Lack of oral tolerance in aging is due to sequential loss of Peyer’s patch cell interactions

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Abstract

Our past studies showed that Peyer’s patches were required for the induction of oral tolerance to the protein antigen ovalbumin (OVA), but not to the hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS). In the present study, the effects of immunosenescence on oral tolerance induction were assessed with these two toleragens. Significant reductions in OVA-specific serum IgG antibody and CD4⁺ T cell responses to subsequent challenge were observed in OVA-fed, young adult mice. Importantly, these reduced anti-OVA antibody responses were associated with delayed-type hypersensitivity, and antigen-induced CD4⁺ Th1- and Th2-type cytokine responses. On the other hand, aged mice fed OVA failed to develop oral tolerance. Thus, CD4⁺ T cells from Peyer’s patches produced selected Th2- but no Th1-type cytokines. The TNP-specific serum IgG antibody and T cell responses were significantly diminished by prior TNBS feeding in young adult, 6- to 8-month-old and 12- to 14-month-old, but not in senescent, 2-year-old mice. Finally, we have directly assessed dendritic cell subsets and T cell responses in Peyer’s patches, and their function in tolerance induction was impaired at an earlier stage of life. These results suggest that lack of oral tolerance to the protein OVA during aging is the result of dysfunctional Peyer’s patches.

Introduction

Age-related deterioration of immune functions has been recognized in many species. In fact, the risk and severity of infections are higher and the susceptibility to certain types of autoimmune disease and cancer are increased with advanced age (1,2). Diminished responses to vaccination also occur in the elderly (2-4). These studies suggest that both dysregulation and an overall decline in host immunity occur in aging. Age-associated alterations in host immunity have been studied extensively in systemic compartments and T cells are considered to be more susceptible to immunosenescence, although dysfunction of the immune system with aging occurs at both B and T cell levels (1,2,5,6).

Age-associated changes in the mucosal immune system are less well understood than are those associated with the systemic lymphoid system. It is now well established that the mucosal immune compartment is functionally separated from its systemic counterpart and forms a unique host defense network termed the common mucosal immune system (7). Mucosal tissues are directly exposed to pathogens and therefore constitute a first line of immunological defense (8). The gastrointestinal tract in the elderly is particularly susceptible to infectious diseases and suggests that mucosal immunity is also affected in aging (3,9). In fact, the severity and mortality caused by the mucosal pathogens influenza virus and Streptococcus pneumoniae are sharply increased in the elderly (4,10,11). A substantial senescence-associated decline in the numbers of lymphoid cells was found in the gut-associated lymphoreticular tissues, e.g. the Peyer’s patches and the draining mesenteric lymph nodes (MLN) (12). Further, in vitro antibody production in cells from Peyer’s patches and MLN were depressed when T cell-dependent, but minimally affected when T cell-independent B cell mitogens were used to assess responsiveness (12). This finding suggested that T cells are more susceptible than...
B cells to immunosenescence in the mucosal compartment. In a recent study, we showed that mucosal and systemic immune responses induced by oral challenge with ovalbumin (OVA) and cholera toxin (CT) as adjuvant were markedly diminished in aged mice, whereas significant immune responses were seen in young adult mice in both mucosal and systemic compartments (13). In addition, our study strongly suggested that the development of age-associated alterations occurs earlier in the mucosal than in the systemic immune compartment (13). These results clearly indicated that both mucosal and systemic immunity were impaired, and that mucosal vaccines are not effective in the elderly (13).

Although the mucosa is the portal of entry for pathogens, its surfaces are also absorbing nutrients indispensable to life and food proteins are actively taken up through these mucosal areas. Inevitably, and in addition to secretory IgA antibody responses, the mucosal immune system has also developed the unique feature of inducing negative responses in the host. This immunologic unresponsiveness has been termed oral tolerance and is considered to be an important physiological arm which controls hypersensitivity to food antigens, commensal intestinal bacteria and self-antigens (14–16). The underlying mechanisms that result in the state of systemic unresponsiveness after oral delivery of antigen are not fully understood; however, the dose of antigen given has been shown to be an important factor (17). For example, repeated low oral protein doses induce cytokine-mediated responses, the mucosal immune system has also developed the unique feature of inducing negative responses in the host. This immunologic unresponsiveness has been termed oral tolerance and is considered to be an important physiological arm which controls hypersensitivity to food antigens, commensal intestinal bacteria and self-antigens (14–16). The underlying mechanisms that result in the state of systemic unresponsiveness after oral delivery of antigen are not fully understood; however, the dose of antigen given has been shown to be an important factor (17). For example, repeated low oral protein doses induce cytokine-mediated active immune suppression characterized by the presence of regulatory T cells, which include the transforming growth factor-β-producing T_{H}3 cells and IL-10-producing T_{H}1 cells (18,19). On the other hand, a high dose of oral antigen leads to T cell clonal deletion or anergy, which is characterized by inhibition of both antibody- and cell-mediated immune responses (20–22).

