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EARLY INDUCTION AND AUGMENTATION OF PARASITIC ANTIGEN-SPECIFIC ANTIBODY-PRODUCING B LYMPHOCYTES IN THE NON-PEYER'S PATCH REGION OF THE SMALL INTESTINE

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1. ABSTRACT

In this study, B lymphocytes from the small intestine of immunized rats were examined for their expression of specific antibodies against *Trichinella spiralis* (TS) antigen. The isotypes of the antigen-specific antibodies on B cells were examined via immunofluorescence microscopy. Monoclonal mouse anti-rat IgE, IgG1, IgG2a, IgG2b, IgG2c, IgA and IgM primary antibodies in conjunction with FITC-conjugated goat anti-mouse Ig secondary antibody and XRITC-conjugated 9D4 *T. spiralis* antigen were used to study the dynamics of the appearance of activated B lymphocytes in the small intestine, Peyer's patch, both the germinal center (PP-GC) and the non-germinal center (PP-NGC), the mesenteric lymph node (MLN), and the spleen. The results demonstrate that activated B cells are elicited by TS in the non-Peyer's patch region of the small intestine to express all isotypes of antibodies against TS antigen. IgG- and IgE-producing cells (Ab-PC) began proliferation only 1 and 2 days after infection, respectively. The strongest response was mounted by the IgE-PC in the lamina propria of the intestine. The response by IgA-PC generated was not only significantly delayed and also much weaker than that of the IgE- and IgG-PC. Peyer's patches failed to be a significant contributor in this immune response. Although this antigen-specific immune response was produced in the MLN and the spleen, it was weaker than that of the small intestine. The study indicates the potential ability of an immunized host to generate an early, yet effective, humoral immunity against *T. spiralis* in the non-Peyer's patch region of the small intestine."

2. INTRODUCTION

Most investigations concerning infection and induction of intestinal immune response with infectious organisms other than TS, to date, have focused on the response of IgA-producing B lymphocytes in the Peyer's patches of the small intestine (1-6). However, intestinal immune responses that take place in areas other than the Peyer's patches are only beginning to be closely studied. Likewise, the dynamics of early activation of intestinal B lymphocytes by TS have yet to be characterized. Since the non-lymphoid regions of the small intestine possess far more disseminated lymphocytes than all of the Peyer's patches combined, and antibodies of the IgG and IgE isotypes are more efficient in many immune responses than IgA, it is therefore important to understand the mechanisms and dynamics of activation of IgG- and IgE-producing B lymphocytes in the non-lymphoid regions of the small intestine.

Helminthic parasites have been observed to augment IgE level in humans and experimental animals, resulting in protective immunity (7-8). But the contribution of IgE to protective immunity in helminthic infections has been fairly elusive, with past research showing no strong evidence as to its role. Recently, however, research has shown direct evidence that IgE (9) as well as IgG (10) are involved in the rapid expulsion of *Trichinella spiralis* infectious larvae by immune rats. Similar immunity involving IgE has also been found in humans (11) and mice (12, 13). Some recent research further shows evidence of IgE-mediated protective immunity induced by *T. spiralis* to take place in the epithelial layer of the gut during rapid expulsion (14). Evidence also shows a rapid dissemination of IgE into the

intestinal tissue and fluids upon production by B cells, and a delayed appearance of the IgE molecules in the blood circulation (15, 16). These findings support the view that intestinal binding of IgE is not due to parasite-induced local damage or inflammation, but is influenced by IL-4, which stimulates the production of IgE antibodies by B lymphocytes (17). It has been demonstrated that not only did IgE increase during TS infection, concomitant augmentation of IgG was also found in serum (18, 19) and anti-TS IgA was detected in serum and mucus scrapings (20). However, whether intestinal B cells in areas other than the Peyer's patches produce these antibodies has not been studied.

There has been some evidence suggesting IgE-bearing cells seen in the gut of nematode-infected rats to be mast cells due to their expression of receptors specific to the Fc portion of IgE (21). Our study has shown, however, that in the intestine, significant increase in mucosal mast cells takes place at least 9 days after activated T helper cells are transferred (22), indicating that they do not affect the early examination of IgE-bearing B cells. B lymphocyte stimulation by IL-4 and the subsequent production of IgE are known to require genetic differentiation after activation by IL-4 in order to express specific immunoglobulin classes. Yet, this process does not necessarily proceed with a heavy chain switch recombination, thus allowing for the earlier appearance of IgE than was previously thought (23). Recently, it has been shown that eosinophils confer potent antibody-dependent cell-mediated cytotoxicity (ADCC) against TS invasion (17). ADCC is most efficient when the target cell is precoated with antibody, as has been observed in the presence of antibodies against TS newborn larvae (24). TS activated T cells have been identified in the non-Peyer's patch region of the intestine of immunized rats (25) and they have been found to activate B cells in the MLN and the spleen (22).

The purpose of this research was to gain a further understanding of the mechanisms through which *T. spiralis* activate antibody-producing B lymphocytes in the non-lymphoid regions of the small intestine. It systematically examined the ability of B lymphocytes from the small intestine of immunized rats to express specific antibodies against *Trichinella spiralis* antigen during an early stage of a primary infection and the host's primary immune response. In addition, the isotypes of specific antibodies produced were identified and the dynamics of appearance of activated B lymphocytes determined. The significance of this study is to reveal the potential ability of an immunized host to generate an early, yet effective, humoral immunity against *Trichinella spiralis* at the site of non-Peyer's patch region of the small intestine that has been traditionally neglected.

