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Changes in Peyer's Patch and Cecal Tonsil B Lymphocytes in Laying Hens Following Challenge with *Salmonella enterica* Serovar Enteritidis

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Abstract: Two trials were conducted to determine B cell changes in Peyer's Patches (PP) and Cecal Tonsils (CT) of specific-pathogen-free Single Comb White Leghorn hens challenged with *Salmonella enterica* Serovar Enteritidis (SE). Prior to challenge and then weekly post challenge, 4 or 3 hens in Trials 1 and 2, respectively, were sacrificed and their intestinal tracts excised. Cells were purified from proximal and distal PP along with both CT and then aliquots of cells were incubated with antibodies to chicken immunoglobulins IgM, IgG and IgA. The B cells expressing the different immunoglobulin isotopes were identified via flow cytometric analysis. B lymphocytes expressing IgM were most prevalent, representing 40-60% and 30-50% of CT and PP B cells, respectively, while 20-30% of CT and PP lymphocytes expressed IgA. Only a small percentage of CT and PP lymphocytes, <10%, expressed IgG. Significantly more IgM+ cells were detected in CT vs proximal and distal PP and proximal PP in trials 1 and 2, respectively. Significantly more IgA+ cells were observed in proximal PP vs distal PP and CT in trial 1 but not in trial 2. Following SE infection, these differences were no longer observed. For IgG+ cells, however, no significant differences between tissues were observed prior to challenge but significantly more IgG+ cells were observed in both PP vs CT at weeks 1 and 3 post challenge in trial 1 and week 1 post challenge in trial 2. These results indicate that B lymphocyte differences do occur in PP vs CT in adult chickens and these populations can change in response to stimuli such as intestinal infection.

Key words: Peyer's patches, cecal tonsils, *Salmonella enterica*

INTRODUCTION

Challenge of adult poultry with *Salmonella enterica* Serovar Enteritidis (SE), will generally result in a disseminated infection which affects a variety of organs but produces little discernable morbidity or mortality in these individuals (Gast, 2008). As a result, flock disease problems due to infections by these organisms are generally rare. However, the problem arises when the organism disseminates to the hen reproductive tissues and enters eggs during formation. Human consumption of these SE-containing eggs can result in serious, potentially life-threatening salmonellosis infection (Braden, 2006). Food-related SE outbreaks stemming from consumption of eggs remain a significant problem in the U.S. (Schroeder *et al*., 2005; Braden, 2006). As a result, the egg industry receives increased pressure to reduce or eliminate these problem organisms from their flocks. The producer has a number of tools available to combat flock SE infection and vaccination can be an important method to increase resistance to SE, especially in older birds. While a fair amount of information is available regarding the development of systemic immunity in the bird following either SE infection or vaccination, much less is known about immunity in the gut. Following ingestion of SE, the organism migrates through the alimentary tract and invades the intestinal mucosa, primarily in the large and small intestine (Barrow *et al*., 1987). Protection of the intestinal tract is mediated via multiple mechanisms. Heterophils and other innate immune mechanisms play an important first line nonspecific defense against invasion (Swaggerty *et al*., 2005). However, as indicated by the ready infection of poultry by *Salmonella*, these defenses generally function to reduce the severity of the infection, not provide total protection. The development of a specific or adaptive immune response provides the individual with a more solid protective defense against infection.

One facet of the adaptive immune response is humoral immunity. The effector mechanisms of this immunity are antibodies, proteins found in the serum and most bodily fluids. Also known as immunoglobulins, these proteins exhibit specific reactivity for a particular component of the invading organism. Protection by secreted antibody is mediated through blocking the ability of the microorganism to bind to its specific attachment site in the gut (O'Farrelly *et al*., 1992), blocking virulence factor activity (Rogers, 1973), enhancement of engulfment and killing by phagocytes (Densen and Mandell, 1980; Nencioni *et al*., 1983), or activation of the complement

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cascade which either directly results in the rupture of the cell or enhances its phagocytosis and killing (Densen and Mandell, 1980; Rus *et al*., 2005).