The nature of antigen and the dose or frequency of antigen feeding have been reported to influence the induction and maintenance of oral tolerance (8,23–27). In addition, host-related factors such as genetic background and the absence of microflora in the gastrointestinal tract also affect oral tolerance induction (28–30). Our recent study showed that organized Peyer’s patches are required for induction of oral tolerance to protein, but not to hapten antigens (31). Further, and most interestingly, the age of the host has been suggested to influence susceptibility to oral tolerance induction. Previous studies reported that neonatal mice do not develop oral tolerance when fed protein antigen (32–34). This finding may partially explain hypersensitivity to food antigens commonly seen in infants. On the other hand, several studies showed that aged mice exhibit a decreased susceptibility to oral tolerance induction, but not to its maintenance (29,35–37). However, these studies mainly assessed oral tolerance induction by analysis of serum antibody titers. Thus, antigen-specific T cell responses were not characterized in these studies, although reductions in antibody production seen in oral tolerance most likely reflected defective T cell help. Further, the mechanisms of impaired oral tolerance induction with advanced age has not been addressed.

In the present study, we assessed oral tolerance to OVA (a Peyer’s patch-dependent antigen) and to 2,4,6-trinitrobenzene sulfonic acid (TNBS) (a Peyer’s patch-independent antigen) in young adult and aged mice in order to elucidate why impairment of oral tolerance occurs during aging. We focused our studies on Peyer’s patches in order to determine if the dysregulation of oral tolerance that occurs during aging was due to functional abnormalities in this mucosal inductive site.

**Methods**

**Mice**

Young adult and aged C57BL/6 mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) and the Jackson Laboratory Animal Resources Center (Bar Harbor, ME) respectively. These mice were transferred to microisolators and maintained in horizontal laminar flow cabinets in the UAB Immunobiology Vaccine Center. All mice were free of pathogenic bacteria and viruses as determined by antibody screening and routine histologic analysis of organs and tissues from sentinel mice. Experiments were performed using young adult (8- to 12-week-old mice) or mice at 6–8 months, 12–14 months or 2 years of age.

**Induction of oral tolerance**

In order to induce OVA-specific immune unresponsiveness, mice were gastrically intubated with 50 mg OVA (Fraction V; Sigma, St Louis, MO) dissolved in 0.25 ml PBS while control mice were given 0.25 ml PBS orally (38). On days 7 and 14 after gastric delivery, mice were s.c. immunized with 100 µg OVA and 1 µg CT (List Biological, Campbell, CA) (13). In some experiments, mice were immunized orally with 1 mg OVA and 10 µg CT on days 7, 14 and 21 after an initial 50 mg dose of OVA (38–40). In order to examine T_{H}1 and T_{H}2 cytokine responses in Peyer’s patches, CD4^+ T cells were isolated 3 days after an initial 50 mg of oral OVA. The mice were sacrificed 7 days after the final immunization, and blood samples and lymphocytes from mucosal and systemic tissues were subjected to further analyses.

For the induction of oral tolerance to 2,4,6-trinitrochloro-1-benzene (TNCS), mice were gastrically intubated with 10 mg of the water-soluble form of this hapten, TNBS (Sigma), in 0.25 ml PBS on days 0 and 7 while control mice were given 0.25 ml of PBS orally. On day 14, the mice were challenged by applying 100 µl of 7% TNCS (Nacalai Tesque, Kyoto, Japan) in acetone and ethanol (acetone:ethanol = 1:3 v/v) on the shaved abdominal skin (31). Specific delayed-type hypersensitivity (DTH) responses to TNCS were measured on day 19 and blood samples were collected on day 28 for analyses of antibody responses to the sensitizing hapten.