3. MATERIALS AND METHODS

3.1. Animals

Male or female SD rats of 6 or more weeks of age were used in all experiments. Rats were purchased from Harlan Sprague Dawley, Inc (Indianapolis, Indiana), housed at the California State University San Bernardino vivarium, and given food and water *ad libitum*. Animals were randomized into seven groups of three. The pooled control group contained a total of six animals.

3.2. Parasite

Trichinella spiralis was received from Dr. R. G. Bell at the Baker Institute for Animal Health, Cornell University, and maintained by serial passage in retired breeder rats of DA or PVG strains that had been infected with 5,000 muscle larvae at least 30 days prior to use.

3.3. Collection of muscle larvae

The procedures used to isolate the larvae from infected rats and to count intestinal worms have been previously described (26, 27). Briefly, infectious muscle larvae were obtained from minced rat carcasses by pepsin hydrochloride digestion at 37°C for 1 hr. Larvae were isolated by pouring the digested fluid through cheesecloth to remove bones and undigested material and then poured through a 200-mesh sieve, which retained the larvae.

3.4. Preparation of adult antigen

Preparation of the adult antigen of *T. spiralis* has been previously described (27). Briefly, the intestines were removed from rats three days after an oral infection with 8000 *T. spiralis* muscle larvae. The collected worms were sonicated to obtain crude larval antigen. A rat monoclonal antibody, 9D4, against *T. spiralis* antigen (28, 29) was used to affinity purify the 9D4 antigen (29), also called the Ts L1 antigen, which was further conjugated with tetramethylrhodamine isothiocyanate (XRITC, Sigma, St. Louis, MO) fluorescent dye. The 9D4 antigen was first diluted in 0.15 M Nacl and further diluted in the conjugation buffer, 0.5 M, pH 9.5, prepared by mixing 5.8 ml of 5.3% Na_2CO_3 with 10 ml of 4.2% $NaHCO_3$. The diluted 9D4 antigen was then mixed with XRITC at a ratio of 30 ug of XRITC per mg of protein antigen and the mixture was gently rotated overnight at 4^{0}C. The conjugate was dialyzed against 0.15 M NaCl in order to remove the free dye. After several changes, the final preparation was dialyzed in PBS and centrifuged to remove unbound material.

3.5. Infection with muscle larvae

Large numbers of *T. spiralis* muscle larvae were collected from the pepsin digest previously described. The muscle larvae were flushed from the sieve with 0.85% NaCl then washed again three times with 0.85% NaCl and counted microscopically. A total of 2000 larvae per 1 ml of saline were administered using a feeding tube to rats lightly anesthetized with halothane.

3.6. Obtaining tissues and cryohistological preparation

In order to analyze the isotypes of antibodies produced by the intestinal B lymphocytes and the quantities and tissue distribution of isotype-specific antibody-producing B cells in the intestine, intestinal tissue (15 cm distal to the duodenum), Peyer's patch (the nearest region to the intestine taken), mesenteric lymph node, and spleen were obtained from normal uninfected rats on days 0 and 5, while the same tissues from rats infected orally with 2,000 *Trichinella spiralis* muscle larvae (a standard low dose) were collected on days 1, 2, 3, 5, and 7 following infection (early phase infection). The fresh tissue was histologically processed by dipping in cold isopentane (2- Methylbutane, Fisher Chemical, Fisher Scientific, Fair Lawn, NJ) and transferring the dissected tissue to an aluminum foil cup containing Optimum Cutting Temperature (OCT) embedding medium (Miles Scientific, Naperville, IL) and then frozen in liquid nitrogen. Tissue samples were stored at -80°C until processed. Frozen sections of 6 um were cut from the intestine, MLN, spleen, and Peyer's patch of infected as well as control rats using a Lab Tek Precision Microtome Cryostat (Lab Tek Instruments Co.,

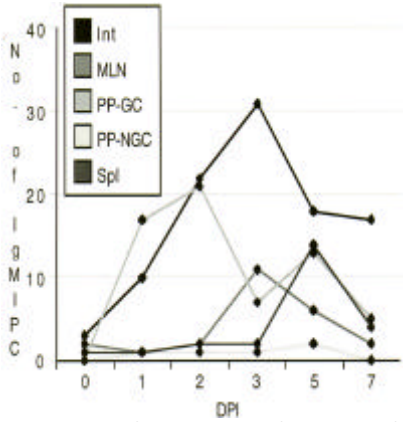

Figure 1. Kinetics of appearance of 9D4-specific IgM-PC in rats infected with *T. spiralis*. Five groups of 3 rats were infected on day 0 with 2,000 muscle larvae orally and their intestinal (INT), mesenteric lymph node (MLN), Peyer's patch (PP-GC: Peyer's patch germinal center; PP-NGC: Peyer's patch non-germinal center), and spleen (SPL) tissues were obtained on days 1, 2, 3, 5, or 7, respectively, with day 0 as the pooled uninfected controls. Same tissues of 3 of these uninfected rats were taken from day 0 and of another 3 taken on day 5. All the tissues were histologically processed and labeled first with monoclonal mouse-anti-rat IgM (H chain specific) antibody, and then with FITC-goat anti-mouse IgG ($F(ab)'_2$) and XRITC-9D4 antigen. Data represent mean numbers (with SD not shown) of 3 rats per infected group and 6 rats in the pooled control group. Comparing to the controls, significance ($p<0.05$) was only found in the number of IgM-PC in the intestine on days 2-5, in the MLN on day 3, in the PP-GC, on days 1-5, and in the spleen, on days 5 and 7.