There are 3 main classes of immunoglobulins in the chicken: IgM, IgG (IgY) and IgA (Davison *et al*., 2008). IgM is the primary immunoglobulin found on B cells and is important in the early immune response, being the predominant antibody detected in serum following infection. Similar to the mammalian immune system, the IgM presence is transient (Hassan *et al*., 1991) and as the immune response progresses, B cells switch from IgM synthesis to producing either IgG or IgA for the secondary immune response. The prevalence of a particular immunoglobulin type relates to location, IgG being found predominantly in the serum while IgA is found in and on, mucosal surfaces (Davison *et al*., 2008). The primary site for initiation of SE infection is the intestinal tract. A more thorough understanding of the development of immunity in this area will help to develop vaccination regimes that provide more effective protection by generating immune responses at the site of infection initiation and abrogate the infection before the organism has a chance to become established.

Peyer's Patches (PP) are organized lymphoid tissues located at various sites along the small intestine (Makala *et al*., 2002). Shown to be important regulators of the gut immune response in mammals (Keren *et al*., 1978), PP received a great deal of attention regarding their pivotal role in providing local protection against intestinal pathogens. In the chicken, however, much less is known about the role of PP in gut immunity. Prior to the development of a staining procedure to allow PP visualization in the chicken gut (Vaughn *et al*., 2006), little research was performed on this lymphoid tissue and these studies required the use of fixed tissue (Befus *et al*., 1980; Burns, 1982). The introduction of the staining procedure enabled researchers to work with fresh, unfixed tissue and allowed them to delve more deeply into the functional role of PP in chicken gut mucosal immunity (Holt *et al*., 2010). As PP represent one of the major lymphoid tissues in the intestinal tract, an understanding of its response to infection by a *Salmonella* organism would provide valuable information regarding its role in protection against the pathogen. Significant changes were observed in T cell populations of Peyer's patches and the Cecal Tonsil (CT), a gut lymphoid tissue located in the large bowel, following hen infection with SE (Holt *et al*., 2010). The current paper details a companion study which examined changes in B cell populations in PP and CT from these same hens.

MATERIALS AND METHODS

Chickens: Single-Comb White Leghorn chickens, 34 and 41 weeks of age in Trials 1 and 2, respectively, were obtained from the specific-pathogen-free flock maintained at the Southeast Poultry Research Laboratory (SEPRL), Athens, GA. The hens were housed in individual adjacent laying cages in an environmentally controlled BSL-2 building at SEPRL and were fed layer ration *ad libitum* throughout the experiment duration. The hens were screened for *Salmonella* prior to the commencement of experiments and found to be *Salmonella*-free. The studies were approved by and conducted under the guidelines of the SEPRL Institutional Animal Care and Use Committee.

Infection: An overnight broth culture of nalidixic-acid-resistant SE, strain SE89-8312, was diluted 10^{-1} in sterile saline and each bird received a dose of 1 ml per os (6.9×10^7 and 5.3×10^7 SE, Trial 1 and 2, respectively). Crop lavage samples were obtained from 4 hens/sampling day (Trial 1) and 3 hens/sampling day (Trial 2) on day 0 and weeks 1-4 as described by Holt *et al*. (2002). Crop samples were kept on ice in 15 ml tubes until they were processed. The birds were then euthanized via CO_2 inhalation and approximately 1 cm of cecum was placed into a tared stomacher bag. The crop and cecum samples were transported back to the laboratory. One milliliter of each crop sample was added to 9 ml Tetrathionate Brilliant Green (TBG) broth. The cecum tissue was diluted 1:10 (w/v) in TBG and then emulsified via stomaching. The crop and cecum TBG samples were incubated for 24 h at 37°C and then a loopful of each sample was plated onto brilliant green agar containing 20 µg/ml nalidixic acid and novobiocin (BGNN). Following 24 h incubation at 37°C, the plates were evaluated for the presence of SE.

