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Differential Effect of Aging on B-Cell Immune Responses to Cholera Toxin in the Inductive and Effector Sites of the Mucosal Immune System

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The age-associated primary immune response of B cells from the Peyer's patches (PP), the lamina propria (LP), the mesenteric lymph nodes (MLN), and the spleen of mice following oral immunization with cholera toxin (CTx) was investigated. The induction of immune responses was assessed in 4-, 11-, and 24-month-old, individual C57BL/6J male mice by determining the number and isotype of anti-CTx ELISPOT-forming cells (SFC) in the PP, LPL, MLN, and spleen and the titer and isotype of serum anti-CTx antibody. The data indicate a significant age-associated decline in immunoglobulin G (IgG) and IgA anti-CTx SFC in the LP B cells but only in IgA anti-CTx SFC in the PP. No decline was seen in the anti-CTx SFC response in the MLN and spleen. Peroral immunization of mice with CTx resulted in a serum anti-CTx antibody response which was predominantly of the IgG class in all three age groups of mice tested. There was no age-associated decline in anti-CTx IgM, IgG, or IgA titers in serum. Isoelectric focusing and affinity immunoblotting revealed several distinct new antibody clonotypes in the immune serum of old mice following oral immunization with CTx. The results indicate a loss of immune responsiveness to CTx following oral immunization in senescent PP and LP B cells. The MLN and spleen B-cell responses were found to be refractory to the loss of immune function with aging. These findings suggest a differential effect of aging in the inductive and effector sites of the mucosal immune system, and the loss of antigen-specific IgA responses at mucosal sites may have adverse effects on the host's defense against potential pathogens.

There is much evidence which indicates a loss of immune competence with aging in humans and in various animal species (14, 38, 39, 41). However, this age-associated loss of immune function is not consistent in different compartments of the immune system (14, 28–31, 38, 39, 41, 42). It appears that mucosal tissues are refractory to the loss of immune function with aging compared with systemic tissues. Studies with humans and animals have shown either an increase or no change in immunoglobulin A (IgA) and IgM levels in serum with age (8, 9, 11, 23, 24). Similarly, no age-related changes were observed in the gut perfusate or luminal IgA and IgM levels (9, 17, 23). In contrast, others have reported age-associated declines of the background immunoglobulin-secreting cells in the murine small intestine (3, 34), in the antigen-specific secretory IgA response in the parotid glands (10), in antigen-specific intraluminal IgA responses (10, 22–24), and in antibody-containing cells in the murine intestine (10, 22, 33).

None of these studies have examined the sequential immune response in the mucosal immune system at both inductive and effector sites following antigen stimulation with aging. In the present study, the age-related changes in the immune response at the inductive and effector sites of the small intestine at the single B-cell level following oral immunization with cholera toxin (CTx) were examined in mice. The results show differential effects of aging on the B-cell responses in different mucosal tissues, despite the existence of a common mucosal immune system (4, 5, 19, 20). The lack of age-related changes in anti-CTx ELISPOT-forming cell (SFC) responses in the mesenteric lymph nodes (MLN), as opposed to a significant decline in the lamina propria (LP) and Peyer's patches (PP), emphasizes its distinct nature as a mucosal site which may not necessarily reflect the actual status of immune responsiveness in mucosal tissues as a whole.

MATERIALS AND METHODS

Animals. C57BL/6J male mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) at 2 months of age or as retired breeders at 8 months of age. After arrival, the mice were housed for a minimum period of 2 to 3 weeks before being used for experiments. Mice were maintained in grouped cages for a certain age group within the same room in an animal care facility until use. Mice had free access to untreated water and Purina Rodent Laboratory Chow. Animal health was monitored on a routine basis, and mice exhibiting symptoms of infection or ill health were excluded from the study, as were mice showing any signs of tumors or pathological abnormalities at the time of autopsy. The mean lifespan of the colony was calculated to be 24 months, with 10 and 50% survival times of 28.4 and 23.2 months, respectively (42).

Antigen and immunization. Purified CTx (Sigma Chemical Co., St. Louis, Mo.) was used as the antigen for immunization. CTx B subunit (CTxB), kindly provided by the Institute Merieux, Lyons, France, was used as coating antigen in isoelectric focusing (IEF) and affinity immunoblotting of immune sera.

Mice were immunized perorally under light ether anaesthesia with 10 μg of CTx in 0.5 ml of phosphate-buffered saline (PBS) through a small feeding tube.

Mice were bled retroorbitally 10, 14, and 16 days after

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immunization and killed by cervical dislocation. The serum was separated immediately and stored at −20°C until assayed. The spleens, MLN, PP, and small intestines were removed for the determination of CTx-specific antibody-secreting cells.

