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RAPIDITY AND MULTIPLICITY OF SYNTHESIS AND EXPRESSION OF IMMUNOGLOBULIN ISOTYPES BY B LYMPHOCYTES IN THE SMALL INTESTINE

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1. ABSTRACT

An immunofluorescence double labeling assay was used to examine the kinetics of intestinal B lymphocytes with concurrent expression of multiple antibody isotypes in the mucosal tissues of rats infected with *Trichinella spiralis* (TS) muscle larvae for 1 to 15 days. As compared to the uninfected controls (day 0), the non-Peyer's patch tissues of the small intestine contained a significantly increased number of dual antibody-expressing B cells as early as 3 days after infection with a maximum proliferation of these B cells on days 7 and 10. These results indicate the rapidity of B cell response in the small intestine. Similar results were observed in the germinal centers of the Peyer's patches. The non-germinal centers of the Peyer's patch tissues showed delayed kinetics in B cell activation which occurred 10 days after infection. Quantification of the total number of B cells in these tissues was also carried out by staining the CD45RA marker on B cells with the OX33 monoclonal antibody. When comparing the total numbers of B cells with the numbers of B cells expressing dual isotypes of antibodies, our results showed the numbers of dual-expressing B cells (IgA:IgE, IgM:IgE, IgG1:IgE, IgG2a:IgE, IgG2b:IgE, and IgG2c:IgE), when combined, were over 7 times that of the total number of OX33-labeled B cells on day 7 in the small intestine. The dual-expressing B cells in the Peyer's patch-germinal center were more than 5 times that of the OX33-labeled B cells on day 15. These results therefore suggest that the dual-expressing B cells most likely synthesized and expressed more than two isotypes of antibodies during the peak days of the humoral response. Such phenomenon was not observed in non-germinal centers of the Peyer's patch tissues.

2. INTRODUCTION

B lymphocytes are major players of humoral immune response against pathogens by producing antibodies (Ab) to interact with specific epitopes on antigens expressed by the pathogen. Once activated, B cells may undergo isotype switching. A general model for this process involves the looping out and deletion of C_H genes at the DNA level (1, 2). An alternative mechanism to deletional-isotype switching involves differential RNA splicing after transcription (3). This RNA splicing process allows the B cell to maintain its entire C region at the DNA level. Therefore the B cells are still able to express other Ab isotypes through production of new RNA transcripts. TS is a well-known parasite shown to activate B cells and induce Ab production (4-6). TS are able to infect the epithelial layer of the intestine in all mammals (7). This infection leads to active immune responses by both T cells and B cells that can lead to the eventual as well as rapid expulsion of the pathogen from the small intestine during primary and secondary immune responses, respectively. Most research on B cell involving intestinal pathogens has revolved around IgA production in the Peyer's patches (8-10); TS research is, only recently, turning to other regions and investigating the possibility of other isotypes of Ab being produced (4-6, 11). Numerous studies have demonstrated that multiple isotypes of

antibodies could be concurrently produced in serum as well as in the mucosal tissues against infectious agents (12-15). However, seldom has there been study addressing whether these concurrently produced various isotypes of antibodies are secreted from the same B lymphocytes.

Wang *et al* (16) showed that OX22- (Th2) T cells led to eosinopoiesis against TS in the intestine. There was a rapid proliferation of Th2 cells within 12hrs of TS infection. The fact that Th2 cells produce cytokines like IL-4 and IL-5 would suggest a wider range of isotypes of Ab (other than IgA) being produced by B cells against this pathogen. Previous studies have shown that IgE was transported from the plasma to intestinal tissue of TS infected rats (4). Wang *et al* (5) have shown an elevated production of all major Ab isotypes by B cells, and more recently a particularly high increase in IgE and IgG1 in the intestine of rats infected with TS (6). The results produced from Wang *et al* (5, 6) suggested the possibility that B cells may be capable of producing more than one Ab isotype on its surface at a given time towards the TS Ag. Few studies have dealt with the possibility of multiple isotype expression by B cells (17, 18), and none of these studies was reproduced *in vivo.* SIgM+ and sIgD+ dual-positive B cells were identified in normal mouse spleen cells (3). After stimulation with lipopolysaccharides and IL-4, coexpression of IgM/IgG1 and IgM/IgE was evident (19). Other studies using various B cell lines revealed coexpression of IgM/IgG1 (20) or sIgM/sIgA (21). Most investigations of B cells producing multiple Ab isotypes used lymphoid tissue or lymphoid cell lines and studied limited variations of Ab isotypes. Thus far only tumor B cells have been shown to have the ability to individually synthesize and express more than two isotypes of Ab (22). This study is the first to examine whether this phenomenon occurs *in vivo* during a natural infection against a pathogen by activated normal B cells, and also whether these B cells are present in lymphoid and nonlymphoid tissues.

Our experiments were to determine first whether there was a rapid proliferation of Ab-producing B cells in the non-Peyer's patch as well as the Peyer's patch regions of the small intestine in rats infected with TS muscle larvae. Secondly, the study examined whether these B cells expressed multiple isotypes of Ab at a given time. Each of the Ab isotypes, i.e., IgA, IgM, IgG1, IgG2a, IgG2b, and IgG2c was identified on B cells by a fluorescent marker (fluorescein), respectively. Along with the first marker was a second fluorescent marker (rhodamine) specific for IgE to determine if dual Ab isotypes (IgA:IgE, IgM:IgE, IgG1:IgE, IgG2a:IgE, IgG2b:IgE, and IgG2c:IgE) were expressed. The total number of B cells was quantified using OX33 monoclonal Ab that labels CD45RA, a pan B cell marker found only on B cells in rats (23). B cells expressing various Ab isotypes were examined in the germinal center and non-germinal center regions of the Peyer's patches, as well as the nonlymphoid region of the small intestine in rats infected with TS.

3. MATERIALS AND METHODS

3.1. Experimental Animals

Male or female pathogen-free AO rats of six to eight weeks of age were randomized into seven groups of six to conduct this experiment. Rats were purchased from Harlan-Sprague Dawley (Indianapolis, IN), and maintained at the California State University San Bernardino vivarium where food and water were distributed *ad libitum*.

3.2. Infection with TS Muscle Larvae

Muscle larvae from infected rats were isolated by digesting minced rat carcasses with 1% pepsin hydrochloride at 37 °C for 1 hr. The digested fluid was first filtered through cheesecloth to remove bones and undigested tissues, and then filtered through a 200 – mesh sieve using 0.85% NaCl to retain the larvae. Muscle larvae were then counted and the concentration adjusted accordingly. Infection was carried out by injecting 2,000 muscle larvae per rat orally using a blunt-end feeding-needle (24).

3.3. Processing of Tissues

The nonlymphoid region of the small intestine, 15 cm distal to the pylorus, and the Peyer's patch tissues, nearest the small intestine, of control and infected rats were obtained on days 0, 1, 3, 5, 7, 10, and 15, respectively. Tissues were cryo-histologically processed using a snap-freezing technique (25). Described briefly, tissues were immersed in isopentane (cooled in liquid nitrogen) for 10 sec and then added along with Histo Prep tissue embedding media (Fisher Scientific, Pittsburg, Pa.) into an aluminum foil cup. Tissues were then frozen and stored at - 80 °C until further processing. Each of the frozen tissues was cut into 6μm sections using a Lab Tek Precision Microtome Cryostat (Lab Tek Instruments Co., Westmont, IL.). The tissue sections were then placed on slides coated with Poly-L-lysine, and allowed to air dry. Slides were then fixed in 2% paraformaldehyde at room temperature for 20 min, and finally washed three times, 5 min each wash, in 1X PBS, pH 7.2.