**Lymphoid cell isolation**

The spleen was removed aseptically and single-cell suspensions were prepared by passage through sterile wire mesh screens as described previously (13,31,38). Peyer’s patches were carefully excised from the intestinal wall and dissociated using the neutral protease enzyme collagenase type V (Sigma) in RPMI 1640 (Cellgro Mediatech, Washington, DC) to obtain single-cell preparations (13,38). Mononuclear cells were collected, washed and resuspended in RPMI 1640.
containing 10% heat-inactivated FCS (Summit Biotechnology, Fort Collins, CO).

Detection of antigen-specific antibody responses in serum

The serum antibody titers to OVA, to the B subunit of CT (CT-B) and to trinitrophenyl (TNP) haptenes were determined by endpoint ELISA as described in detail elsewhere (31,38). The CT-B was purified from *Bacillus brevis* expressing the plasmid for c-CT-B (kindly provided by Dr Yoshikazu Yuki, JCR Biopharmaceuticals, San Diego, CA). Briefly, Falcon Microtest III assay plates (Becton Dickinson, Oxnard, CA) were coated with a solution of OVA (1 mg/ml), CT-B (5 μg/ml) or TNP-coupled BSA (TNP-BSA) (0.1 mg/ml) in PBS and incubated overnight at 4°C. After blocking with 1% BSA in PBS for 2 h at 25°C, serial dilutions of serum samples were added to each well. Following incubation for 2 h at 25°C, horseradish peroxidase (HRP)-labeled goat anti-mouse μ, γ or α heavy chain-specific antibodies (Southern Biotechnology Associates, Birmingham, AL) were added to wells and incubated for 2 h at 25°C. For analysis of IgG1, IgG2a, IgG2b and IgG3 antibody subclasses, biotin-conjugated mAb specific for γ1 (G1-6.5), γ2a (R19-15), γ2b (R12-3) or γ3 (R40-82) (BD PharMingen, San Diego, CA) were used. A HRP-labeled anti-biotin antibody (Vector, Burlingame, CA) was used for detection. The color reaction was developed with 0.1 M citrate phosphate buffer (pH 4.2) containing 0.01% H_{2}O_{2}. Endpoint titers were expressed as the last dilution yielding an optical density at 415 nm (OD415) of > 0.1 units above negative control values after a 15-min incubation.

Measurement of DTH responses

To assess DTH responses which were specific for OVA in vivo, 10 μg OVA in 20 μl PBS was injected into the left ear pinna and PBS alone (20 μl) was administered to the right ear pinna as a control (31,38). For the assessment of TNCB-specific DTH responses, mice were sensitized by applying 20 μl 0.8% TNCB in olive oil on both sides of the right ear and the same amount of olive oil on both sides of the left ear as a control (31). The thickness of the ear was measured 24 h later with an upright dial thickness gauge (Peacock; Ozaki, Tokyo, Japan). The DTH response was expressed as the increase in ear swelling after challenge with antigen after subtraction of swelling in the control side.

**CD4**^+^ T cell proliferation assay

CD4^+^ T cells were purified by the MACS system (Miltenyi Biotec, Auburn, CA) as previously described (31,38). Briefly, splenic or Peyer’s patch cells were incubated with biotin-conjugated anti-mouse CD4 mAb (GK 1.5) and subsequently with streptavidin-conjugated microbeads. The CD4^+^ T cell population was enriched following passage through a magnetized column. The isolated CD4^+^ T cells were >97% pure and >99% viable. Purified CD4^+^ T cells were cultured with 1 mg/ml of OVA in the presence of T cell-depleted irradiated (3000 rad) splenic feeder cells from naive syngeneic mice in 24- and 96-well tissue culture plates (Corning Glass Works, Corning, NY) for 4–5 days. An aliquot of 0.5 μCi [³H]thymidine (Amersham, Arlington Heights, IL) was added during the last 18 h and amounts of [³H]thymidine incorporated into dividing cells measured by scintillation counting.