Preparation of lymphoid cells. Single-cell suspensions were prepared in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS) (GIBCO) and 0.1 mg of DNase per ml from the spleens, MLN, and PP. PP were excised from the serosal surface of the thoroughly washed small intestine. Organs were gently teased in medium, and passed through 100-μm polypropylene mesh to get a single-cell suspension. The cells were washed twice by centrifugation (150 × *g*) with cold medium at 4°C and suspended in ice-cold medium. All cellular work was performed on ice unless otherwise mentioned. Viable cell counts were made by the trypan blue exclusion method. The average concentrations of cells recovered were (in 10^6 cells per ml) 10.9 ± 6.5 for the MLN, 3.9 ± 1.3 for the PP, and 57.4 ± 19 for the spleens of 4-month-old mice, and 10.5 ± 6.4 for the MLN, 4.9 ± 2.2 for the PP, and 42.3 ± 15 for the spleens of 24-month-old mice.

Intestinal LP lymphocytes were prepared by a modification of the method described by Davies and Parrott (7). Briefly, the small intestine was removed from the gastroduodenal to the ileo-caecal junction, and the fecal contents were washed thoroughly with 50 ml of cold calcium-magnesium-free Hanks balanced salt solution (CMF-HBSS) (GIBCO) by using a 50-ml syringe. All visible PP and fat tissue were removed. The intestine was opened longitudinally and was cut into 0.5- to 1.0-cm pieces. The pieces were washed five to six times with cold CMF-HBSS and incubated for 75 to 90 min in 25 ml of CMF-HBSS containing 5 mM EDTA disodium salt (GIBCO) in a polypropylene conical flask at room temperature in a shaking water bath at a rate of 150 strokes per min. The EDTA-containing CMF-HBSS medium was changed every 15 min during the incubation. This treatment effectively removed the epithelial cells, as confirmed histologically by hematoxylin-eosin staining of the intestinal segments. The pieces were washed once with CMF-HBSS and incubated for 20 min with shaking in 25 ml of DMEM with 10% FCS to inactivate the remaining EDTA. The fragments were incubated in 25 ml of DMEM containing 10% FCS, 0.1 mg of DNase per ml, and 40 U of collagenase (type C-2139; Sigma Chemical Co., St. Louis, Mo.) per ml at 37°C in the water bath at 150 strokes per min for 90 min. The supernatant containing LP lymphocytes was collected, and the remaining digested tissues were mechanically disrupted by passage through 100-μm polypropylene mesh. The cell suspension was pooled and filtered through cotton wool and washed three times with cold DMEM with 10% FCS and 0.1 mg of DNase per ml. Cells were purified by centrifugation on Percoll (Pharmacia, Uppsala, Sweden) discontinuous gradients of 40% (1.050 g/ml; refractive index, 1.341) and 72% (1.092 g/ml; refractive index, 1.349), made by diluting isotonic stock Percoll solution with 1× DMEM. Highly viable cells (>95%) were recovered from the interface, washed twice, and kept on ice until used. This procedure yielded an average of 2.5 × 10^6 viable LP lymphocytes per mouse. The average concentration of cells recovered were (in 10^6 cells per ml) 1.1 ± 1.0 for LP lymphocytes of 4-month-old mice, and 3.0 ± 3.8 for LP lymphocytes of 24-month-old mice.

ELISPOT assay. CTx-specific antibody-secreting cells were enumerated by the ELISPOT assay of Czerkinsky et al. (6). Briefly, a 96-well enzyme immunoassay microtiter plate (Flow laboratories) was coated with ganglioside GM1 (Sigma Chemical Co.) (3 nmol/ml) in PBS by overnight incubation at room temperature. The plate was washed three times with PBS, and then purified CTx (3 μg/ml in PBS) was added and the plate was incubated for 2 h at room temperature. The plate was washed three times with PBS–Tween 20 (0.05%) and blocked by incubating for 1 h at 37°C with PBS-Tween 20 containing 1% bovine serum albumin (BSA). The plates were then washed twice with PBS-Tween 20 and three times with PBS alone. Aliquots (100 μl) of cell suspension were added in duplicate to each well and incubated in a vibration-free incubator at 37°C in 5% CO_2 and 100% humidity for 4 h. After incubation, the cells were washed off by rinsing three times with PBS-Tween 20. Isotype-specific anti-CTx antibody-secreting cells (measured as SFCs) were then detected by incubating with Fc-specific anti-mouse IgG, IgM, and IgA antibody conjugated with alkaline phosphatase (Sigma Chemical Co.). Aliquots (100 μl) of conjugate (1:1,000) in PBS-Tween 20 containing 0.25% BSA were added to each well, and the plate was incubated overnight at room temperature. Following incubation, the plate was washed three times with PBS-Tween 20, and the spots were developed by adding 1 mg of 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidinium (BCIP) substrate per ml in 2-amino-2-methyl-1-propanol buffer (pH 10.5). Each blue spot, representing a single antibody-secreting cell (SFC), was then counted under a microscope. The reaction was stopped by adding 50 μl of 3 M NaOH. The number of antibody-secreting cells was expressed as the number of SFC per 10^8 cells.