Westmont, IL), placed on slides coated with poly-L-lysine, and allowed to air dry. Three sections were prepared for each of the tissue samples and then fixed with 2% paraformaldahyde at room temperature for 20 min, washed in PBS pH 7.2 for 5x for 5 min each time.

3.7. Immunofluorescence assay (IFA)

A double labeling immunofluorescence assay was used to reveal the distribution of different B cell populations located within the tissue sections on the slides. The dynamics of the various isotypes of antibodies produced by B cells, specifically IgE, IgA, IgM, IgG1, IgG2a, IgG2b, and IgG2c, were determined by quantification of the frozen sections using immunofluorescence microscopy. The sections were incubated for 1 hr at room temperature with 1:500 diluted monoclonal mouse anti-rat IgE, IgG1, IgG2a, IgG2b, IgG2c, IgA, and IgM (Zymed Labs Inc., San Francisco, CA). These monoclonal antibodies were H chain specific and in the $F(ab)_2'$ form to prevent non-specific binding to the Fc receptors. The primary antibody was aspirated off and the slides were then washed with PBS pH 7.2 three times for 5 min each. Fluorescein (FITC) conjugated goat anti-mouse Ig ($F(ab)'_2$) and rhodamine (XRITC) conjugated 9D4 *T. spiralis* antigen were used in a 1:1000 dilution. The FITC-Ig and XRITC-9D4 were mixed 1:1. Both incubations were done by placing 20 µl drops on the sections. Incubation with fluorescent conjugates was done in the dark for 1 hr at room temperature. The slides were then washed again with PBS pH 7.2 three times for 5 min each and mounted in 60% (v/v) glycerine-PBS, or washed a fourth time in dH_2O pH 7.2 and mounted with Fluorsave (CalBio Chemical Corp., La Jolla, CA). The conjugated antibodies were visualized using a Nikon optiphot biological microscope with an episcopic-fluorescence attachment EF-D containing a B2A 495nm filter specific for FITC and a G2A 546nm filter specific to XRITC. Double labeled cells in at least 20 villus crypt units (VCU) per intestine, and 20 fields (400x) per section for the spleen, Peyer's patch, and mesenteric lymph node were enumerated and data recorded.

3.8. Statistical analysis

The significance of differences in mean values was first determined by comparing independent means using analysis of variance. Days of infection producing significant F values were further analyzed by Newman-Keul's multiple-range test for each day, as compared to the pooled control and to the previous day of infection. Probability levels less than 0.05 were considered significant.

4. RESULTS

To determine the kinetics of the appearance of 9D4 antigen-specific IgA-, IgG1-, IgG2a-, IgG2b-, IgG2c-, IgE-, and IgM-producing B lymphocytes (Ig-PC), SD rats were infected with 2,000 *T. spiralis* muscle larvae on day 0, and tissue samples were taken from the small intestine , Peyer's patch, mesenteric lymph node, and the spleen on days 1, 2, 3, 5 and 7. Control samples were taken on days 0 and 5 from uninfected rats. Frozen sections of the tissues were double labeled with monoclonal mouse anti-rat specific immunoglobulin, followed by fluorescein (FITC)-conjugated goat anti-mouse nonspecific Ig- and rhodamine (XRITC)-conjugated monoclonal antibody affinity purified *T. spiralis* 9D4 antigen. The number of 9D4 antigen-specific Ig-PC was quantified by immunofluorescence microscopy.

4.1. Kinetics of appearance of 9D4 antigen-specific IgM-producing B lymphocytes

The tissues described above were labeled with both XRITC-conjugated 9D4 antigen and the monoclonal mouse anti-rat IgM antibody plus the FITC-conjugated goat anti-mouse Ig to determine the number of 9D4 antigen-specific IgM-producing B cells. The results show (figure 1) that in the non-Peyer's patch region of the small intestine, the number of 9D4-specific IgM-PC per VCU increased significantly from day 2 (22 $\pm$ 3) onto day 5 (18 $\pm$ 4) as compared to the pooled controls (3 $\pm$ 2), with day 3 being the peak response (31 $\pm$ 5). In the germinal centers of the Peyer's patch, significant increase was detected from day 1 onto day 5, with days 1, 2, 3 and 5 showing 17 $\pm$ 2, 21 $\pm$ 2, 7 $\pm$ 4, and 11 $\pm$ 7 double labeled cells, respectively, as compared to the control of 0 $\pm$ 1. Throughout the duration of examination, no significant increase in the number of 9D4-specific IgM-expressing B lymphocytes was observed in the non-germinal centers of the Peyer's patch. The number was augmented significantly only on day 3 (11 $\pm$ 7) for the MLN, and on days 5 (14 $\pm$ 1) and 7 (4 $\pm$ 1) for the spleen.