Peyer's patch and cecal tonsil processing and staining: Intestines, approximately 5 cm cranial to the Meckel's diverticulum to 2.5 cm caudal to the ileocecal junction, were removed from the sacrificed hens alluded to above and the lumen of each thoroughly flushed with distilled water. Both CT were extirpated and combined into a tube containing chilled RPMI-1640 tissue culture media (Sigma Chemical Company, St. Louis, Missouri). The PP were visualized using a previously described staining procedure (Vaughn *et al*., 2006) that involved infusing aqueous eosin-Y (Hema 3, Fisher Scientific, Suwanee, Georgia) into the gut lumen followed by diluted crystal violet stain (Sigma). Two PP could be detected: the proximal PP located 3-6 cm caudal to the Meckel's diverticulum and the distal PP located 7-10 cm cranial to the ileocecal junction (Fig. 1). Both PP were removed and placed into separate tubes of chilled RPMI-1640 media. Lymphocytes from CT and both PP were processed as described by Holt *et al*. (2010). Briefly, tissue was abraded through a 70 µm nylon-strainer (Fisher Scientific) and collected into 4 ml RPMI-1640. A further 2 ml media was used to rinse any remaining cells through the strainer. Following a centrifugation at

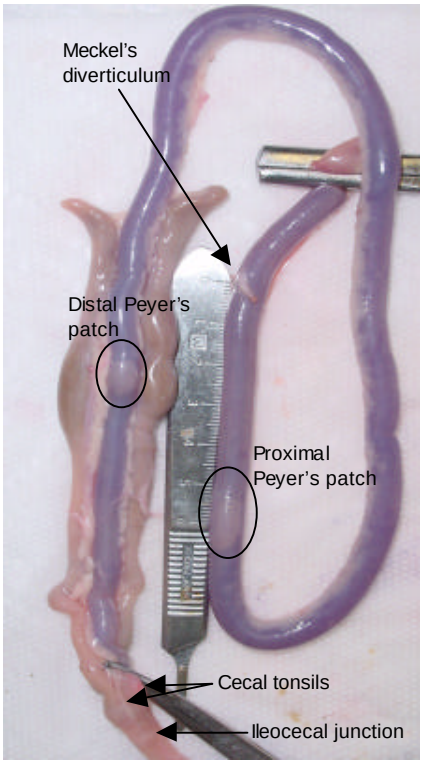

Fig. 1: Location of ileal distal and proximal Peyer's patch tissues in relation to cecal tonsils following staining procedure

1500 rpm, the cells were washed and centrifuged two more times. Viable lymphocyte counts were made using trypan blue exclusion and the cells were diluted to a working concentration of 2 x 10^6 viable cells/ml. One hundred microliters of cells were aliquoted and stained using cocktails containing: anti-chicken Bu1b coupled to biotin (1.2 µg antibody/10^5 cells) plus anti-chicken IgM, IgG, or IgA coupled to R-phycoerythrin (R-PE, 0.4 µg antibody/10^5 cells). All antibody reagents were purchased from Southern Biotech, Birmingham, Alabama. The cells were stained at 4°C in the dark for 30 min and then washed 1x. Streptavidin-labeled Cyanine 5 (Cy5, 1 µg/10^5 cells) was added to each cell preparation, the cells were incubated a further 15 min at 4°C in the dark and washed 2x. At the completion of the staining and washing procedure, the cells were resuspended in 400 µl PBS/1% paraformaldehyde and allowed to fix overnight at 4°C. Flow cytofluorometry was performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California). Four parameters, Forward Light Scattering (FSC), Sideward Light Scattering (SSC), PE (FL2) and Cy5 (FL4) were collected on the cells and evaluated using FlowJo analysis software (Tree Star Inc., Ashland, Oregon). Compensation for spectral overlap of each fluorochrome was performed using CompBead anti-mouse IgG 6 beads (Becton Dickinson) incubated with the appropriate fluorochrome-labeled antibody reagent.