3.4. Immunofluorescence Staining

A double–labeling immunofluorescence assay was used in order to show the different isotypes of antibodies expressed by B cells located within the tissues. All antibody probes were purchased from Accurate Chemicals and Scientific Corp. (Westbury, NY.), or Zymed Labs Inc. (San Francisco, CA). The optimal dilution of each of the antibody probes was previously determined (5). Each tissue section was stained for 1 hr at room temperature with 20 μl per section of 1:500 diluted monoclonal mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b, or IgG2c, respectively. Following incubation, this primary antibody was aspirated off, and slides washed three times in 1X PBS, pH 7.2, for 5 min. each wash. Then 20 μl of fluorescein isothiocyanate (FITC) conjugated goat anti-mouse Ig [$F(ab)_2$'], and goat anti-rat IgE labeled with rhodamine isothiocyanate (XRITC) [$F(ab)_2$'] were added to each slide in a 1:1000 dilution and mixed together in a 1:1 ratio. These Ab were H chain specific and in the $F(ab)_2$' form to prevent non-specific binding to the Fc receptors. Normal rat serum was used at a dilution of 0.1% in the antibody preparation to prevent non-specific binding. This fluorescence staining was incubated under dark conditions at room temperature for 1 hr. Slides were once again washed three times in 1X PBS, pH 7.2. Each slide was then mounted with fluorosave to prolong the staining

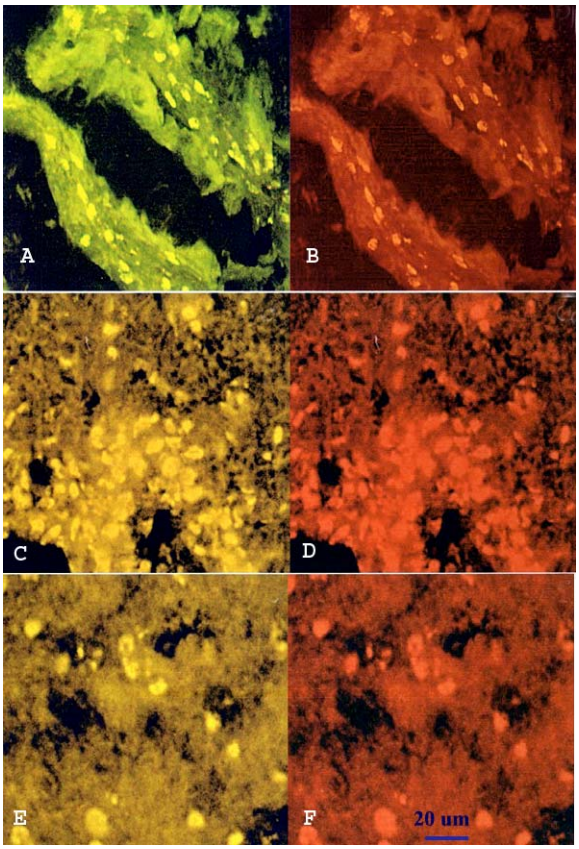

Figure 1. DEBC in the Small Intestine, PP-GC, and PP-NGC of *Trichinella spiralis* Infected Rats: Tissues were labeled with monoclonal mouse anti-rat IgG1 Ab, and FITC-conjugated goat anti-mouse IgG [H&L chain specific $(F(ab)'_2$ fragment)]: XRITC-conjugated goat anti-rat IgE Ab [H chain specific $(F(ab)'_2$ fragment)] in a 1:1 ratio. (a,c, and e) IgG1-expressing B cells localized within a field (VCU in the small intestine) as observed @ 400X magnification using a 495nm filter specific for viewing FITC. (b,d, and f) IgE-expressing B cells localized within the identical field (or VCU) as observed @ 400X magnification using a 546nm filter specific for viewing XRITC. (a, b) Small intestine; (c, d) PP-GC; (e, f) PP-NGC. The intestinal tissue was obtained on day 7, and PP tissues were obtained on day 10. The diameters of the labeled cells are between 10-12μm.

effect, and a coverslip added. Antibody labeled B cells were viewed using a Nikon Optiphot Biological Microscope with an Episcopic-Fluorescence attachment EF-D containing a B2A 495 nm filter specific for viewing FITC, and a G2A 546 nm filter specific for viewing XRITC. Single and double-labeled cells of at least 10-20 VCU in the small intestine or 10-20 fields per Peyer's patch tissue (400X) were counted for each rat.

3.5. OX33 Staining

OX33 is a monoclonal antibody that identifies a pan-B cell marker, CD45, on rat B cells (OX33 is a gift from Oxford University to Dr. Ching-Hua Wang). This antibody has been conjugated with XRITC in this laboratory (6). Serial sections of the same tissues used above were prepared according to the above procedure and used for OX33 staining with an optimally diluted mouse anti-rat OX33 monoclonal antibody labeled with XRITC for 1hr at room temperature. The OX33 was then aspirated off and washed three times with 1X PBS, pH 7.2, for 5 min. per wash. Overall B cell numbers were quantified using the fluorescent microscope as described previously.

3.6. Statistical Analysis

The day specific means of different dual-Ab expressing B cell combinations were compared using a two-factor ANOVA. The day specific means of dual Ab-expressing B cells and single Ab-expressing B cells were compared using a two-factor ANOVA. The means of OX33-Ab staining data were compared using a one-factor ANOVA for each tissue tested. Effects resulting in significant F values were then subjected to a Student-Newman Keuls test to determine significant differences between means. Values of q with $p<0.05$ were considered significant.

4. RESULTS

To determine the expression of Ab by B cells, AO rats were infected with 2,000 TS muscle larvae on day 0. Tissues of the small intestine and the Peyer's patches were obtained on days 0 (control), 1, 3, 5, 7, 10, and 15 of infection. Tissues were processed and labeled with monoclonal mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b, or IgG2c Ab, respectively, followed by FITC-conjugated goat anti-mouse Ig- and XRITC-conjugated goat anti-rat IgE Ab. The appearances of Ab-expressing B cells were quantified as either single Ab-expressing B cells (SEBC) or dual Ab-expressing B cells (DEBC) by immunofluorescence microscopy. B cells expressing IgE appeared bright-red when using a 546nm filter, and the IgA-, IgM-, IgG1-, IgG2a-, IgG2b-, and IgG2c- expressing B cells appeared bright-green when using the 495nm filter. Figure 1 shows the color signals that are produced by this fluorescence labeling technique.

4.1. Kinetics of the Appearance of DEBC and SEBC in the Non-Peyer's Patch Region of the Small Intestine

Intestinal samples labeled with specific monoclonal mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b, and IgG2c Ab, respectively, and goat anti-rat IgE Ab showed a significant increase in the number of double-labeled B cells per villus crypt unit (VCU) by day 3 of infection (Figure 2a-f). This significance was maintained for the remainder of the experiment as compared to the day 0 controls. There were no significant differences between different dual Ab-combinations on respective days of infection throughout the duration of the experiment. The number of IgA:IgE-DEBC per VCU significantly increased by day 3 (3.4±1.15), with a maximum on day 7 (28.13±3.52) compared to the controls (0.30±0.45) (Figure 2a). On day 0, IgM:IgE-DEBC in the small intestine were less than (0.08±0.11 per VCU) that of IgM-SEBC (0.23±0.14 per VCU), although the differences were not significant. IgM:IgE-DEBC were significantly increased in number as of day 3 of infection (3.83±2.02) with the highest number of B cells per VCU detected on day