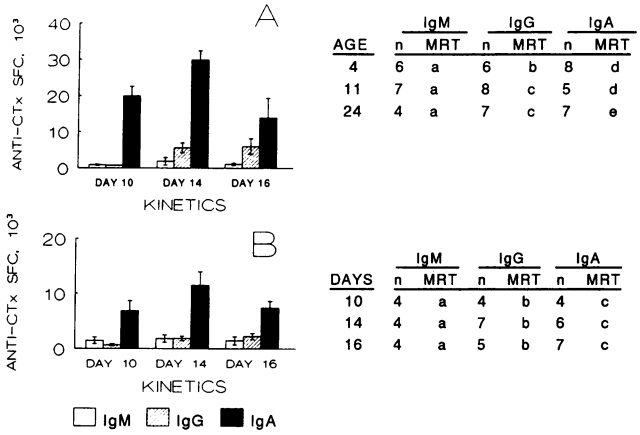
Serum anti-CTx antibody titer determination. Serum isotype-specific anti-CTx antibody was determined by enzyme-linked immunosorbent assay (ELISA) according to the method of Voller et al. (37). Optimal serum antibody activity was previously determined on day 14 after oral immunization with CTx. The 96-well enzyme immunoassay microtiter plate was coated first with ganglioside GM1 (3 nmol/ml) and then with CTx (3 μg/ml), as described above for the ELISPOT assay. Serum samples from individual mice were serially plated in twofold dilutions (100 μl per well) and incubated overnight at room temperature. Solid-phase bound antibody was detected by Fc-specific anti-mouse IgM-, IgG-, or IgA-alkaline phosphatase conjugates. The conjugates were incubated for 2 h at 37°C in a humid chamber. Following washing after incubation, the reaction was developed by adding 100 μl of *p*-nitrophenylphosphate substrate (Sigma Chemical Co.) in diethanolamine buffer, pH 9.5, at a concentration of 1 mg/ml. The substrate was incubated for 1 h at room temperature. The reaction was stopped by adding 50 μl of 3 mM NaOH to each well. The reaction was read in a Titertek enzyme immunoassay plate reader at 410 nm. The highest dilution of serum which gave an optical density reading of 0.4 above the negative control (normal mouse serum) was taken as the anti-CTx titer.

IEF and affinity immunoblotting. IEF of the sera was performed in 1% agarose gels across a broad pH gradient (pH 3 to 10) as described by Schibeci et al. (21). The focused serum samples were then transferred to nitrocellulose paper by affinity immunoblotting as described by Kinsley and Rodkey (18). The nitrocellulose membrane was first coated with CTxB (10 μg/ml in 0.5 M $NaHCO_3$) by incubating overnight at room temperature. After rinsing four times with PBS, the free sites on the membrane were blocked with PBS containing 1% Tween 20 and 1% BSA. The CTx-coated membrane sheet was gently rolled onto the gel and incubated in a moist chamber for 15 min at 37°C. Bound isotype-

specific anti-CTx antibody was detected by incubating the blot with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin specific for IgM, IgG, or IgA (Sigma Chemical Co.). The reaction was developed with a nitroblue tetrazolium-BCIP substrate mixture in ethanolamine buffer, pH 9.5.

Inhibition of affinity immunoblotting was done by saturating the CTx-coated nitrocellulose membrane with excess free CTxB (2.5 mg/ml) just prior to overlaying the gel to transblot the antibody molecules.

Statistics. Data were square root transformed to achieve homogeneity of variance as determined by Bartlett's Test (1). Results were analyzed by using a two-way analysis of variance (ANOVA) test to determine whether the main effects of age and tissue source were significantly different. The interaction between age and tissue source was also tested. A significant interaction would show that the two tissues were not affected by age to the same extent. When the main effect of age was significant, then the one-way ANOVA test and the multiple range test (MRT) were used to determine at what age the SFC responses changed significantly. The MRT was used to assess relatedness by using Fisher's Least Significant Difference Test when the one-way ANOVA was $P < 0.05$ and by using Tukey's Honest Significant Difference Test when the one-way ANOVA was $P \geq 0.05$.



AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	6	a	6	b	8	d
11	7	a	8	c	5	d
24	4	a	7	c	7	e

DAYS	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
10	4	a	4	b	4	c
14	4	a	7	b	6	c
16	4	a	5	b	7	c

FIG. 1. Kinetics of anti-CTx SFC response in the LP from 4-month-old (A) and 24-month-old (B) mice to oral immunization with CTx. The results are shown as the means of anti-CTx SFC per 10^8 nucleated cells ± standard error. *n* refers to the number of individual mice tested for each kinetic day group from 2 to 3 independent experiments. The MRT results are represented by letters a, b, and c. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.

RESULTS

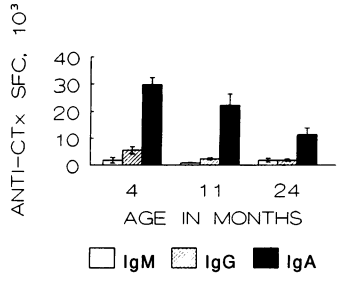
Kinetics, isotype, and specificity of anti-CTx response after peroral immunization. To examine age-related changes in the primary antibody response in the gut-associated lymphoid tissue (GALT), mice of different age groups were immunized perorally with CTx and assayed 10, 14, and 16 days after immunization for antibody-secreting cells in the PP, MLN, LP, and spleen by the ELISPOT assay. The specificity of the anti-CTx Elispots (the SFCs) was determined by competitive inhibition of spots by including CTxB in the incubation medium. SFCs were inhibited in a dose-dependent manner (data not shown). In addition, no SFCs were detected when the plate was coated with an irrelevant antigen (e.g., BSA) or when unprimed B cells were tested on CTx-coated plates. A single oral immunization with CTx was able to induce measurable primary anti-CTx IgM, IgG, and IgA SFCs in the PP, MLN, LP, and spleen. In Fig. 1A, the peak response for IgM, IgG, and IgA anti-CTx SFCs was found to be at 14 days after oral immunization with CTx in the LP for the 4-month-old age group.

Analysis of the data by using a one-way ANOVA indicated that kinetics of the response did not appear to have any significant effect on IgM ($P = 0.6081$) and IgG ($P = 0.0887$) anti-CTx SFC responses but indeed had a significant effect on IgA ($P = 0.0203$) responses. For IgA anti-CTx SFCs, the response at 14 days (letter d) was significantly greater than at 16 days (letter e) but no different from the 10-day response (letter d). Significant differences among the kinetics of individual days as detected by the MRT are indicated by different alphabetical letters in separate columns (Fig. 1).

For the 24-month-old age group (Fig. 1B), peak responses occurred at 14 days after immunization with CTx. From the one-way ANOVA, kinetics had no significant effect at the 95% confidence level over the entire period of days examined for the three isotypes. It is noteworthy that the kinetic responses of the PP and MLN anti-CTx SFCs showed the best responses 14 days after oral immunization with CTx, while splenic anti-CTx SFC responses were best at 16 days (data not shown).

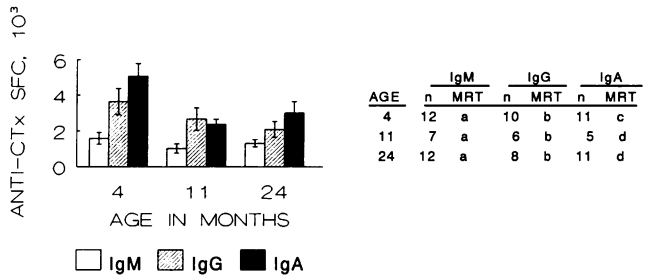
Effect of aging on the anti-CTx SFC responses in the LP, PP, MLN and the spleen. There was a significant age-related decrease of IgA anti-CTx SFC responses in both the LP (Fig. 2) and the PP (Fig. 3) 14 days after oral immunization with CTx. In addition, IgG anti-CTx SFC responses in the LP significantly declined after 11 months of age compared with the young group (Fig. 3). It is noteworthy in Fig. 3 that IgM and IgG anti-CTx SFC responses in the PP did not exhibit an age-related decline. In the MLN and the spleen, IgM, IgG, and IgA anti-CTx SFC responses did not decline with age (Fig. 4 and 5, respectively). There was no significant age-related decline of anti-CTx IgM response in any tissues examined.