Cytokine analysis

Cytokine levels in splenic CD4^+^ T cell culture supernatants were determined by a cytokine-specific ELISA as described previously (13,31,38). Culture supernatants were collected on day 2 for IL-2, and on day 5 for IFN-γ, IL-4, IL-5, IL-6 and IL-10 for analysis of secreted cytokines respectively. In some experiments, Peyer’s patch CD4^+^ T cell culture supernatants were collected on day 4. The immunoplates (Nunc, Naperville, IL) were coated with anti-IL-2 (clone JES6-1A12), anti-IFN-γ (clone R4-6A2), anti-IL-4 (clone BVD4-1D11), anti-IL-5 (clone TRFK-5), anti-IL-6 (clone MP5-20F3) or anti-IL-10 (clone JES5-2A5) (BD PharMingen) mAb. After blocking with 3% BSA in PBS, serial 2-fold diluted samples and standards were added to wells, and incubated overnight at 4°C. The wells were washed and then incubated with biotinylated anti-IL-2 (clone JES6-5H4), anti-IFN-γ (clone XMG 1.2), anti-IL-4 (clone BVD6-24G2), anti-IL-5 (clone TRFK-4), anti-IL-6 (clone MP5-32C11) or anti-IL-10 (clone JES5-16E5) mAb for detection respectively. After incubation overnight at 4°C, HRP-labeled goat anti-biotin antibody (Vector) was added and incubated for 1 h at 25°C. The color reaction was developed with 1.1 mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma) in 0.1 M citrate phosphate buffer (pH 4.2) containing 0.01% H_{2}O_{2}. The minimal detectable level of each cytokine was 0.98 pg/ml for IL-2, 78.10 pg/ml for IFN-γ, 2.34 pg/ml for IL-4, 1.95 pg/ml for IL-5, 19.53 pg/ml for IL-6 and 390.60 pg/ml for IL-10.

Immunohistochemistry of the small intestine

Samples from the small intestine, including Peyer’s patches, were removed by blunt-end dissection, snap-frozen and 4-μm frozen sections prepared. These sections were fixed with cold acetone, blocked with 1% BSA and then incubated with biotin–anti-mouse CD11c (clone R4-6A2), anti-IL-2 (clone JES6-1A12), anti-IFN-γ (clone R4-6A2), anti-IL-4 (clone BVD4-1D11), anti-IL-5 (clone TRFK-5), anti-IL-6 (clone MP5-32C11) or anti-IL-10 (clone JES5-16E5) mAb for detection respectively. After incubation overnight at 4°C, HRP-labeled goat anti-biotin antibody (Vector) was added and incubated for 1 h at 25°C. Bound FDC-M1 mAb was detected with FITC-labeled anti-rat IgG antibody (Southern Biotechnology Associates). The CD11c^+^ and PNA^+^ cells were detected with tetramethyl rhodamine– and FITC–streptavidin respectively (41). Sections were examined with a fluorescence microscope (BXS50/BXFLA; Olympus, Tokyo, Japan) equipped with a digital image capture system (Olympus).

**Statistics**

The data are expressed as the mean ± SD and mouse groups were compared with control mice using Student’s t-test with StatView software (Abacus Concepts, Berkeley, CA) designed for Macintosh computers. *P* < 0.05 was considered significant.

**Results**

Lack of oral tolerance to OVA in aging

To address the effects of aging on oral tolerance, young adult (8–12 weeks old), and 6- to 8-month-old, 12- to 14-month-old and 2-year-old mice were fed a high dose of OVA prior to systemic challenge. One week after systemic immunization...
Fig. 1. OVA (A)- or CT-B (B)-specific IgG and OVA-specific IgG subclass antibody (C) responses in serum of young adult and aged mice fed OVA (filled squares) or PBS (open squares) before systemic challenge. Mice were gastrically intubated with 0.25 ml PBS or 50 mg OVA on day 0. On days 7 and 14 after intubation, these mice were s.c. immunized with 100 μg OVA and 1 μg CT as adjuvant. Antigen-specific antibody responses were determined 7 days after the final challenge. The results are expressed as the mean titer ± SD for 12–15 mice in each experimental group. *P < 0.001 when compared with control mice.
with OVA and CT as adjuvant, OVA-specific serum antibody responses were assessed. Young adult mice fed OVA showed significantly reduced serum IgG antibody responses when compared with PBS-fed mice (Fig. 1A, \( P < 0.001 \)). In contrast, no reductions in OVA-specific IgA antibody responses were seen in the three groups of aged mice initially fed OVA. Furthermore, enhanced OVA-specific IgG antibody responses were seen in aged mice fed OVA when compared with PBS-fed mice, although the differences were not significant between these two groups (Fig. 1A). These results suggest that oral tolerance to OVA is impaired during aging and this impairment occurs as early as 6–8 months of age. OVA-specific serum IgM antibody responses were unaffected by OVA feeding prior to systemic challenge (data not shown). To ensure that reduced IgG antibody responses to OVA were the result of OVA feeding, we also assessed serum IgG antibody responses to the B subunit of CT (CT-B), which is the immunogenic determinant of this molecule. Our results showed that comparable levels of CT-B-specific serum IgG antibody responses were induced in both PBS- or OVA-fed mice in all four groups (Fig. 1B). This result shows that systemic unresponsiveness to oral OVA is induced in young adult, but not in aged mice.