Statistical analysis: Statistical analyses were performed using GraphPad Instat (GraphPad Software, Inc., San Diego, CA). Unpaired t tests were performed on comparisons between the percentage of SE in crop vs cecum at each time point. Analysis of Variance (ANOVA) with Tukey's multiple comparison test procedures were conducted for comparing B cell subpopulations between PP and CT tissues at each sample point, examining changes in B cell populations within same tissue types over time. Significance differences were expressed at $p<0.05$.

RESULTS

SE tissue colonization: Both crop and ceca were 100% culture positive for SE at one week post challenge in Trial 1 (Fig. 2a) and remained positive in the crop through week 3 while the presence of SE in the cecum declined at week 2 and no SE was detected in the cecum by week 3 ($p<0.01$). In Trial 2, both tissues were 100% SE culture positive at one and two weeks post challenge and the percentages decreased thereafter (Fig. 2b).

Isotype expression within tissues: The predominant B cell in the PP and CT in both trials were those expressing IgM on their surfaces followed by IgA then IgG. In Trial 1, B cells expressing the different immunoglobulin isotopes remained relatively constant in each tissue over time except that the IgM+ B cells in the distal PP were significantly increased ($p<0.05$) at week 2 post challenge compared with distal PP prior to challenge (Fig. 3b). In Trial 2, IgG-expressing cells were significantly increased in both proximal (Fig. 3d) and distal PP (Fig. 3e) at one week post challenge compared with week 2-4 ($p<0.05$).

Isotype expression between tissues: The percentage of IgA+ cells was significantly higher ($p<0.05$) in proximal PP compared with distal PP and CT prior to challenge in Trial 1 (Fig. 4a) but following SE challenge, this percentage decreased and was no longer different from those observed for distal PP and CT. A similar difference in pre-challenge proximal PP % IgA+ cells was not observed in Trial 2 (Fig. 4d). In Trial 1, the percentage of IgG+ cells increased in proximal and distal PP at one week post challenge to levels significantly higher than in CT (Fig. 4b). Similar results were observed in a repeat trial (Fig. 4e). The percentages decreased 2 weeks post challenge in Trial 1 so that all the percentage of IgG+ cells were similar in all three tissues but then increased a second time in the PP such that the percentages were significantly higher than in CT ($p<0.05$).

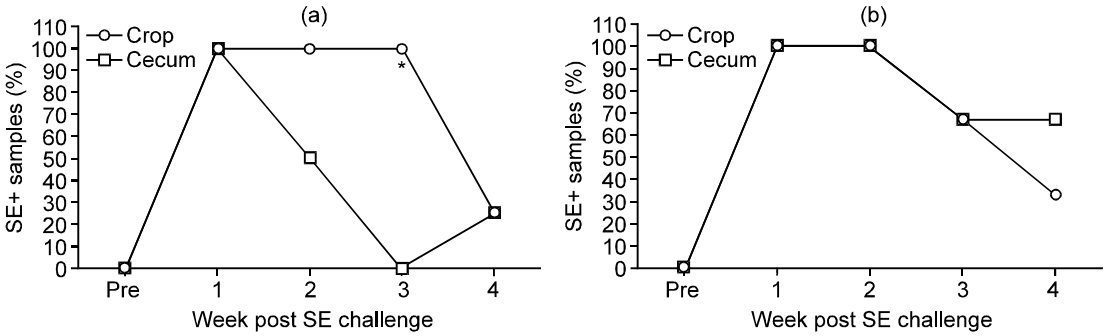

Fig. 2: Recovery of *S. enteritidis* (SE) from crop (open circle) and cecum (open squares) in trial 1 (a) and trial 2 (b). Results represent the % recovery of SE from the two tissues at various times post challenge. * = Significantly different recovery between crop vs cecum ($p<0.05$)