Peroral immunization of mice was found to give IgA anti-CTx SFC which was the significantly predominant re-



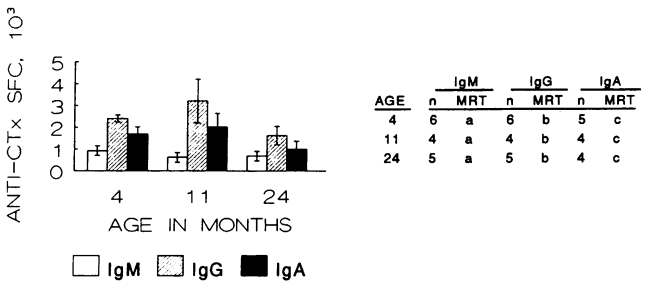
AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	6	a	6	b	8	d
11	7	a	8	c	5	d
24	4	a	7	c	7	e

FIG. 2. Effects of aging on the anti-CTx SFC response in the LP from 4-, 11-, and 24-month-old mice, 14 days after oral immunization with CTx. The results are shown as the means of anti-CTx SFC per 10^8 nucleated cells ± standard error. *n* refers to the number of individual mice tested for each age group from 2 to 4 independent experiments. The MRT results are represented by letters a, b, c, d, and e. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.



AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	12	a	10	b	11	c
11	7	a	6	b	5	d
24	12	a	8	b	11	d

FIG. 3. Effects of aging on the anti-CTx SFC response in the PP from 4-, 11-, and 24-month-old mice, 14 days after oral immunization with CTx. The results are shown as the means of anti-CTx SFC per 10^8 nucleated cells ± standard error. *n* refers to the number of individual mice tested for each age group from 3 to 6 independent experiments. The MRT results are represented by letters a, b, c, and d. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.



AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	6	a	6	b	5	c
11	4	a	4	b	4	c
24	5	a	5	b	4	c

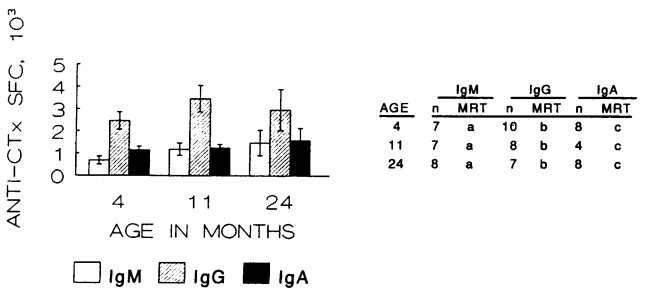
FIG. 5. Effects of aging on the anti-CTx SFC response in the spleen from 4-, 11-, and 24-month-old mice, 16 days after oral immunization with CTx. The results are shown as the mean of anti-CTx SFC per 10^8 nucleated cells ± standard error. *n* refers to the number of individual mice tested for each age group from 2 to 3 independent experiments. The MRT results are represented by letters a, b, and c. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.

sponse in the LP. IgA SFC responses in the PP, MLN, and spleen were found to be not significantly different from one another but were higher in the PP than in the MLN and spleen. IgM and IgG SFC responses in the LP, PP, MLN, and spleen were also found to be not significantly different. The anti-CTx responses in the spleen were slightly lower in magnitude than in the mucosal tissues.

From the one-way ANOVA, aging had a significant effect (at the 95% confidence level) on the anti-CTx responses in the LP for IgA ($P = 0.0007$) and IgG ($P = 0.0071$) but had no significant effect on the IgM response over the entire age span examined. Aging had a significant effect (at the 95% confidence level) on the anti-CTx response in the PP for IgA ($P = 0.0280$) but had no significant effect on IgM and IgG responses over the entire age span examined. The significant interactions observed in a two-way ANOVA between age and tissue for anti-CTx SFC responses indicated that, with aging, SFC responses differ depending on the tissue source. IgG and IgA anti-CTx SFC responses were significantly different among the tissue sources in a two-way ANOVA, whereas there were no significant differences in IgM SFC responses.

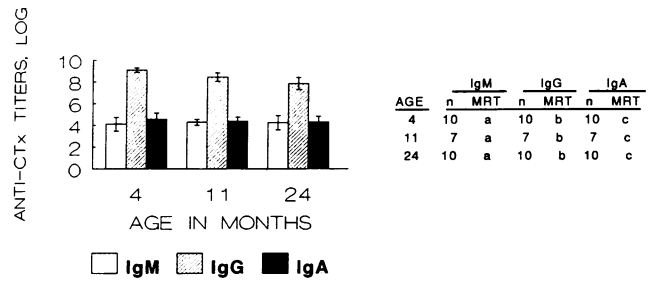
Serum anti-CTx antibody titers. Peroral immunization of mice with CTx was found to induce predominantly IgG anti-CTx antibody responses in serum in all age groups (Fig. 6). Although IgM and IgA anti-CTx titers were 4 to 5 log units lower than IgG titers, no age-related differences were observed. From the one-way ANOVA, aging had no significant effect (at the 95% confidence level) on the anti-CTx antibody titers for IgM, IgG, and IgA over the entire age span examined.