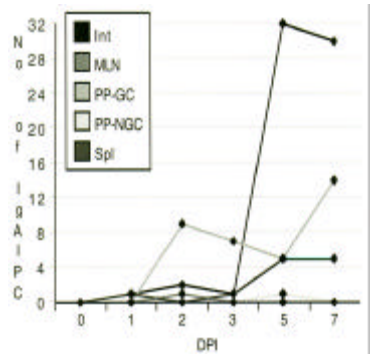

Figure 2. Kinetics of appearance of 9D4-specific IgA-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgA (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine and the spleen on days 5 and 7.

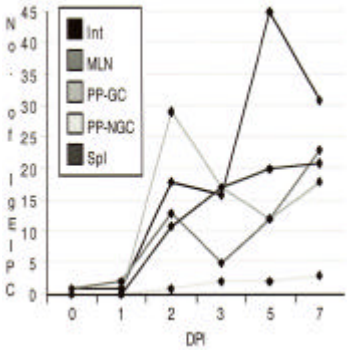

Figure 3. Kinetics of appearance of 9D4-specific IgE-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgE (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine and the PP-GC from day 2 through 7, in the MLN on days 2 and 7, and in the spleen from day 3 to 7.

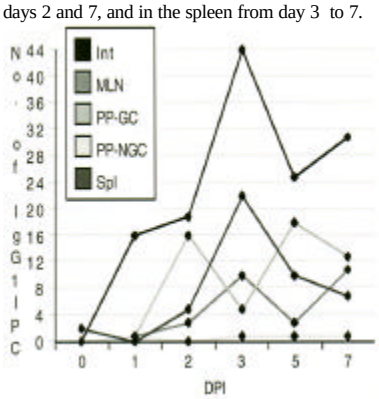

Figure 4. Kinetics of appearance of 9D4-specific IgG1-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgG1 (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine from day 1 through 7, in the MLN on days 3 and 7, in the PP-GC on day 5 alone, and in the spleen on day 3 alone.

4.2. Kinetics of appearance of 9D4 antigen-specific IgA-producing B lymphocytes

When the same tissues were stained with both the XRITC-conjugated 9D4 antigen and the FITC-labeled goat anti-mouse antibody in addition to the monoclonal mouse anti-rat IgA antibody, insignificant results were obtained in tissues on almost all days examined with the exception of days 5 (32 $\pm$ 9) and 7 (30 $\pm$ 9) for the small intestine, and on the same days, both days 5 and 7, showing 5 $\pm$ 1 9D4-specific IgM B cells in the spleen (figure 2). The Peyer's patch revealed 9D4-specific IgA-PC in two different cell populations. Germinal center populations (PP-GC) contained some cells with immunoglobulins aggregated in clumps exhibiting a bright uneven signal, but showed insignificant numbers of IgA-PC to be present for the duration of the infection as mentioned above (0 $\pm$ 0 on days 0 and 1, 9 $\pm$ 5 on day 2, 7 $\pm$ 2 on day 3, 5 $\pm$ 2 on day 5, and 14 $\pm$ 10 on day 7 of infection). In the non-germinal center regions (PP-NGC), one could barely detect any change in the infected rats from the pooled control throughout the experiment (0 $\pm$ 0 IgA-PC on days, 0, 1, 2, 3 and 7, and 1 $\pm$ 1 per field of vision on day 5 of infection). Similar results were found in the mesenteric lymph node (0 $\pm$ 0 on days 0 and 3, 1 $\pm$ 0 on day 2, and 0 $\pm$ 1 per field of vision on days 1, 5 and 7 of infection).

4.3. Kinetics of appearance of 9D4 antigen-specific IgE-producing B lymphocytes

As shown in figure 3, the non Peyer's patch region of the intestinal samples labeled with monoclonal mouse anti-rat IgE and 9D4 antigen showed a significant increase in 9D4-specific IgE-PC beginning on day 2 of infection (18 $\pm$ 9 per VCU) and maintained the significant pattern of augmentation for the remainder of the experiment (16 $\pm$ 9 on day 3, 45 $\pm$ 9 on day 5, and 31 $\pm$ 14 on day 7 of infection), as compared to the pooled control (1 $\pm$ 3). Another significant increase was observed on day 5 of infection, as compared to day 3, whereas a significant decrease was observed on day 7 of infection, as compared to day 5 while maintaining significance from the pooled control. The Peyer's patch germinal centers (figure 3) also showed significant numbers of 9D4-specific IgE-PC from day 2 onward (29 $\pm$ 16 on day 2, 17 $\pm$ 12 on day 3, and 12 $\pm$ 5 and 18 $\pm$ 8 for days 5 and 7, respectively), as compared to the pooled control (0 $\pm$ 1). The non-germinal center regions of the Peyer's patch failed to show any significant increase in IgE-PC throughout the duration of the experiment, as compared to the pooled control (0 $\pm$ 1 on days 0 and 1, 1 $\pm$ 1 on day 2, 2 $\pm$ 0 on day 3, 2 $\pm$ 1 on day 5, and 3 $\pm$ 3 on day 7 of infection). Significant increase in 9D4-specific IgE-PC occurred in the mesenteric lymph node (figure 3) on days 2 (13 $\pm$ 0) and 7 of infection (23 $\pm$ 10 per field of vision), as compared to the pooled control (1 $\pm$ 0), with day 5 (12 $\pm$ 6) showing insignificant result. The spleen (figure 3) showed a significant increase in the mean on day 7 of infection, figure 4). The Peyer's patch, though, only showed a significant increase of 9D4-specific IgG1-PC in the germinal center regions on day 5 of infection (18 $\pm$ 7), as compared to the pooled control (0 $\pm$ 1), and the number dropped to insignificance again on day 7 of infection (13 $\pm$ 6 per field). Non-germinal center regions of the Peyer's patch did not significantly differ from the pooled control (00) throughout the entire period examined (figure 4). In the mesenteric lymph node, a significant increase was first number of antigen-specific IgE-PC from day 3 of infection (17 $\pm$ 9 per field), and remained significant throughout the remainder of the experiment, as compared to the pooled control (0 + 1 on day 1, 20 $\pm$ 16 on day 5, and 21 $\pm$ 13 per field of vision on day 7 of infection).