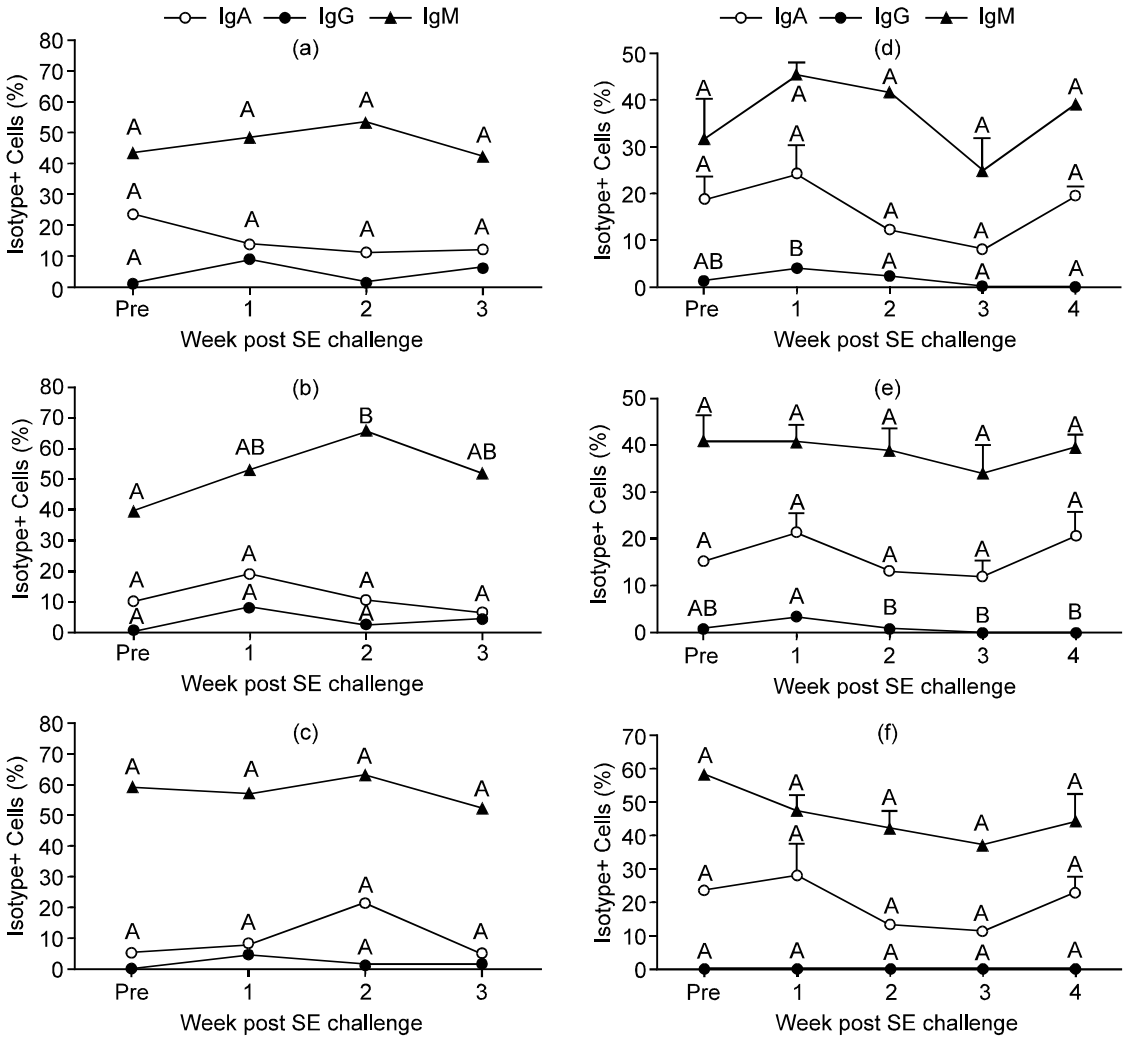

Fig. 3: Comparison of within-tissue changes of B cells expressing IgA (open circle) IgG (filled circle) and IgM (filled triangle) in proximal PP (a, d), distal PP (b, e) and CT (c, f) at various times post SE challenge. Results represent %B cells expressing the different immunoglobulin isotopes in trial 1 (a-c) and trial 2 (d-f). Letter change within a line represents significantly different population counts within a tissue on different days in relation to SE infection ($p<0.05$)