Analysis of serum antibody clonotypes by IEF. Since anti-CTx antibody titers in serum 14 days after oral immunization were found not to decline with aging, analysis of serum antibody clonotypes was conducted by IEF and affinity immunoblotting of CTx immune serum from young and old mice. The data revealed several distinct new IgG clonotypes in the immune serum of older mice (Fig. 7). However, there occurred some loss of clonotypes in the immune serum of older mice compared with those of the young group. Similar patterns were also observed for anti-CTx IgM and IgA. Thus, peroral immunization appeared to induce distinct new IgG anti-CTx clonotypes, as revealed by IEF. The speci-



AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	7	a	10	b	8	c
11	7	a	8	b	4	c
24	8	a	7	b	8	c

FIG. 4. Effects of aging on the anti-CTx SFC response in the MLN from 4-, 11-, and 24-month-old mice, 14 days after oral immunization with CTx. The results are shown as the mean of anti-CTx SFC per 10^8 nucleated cells ± standard error. *n* refers to the number of individual mice tested for each age group from 2 to 4 independent experiments. The MRT results are represented by letters a, b, and c. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.



AGE	IgM n	IgM MRT	IgG n	IgG MRT	IgA n	IgA MRT
4	10	a	10	b	10	c
11	7	a	7	b	7	c
24	10	a	10	b	10	c

FIG. 6. Effects of aging on serum anti-CTx antibody titers from 4-, 11-, and 24-month-old mice, 14 days after oral immunization with CTx. The results are shown as natural log of serum anti-CTx antibody titers as determined by ELISA. The highest dilution of serum which gave an optical density at 410 nm of ≥0.4 above the control (normal mouse serum) was taken as the anti-CTx titer. *n* refers to the number of individual mice tested for each age group from 4 to 8 independent experiments. The MRT results are represented by letters a, b, and c. The letters indicate whether or not the results at each group are significantly different from one another. If the results are not significantly different, they are assigned the same letter.

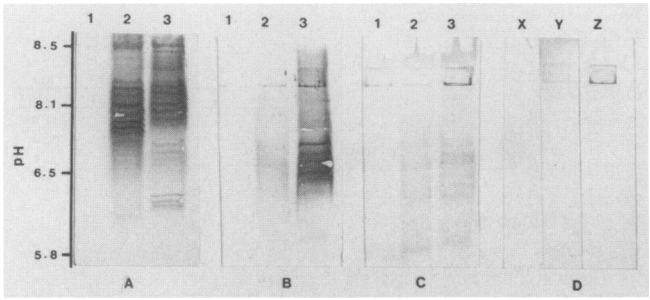


FIG. 7. IEF and affinity immunoblots of serum anti-CTx antibodies from 4- and 24-month-old mice, 14 days after oral immunization with CTx. A nitrocellulose membrane blot was coated with CTxB (2.5 mg/ml). Panel A, IgG anti-CTx antiserum; panel B, IgM anti-CTx; panel C, IgA anti-CTx; and panel D, IgG (lane X), IgM (lane Y), and IgA (lane Z) anti-CTx antiserum blocked with soluble CTxB. Lane 1, nonimmune serum; lane 2, CTx immune serum from 4-month-old mice; and lane 3, CTx immune serum from 24-month-old mice.

ficity of the anti-CTx clonotypic bands was determined by inhibiting the development of antibody bands by saturating the CTx-coated nitrocellulose membrane with excess CTxB just prior to overlaying the gel. No focused bands were observed following such treatment.

DISCUSSION

The results of the present studies show that a single oral immunization of mice with CTx induces measurable antibody-secreting B cells in the PP, MLN, LP, and spleen. There have been no previous studies that examined the age-associated antigen-specific responses in both inductive and effector sites of the GALT simultaneously. The predominant anti-CTx isotype in the PP and LP was found to be IgA, followed by IgG and IgM. The findings have shown that there was a defect in the induction of antigen-specific IgA anti-CTx SFC B cells with age in the PP, as well as of IgG and IgA anti-CTx SFC in the LP. There was, however, no age-related decline in IgM anti-CTx SFC response in the PP or LP. In contrast, there was no age-related decline of IgM, IgG, or IgA anti-CTx SFC responses in the MLN or spleen. We did not observe any difference in the peak anti-CTx SFC immune response in any tissues with aging, except in the spleen, which indicated that there was no delay in the homing of the antigen-specific B cells to the respective mucosal sites.