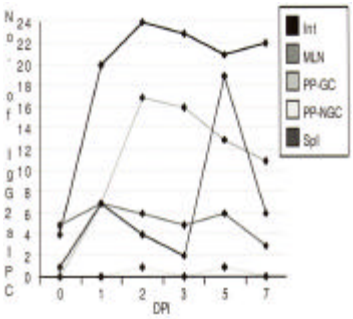

Figure 5. Kinetics of appearance of 9D4-specific IgG2a-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgG2a (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine and the PP-GC from day 2 through 7, and in the spleen on days 1, 5 and 7.

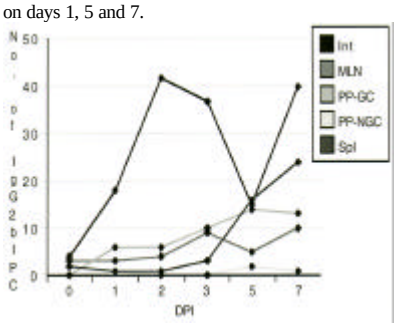

Figure 6. Kinetics of appearance of 9D4-specific IgG2b-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgG2b (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine from days 2, 3 and 7, in the MLN on days 3 and 7, in the PP-GC and the spleen on days 5 and 7.

to climb higher for the following days, with day 3 showing 33 ± 3, day 5, 34 ± 3 and day 7, 38 ± 5 antigen-specific IgG2c-PC per VCU. No dip was observed throughout this

4.4. Kinetics of appearance of 9D4 antigen-specific IgG1-producing B lymphocytes

Intestinal samples taken from infected rats and labeled with monoclonal mouse anti-rat IgG1 and the 9D4 antigen showed a significant increase in 9D4-specific IgG1-PC as early as day 1 of infection (16 ± 6). This increasing trend peaked on day 3 (44 ± 10), as compared to the pooled control (0 ± 0). Although it declined from the peak for the remaining period examined, but it was still significantly above the control (25 ± 4 on day 5 and 31 ± 12 per VCU ± observed on day 3 (10 ± 5), comparing to the pooled control (0 ± 1). A significant decrease in 9D4-specific IgG1-PC numbers was then observed on day 5 of infection (3 ± 4), as compared to day 3. However, day 7 showed another increase to a significant mean (11 ± 4), as compared to the pooled control and day 5 of infection (figure 4). In the spleen (figure 4), significantly enhanced 9D4-specific IgG1-PC numbers were only detected on day 3 of infection (22 ± 8) over that of the pooled control (2 ± 4).

4.5. Kinetics of appearance of 9D4 antigen-specific IgG2a-producing B lymphocytes

The results presented in figure 5 showed that the intestinal tissues labeled with monoclonal mouse-anti-rat IgG2a and the 9D4 antigen had a pooled control mean of 4 ± 5 9D4-specific IgG2a-PC per VCU. On day 1, the number was 20 + 18, an insignificant result. Beginning on day 2, however, 24 ± 8 of antigen-specific IgG2a-PC were enumerated per VCU, which was significantly higher than the control. Similar level of augmentation was revealed on the following days examined, with 23 ± 7 on day 3, 21 ± 9 on day 5, and 22 ± 11 per VCU on day 7 of infection, all of which were significantly higher than the control. The Peyer's patch germinal centers (figure 5) maintained significant numbers of 9D4-specific IgG2a-PC throughout the experiment, as compared to the control (0 ± 0 on day 0, 7 ± 2 on day 1, 17 ± 7 on day 2, 16 ± 2 on day 3, 13 ± 4 on day 5, and 11 ± 2 per field of vision on day 7 of infection). Non-germinal center of the Peyer's patch showed insignificant numbers of 9D4-specific IgG2a-PC for the duration of the experiment, as compared to the pooled control (0 ± 0 on days 0, 1, 3 and 7, 1 ± 1 on day 2 and 1 ± 2 per field of vision on day 5). Similar insignificant kinetics were found in the mesenteric lymph node (figure 5) throughout the duration of the experiment, with 5 ± 4 on day 0, 7 ± 2 on day 1, 6 ± 1 on day 2, 5 ± 4 on day 3, 6 ± 3 on day 5, and 3 ± 3 per field of vision on day 7 of infection. The spleen (figure 5) showed a significant mean number of antigen-specific IgG2a-PC on day 1 of infection (7 ± 2 per field of vision), as compared to the pooled control (1 ± 1). A significant decrease in mean number was observed by day 3 (2 ± 1), as compared to that of day 1. This was followed by another significant increase on day 5 (19 ± 1). Again, the mean number of 9D4-specific IgG2a-PC dropped on day 7 (6 ± 2) but remained significant from the pooled control.