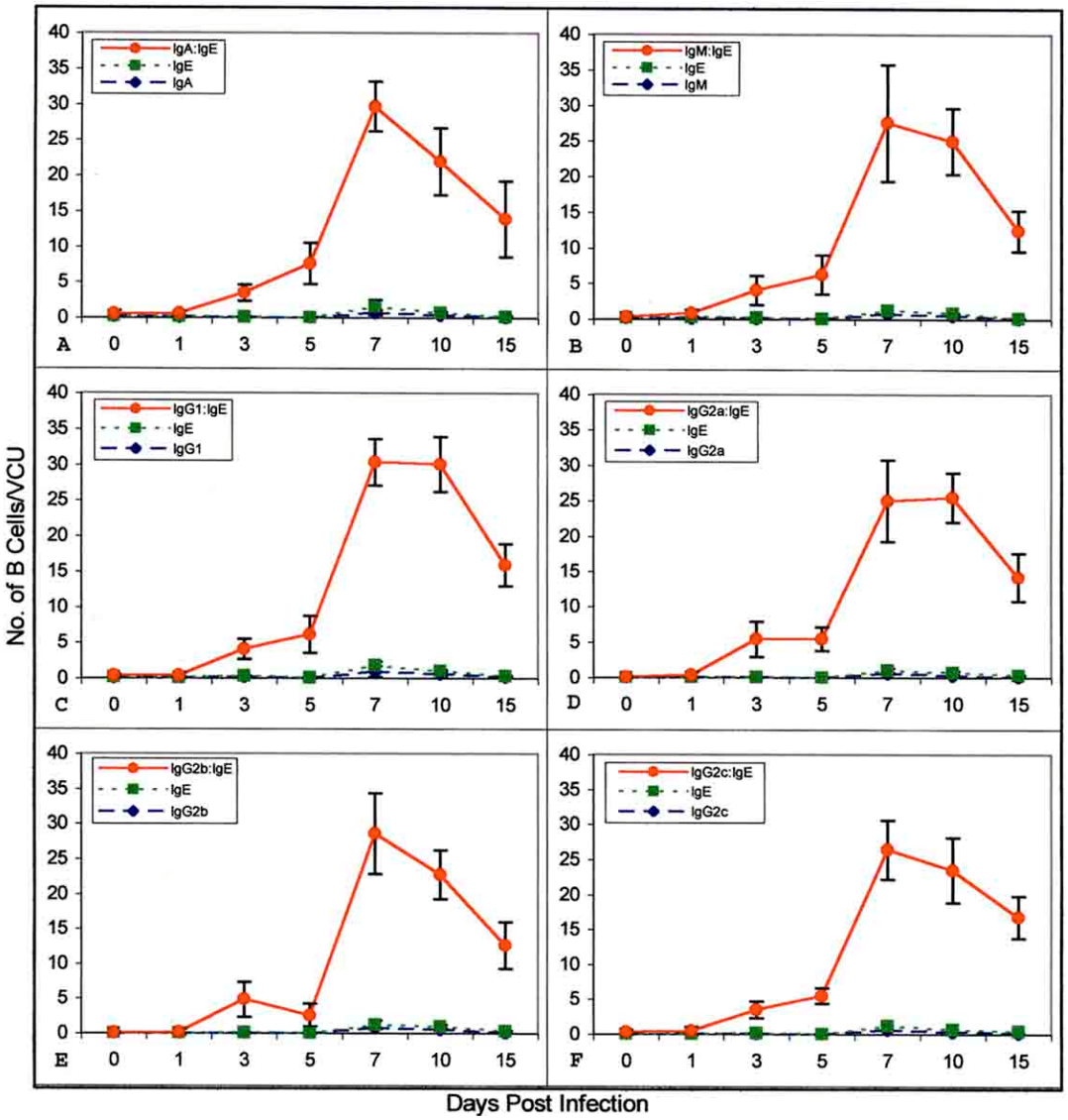

Figure 2. Kinetics of the Appearance of DEBC and SEBC in the Small Intestine: Rats were infected with 2,000 TS muscle larvae on day 0. Samples of the small intestine 15cm from the pylorus were removed on the indicated days post infection and immunohistochemically processed to reveal DEBC or SEBC. Data represent means ± standard deviation (SD) of FITC and/or XRITC labeled cells per VCU in the small intestine of 6 rats per day. Kinetics of (a) IgA:IgE-DEBC, IgA-SEBC, or IgE-SEBC; (b) IgM:IgE-DEBC, IgM-SEBC, or IgE-SEBC; (c) IgG1:IgE-DEBC, IgG1-SEBC, or IgE-SEBC; (d) IgG2a:IgE-DEBC, IgG2a-SEBC, or IgE-SEBC; (e) IgG2b:IgE-DEBC, IgG2b-SEBC, or IgE-SEBC; (f) IgG2c:IgE-DEBC, IgG2c-SEBC, or IgE-SEBC are presented. All intestinal samples labeled with the respective DEBC showed a significant increase ($p<0.05$) per VCU by day 3-post infection and was maintained for the remainder of the experiment as compared to the controls.

7 (26.32±8.2) and day 10 (24.15±4.61) (Figure 2b). IgG1:IgE-DEBC showed augmentation of DEBC with day 7 (28.53±3.26) and day 10 (29.01±3.87) being the peak days of proliferation (Figure 2c). A significant increase of IgG1:IgE-DEBC over the day 0 controls (0.21±0.18) occurred on day 3 of infection (3.79±3.87). IgG2a:IgE, IgG2b:IgE, and IgG2c:IgE-DEBC also showed significant increases from day 3 of infection (5.35±2.50, 4.79±2.28, 3.38±1.22, respectively) through the end of the study (figures 2d, 2e, and 2f, respectively). IgG2a:IgE-DEBC revealed the maximum proliferation on day 10 of infection (24.76±3.46), whereas IgG2b:IgE-DEBC, and IgG2c:IgE-DEBC showed a maximum proliferation on day 7 of infection (27.39±3.06 and 25.23±4.22). All of the dual Ab-combinations shown in this experiment had a significant decrease per VCU on day 15 of infection as compared to the peak days of augmentation on day 7 and day 10, but the numbers of B cells were still significantly increased over the controls. When comparing the number of B cells expressing the respective IgE-, the IgA-, IgM-, IgG1-, IgG2a-, IgG2b-, and IgG2c-SEBC, with that of DEBC labeled with both IgE and the respective second Ab of IgA,