Schmucker et al. (22) have shown similar declines of anti-CTx antibody-containing cells in the small intestinal LP of aged rats. They also found that the α-CTx-IgA titer in the bile of immunized, aged rats was markedly reduced (fivefold lower than those of young rats) 5 days following secondary immunization. However, α-CTx-IgA levels in nonimmunized rats were negligible, and age-related shifts in other antibody titers (α-CTx IgG and IgM) were not significant (22). A decreased proportion of IgG and IgA antibody-containing cell in the secretory tissues of aged rats was also reported by Ebersole and Steffen (10). However, others have observed either an age-related increase or no change in the gut perfusate or luminal IgA, IgG, and IgM levels (9, 17, 23, 24), while the binding capacities of natural intraluminal IgG antibody (24) or purified intestinal dimeric IgA for outer membrane antigens of normal habitant bacteria were markedly reduced in the old animals. In our studies, it appears that an age-related decline of anti-CTx IgA SFC in the PP implicates an age-related dysfunction in the inductive sites of the GALT, with a consequential age-related decline of antibody-secreting cells in the LP. Our findings are in agreement with the results of Schmucker et al. (22) and Ebersole and Steffen (10). However, MLN and splenic anti-CTx SFC responses were found not to decline in old animals following oral immunization with CTx. These latter findings clearly demonstrate that different immunoregulatory mechanisms operate in different mucosa-associated tissues.

The decline of anti-CTx IgA SFC in PP and LP in our studies may be due to the intrinsic defects of B cells in PP (17) or an alteration in the T-helper function with age (10). Preliminary studies in our laboratory have indicated normal distribution of $L3T4^+$ cells and interleukin-4- (IL-4) and interferon-γ (INF-γ)-secreting T cells in the spleen, MLN, and PP of aged mice. Other studies have examined the proportion of T cells in the spleen and GALT with aging. IL-2 receptor (IL-2R) expression by $Thy\text{-}1^+$ PP lymphocytes was not significantly reduced with age (12). A total of 70% of PP lymphocytes from old mice expressed IL-2R, compared with 80% of $Thy\text{-}1^+$ lymphocytes from young mice. Levels of IL-2R expression as determined by fluorescence intensity were reduced with age on splenic lymphocytes but not on PP lymphocytes. The percentages of $CD4^+$ and $CD8^+$ lymphocytes expressing IL-2R after activation with concanavalin A (Con A) were equally reduced with age in the splenic lymphocyte population. Expression of IL-2R by either T-lymphocyte subset was unaffected by age in the PP. One study has examined the effects of age on T-lymphocyte subsets in detail. The lymphocyte populations of the PP from A/J mice were compared at 3 to 4 and 18 months of age (36). There was no change with age in the numbers of $Thy\text{-}1^+$ or $L3T4^+$ or surface Ig-bearing lymphocytes, but there was a decrease in the percentage of $Lyt\text{-}2^+$ lymphocytes. The percentage of lymphocytes with Fc receptors for IgM and for IgG2a decreased with age, while there was an increase in the percentage with Fc receptors for IgG2b. Ernst et al. (13) examined the effects of age on the percentage of $Thy\text{-}1^+$ lymphocytes in the PP of C57BL/6 mice and found no significant change between 2 and 20 months of age.

Recent studies in rhesus monkeys support the categorization of mucosal immune system into inductive and effector sites containing different populations of lymphocytes. T lymphocytes in the MLN and LP express higher levels of IL-4 and IL-5 (Th2 helper subset) than do peripheral T lymphocytes (16). Populations in the MLN and LP, however, are not identical. MLN lymphocytes had less mRNA for IL-2 and IFN-γ than did LP lymphocytes. LP T lymphocytes showed higher helper activity than MLN lymphocytes in pokeweed mitogen-stimulated cultures. These studies suggest that T lymphocytes in inductive sites such as the MLN have mixed populations consisting of naive cells (producing low levels of IFN-γ and IL-2) and differentiated cells (producing high levels of IL-4 and IL-5). In effector sites, such as the LP, more differentiated memory cells predominate. These cells are capable of producing high levels of IL-2, IFN-γ, IL-4, and IL-5 and have a higher capacity for helper activity than do naive T lymphocytes. Recently, Taguchi et al. (32) have shown that although significant numbers of IFN-γ-secreting cells following Con A activation were seen in the LP, the frequency of IL-5-secreting cells was always higher (Th1:Th2 among LP lymphocytes, 1:3). In the intraepithelium, the PP, and the spleen, equal numbers of IFN-γ- and IL-5-secreting cells were seen following Con A activation. Thus, even within the

mucosal immune system, lymphocyte populations may show compartmentalization.

