 and 60, when the S:P ratios were 1.3 and 1.0, respectively. Control animals remained serologically negative. The percentage of $CD4^+$ T cells was less ($P < 0.01$) in control pigs than vaccinated pigs at day 3. In contrast, numbers of $CD8^+$ and $CD4^+CD8^+$ T cells were greater ($P < 0.01$) in control pigs than in vaccinated pigs at days 3 and 7. After day 7, few differences in immune cell types were evident between the groups. Differences in lymphocyte populations could not be solely attributed to vaccination, due at least in part, to the confounding influence of weaning. It was difficult to distinguish the influence of vaccination from the impact of weaning on peripheral immune cell populations.

1. Introduction

Vaccines routinely are used to minimize the effects of various pathogens in commercial pig production. Most studies compared vaccinated to control groups by assessing the overall health of pigs, gross and histopathological lesions, and antibody titers to the pathogen or antigen (Okada et al., 1999; Pallares et al., 2000; Haruna et al., 2006). Fewer studies evaluated the effects of vaccination on cell mediated immunity or immune cell populations. Vaccination and challenge with porcine reproductive and respiratory virus (PRRS) altered $CD4^+$ and $CD8^+$ percentages of peripheral blood lymphocytes (PBL), and interferon-γ (IFN-γ) production (Martelli et al., 2007, 2009). In another study, PRRSV vaccination resulted in higher IFN-γ staining and expression (Charerntantanakul et al., 2006). Conversely, *Hemophilus parasuis* vaccination had no effect on the percentages of T-cell subsets (de la Fuente et al., 2009).

One report indicated that vaccination against *Mycoplasma hyopneumoniae* (MHYO) or pathogen challenge failed to induce a nonspecific change in peripheral $CD4^+$ and $CD8^+$ cells (Thacker et al., 2000). In contrast, the same investigation revealed that INF-γ secreting cells were important to the protective response. Overall, a local mucosal humoral and a systemic cell mediated immune response were considered critical to controlling mycoplasmal pneumonia in pigs (Thacker et al., 2000). The aforementioned failure to detect changes in peripheral lymphocyte populations is contrary to other investigations with other pathogens such as PRRSV (Martelli et al., 2007). The porcine immune system is unique in the expression of $CD4^+CD8^+$ (double positive) lymphocytes. These cells are associated with immunological memory due to their gradual increases with age, the expression of memory phenotype and their ability to respond to recall viral antigen (Hernandez et al., 2001). With the inconsistency in results from published reports on porcine immune responses, it is difficult to predict the response associated with vaccination and antigen challenge. Therefore, the primary aim of this study was to investigate immune cell populations following an intramuscular injection with a commercial vaccine for MHYO. Although it originally was not planned, this study also examined changes in peripheral immune cell populations following weaning.

2. Materials and methods

2.1. Animals and housing

The North Carolina State University Institutional Animal Care and Use Committee approved all experimental procedures involv-

* Corresponding author. Tel.: +1 919 513 6370; fax: +1 919 513 6464.
E-mail address: glen_almond@ncsu.edu (G.W. Almond).
[1] Present address: United States Military Academy, West Point, New York 10996-1905, USA.

0034-5288/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2010.11.005

ing animals in this study. Twenty-four piglets were selected from three crossbred (Large White × Landrace × Hampshire) sows (⩾2nd parity) at the NC State University Teaching Animal Unit. The sows had not received vaccinations of any kind in the previous six months and exhibited no signs of illness during gestation or lactation. The study was conducted from January through March, 2009. Sows were maintained in conventional farrowing stalls with ad libitum feed and access to nipple drinkers. Farrowing room temperatures were between 21 and 26 °C. Supplemental heat for the piglets was provided by two heat lamps in each farrowing stall. Piglet processing occurred within 72 h after birth and included iron injection, tail docking, teeth clipping, and castration of the males.

Each nursery pen was equipped with two nipple drinkers and pigs received feed ad libitum through conventional feeders (Hog Slat, Newton Grove, NC, USA). Ventilation and temperature were controlled with a side-wall baffle system that relied on two, variable speed exhaust fans for cooling and normal air circulation and a gas-heater located in the end of the room. The feed met or exceed established NRC guidelines (National Research Council, Subcommittee on Swine Nutrition, 1998).

2.2. Experimental design

Pigs were weaned (day 0 of the study) at 16 (±1) days of age and randomly assigned to treatment groups. Pigs ($n = 16$) in the treatment group (VAC) were vaccinated intramuscularly with MHYO bacterin, (PneumoSTAR® Myco, Novartis Animal Health, Greensboro, NC). Control (CNTL) pigs ($n = 8$) were not vaccinated, but were injected intramuscularly with 1 mL of saline, which is the equivalent volume of the vaccination. The initial blood samples (day 0) were collected within two hours after weaning, and immediately prior to the vaccination or saline injections. VAC pigs were placed in one of three nursery pens, and CNTL pigs were placed in one nursery pen. All nursery pens were in the same room, and there were approximately 12 pigs per pen (some pigs per pen were not included in the study). Pigs remained in the same pens for the remainder of the study.