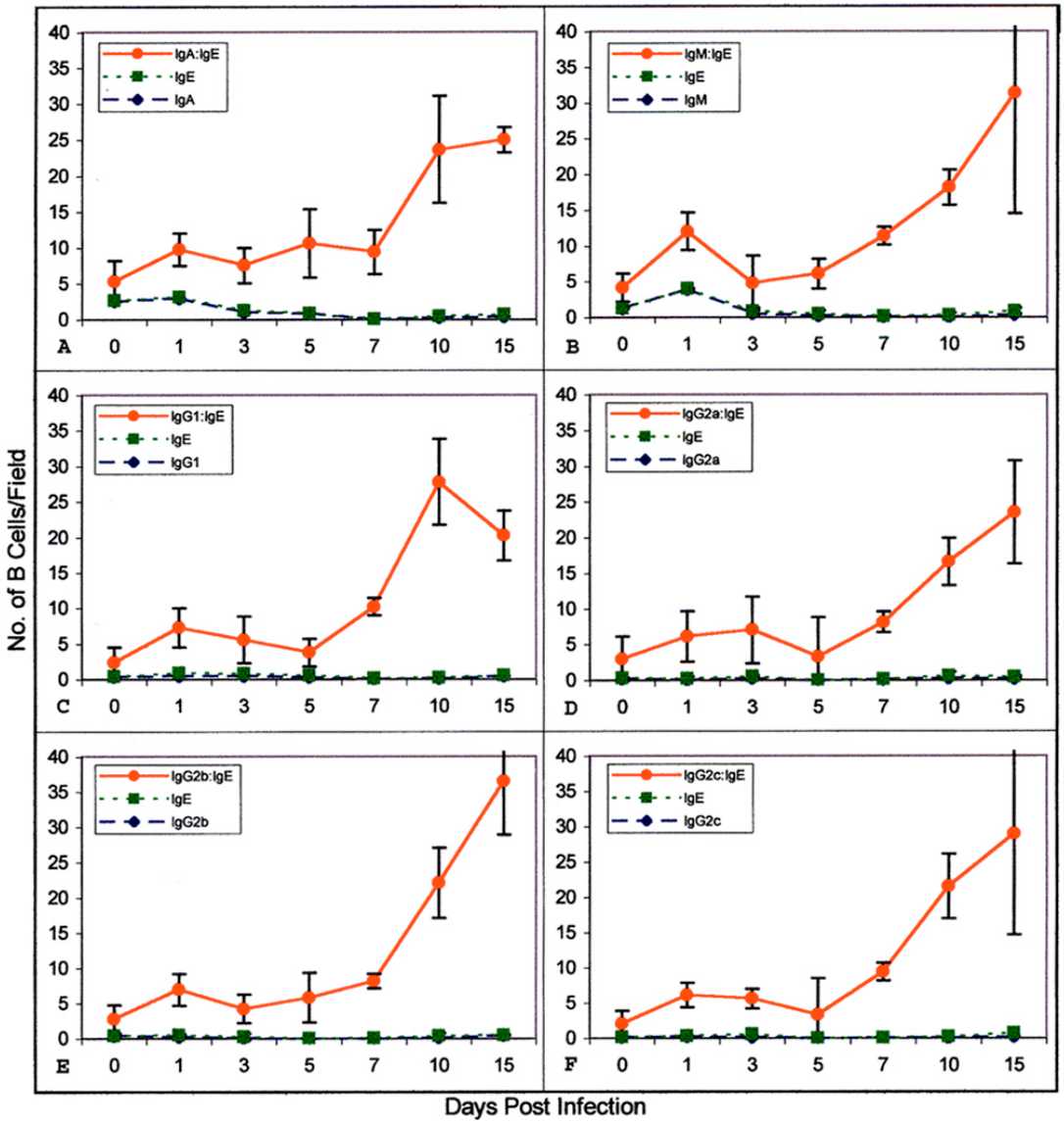

Figure 3. Kinetics of the Appearance of DEBC and SEBC in the PP-GC: The experimental procedure is as described in Figure 2, with the exception of the tissue used was from the Peyer's patches, and the B cells were counted per field @400X. Data represent mean ± SD of FITC and/or XRITC labeled cells per field of PP-GC tissue of 6 rats per day. Kinetics of (a) IgA:IgE-DEBC, IgA-SEBC, or IgE-SEBC; (b) IgM:IgE-DEBC, IgM-SEBC, or IgE-SEBC; (c) IgG1:IgE-DEBC, IgG1-SEBC, or IgE-SEBC; (d) IgG2a:IgE-DEBC, IgG2a-SEBC, or IgE-SEBC; (e) IgG2b:IgE-DEBC, IgG2b-SEBC, or IgE-SEBC; (f) IgG2c:IgE-DEBC, IgG2c-SEBC, or IgE-SEBC are presented. Significant increases ($p<0.05$) of the various DEBC over the respective controls varied from day 5 to day 7, but once significance was determined, it continued for the remainder of the experiment.

IgM, IgG1, IgG2a, IgG2b, and IgG2c, there were significant increases for all of the DEBC over the respective SEBC as of day 3 of infection, and such significance remained for the entire duration of the experiment (Figure 2a-f).

4.2. Kinetics of the Appearance of DEBC and SEBC in the Germinal Center Region of the Peyer's Patches

Samples of the germinal center regions of the Peyer's patches (PP-GC) were labeled with specific monoclonal mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b, and IgG2c, respectively, and goat anti-rat IgE. The results showed that the number of IgA:IgE-DEBC (9.78±4.78) was significantly increased as compared to the controls (2.65±2.85) as of day 5 of infection, and it remained significantly increased for the remainder of the experiment. Significant increase was also found on day 10 (23.16±7.46) and day 15 (24.32±1.75) over the day 5 infected rats, and these were the peak days of B cell proliferation (Figure 3a). IgM:IgE-DEBC showed a sharp significant increase as of day 1 of infection (8.03±2.62). On day 3, this number (3.97±3.85) returned to the normal levels. On day 7 (11.22±1.28) the DEBC were again significantly increased

over the control, and remained increased for the remainder of the experiment (Figure 3b). IgG1:IgE-DEBC (10.04±1.23) and IgG2a:IgE-DEBC (7.98±1.49) showed similar results in B cell proliferation, with a significant increase over the controls (1.98±2.15 for IgG1:IgE-DEBC; 2.71±3.11 for IgG2a:IgE-DEBC) as of day 7 of infection and such kinetics remained throughout the duration of the experiment (Figure 3c-d). IgG2b:IgE-DEBC and IgG2c:IgE-DEBC showed similar ranges of B cell proliferation to that of the IgM:IgE-DEBC. These two combinations showed a sharp significant increase as of day 1 of infection (6.50±2.25 for IgG2b:IgE-DEBC; 5.84±1.75 for IgG2c:IgE-DEBC), followed by a decrease on day 3 (3.97±2.03 for IgG2b:IgE-DEBC; 5.08±1.39 for IgG2c:IgE-DEBC) of infection that was not significantly increased over the respective controls. On day 7 (8.14±1.03 for IgG2b:IgE-DEBC; 9.37±1.25 for IgG2c:IgE-DEBC) the DEBC were once again significantly increased over the control, and remained increased for the remainder of the experiment (Figure 3e-f). IgA-SEBC and IgM-SEBC were significantly higher than the other SEBC on day 1 and day 3 of infection (Figure 3a-b), but became similar, with no significant differences, to the other SEBC as the days of infection proceeded. As compared to the IgA:IgE-DEBC (6.28±2.46), the number of IgA-SEBC (1.05±0.56) was significantly less than the DEBC as of day 3 of infection (Figure 3a). The kinetics of the numbers of IgM-SEBC (0.56±0.70) were similar to that of IgA-SEBC, with day 3 being the first day where there was a significant decline as compared to the IgM:IgE-DEBC (3.97±3.85) (Figure 3b). The numbers of IgG1-, IgG2a-, IgG2b-, and IgG2c-SEBC were all significantly less than that of the respective DEBC for the entire experiment (Fig. 3c-f). IgE-SEBC for each group of rats was also significantly less in number than that of the respective DEBC for the entire experiment (Figure 3a-f).

4.3. Kinetics of the Appearance of DEBC and SEBC in the Non-Germinal Center Region of the Peyer's Patches

Samples of the non-germinal center regions of the Peyer's patches (PP-NGC) labeled with specific monoclonal mouse anti-rat IgA, IgM, IgG1, IgG2a, IgG2b, and IgG2c Ab, respectively, and goat anti-rat IgE showed a significant increase in the number of double labeled cells per field by day 7 of infection (Figure 4a-f). This significance was maintained for the remainder of the experiment as compared to the day 0 controls. There were no significant differences between different dual Ab-combinations on respective days throughout the duration of the experiment. IgA:IgE-DEBC showed significant increase over the control (0.11±0.08) on day 7 of infection (2.37±1.18), with a maximum value at day 10 (3.65±1.00) (Figure 4a). Similar kinetics of proliferation were obtained in IgM:IgE-DEBC with day 7 being significantly greater than day 0 (2.56±1.06; 0.04±0.18), and day 10 (2.86±2.67) being the maximum proliferation of DEBC (Figure 4b). Significant increases in the number of IgG1:IgE-DEBC occurred on day 7 of infection (2.78±2.17) when compared to the control (0.07±0.08), and this was also the peak day of proliferation (Figure 4c). IgG2a:IgE-DEBC showed maximum augmentation on day 7 (2.07±2.05), which was the first day of significant increase over the control (0.09±0.06) (Figure 4d). Similar numbers to day 7 IgG2a:IgE-DEBC were also found on day 15 (2.50±1.08). IgG2b:IgE-DEBC as with IgG1:IgE-DEBC had the highest increase on day 7 (3.76±1.22) of infection, and this was also the first day of significant increase over the control (0.03±0.13) (Figure 4e). The IgG2c:IgE-DEBC was increased significantly on day 7 (2.29±2.76), and this was also the peak day of proliferation (Figure 4f). When comparing the respective IgE-, the IgA-, IgM-, IgG1-, IgG2a-, IgG2b-, and IgG2c-SEBC, and the IgA:, IgM:, IgG1:, IgG2a:, IgG2b:, and IgG2c:IgE-DEBC, all of the DEBC were significantly increased over the respective SEBC as of day 7 of infection, and such significant increases remained for the duration of the experiment (Figure 4a-f).

4.4. Kinetics of the Appearance of OX33-Labeled B Cells in the Small Intestine, PP-GC, and PP-NGC

To quantify the total B cells in the above tissues, serial sections from the above tissues obtained from the same rats were labeled with monoclonal mouse anti-rat OX33 Ab conjugated with XRITC. The OX33 Ab is used to identify the CD45RA molecule that is found on all rat B cells. The kinetics of appearance of B cells were examined by immunofluorescence microscopy and the $OX33^+$ cells appeared bright red when using a 546nm filter (Figure 6).

A significant increase in the number of $CD45RA^+$ B cells was found in the small intestine by day 3 of infection (7.57±2.15), as compared to the day 0 controls (0.52±0.76). Such augmentation continued throughout the entire experiment, with the greatest number of B cells detected on day 15 after infection (31.87±5.72) (Figure 5a). The lowest degree of B cell proliferation was detected in the PP-NGC (Figure 5b) as compared to the small intestine and the PP-GC. Significant increase over the control of the PP-NGC took place on day 5 (7.87±3.12), followed by a slight decrease on day 7 (7.08±4.84) that was not significantly different from the control (2.01 ±1.47). In the PP-NGC, significant increase in B cell number over the control was again demonstrated on day 10 (13.21±2.24) after infection, which was also the peak day of proliferation. The PP-GC exhibited a high number of B cells in the control (9.35±3.43) as compared to controls of the small intestine and PP-NGC, and infected rats showed a significant increase over the controls on day 3 (18.23±4.94) (Figure 5c). Such proliferation was maintained throughout the remainder of the experiment with a maximum number of B cells detected on day 15 (30.71±7.15).