4.6. Kinetics of appearance of 9D4 antigen-specific IgG2b-producing B lymphocytes

The non-Peyer's patch region of the small intestinal tissues labeled with monoclonal mouse anti-rat IgG2b and the 9D4 antigen contained a significantly increased number of the 9D4-specific IgG2c-PC per VCU on days 2 (42 ± 25), 3 (37 ± 6) and 7 (40 ± 16) over the pooled control (4 ±3), with days 1 (18 ± 15) and 5 (15 ± 15) being insignificant due to the large variations (figure 6). The Peyer's patch germinal centers (figure 6) showed significant numbers of 9D4-specific IgG2b-PC on days 5 (14 ± 4) and 7 (13 ± 10) of infection, as compared to the pooled control (0 ± 0). The non-germinal center regions did not show any significant increase in the same cells for the duration of the experiment, as compared to the pooled control (0 ± 0). The mesenteric lymph node (figure 6) harbored significantly increased numbers of 9D4-specific IgG2b-PC over the control (3 ± 1) on days 3 (9 ± 3) and 7 (10 ± 1) only. There was a significant decline in the number of antigen-specific IgG2b-PC on day 5 (5 ± 0). The spleen (figure 6) presented a significant increase in 9D4 directed IgG2b-PC on days 5 (16 ± 14) and 7 (24 ± 1) only, as compared to the pooled control (2 ± 1).

4.7. Kinetics of appearance of 9D4 antigen-specific IgG2c-producing B lymphocytes

When the non Peyer's patch tissues of the small intestine were examined after they had been labeled with monoclonal mouse anti-rat IgG2c and the same antigen used throughout the experiment, a similar pattern of kinetics was observed. As early as 2 days after infection, the number of 9D4-specific IgG2c-PC jumped significantly (27 ± 8) over the controls (2 ± 3). Such significant proliferation continued

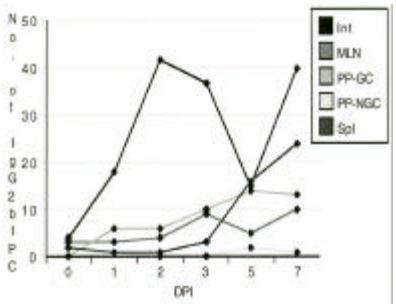

Figure 7. Kinetics of appearance of 9D4-specific IgG2c-PC in rats infected with *T. spiralis*. The experimental procedure is as described in figure 1, except the primary antibody used was monoclonal mouse anti-rat IgG2c (H chain specific). Comparing to the controls, significance ($p<0.05$) was only found in the intestine from day 2 through 7, in the MLN on days 2 and 7, in the PP-GC on days 3 and 5, and in the spleen on days 5 and 7.

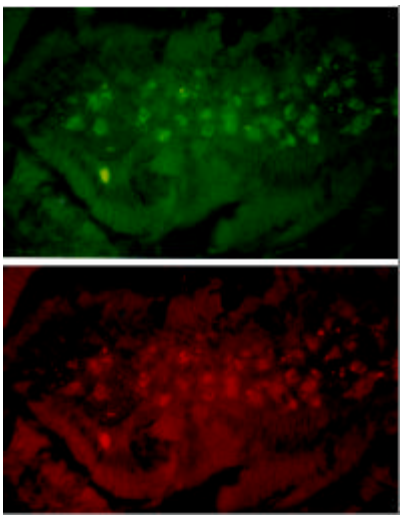

Figure 8. Exhibition of 9D4 antigen-specific IgE-expressing cells in the lamina propria of the small intestine. The experimental procedures are described in figures 1 and 3. The intestinal tissue shown in these photographs was obtained from rat 5 days after infection with 2,000 muscle larvae of *T. spiralis*. The cryosection of the intestinal tissue was first labeled with monoclonal mouse anti-rat IgE (H chain specific) antibody and then, further labeled with FITC-goat anti mouse Ig ($F(ab)'_2$) and XRITC-9D4 antigen. Figure 8a, the one shows the green color, reveals IgE-producing cells labeled with the FITC dye in one villus crypt unit (VCU). Almost all the labeled cells occur in the lamina propria of the intestinal tissue. Figure 8b, the one shows the red color, demonstrates that the same cells in the identical VCU labeled with the FITC dye were also labeled with the XRITC dye, indicating that these cells were expressing IgE antibody molecules on the surface membrane that were specifically binding the 9D4 antigen. The size of the labeled cells range from 12 to 16 um in diameter.