2.3. Collection of blood samples and serology

Piglets were restrained and bled in a supine position with blood samples (12 mL/sample) collected by vena cava puncture into Vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). Blood samples were collected at weaning and vaccination (day 0) and then on days 3, 7, 14, 30 and 60. Complete blood counts (CBCs) and flow cytometry were completed at each blood collection day. An additional aliquot (<5 mL) of blood was collected at each collection to provide serum for the *M. hyopneumoniae* serology. Serum samples were sent to the Iowa State University Veterinary Diagnostic Laboratory. The diagnostic laboratory used the IDEXX HerdChek® ELISA for *M. hyopneumoniae*. This indirect ELISA uses the following equation to determine the SP ratio; equation: sample optical density (OD) − negative control OD ÷ positive control OD − negative control OD.

2.4. Differential blood leukocyte concentrations

Whole blood samples were submitted to Veterinary Hospital Clinical Pathology Service (North Carolina State University) for CBCs. This facility uses a Siemens Healthcare Diagnostics Advia 120 for quantification of blood leukocytes. Differential blood leukocyte concentrations were obtained for porcine blood and reported as absolute numbers of neutrophils and lymphocytes.

2.5. Flow cytometry

Flow cytometry was performed, using previously established procedures (Feng et al., 2002) with minor modifications, to assess differences in immune cell populations in peripheral blood after MHYO vaccination and weaning. The following antibodies were utilized in one or two color flow cytometry and at the noted dilutions to identify the following cell types: $CD4^+$ T cells (CD4α, mouse IgG_{2b}, MCA1749F, AbD Serotec, Oxford, UK) at 1:100 dilution with R-phycoerythrin (R-PE) and $CD8\alpha^+$ T cells (CD8α, mouse IgG_{2a}, MCA1223, AbD Serotec, Oxford, UK) at 1:100 dilution with Fluorescein (FITC); $CD3^+$ T cells (CD3e, mouse $IgG_{2b}\kappa$, BB238E6, Southern Biotech, Birmingham, AL) at 1:50 dilution with Allophycocyanin (APC); mature B cells (CD21, mouse $IgG_1\kappa$, BB6-11C9.6, Southern Biotech, Birmingham, AL) at 1:200 dilution per tube with R-PE; activated T and B cells (CD25, mouse $IgG_{2b,}$ MCA1736, AbD Serotec, Oxford, UK) at 1:200 dilution with FITC; and IgG Fc Receptor (AAI41F, goat IgG, AbD Serotec, Oxford, UK) at 1:100 dilution with FITC.

Briefly, antibodies were diluted at the listed dilutions in phosphate buffered saline (PBS) to a volume of 100 μL. Whole blood was centrifuged for 10 min at 400*g* and serum was removed. Blood cells were reconstituted to original volume with PBS. Then, 100 μL was added to each staining series as well as a cells only tube with only PBS. Blood was incubated for 20 min in the dark at room temperature. Cells were washed with 3 mL PBS and centrifuged for 10 min at 400*g*. Red blood cells were lysed with 1 mL FACS lysing solution (Beckton Dickinson, San Jose, CA) for 10 min at room temperature twice and washed with 2 mL PBS after each lyse and centrifuged for 10 min at 400*g*. Cells were resuspended in 200 μL PBS/2% Fetal Bovine Serum (FBS). Samples were analyzed by FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CELLQuest Pro software with 20,000 events and gating based upon forward and side scattering. For each cell type, the color cytometric evaluation provides a percentage of all cells. Using the WBC results of absolute numbers of cells, the number of a specific cell type was determined. The two color method was used to determine the double positive cells.

2.6. Statistical analysis

All data were analyzed utilizing the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). The initial statistical model included time, treatment, and the treatment by time interaction. The individual variation within animals was not statistically different from the residual error ($P > 0.10$) for the study. This indicated that the variations among samples within pig were not a significant source of variation in the study and samples taken from the same animal over time were essentially independent. Consequently, a statistical model examining the effect of time and treatment as independent sources of variation was used. Next, all data were tested for the interaction between time and treatment. Main effects of treatment and time were analyzed when the interaction was not significant ($P > 0.10$). Data were analyzed for the main effect of treatment at each time point. All data are presented as least squares means ± SEM.

3. Results

3.1. Immune cells

The percentage of $CD4^+$ T cells of the PBL tended to be different ($P = 0.0663$) between treatments, while treatments did not differ ($P = 0.3562$) for the number of $CD4^+$ T cells (Fig. 1). Three days after