5. DISCUSSION

Intestinal B lymphocytes, in response to TS infection, have been shown to produce nearly all Ab isotypes during the immune response. Bell *et al* (11) showed that three isotypes of IgG (IgG1, IgG2a, and IgG2c) in rats were actively produced by B cells against this parasite. Other researchers have shown dramatic increases of IgA (10, 26), or significant increases of IgE (4, 5) against the same parasite. Based on our recent findings

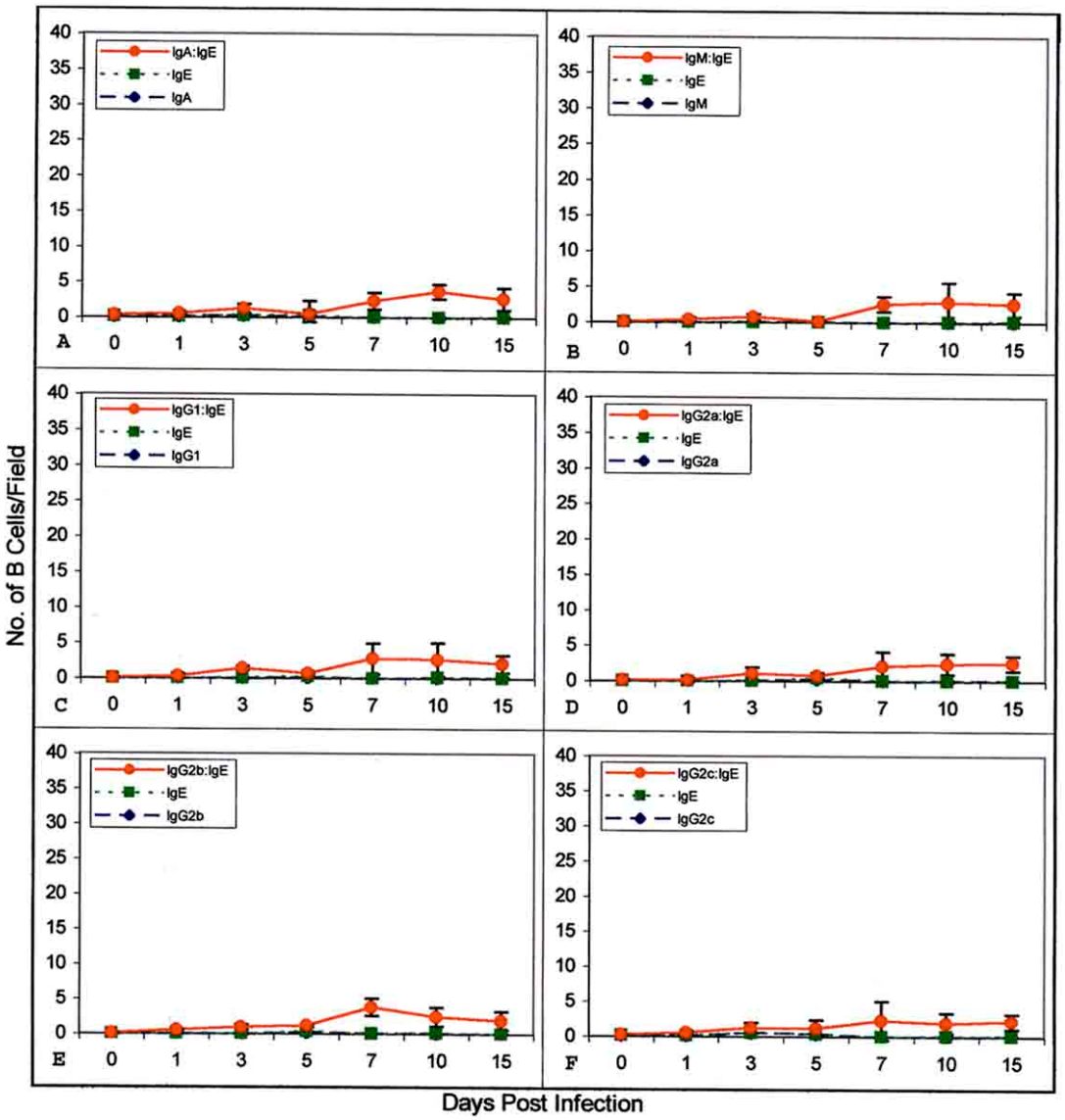

Figure 4. Kinetics of the Appearance of DEBC and SEBC in the PP-NGC: The experimental procedure is as described in Figure 2, with the exception of the tissue used was from the Peyer's patches, and the B cells were counted per field @400X. Data represent mean ± SD of FITC and/or XRITC labeled cells per field of PP-NGC tissue of 6 rats per day. Kinetics of (a) IgA:IgE-DEBC, IgA-SEBC, or IgE-SEBC; (b) IgM:IgE-DEBC, IgM-SEBC, or IgE-SEBC; (c) IgG1:IgE-DEBC, IgG1-SEBC, or IgE-SEBC; (d) IgG2a:IgE-DEBC, IgG2a-SEBC, or IgE-SEBC; (e) IgG2b:IgE-DEBC, IgG2b-SEBC, or IgE-SEBC; (f) IgG2c:IgE-DEBC, IgG2c-SEBC, or IgE-SEBC are presented. All PP-NGC samples labeled with respective DEBC showed a significant increase ($p<0.05$) per field by day 7 post infection and were maintained for the remainder of the experiment as compared to the controls.

(5, 6), it was shown that IgE-producing B cells produced the strongest response against the 9D4 antigen of TS. Therefore, IgE was used as a common Ab label for testing the dual Ab expression on B cells in this experiment. Quantitative analysis of DEBC and SEBC from the small intestine, PP-GC, and PP-NGC of AO rats are reported. Using a double-labeling immunofluorescence assay, IgA, IgM, IgG1, IgG2a, IgG2b, or IgG2c, respectively, combined with the common IgE Ab were detected on B cell membranes. A separate immunofluorescence assay was also carried out to quantify total B cell numbers in serial tissue sections within the same tissues to examine the possibility that B cells in rats may be expressing multiple Ab isotypes on the surface during TS infection.

A significant increase in the expression of two Ab isotypes on the surface of B cells over the controls and the SEBC in the non-Peyer's patch region of the small intestine occurred as of day 3 post infection for all of the isotypes tested (Figure 2). This shows that B cells were activated to proliferate and produce Ab in the small intestine very early after infection with TS. Most B cells

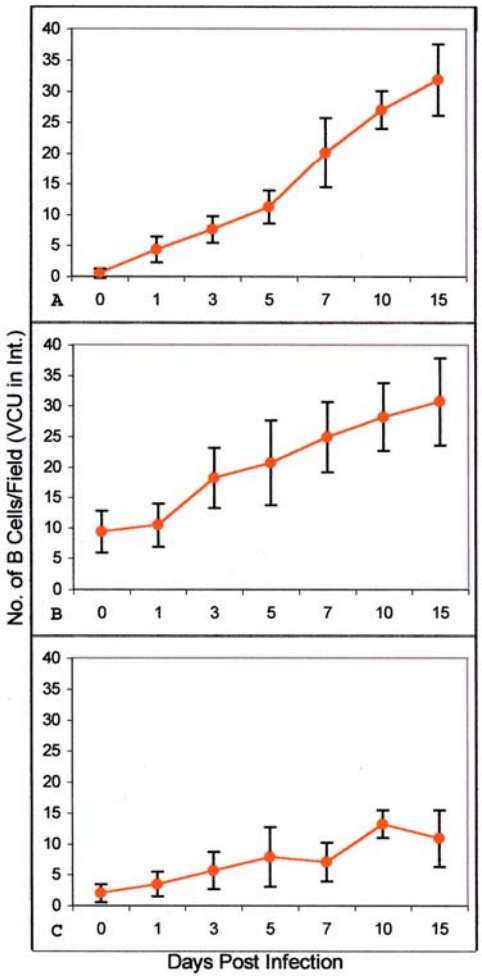

Figure 5. Kinetics of the Appearance of CD45RA-Expressing B Cells Detected by OX33 Monoclonal Ab in the Small Intestine and Peyer's Patches (NGC and GC): The experimental procedure is as described in Figure 2, except the serial sections obtained from the same tissues of the same rats were immunohistochemically processed to reveal $CD45RA^+$ B cells. Data represent means ± SD of OX33 labeled B cells per VCU (or field) of 6 rats per day. The total number of B cells in (a) the small intestine significantly increased ($p<0.05$) over the controls as of day 3 post infection; (b) PP-GC significantly increased ($p<0.05$) over the controls as of day 3 post infection; (c) PP-NGC significantly increased ($p<0.05$) over the controls on days 5, 10, and 15 post infection.