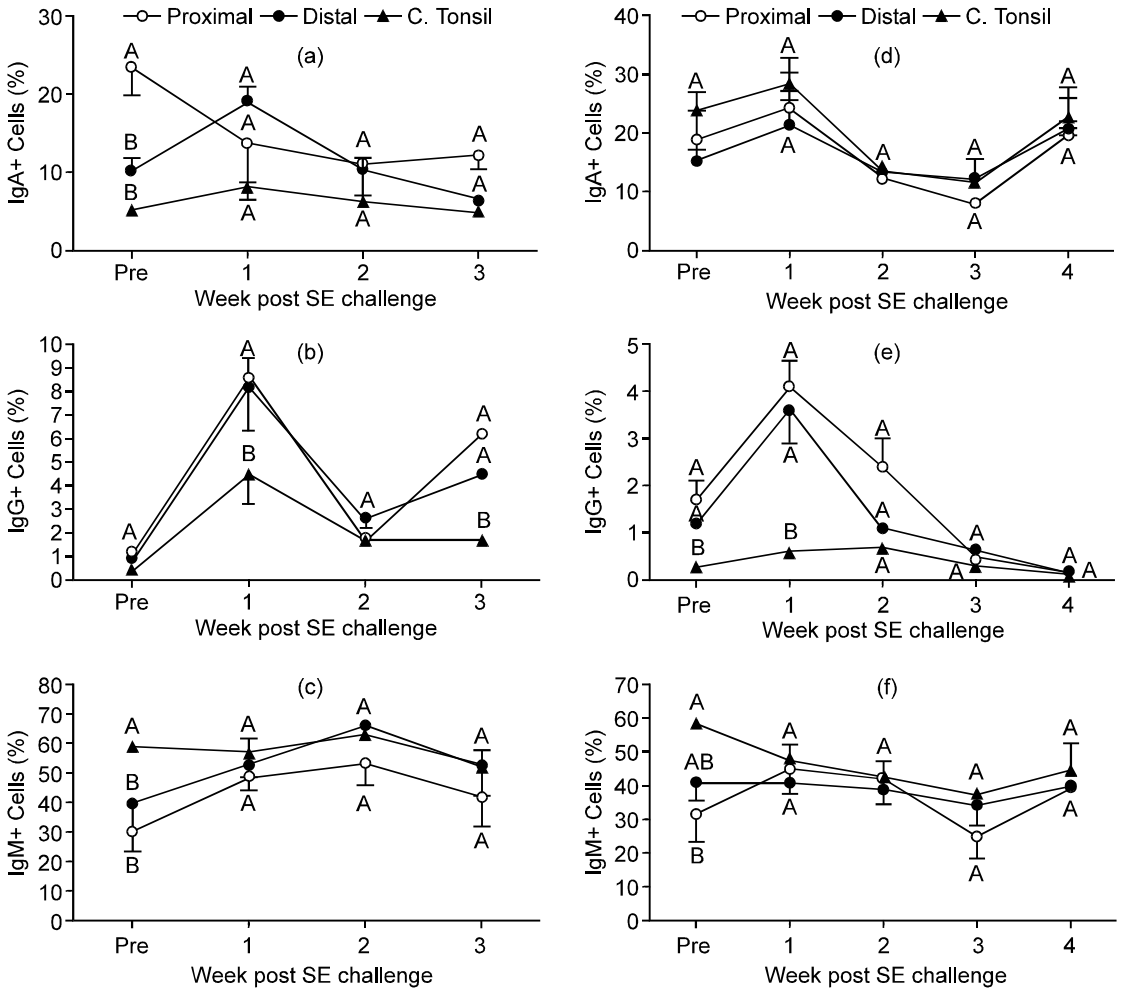

Fig. 4: Comparison of between-tissue changes of B cells expressing IgA (a, d), IgG (b, e) and IgM (c, f) in proximal (open circle), distal (filled circle) and cecal tonsils (filled triangle) at various times post SE challenge. Results represent % each tissue expressing a particular isotype in trial 1 (a-c) and trial 2 (d-f). Letter change at a particular time point represents significantly different population counts between tissues on different days in relation to SE infection ($p<0.05$)

