Veterinary Immunology and Immunopathology 160 (2014) 281–287

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

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

Research paper

Quantitative immunohistochemical assessment of IgA, IgM, IgG and antigen-specific immunoglobulin secreting plasma cells in pig small intestinal lamina propria

C. Bianco[a,*], V. Felice[a], S. Panarese[a], R. Marrocco[a], F. Ostanello[a], B. Brunetti[a], L.V. Muscatello[a], G. Leotti[b], T. Vila[c], F. Joisel[c], G. Sarli[a]

[a] Department of Veterinary Medical Sciences, Ozzano Emilia, BO, Italy
[b] Merial Italia SpA, Milan, Italy
[c] Merial S.A.S., Lyon, France

ARTICLE INFO

Article history:
Received 28 November 2013
Received in revised form 13 March 2014
Accepted 29 May 2014

Keywords:
Swine
PCV2
Immunity
Vaccination
Intestinal mucosa
Image analysis

ABSTRACT

Intestinal immune response plays an important defensive role for pathogens, particularly for those transmitted by the oro-faecal route or for foecal shedding modulation. This work examined three parts of intestine from twelve gilts experimentally infected with PCV2-spiked semen, six vaccinated (V group) and six unvaccinated (NV group) against PCV2, 29 and 53 days post infection (DPI). An immunohistochemical investigation for IgA-, IgG- and IgM-antibody bearing plasma cells (PCs) was run on intestinal samples coupled with a sandwich immunohistochemical method to reveal anti-PCV2 antibody-secreting PCs. Plasma cell density was compared in the two groups of animals at 29 and 53 DPI. The IgA, IgG and IgM PC density did not differ between groups but displayed an increase from the upper (villus) to the lower part of the crypts while a decreasing trend in PC density was identified from duodenum to ileum. In the NV group, no increase in anti-PCV2 PC density was demonstrable in the two sampling moment: the amounts of *lamina propria* PCV2-specific antibody-producing PCs remained constant, 10.55 ± 4.24 and 10.06 ± 5.01 at 29 DPI and 53 DPI, respectively. In the V group a significant increase in PCV2-specific antibody-producing PCs was observed over time. The amounts of PCV2-specific antibody-producing PCs increased from 9.37 ± 13.36 at 29 DPI to 18.76 ± 15.83 at 53 DPI. The data on IgA, IgM and IgG PC counts can be considered reference values in a population of adult pigs. The sandwich method can be proposed as a technique able to identify specific antibody-secreting PCs in formalin-fixed paraffin-embedded tissues. A practical application of the sandwich method is the demonstration of a "booster-like" response of the *lamina propria* in vaccinated compared to unvaccinated animals. After virus challenge, vaccination induced an increase in the number of PCs containing specific anti-PCV2 antibodies at the level of intestinal mucosa.

1. Introduction

The gastrointestinal tract's natural defence mechanisms include the antibacterial properties of gastric and intestinal secretions, a mucus-coated epithelial surface, and the secretion of cytokines and chemokines. The mechanism of

* Corresponding author at: Department of Veterinary Medical Sciences – DiMeVET, Via Tolara di Sopra, 50 Ozzano dell'Emilia, BO, Italy. Tel.: +39 051 20 9 7954; fax: +39 051 20 9 7968.
E-mail address: carlo.bianco3@unibo.it (C. Bianco).

http://dx.doi.org/10.1016/j.vetimm.2014.05.014
0165-2427/

intestinal defence that receives most attention, however, is the intestinal immune system, which is usually considered to have the largest accumulation of antibodies in the body (Burkey et al., 2009).

Broadly speaking, the organization of the mucosal immune system can be divided into: (a) inductive sites and, (b) effector sites. Inductive sites are where the uptake of antigens from the mucosal surface and the priming of naïve T and B lymphocytes occurs. The other sites recruit several effector mechanisms including the production of secretory immunoglobulin A (SIgA) antibodies (Brandtzaeg et al., 2008). The inductive sites of mucosal immunity include well-defined tissues such as the mucosal-associated lymphoid tissue (MALT) (Liebler-Tenorio and Pabst, 2006) and the loco-regional lymph nodes. The effector sites encompasses different and not well-delimited compartments, such as the *lamina propria* of the various mucous membranes that should not be considered part of the MALT (Brandtzaeg et al., 2008). The organization of the immune system in the intestinal mucosa includes non-encapsulated and concentrated lymphoid tissue (Peyer's patches of the ileum and jejunum) (Burkey et al., 2009) constituting the inductive sites that also include part of the epithelium associated with the follicles (Sato and Iwasaki, 2005). A diffuse non-encapsulated compartment, including cells (lymphocytes, plasma cells, macrophages, dendritic cells, mast cells) located outside Peyer's patches but residing in the *lamina propria*, represents the effector sites where effector mechanisms of cell-mediated and humoral immunity take place (Tizard, 2013). Plasma cells are located mainly around the intestinal crypts and form the secretory component of the mucosal immune system, devoted to the production of protective humoral factors acting on the mucosal surface.