become activated to undergo clonal proliferation through interactions with T helper cells, and Wang *et al* (27) showed that intestinal T cells are actually activated as early as 12 hrs post infection with the same parasite. Therefore, it takes less than 2 days for the intestinal T cells to activate B cells in the intestine. These B cells then immediately start expressing many Ab isotypes on the surface and most likely are secreting these Ab into the mucosal tissues. The concentration of B cells per VCU of the small intestine of infected rats was higher than those found per field in all of the lymphoid tissues studied (figures 2-4). However, in normal rats, the density of B cells in the lymphoid tissues, such as the Peyer's patch, is much higher than in the nonlymphoid tissues such as the small intestine (figures 2-4). This shows that the B cells, which are present in the villi of the small intestine, are actively proliferating to fight off the infection even before the other surrounding lymphoid tissues are showing signs of B cell activation and proliferation. Therefore, the nonlymphoid region of the small intestine does have an enormous capability to mount a rapid and an effective immune response against TS infection.

Although B cell activation and response were observed in the Peyer's patch tissues as well, the kinetics were not only delayed, but the quantity and strength of proliferation were also weaker for certain Ab isotypes than that in the non-lymphoid region of the small intestine. Within the non-germinal center region of the Peyer's patches, there was a relatively low rate of proliferation with a significant increase only 7 days after infection (Figure 3). Most of the B cell proliferation in the Peyer's patches occurred within the germinal center region. Within the germinal centers, a high number of Ab-expressing B cells was identified, with variable significant rates of Ab-expressing B cells first found from day 5 for IgA:IgE-DEBC to day 7 for the other Ab isotypes (Figure 4). The kinetics of DEBC proliferation in this region were delayed by 3 to 4 days compared to that of the small intestinal villi, but the total number of B cells seemed to be comparable on the peak days of infection. Considering the density of B cells in the lymphoid tissues of the Peyer's patches is normally much higher than that of the small intestinal tissue, it is not difficult to realize that the strength of B cell proliferation in the nonlymphoid intestinal tissue is much stronger than that of the Peyer's patches.

Within the different tissues examined throughout the duration of the experiment, it was found that B cells expressed all of the different Ab isotypes. In the villi of the small intestine and PP-NGC, no significant quantitative differences between the numbers of B cells expressing different dual Ab-isotypes were revealed during each day after infection. Therefore these results suggest that these B cells were activated to synthesize all of the different Ab isotypes to fight off the TS infection in both the lymphoid and the nonlymphoid tissues. Theoretically, the greater the variety of a response to a specific pathogen, the greater the chance of destroying the pathogen before extensive damage occurs. With greater variety of Ab produced against TS, then the more effective the immune response. In the PP-GC, B cells also expressed all of the multiple isotypes, but different isotypes showed varying kinetics throughout the infection. In the controls there was a higher number of IgA- and IgM-expressing B cells, but as the infection took place and proceeded through time, similar quantities of the IgA or IgM-expressing B cells were found as compared to that of other Ab-expressing B cells. This shows that under normal conditions, the PP-GC produce B cells that mostly express IgA and IgM, which would confirm many previous studies (26, 28, 29). However, once an infection of TS occurs, B cells are quickly stimulated to express various Ab isotypes. Although IgE-expression was not measured alone in the tissues in these experiments, it was found in high

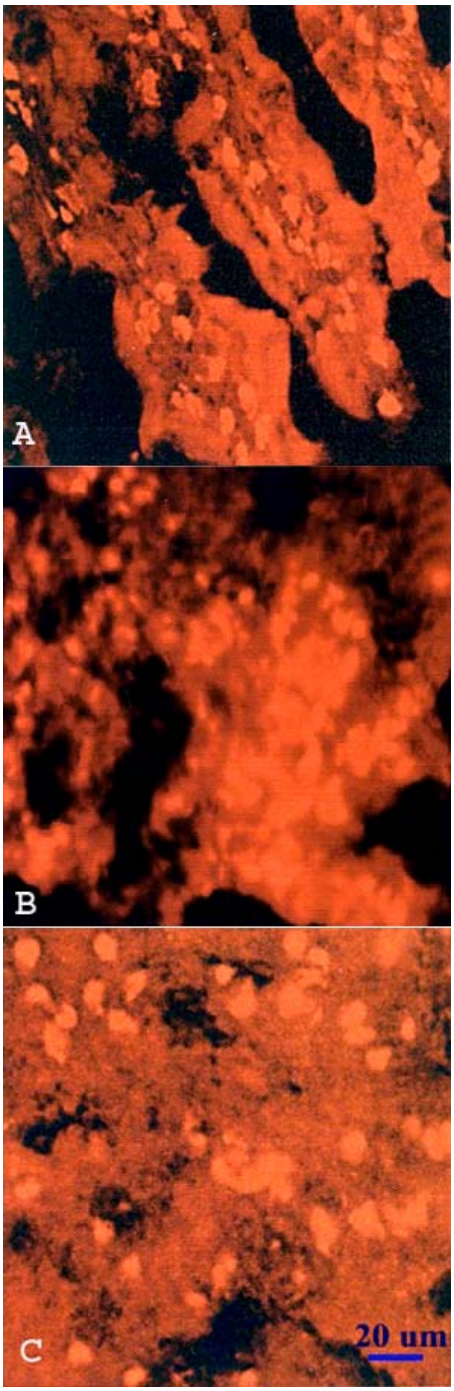

Figure 6. Appearance of CD45RA Positive B Cells Detected by OX33 Ab in the Small Intestine, PP-GC, and PP-NGC of *Trichinella spiralis* Infected Rats: Tissues were labeled with monoclonal mouse-anti-rat OX 33 Ab conjugated with XRITC. $CD45RA^{+}$ B cells localized within a field (VCU in the small intestine) from (a) small intestine, (b) PP-GC, and (c) PP-NGC were observed @ 400X magnification using a 546nm filter specific for viewing XRITC. The diameters of the labeled cells are between 10-12μm.

amounts on the DEBC that would indicate that B cells against this parasite were also actively producing the IgE. Most mucosal B cell research revolves around IgA production within the Peyer's patches of the small intestine (8, 30, 31), but in this experiment it was shown that B cells expressed all of the different isotypes after infection with TS. It has also been shown that IgA is not the only isotype produced, and that IgA does not even appear to be the major isotype produced against this infection in any of the tissues tested (6). As would be expected from a parasitic infection and from previous studies (4, 5), IgE would appear to be the major single Ab produced by B cells against TS. However, in these experiments, all of the other Ab isotypes are also expressed in similar high quantities to that of IgE as well. Therefore our results from these experiments showed that no single Ab isotype produced by B cells dominated any other isotype against this infection.

The various Ab isotypes expressed by B cells were demonstrated through the use of double-labeling of the B cells for two Ab isotypes. In the small intestine, significant increase of DEBC first occurred on day 3 after infection with TS, and the peak days were on days 7 and 10. Based on the amount of DEBC that were counted per VCU on average (between 21.26±4.71 and 29.01±3.87) for each of the dual Ab-labeled combinations, it would appear that each villus would become overwhelmed with B cells alone and not leave any space for other cells of the immune system to function. For this reason a total B cell count, using OX33 monoclonal Ab to label CD45RA found only on B cells in rats, was taken to determine the quantities of B cells within each VCU. For the controls and the first 3 days post infection, no differences were found between the total number of B cells and the total number of DEBC for each of the combinations. When analyzing and comparing all the different combinations of DEBC for each of the peak days post infection (day 7 and day 10), the combined average number of DEBC per VCU considerably outnumbered that of the expected total B cell numbers from the OX33 labeled cells (Figure 5). The total combined DEBC on day 7 after infection was more than seven times that of the detected total number of OX33-labeled B cells. DEBC on day 10 post infection when combined was over five times that of the actual total number of B cells revealed through OX33 labeling. This indicates that these B cells are not only expressing two Ab isotypes on the surface as was shown, but are most likely expressing more than just the two isotypes of Ab during TS infection. This phenomenon could lead to a greater variation and efficiency in response to the TS Ag, and a much more effective expulsion of the parasite from the rat intestine. As of day 15 after infection, the total combined number of DEBC in the small intestine was < 3 times over that of the OX33 labeled B cells. This decreasing number of possible multiple Ab-expressing B cells in the non-Peyer's patch regions of the small intestine from the peak day 7 through day 15 is most likely due to an increase in B cells that have switched to producing less variety of Ab isotypes to allow for a more specific response against a TS Ag. Another reason for this reduced number of possible multiple Ab-producing B cells could be that more Ab producing B cells

have entered the mode of memory B cells as the infection continues.