Murine IL-6-responsive B cells have now been found in the PP (2). In this case, the addition of IL-6 to these cells selectively stimulated the production of IgA without inducing any proliferation. IL-6 was found to act on cycling membrane IgA^+ ($mIgA^+$) and not on $mIgA^-$ B cells, suggesting that the major role of IL-6 is to induce the terminal differentiation of B cells into plasma cells (35).

In the present studies, an age-related decline in the Th2 subset in the effector sites of the GALT (e.g., the LP) may contribute to the observed decline of LP B-cell SFC responses to CTx. In a recent study, we have found that Th2-cell subsets producing IL-5 may be the predominant helper T-cell population producing IL-5 in the PP, whereas the MLN and spleen contain both Th1 and Th2 populations producing IFN-γ and IL-4 (15). With aging, there appeared to be a proportional shift in the spleen and MLN toward more Th2 cells in the helper-cell populations producing IL-5 than that seen in Th1-cell populations producing IFN-γ. There was a significant age-related decline in the percentage of cytoplasmic IL-5-containing Th2-cell populations in the PP. It would appear from these findings that the observed age-related decline of IgA anti-CTx SFC in the LP and PP may be due to a loss in the number of IL-5-producing Th2-helper cells with aging. An alternate possibility is a relative deficiency of IL-6 in the mucosa with aging.

The age-associated decline of the anti-CTx SFC responses in the PP and LP is in contrast to our previous observation of no change in the anti-CTx SFC responses in the MLN and bronchial lymph nodes following intraperitoneal immunization with other antigens (30, 31, 41, 42). However, following oral immunization with trinitrophenylated bovine γ-globulin, we had previously observed either no age-related decline or an increase in the anti-trinitrophenyl antibody-secreting cells in the MLN and spleen (40, 42). In the present studies, the anti-CTx SFC responses in the MLN and spleen following oral immunization were also unaffected with age for all isotypes examined. This lack of age-related declines of anti-CTx responses in the MLN and spleen was not the case for the LP and PP, in which there were significant age-related declines in IgA anti-CTx SFCs. Similar observations of age-related declines in antigen-specific intraluminal IgA responses (10, 22–24) and antibody-containing cells in the murine intestine (10, 22, 33) have also been made. We believe that this differential effect of aging on the B-cell responses in mucosal tissues indicate an important role of local factors in regulating the immune response at individual mucosal sites, despite the existence of a common mucosal immune system (4, 5, 19, 20). The distinct nature of the MLN as opposed to the PP with regard to the presence of lymphocyte-homing molecules may contribute to the ability of MLN lymphocytes to respond differently from those of the PP. The high endothelial venules of the MLN express both MECA-79, a homing molecule predominantly present in peripheral lymph nodes, and the MECA-367 molecule, which is present in the PP. PP high endothelial venules express only MECA-367 (27). Further studies are required to elucidate age-associated changes in the homing pattern of lymphocytes and their influence on the immune response at each of these sites.

We also observed no age-related decline of anti-CTx titers in serum after oral immunization, which is consistent with the anti-CTx SFC response data. The predominant anti-CTx isotype in serum was IgG. Our findings are in agreement with the observations of Smith et al. (26), who also found no change in antibody titers in serum with age following primary and secondary immunization. However, IEF of anti-CTx immune sera revealed the expression of several distinct clonotypes in the older group compared with the young group. It is noteworthy that others have found silent T-cell clones in young mice which were activated in older mice (25). The significance of the increased activation of immunocompetent lymphocytes is not well understood, but it may contribute to the immunological alteration leading to increased autoreactivity with aging.

At the single-cell level, the present study has shown that the aging process can differentially affect the capability of mucosal sites to respond to antigen following oral immunization. The lack of age-related changes in the specific anti-CTx SFC responses in the MLN, as opposed to declines in the LP and PP, emphasize its distinct nature as a mucosal site which may not necessarily reflect the actual status of immune responsiveness in mucosal tissues as a whole. The lack of age-related IgG anti-CTx titers in the serum may have a more predominant role in the periphery. A loss of anti-CTx SFC responses in the LP and PP with aging following oral immunization with CTx may have adverse effects on the host's defense against potential pathogens at the mucosal surfaces.

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