The intestinal mucosa is exposed to heavy loads of commensal and pathogenic microorganisms and since it is the first line of defence, mucosal immunization is considered an interesting target for vaccination (Kim et al., 2012). Therefore the intestinal mucosal immune system has attracted much research interest in recent years. Mucosal responses are more difficult to assess than systemic responses. Mucosal immunity assays can be run through quantifying cell subpopulations (mononuclear cells, neutrophils) or by analyzing immune-related molecules (cytokines, immunoglobulins). There is no gold standard for evaluating secretory immunity in mucosal surfaces. The humoral mucosal immune system can be examined with the same techniques used for serology. A critical point is the sampling of secretions, which may be affected by serum or blood contamination, or excessive dilution by other contaminants (ingesta or enzymes) (Guy, 2002). Flushing the mucosal surface does not always allow the collection of immunoglobulins contained in the mucosal surface film, if the film is highly viscous. In these cases, scraping the mucosal surface ensures a more suitable sample for analysis compared to simple flushing, but can increase serum leakage.

Because the efficiency of the intestinal mucosal immune system is useful for diseases where the oro-faecal route is important, such as the porcine circovirus diseases (PCVDs) (Rose et al., 2012), we aimed to devise a new, objective and quantitative method, a sandwich immunohistochemical technique, to disclose anti-PCV2 antibody-secreting PCs *in situ* in the intestinal *lamina propria*. We used a morphometric method coupled with immunohistochemistry to investigate the intestinal IgA-, IgM- and IgG-producing PCs and PCV2-specific antibody-secreting PCs *in situ* in six vaccinated (V) and six unvaccinated (NV) gilts experimentally infected with PCV2-spiked semen.

2. Materials and methods

2.1. Samples

The samples examined in this study come from a previous experiment (Sarli et al., 2012). In brief, six gilts vaccinated (V group) by two injections of 2 ml IM with a commercial inactivated vaccine (CIRCOVAC®, Merial, Lyon, France) against porcine circovirus type 2 virus (PCV2) and six unvaccinated gilts (NV group) were experimentally infected with semen supplemented with 0.2 ml of a PCV2b suspension containing $10^{4.4}$ $TCID_{50}$/50 μl. The time of insemination was set as 0 days post infection (DPI). At 29 DPI intestinal samples were collected (duodenum, jejunum, ileum) from gilts resulting empty by ultrasonography (three of the V group and three of the NV group), while at 53 DPI were collected samples from pregnant sows (three of the V group and three of the NV group).

2.2. Immunohistochemical stainings

Samples were available as formalin-fixed and paraffin wax-embedded tissues. Five consecutive sections (4 μm thick) from each sample were stained with haematoxylin and eosin (HE) and by immunohistochemistry (IHC) using antibodies specific to porcine IgG, IgA and IgM. The last section was used for the detection of PCV2-specific antibody-producing PCs. On the basis of the technique proposed by Kipar et al. (1998) a sandwich IHC method to reveal anti-PCV2 antibody-secreting PCs was standardized. This method uses a PCV2 suspension as preliminary step followed by a classical IHC procedure employing an anti-PCV2 immunoglobulin (Mab F217, provided by Dr. G. Allan, Belfast, UK) as primary antibody (Table 1).

As positive controls to assess the specificity of the immunohistochemical procedure for anti-IgG, IgM and IgA stains, sections of a normal swine lymph node were used following the same protocols; as negative control, the primary antibodies were replaced with one of irrelevant specificity. In the sandwich method, negative controls were obtained (1) by the replacement of the primary antibody with another of irrelevant specificity or, (2) omitting the viral suspension step.

2.3. Quantitative analyses of PCs

The quantitative analysis of PCs was conducted according to the method of Waly et al. (2001). PC counts were performed in three anatomo-functional areas of the intestinal *lamina propria*: (1) the *lamina propria* of the villi (area 1), (2) the *lamina propria* of the upper part (area 2) and, (3) lower part (area 3) of the crypt (Fig. 1). For each area three to six photographs were randomly acquired with a Leica TM