DISCUSSION

Paratyphoid salmonellae such as SE are enteric organisms and preferentially colonize and invade the alimentary tract. This is reflected in the current study where both the crop and the cecum were 100% colonized at one week post challenge and in trial 1, the crop remained colonized longer than the cecum. Similar extended colonization of the crop vs the cecum has been reported previously (Barrow *et al*., 1988; Hargis *et al*., 1995). Because of the predilection of salmonellae for the alimentary tract, the development of immunity in the gut is crucial to the protection against or resolution of an infection by SE. Organs such as CT and PP play an important role in this protection by providing sites of concentrated lymphoid tissue to amplify and regulate immunity in the gut (Keren *et al*., 1978; Sasai *et al*., 2000; Makala *et al*., 2002). Because of the difficulty in locating PP in the chicken, however, few studies of this tissue have been published and as a result, little information is available regarding physiologic and functional characteristics of chicken PP. A recent staining procedure developed in our laboratory enables researchers to locate chicken PP in the ileum and conduct studies on the tissue and its constituent cells. Comparing T lymphocyte populations from PP with those from CT, Holt *et al*. (2010) showed that the tissues exhibited similar gross T cell populations in uninfected hens versus hens infected with SE. However, some differences were observed, especially in the ratio of CD4/CD8 T cells which was higher in CT than PP and this was accentuated at one week post challenge.

Similarly, tissue differences in B cell isotype expression were observed and this was affected by infection. Minimal differences in the percentage of IgG+ B cells could be observed between the two PP and CT prior to challenge but both PP exhibited significantly greater IgG+ B cell percentages at one week post challenge compared with CT (Fig. 4b,e). These differences decreased to nonsignificant levels by 2 weeks post challenge but increased to significant levels at week 3 in one trial. The significance of this observation remains to be determined considering that the IgG+ B cell population is the lowest in these tissues. However, Hassan *et al*. (1991) showed that a significant IgG anti-*Salmonella* response could be detected in the guts of chickens infected with *S. typhimurium* indicating that while the density of IgG+ B cells is low in these tissues, their role in gut immunity may still be important. B cells expressing IgM antibodies were significantly higher in CT compared with the PP prior to infection but shifted to nonsignificant levels following the SE challenge (Fig. 4c,f). The shift in percentage of cells expressing this isotype following challenge is probably a reflection of isotype switching that occurs in B cells following antigenic stimulation (Shikina *et al*., 2004; Stavnezer and Amemiya, 2004).

Infection by paratyphoid salmonellae such as SE can occur at many stages of a chicken's lifespan and severity of infection depends upon the age of the individual. In very young birds, the infection can be severe, resulting in significant morbidity and mortality (Gast and Holt, 1998) and dramatic depression of the immune response against the infecting organism (Holt *et al*., 1999). Significant changes in T cell subsets could be observed in the blood (Berndt and Methner, 2001), spleen (Sasai *et al*., 1997), the cecal mucosa (Berndt and Methner, 2001; Berndt *et al*., 2007) or jejunum mucosa (Van Hemert *et al*., 2007) of young chicks challenged with *Salmonella*. Changes in cecal tonsil B cell subpopulations of chicks following SE challenge however, were less dramatic (Sasai *et al*., 2000) and resembled those observed in the current study. These results indicate that B cell populations, at least in gut lymphoid tissues, are more refractory than T cells to effects of gut infections by paratyphoid salmonellae.

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Nomenclature: FITC, Fluoresceine isothiocyanate; PBS, Phosphate buffered saline; PP, Peyer's patch; R-PE, R-phycoerythrin; S., *Salmonella*; IgM, Immunoglobulin M; IgA, Immunoglobulin A; IgG, ImmunoglobulinG; APC, Allopyocanin; XLT4, Xylose lysine tergitol-4 agar; SE, *Salmonella enterica* serovar Enteritidis; BSL-2, Biosafety level-2.

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