period (figure 7). The germinal centers of the Peyer's patch showed a significant increase on days 3 (15 $\pm$ 11) through 5 after infection (14$\pm$7), followed by a decrease in mean number of 9D4-specific IgG2c-PC to insignificance by day 7 (5 $\pm$ 1), as compared to the pooled control (1 $\pm$ 1, figure 7). Once again, no significant change in kinetics of the numbers of 9D4-specific IgG2c-PC was demonstrated in the non-germinal center regions of the Peyer's patch. The mesenteric lymph node showed a significant increase in these cells on days 2 (11 $\pm$ 3) and 7 of infection (23 $\pm$ 7), as compared to the pooled control (2 $\pm$ 2). In between these two dates, a decline was observed (figure 7). No significant augmentation was demonstrated in the spleen until the last two days examined, with day 5 having 18 $\pm$ 4 and day 7, 35 $\pm$ 10 double labeled cells against the controls (0 $\pm$ 0).

An example of the color signals produced by this labeling technique can be seen in figure 8, which contains photographs of samples of the non Peyer's patch intestinal tissue labeled with monoclonal mouse anti-rat IgE primary antibody and the FITC-conjugated goat anti-rat Ig in combination with the XRITC-conjugated 9D4 antigen on day 5 of infection. Figure 8a shows the cells positively labeled with the FITC dye, indicating the IgE-PC, and figure 8b reveals the same cells in the identical field of examination showing the XRITC emission, indicating the 9D4 antigen-specificity of these cells. In both micrographs, the labeled cells were found in the lamina propria of the small intestine.

5. DISCUSSION

The experiments reported here quantitatively examined the non-Peyer patch tissues of the small intestine, Peyer's patch, mesenteric lymph node, and spleen of rats for the presence of B lymphocytes, expressing IgM, IgA, IgE, IgG1, IgG2a, IgG2b, and IgG2c antibody isotypes that were specific for monoclonal antibody affinity purified *T. spiralis* 9D4 antigen using a double labeling immunofluorescence assay. The results showed that merely 1 day after infection, 9D4 antigen-specific IgG1-PC first appeared in significant numbers in the lamina propria of the non-Peyer's patch tissue of the small intestine (figure 4). This was followed by drastic increases of antigen-specific IgM- (figure 1), IgE- (figure 3), and the rest of the subtypes of IgG-PC (figures 5-7) 24 hr later. This is the first piece of evidence demonstrating such an almost immediate B cell response after *T. spiralis* infection that is directly taking place right in the tissues of the small intestine that are not associated with the Peyer's patches.

It has been demonstrated that it takes some 12 hr before the first T cells to become activated in these tissues (25), and the 9D4 antigen is a glycosylated protein preparation (28, 29). Therefore, the B cells examined here most likely need the stimulation from the T cells due to the antigen being T cell-dependent. The fact that the 9D4-specific IgG1-PC increased 16 times (figure 4) over the control within 12 hr after T cells became activated, strongly demonstrates the immediacy of the interaction between the T and B cell populations in the tissues and the efficiency of this immune response. The proliferative response by IgE-PC increased 18 times (figure 3), and that of the IgM-, IgG2a-, IgG2b- and IgG2c-PC climbed up 7, 6, 10, and 13 times, respectively (figures 1, 5-7), all by 24 hr, over the controls. This further reveals the rapidity of the immune response in a much broader scope. When the kinetics are scrutinized in more detail, one can see that IgG2b-PC reached its peak only 2 days after infection, which was 10 times of the control. This was followed by a decline on day 5 and then a rebound by day 7 (figure 6). Whether this suggests a short half-life or a dissemination or migration of these cells needs to be further investigated. IgM- and IgG1-PC reached their peak 24 hr after IgG2b-PC, producing a 10x and 44 x augmentation, respectively (figures 1, 4)). IgE-PC climbed to the peak on day 5, which was 45 times over the control (figure 3), while IgG2c-PC produced the highest proliferation on day 7 (19 times of the control), the last day examined (figure 7). Although these kinetic curves do not necessarily correlate with the quantities of antibodies ultimately secreted by these B cells, the magnitude of

enhancement in the numbers of these Ab-PC only days after infection once again demonstrates the immunological competency of the non-Peyer's patch tissues of the small intestine. Our results correspond with previous evidence of high levels of serum IgG and IgE antibodies (9-11, 14, 18, 19) and evidence of rapid increase of IgE in intestinal fluids (15, 16) after *T. spiralis* infection. Therefore, it is more than likely that there is such correlation between the numbers of Ab-PC and the quantities of antibodies produced *in vivo*. In fact, when examining these double labeled cells as shown in figures 8a and 8b, one cannot help but noticing that aside from the labeled cells, there are quite a few small aggregated clumps within the lamina propria showing both the green and red color. Most likely, these are immune complexes formed between the secreted IgE antibodies and the 9D4 antigens, indicating that these IgE molecules are indeed produced by the IgE-bearing B cells in response to the parasitic challenge in the small intestine.

No proliferation of 9D4-specific IgA-PC was observed in the intestine until 3 to 4 days later as compared to the other isotypes of Ab-PC (figure 2). This shows that at least in this immune response, e. g. a multiple isotypic B cell response, IgA is not as important as IgG- and IgE-PC. This result is contrary to many of previous studies, in which IgA has always been found to be a vital immunological factor in the mucosal immune response against various pathogens and stimuli (1-6). Since IgA-PC has been associated with the function of the Peyer's patches, and Peyer's patches are not the tissue sites taken by *T. spiralis* as its niche, it is reasonable to believe that when Peyer's patches are not stimulated directly by a pathogen, IgA-PC are not generated in abundance.