To further characterize the impairment in oral tolerance, we assessed serum IgG subclass antibody responses. In young adult mice, where oral tolerance to OVA was induced, marked reductions in IgG1 antibody responses were seen prior to systemic challenge when compared with PBS-fed mice (Fig. 1C, \( P < 0.001 \)). On the other hand, IgG2a, IgG2b and IgG3 antibodies were unaffected by prior OVA feeding. These findings were in agreement with our previous study which showed that prior OVA feeding significantly reduced IgG1, but not IgG2a, IgG2b or IgG3, antibody production induced by subsequent challenge with OVA in complete Freund’s adjuvant (42). In contrast, such reductions in IgG1 subclass antibody levels observed in young adult mice were not seen in the three groups of aging mice. Again, an enhancement in IgG1 antibody responses was seen in aging mice fed OVA when compared with PBS-fed mice, although the differences were not significant (Fig. 1C). On the other hand, no changes were seen in other IgG subclass antibody responses (Fig. 1C). We also confirmed that IgG subclass antibody titers specific for CT-B were comparable between PBS- and OVA-fed mice in all four aging groups (data not shown). These results suggest that the IgG1 antibody response is highly susceptible to high-dose oral tolerance in young adult, but not in 6- to 8- and 12- to 14-month-old mice.

**Transitional responses and loss of oral tolerance**

Reductions in antibody responses induced by oral tolerance most likely reflect impairment of T cell help. Thus, we next determined whether T cell tolerance induced by a single high oral dose of OVA was impaired in aging. We first assessed OVA-specific DTH responses in young adult and aged mice given PBS or OVA orally before systemic challenge. As expected, markedly diminished OVA-specific DTH responses were induced in young adult mice fed OVA when compared with PBS-fed mice (Fig. 2, \( P < 0.001 \)). In contrast, DTH responses were comparably induced in all groups of aging mice fed OVA when compared with PBS-fed mice (Fig. 2). Furthermore, splenic CD4+ T cells from young adult, PBS-fed mice underwent marked proliferation in response to OVA, while CD4+ T cell responses were diminished in OVA-fed, young adult mice (Fig. 3, \( P < 0.001 \)). In contrast, splenic CD4+ T cell proliferative responses of 6- to 8-month-old mice fed OVA were not significantly reduced when compared with those of PBS-fed mice. Although splenic CD4+ T cells from most of the PBS-fed 12- to 14-month-old mice responded to OVA, some mice failed to respond (Fig. 3), suggesting that a decline in systemic T cell responses was occurring at this age. Further, prior OVA feeding did not affect the CD4+ T cell proliferative responses to subsequent systemic challenge with OVA and CT as adjuvant in 12- to 14-month-old, when compared with PBS-fed mice. Of interest, splenic CD4+ T cells from 2-year-old mice fed PBS were lowly responsive to OVA, while responses were enhanced in some mice fed OVA prior to systemic challenge (Fig. 3, \( P < 0.05 \)). These findings suggest that T cell immune responses markedly decline in this age group and a high oral dose of OVA elicits positive rather than negative immune responses to OVA. These results indicate that T cell tolerance induced by a single high oral dose is impaired as early as 6–8 months of age. In addition, defective T cell immune responses occur in the later stages of life.

**Lack of T cell-specific oral tolerance in aged mice**

It is generally accepted that a single high oral dose of protein induces antigen-specific clonal anergy and/or deletion characterized by reductions in T and B cell responses and cytokine production by CD4+ T cells in young adult mice (20–22). Thus, we addressed whether aging affects T h1- and T h2-type cytokine responses. Splenic CD4+ T cells from young adult and aged mice fed PBS before systemic challenge produced high levels of IFN-\( \gamma \) in addition to the T h2-type cytokines IL-4, IL-5, IL-6 and IL-10, but essentially no IL-2 in response to OVA (Fig. 4A and B). In young adult mice, cytokine production by splenic CD4+ T cells was dramatically diminished by prior OVA feeding before systemic challenge (Fig. 4A and B). In contrast, prior OVA feeding did not induce marked reductions in cytokine responses of splenic CD4+ T cells in aged mice (Fig. 4A and B). In addition, enhanced cytokine production was seen in 2-year-old mice fed OVA when compared with PBS-fed aged mice (Fig. 4A and B). These results indicate that the anergic state induced by a high oral dose of OVA, which is normally observed in young adult mice, is lost with aging. Further, the results obtained in the above experiments demonstrate that the induction of oral tolerance to OVA, which requires the presence of functional Peyer’s patches, is established only in young adult mice and is impaired as early as 6–8 months of age.