Similar phenomenon of potential multiple Ab-expression was also present in the lymphoid tissues. When comparing the sum of DEBC of the various lymphoid tissues to the OX33-labeled B cells in these tissues on specific days of infection, our data suggest once again more than two isotypes being expressed on the surface of B cells. This not only confirms what was found in the nonlymphoid region of the small intestine, but also strengthens the theory proposed above that these DEBC are synthesizing more than just two isotypes at any particular point in time. The kinetics of the number of $OX33^+$ B cells in the lymphoid tissues were similar to that found in the proliferation of the Ab-expressing B cells, which generated a delayed response to the TS infection compared to that of the nonlymphoid region of the small intestine. DEBC in the PP-NGC, though present, remained relatively low throughout the entire experiment. When comparing the number of DEBC to that of the OX33 labeled cells, no multiple-expression was evident. In contrast, the PP-GC demonstrated similar kinetics of the numbers of DEBC to that of the small intestinal villi. On day 7, the combined dual Ab-bearing B cells was greater than two times that of the total B cells quantified using the OX33 monoclonal Ab. Within the PP-GC, however, there was a steady increase of the combined number of DEBC through day 10, which was more than four times over the number of OX33 labeled cells, and by day 15, it became greater than five times that of the OX33 labeled B cells. These results show that the B cells in the PP-GC also conceivably produced multiple Ab isotypes by day 7 post infection. The number of DEBC in the PP-GC though was less than that found in the non-Peyer's patch region of the small intestine and the dynamics of appearance of these B cells was also more delayed. DEBC in the small intestine are likely activated to proliferate within this, normally, nonlymphoid region without the need from B cells in the Peyer's patches migrating into this region.

Most studies about B cells research focus on B cells producing one Ab isotype at a time only. There have been only few studies involving DEBC (32-34), and no studies to date on the possible expression of multiple Ab isotypes by B cells, particularly on such kinetics during a natural infection against an intestinal parasite, or any other pathogen. In this study, activated B cells expressing dual Ab isotypes on the surface were stimulated by TS and were identified and quantified using the double-labeling immunofluorescence assay. Based on the results presented here, it appears that these B cells most likely expressed multiple isotypes. This strongly suggests that in these intestinal B cells, there is tremendous RNA alternative splicing activities following TS antigenic stimulation and activation of T helper cells. DNA gene rearrangement and recombination events may also occur, but at a much lower rate as indicated by the extreme low level of SEBC in these tissues. Other studies using different cell lines *in vitro* have also suggested alternative RNA splicing in mouse (18, 33) and human cells (20, 35, 36). It remains to be proven that active RNA splicing is the supporting mechanism for this type of intestinal immune response.

A possible problem that may have skewed some of the results of this research is that of Ab binding to the FcR of cells involved in the nonspecific immune responses. Neutrophils, eosinophils, basophils, and Macrophages were not tested for in this study at any point during infection. B cells could have secreted out Ab, which could in turn bind to the FcR of these cells. Ab probes that were used would then bind to the Fc region of the Ab connected to the cells FcR, thereby giving a false positive signal and counted as an Ab-expressing B cell. These occurrences, though, were conceivably extremely minimal, because most nonspecific cells in rats have only been shown to express one type of FcR on the surface (37, 38), or their expression was not consistent with the dual-Ab tested for (39-41). Neutrophils have FcR for IgG and IgA (Isashi *et al* 1995), but have not been found to bind IgE. Eosinophils and basophils express FcR only for IgE (37), and Macrophages only subtypes of IgG (38). These cells would then only allow for an increase in the number of SEBC, which remained very low in all of the tissues throughout the experiment. The only exception found to date is that of mast cells that have the capability of expressing FcR for IgE and IgG2a in rats (37). In other experimental systems such as mouse and human, FcR specific to more Ab isotypes have been shown (38, 40, 41).

In conclusion, our experimental results revealed the rapidity of B lymphocyte activation during a specific anti-parasitic immune response in the small intestine. Our results also suggest that multiple isotypes of specific antibodies were concomitantly synthesized and expressed by these B cells, indicating a highly efficient and effective intestinal immune response produced by the host.

6. ACKNOWLEDGMENT

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7. REFERENCES

1. Chen J, F.W. Alt: Gene Rearrangement and B-Cell Development. *Curr Opin Immunol* 5, 194-200 (1993)

2. Matsuoka M, K. Yoshida, T. Maeda, S. Usuda & H. Sakano: Switch Circular DNA Formed in the Cytokine-Treated Mouse Splenocytes: Evidence for Intramolecular DNA Deletion in Immunoglobulin Class Switching. *Cell* 62, 135-142 (1990)

3. Perlmutter A.P, W. Gilbert: Antibodies of Secondary Response can be Expressed Without Switch Recombination in Normal Mouse B Cells. *Proc Natl Acad Sci* USA 81**,** 7189-7193 (1984)

4. Negrao-Correa D, L.S. Adams & R.G. Bell: Intestinal Transport and Catabolism of IgE: A Major Blood-Independent Pathway of IgE Dissemination During a *Trichinella spiralis* Infection in Rats. *J of Immunol* 157**,** 4037-4044 (1996)

5. Wang CH, E.M. Richards, R.D. Block, E.M. Lezcano & R. Gutierrez: Early Induction and Augmentation of Parasitic Antigen-Specific Antibody-Producing B Lymphocytes in the Non-Peyer's Patch Region of the Small Intestine. *Front in Biosci.* 3, a58-65 (1998)

6. Wang CH, E.M. Richards & R.G. Bell: Rapid Anti-Helminthic Response of B Lymphocytes in the Intestinal Mucosal Tissues of Rats. *Cellular Immunology* 193, 59-70 (1999)

7. Hickman C.P, L.S. Roberts & F.M. Hickman: *Integrated Principles of Zoology*. Times Mirror/Mosby College Pub St. Luis, MO (1984)

8. Spencer J, T. Finn & P.G. Isaacson: Human Peyer's Patches: an Immunohistochemical Study. *Gut* 27**,** 405-410 (1986)

9. Jeska E.L. & M. Stankiewicz: Responses of NFR/N Inbred Mice to Very Low-Dose Infections with *Ascaris suum*. *Int J Parasit* 19 (1), 85-89 (1989)

10. Brown P.J, R.G. Bruce: Kinetics of IgA Plasma Cells in the Intestine of NIH Mice Infected with *Trichinella spiralis*. *Res Vet Sci* 46, 187-191 (1989)

11. Bell R. G, J.A. Appleton, D.A. Negrao-Correa & L.S. Adams: Rapid Expulsion of *Trichinella spiralis*in Adult Rats Mediated by Monoclonal Antibodies of Distinct IgG Isotypes. *Immunology* 75**,** 520-527 (1992)

12. Karlsen T.H, H. Sommerfelt, S. Klomstad, P.K. Anderson, T.A. Strand, R.J. Ulvi, C. Ahren & H.M. Grewal: Intestinal and Systemic Immune Responses to an Oral Cholera Toxoid B Subunit Whole-Cell Vaccine Administered During Zinc Supplementation. *Infect Immun* 71, 3909-3913 (2003)

13. Novak J, Z. Csiki, J. Sebesi, A. Takats, P. Demeter & S. Sipka: Elevated Level of Helicobacter Pylori antibodies in Henoch-Schonlein Purpura. *Orv Hetil.* 144, 263-267 (2003)