In this study, the numbers of Ab-PC were quantified in two separate regions in the Peyer's patches, the germinal centers and the non-germinal tissue. It has been revealed that the latter region of the Peyer's patches played no role in all responses by B cells during the entire period studied (figures 1-7). However, in the germinal centers, a totally different kinetics appeared. Barely one day after infection, 9D4-specific IgM- and IgG2a-PC increased significantly over the controls by 17 and 7 times, respectively (figures1, 5). IgE-PC increased 29 times by day 2 (figure 3), which tapered off during the rest of the study period. A comparison of the patterns of kinetics of IgE-PC found in the intestine and in the PP-GC reveals a seemingly opposite trends, in which the former tissue generated a significant ascending curve, whereas the latter produced a gradual descending curve with days post infection (figure 3). First, this suggests that the *T. spiralis* antigen can quickly diffuse into the germinal centers of the Peyer's patches even though they are not the preferred sites for the parasitic invasion. Once the antigenic stimulation occurs, activated by T cells, Ab-PC like IgM-, IgG2a- and IgE-PC begin proliferating. Second, no statistical difference was found when comparing the numbers of IgE-PC in these two tissues on days 2 and 3. However, on days 5 and 7, intestinal tissue showed significantly higher numbers of IgE-PC than PP-GC. On day 5, the number found in the intestine per VCU was nearly 4x that of the PP-GC per field of view, and on day 7, it was nearly 2x that of the PP-GC (figure 3). The PP contains far higher density of lymphocytes than the non-Peyer's patch tissues of the small intestinal tissues. The data collected reflect numbers of Ab-PC per VCU in the intestine and that of Ab-PC per field of vision in the PP. As tissues, the former contains loosely associated and various types of cells, including lymphocytes, and the latter harbors densely packed lymphocytes. This shows that in this particular immune response, the intestine plays a leading role in generating Ab-PC against the invading parasite, not the Peyer's patches. The responses elicited by the other Ab-PC in the PP-GC were also weaker and significantly delayed as compared to that of the intestine, e.g., IgG2b- and IgG1-PC occurred 3 and 4 days later than those in the intestine, respectively (figures 4, 6). Once again, no IgA-PC response was observed in the PP-GC throughout the period studied (figure 2). Substantiating the point that IgA is not important in this immune response, these results also indicate that whatever IgA-PC produced in the intestine, they were produced by the intestinal tissues, not first by the PP, then migrated intestinally into the lamina propria. They also suggest that the parasitic antigen used is not an IgA inducing antigen.

In the MLN, no IgA- and IgG2a-PC were elicited (figures 2, 5). A significant response, yet 3x as weak as that of the intestine, was generated by IgM-PC in the MLN on day 3 only (figure 1). All the other Ab-PC produced significant yet weaker responses against the parasitic antigen, too (figures 3, 4, 6, 7). One interesting bi-peak phenomenon was observed in the response curves of IgE-, IgG2c-, IgG1, and IgG2b-PC. The first two Ab-PC showed a peak on day 2 and a peak on day 7, separated by a dip, and the last two Ab-PC had their first peak on day 3 and second peak on day 7, intervened with a similar decline (figures 3, 4, 6, 7). Similar pattern of response was also demonstrated in the IgM-PC in the intestine (figure 1) and the IgG2a-PC response in the spleen (figure 5). The difference between the peak and the valley is statistically significant. One reasonable explanation for this phenomenon is that these B cells, at least a proportion of them, undergoes migration from the intestine to the general circulation via the lymphatics and blood. Similar pattern of migration has been noticed among intestinally activated T cells using the same experimental system (25), and it is not surprising that these B cells may also hold the same pattern. When the responses by Ab-PC were examined in the spleen, it is demonstrated that although all isotypes of Ab-PC augmented their responses, they were weaker and delayed than the intestine (figures 1-7). Since the spleen is anatomically away from the antigenic stimulation site, these results are understandable.

Comparing the kinetics of appearance of Ab-PC in all tissues examined, it appears that nearly all of the different isotypic B lymphocytes become activated in non-Peyer's patch regions of the intestine either at the same time, or significantly earlier than in the Peyer's patch regions, except IgM-, and IgG2a-PC. These results strongly support the idea that most of the *T. spiralis* antigen-specific B lymphocytes are not derived from the Peyer's patches. In stead, they are originated, stimulated, and activated within the lamina propria of the intestine.

The significance of this study is that this is the first time intestinal B lymphocytes specific for a parasitic antigen have been visualized and quantified at such an early stage of a primary infection. The antigen-specific IgE-PC first appeared as early as 48 hr, and IgG1-PC just 1 day after infection, both occurred not only sooner than the appearance of IgA-PC, but also in greater overall numbers than IgA-PC in the Peyer's patch, mesenteric lymph node and the spleen. The study, therefore, demonstrates that the small intestine, when challenged, is capable of exerting an immediate and yet highly efficient immune response encompassing multiple cellular populations to protect its host.

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