14. Kang S.M, Q Yao, L. Guo & R.W. Compans: Mucosal Immunization With Virus-Like Particles of Simina Immunodeficiency Virus Conjugated With Cholera Toxin subunit B. *J Virol* 77, 9823-9830 (2003)

15. Tsuji N, K. Suzuki, H. Kasuga-Aoki, T. Isobe, T. Arakawa & Y. Matsumoto: Mice Intranasally Immunized With a Recombinant 16-Kilodalton Antigen From Roundworm Ascaris Parasites Are Protected Against Larval Migration of Ascaris Suum. *Infect Immun* 71, 5314-5323 (2003)

16. Wang CH, M. Korenaga, A. Greenwod & R.G. Bell: T-Helper Subset Function in the Gut of Rats: Differential Stimulation of Eosinophils, Mucosal Mast Cells and Antibody-Forming Cells By $OX8^-$ $OX22^-$ and $OX8^-$ $OX22^+$ cells. *Immunology* 71, 166-175 (1990)

17. Fujieda S, Y.Q. Lin, A. Saxon & K.Zhang: Multiple Types of Chimeric Germ-Line Ig Heavy Chain Transcripts in Human B Cells. *J of Immunol* 157, 3450-3459 (1996)

18. Shimizu A, M.C. Nussenzweig, H. Han, M. Sanchez & T. Honjo: Trans-Splicing as a Possible Molecular Mechanism for the Multiple Isotype Expression of the Immunoglobulin Gene. *J Exp Med* 173, 1385-1393 (1991)

19. Snapper C.M, F.D. Finkelman & W.E. Paul: Differential Regulation of IgG1 and IgE Synthesis by Interleukin-4. *J Exp Med* 167, 183-196 (1988)

20. Nolan-Willard M, M.T Berton & P. Tucker: Coexpression of μ and γ1 Heavy Chains can occur by a Discontinuous Transcription Mechanism by the Same Unrearranged Chromosome. *Proc Natl Acad Sci* 89, 1234-1238 (1992)

21. Kunimoto D.Y, M.C. Sneller, L. Claflin, J.F. Musinski & W. Strober: Molecular Analysis of Double Isotype Expression in IgA Switching. *J Immunology* 150, 1338-1347 (1993)

22. Kinashi T, T. Godal, Y. Yoma, N.R. Ling, Y. Yaoita & T. Honjo: Human Neoplastic B
Cells Express More Than Two Isotypes of Immunoglobulins Without Deletion of Heavy-Chain Constant-Region Genes. *Genes Dev* 1, 465-470 (1987)

23. PharMingen Research Products Catalog. *Rat Cell Surface Molecules; CD45RA*. A Becton Dickinson Co: pg. 154. San Diego, Ca (1998)

24. Wang CH & R.G. Bell: Antibody-Mediated *in vivo* Cytotoxicity to *Trichinella spiralis* Newborn Larvae in Immune Rats. *Parasite Immunology* 10, 293-308 (1988)

25. Lincoln C: Technical Excellence In Muscle Biopsy Preparation. *Microscopy Today* 98 (3), 10 (1998)

26. Van Loveren H, A.D. Osterhaus, J. Nagel, H.J.Schuurman & J.G. Vos:Detection of IgA Antibody-Producing Cells Specific for Ovalbumin or *Trichinella spiralis* in the Rat. *Scand J Immunol* 28 (3), 377-381 (1988)

27. Wang CH, M. Korenaga, F.R. Sacuto, A. Ahmad & R.G. Bell: Intraintestinal Migration to the Epithelium of Protective, Dividing, Anti-*Trichinella spiralis* $CD4^+$ $OX22^-$ Cells Requires MHC Class II Compatibility. *J of Immunol* 145 (3), 1021-1028 (1990)

28. Brandtzaeg P: The Human Intestinal Immune System: Basic Cellular and Humoral Mechanisms. *Baillieres Clin Rheumatol* 10 (1), 1-24 (1996)

29. Ehrhardt R.O, B. Gray, R. Duchmann, J.K. Inman & W.Strober: Reciprocal Regulation of Mucosal Surface IgA+ B Cells By Ig Receptor Cross-Linking and CD40 Ligand. *J Immunol* 157 (4), 1397-1405 (1996)

30. Kawanishi H, L.E. Saltzman & W. Strober: Mechanisms Regulating IgA Class-Specific Immunoglobulin Production in Murine Gut Associated Lymphoid Tissues. *I.* T cells Derived From Peyer's

Patches that Switch sIgM B Cells to sIgA B Cells *in vitro*. *J Exp Med* 157, 433-450 (1983a)

31. Hornquist C.E, L. Ekman, K.D. Grdic, K. Schon & N.Y. Laycke: Paradoxical IgA Immunity in CD4-Deficient Mice. Lack of Cholera Toxin-Specific Protective Immunity Despite Normal Gut Mucosal IgA Differentiation. *J Immunology* 155, 2877-2887 (1995)

32. Chen Y.W, C. Word, V. Dev, J.W. Uhr, E.S Vietta & P.W. Tucker: Double Isotype Production by a Neoplastic B Cell Line. II. Allelically Excluded Production of μ and γ1 Heavy Chains Without C_H Gene Rearrangement. *J Exp Med* 164, 562-579 (1986)

33. Wu CJ, J.T. Karttunen, D.H. Chin, D. Sen & W. Gilbert: Murine Memory B Cells are Multi-Isotype Expressors. *Immunology* 72 (1), 48-55 (1991)

34. Goodman D.J. & S. Gerondakis: Simultaneous Expression of Germline Gamma1 and Epsilon Immunoglobulin Heavy Chain Transcripts in Single Murine Splenic B-Cells. *Mol Immunol* 34 (12-13), 919-927 (1997)

35. Akahori Y, Y. Kurosawa, Y. Kamachi,, S. Torii & H. Matsuoka: Presence of Immunoglobulin (Ig) M and IgG Double Isotype-Bearing Cells and Defect of Swithch Recombination in Hyper IgM Immunodeficiency. *J Clin Invest* 85, 1722-1727 (1990)

36. Caldwell J, P. McElhone, J. Brokaw, R. Anker & B.A. Pollak: Co-Expression of Full Length and Truncated Ig Mu-Chains in Human B Lymphocytes Results from Alternative Splicing of a Single Primary RNA Transcript. *J Immunol* 146 (12), 4344-4351 (1991)

37. Alber G, U. M. Kent & H. Metzger: Functional Comparison of Fc Epsilon RI, Fc Gamma RII, and Fc Gamma RIII in Mast Cells. *J Immunol* 149 (7), 2428-2436 (1992)

38. Ravetch J.V. & J. Kinet: Fc Receptors. *Annu Rev Immunol* 3, 457-492 (1991)
18. Shimizu A, M.C. Nussenzweig, H. Han, M. Sanchez & T. Honjo: Trans-Splicing as a Possible Molecular Mechanism for the Multiple Isotype Expression of the Immunoglobulin Gene. *J Exp Med* 173, 1385-1393 (1991)

39. Isashi Y, M. Tamakoshi, Y. Nagai, M. Murakami & T. Uede: The Rat Neutrophil Low-Affinity Fc Receptor for IgG: Molecular Cloning and Functional Characterization. *Immunol Lett* 46 (1-2), 157-163 (1995)

40. Unkeless J.C, E. Scigliano & V.H. Freedman: 1988. Structure and Function of Human and Murine Receptors for IgG. *Annu Rev Immunol* 6, 251-281 (1988)

41. Takizawa F, M.Adamczewski & J.P. Kinet: Identification of a Low Affinity Receptor for Immunoglobulin E on Mucosal Mast Cells and Macrophages as Fc Gamma RII and Fc Gamma RIII. *J Exp Med* 176 (2), 469-475 (1992)

Abbreviations: *Trichinella spiralis* (TS), Antibody (Ab), Antigen (Ag), Dual antibody-expressing B cells (DEBC), Single antibody-expressing B cells (SEBC), fluorescein isothiocyanate (FITC), rhodamine isothiocyanate (XRITC), Peyer's Patch (PP), Germinal Centers (GC), Non-Germinal Centers (NGC), Villus crypt unit (VCU).

Key Words: *Trichinella spiralis*, B lymphocytes, B cells, Antibody, Antigen, fluorescein isothiocyanate, rhodamine isothiocyanate, OX33, CD45RA, Small intestine, Peyer's Patch, Germinal Center, Villus crypt.

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