**Lack of oral tolerance to hapten in aging**

During the course of our study, we noted that the Peyer’s patches diminished in size with aging. This finding supports earlier work which showed that the weight and cell numbers of Peyer’s patches were reduced in aged mice (12). Based upon these findings, we hypothesized that loss of Peyer’s patch function may cause the impairment in oral tolerance seen in 6- to 8- and 12- to 14-month-old mice. Thus, we assessed oral tolerance induction to the hapten TNBS in young adult and
Impairment of oral tolerance in aging

aged mice. In this regard, our recent study showed that functional Peyer’s patches are essential for induction of oral tolerance to protein antigens such as OVA, but not to the hapten, TNBS (31). In this study, mice were given either oral PBS or 10 mg of TNBS twice at 1-week intervals and subsequently challenged by applying TNCB to their shaved abdominal skin. The levels of TNP-specific serum IgG antibodies and DTH responses were determined. Young adult mice fed TNBS showed significantly diminished IgG anti-TNP antibody responses when compared with PBS-fed mice (Fig. 5A, P < 0.001). In addition and more interestingly, significant reductions in TNP-specific IgG antibody responses were also seen in 6- to 8- and 12- to 14-month-old mice (Fig. 5A, P < 0.001 and P < 0.01 respectively). In contrast, no significant reductions in IgG anti-TNP antibody responses were seen in 2-year-old mice fed TNBS (Fig. 5A).

To further characterize reduced TNP-specific IgG antibody responses, we assessed serum IgG subclass antibody responses. In young adult mice, anti-TNP antibody responses in all four subclasses were diminished when compared with those mice fed PBS (Fig. 5B, P < 0.001). Further, significant reductions in TNP-specific IgG1, IgG2a, IgG2b and IgG3 subclass antibody responses were also

Fig. 2. The effects of prior OVA feeding on DTH responses in young adult and aged mice systemically challenged with OVA and CT. An aliquot of 20 μl PBS or 10 μg OVA in PBS was injected into the right or left ear pinna respectively of mice fed either OVA (filled squares) or PBS (open squares). Ear swelling was measured 24 h later and DTH responses were expressed as increase in ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS. The results are expressed as the mean ± SD for 12–15 mice in each experimental group. *P < 0.001 when compared with control mice.

Fig. 3. Comparison of splenic CD4+ T cell proliferative responses between young adult and aged mice fed OVA (filled circles) or PBS (open circles) prior to systemic challenge with OVA and CT. Purified CD4+ T cells were cultured with 1 mg/ml OVA in the presence of T cell-depleted irradiated (3000 rad) splenic feeder cells from naive, syngeneic mice in 96-well tissue culture plates for 5 days. An aliquot of 0.5 μCi [3H]thymidine was added during the last 18 h and [3H]thymidine incorporated by cells was measured by scintillation counting. The results represent data taken from individual mice. n.s. indicates not significant.

Fig. 4. Continued on facing page.
observed in 6- to 8-month-old mice fed TNBS (Fig. 5B, \( P < 0.001 \), \( P < 0.001 \), \( P < 0.001 \) and \( P < 0.01 \) respectively). In contrast, only IgG1 subclass antibody responses specific for TNP were affected in 12- to 14-month-old mice fed TNBS when compared with PBS-fed mice (Fig. 5B, \( P < 0.001 \)). No reduction in IgG subclass antibody responses was seen in aged 2-year-old mice.

We next assessed DTH responses to TNCB in mice given oral TNBS. These DTH responses were significantly reduced in young adult, 6- to 8- and 12- to 14-month-old mice (Fig. 6, \( P < 0.001 \), \( P < 0.01 \) and \( P < 0.05 \) respectively), while slight reductions in DTH responses to TNCB were seen in 20- to 24-month-old mice (Fig. 6). These results strongly suggest that T cell tolerance to TNCB is induced in mice up to 12–14 months of age; however, aged mice are resistant to tolerance induction. Further, these results indicate that oral tolerance to TNBS, which could be established in the absence of functional Peyer’s patches, is induced in mice up to 1 year of age, while oral tolerance to OVA is induced only in young adult mice.

**Fig. 4.** OVA-specific Th1 (A)- and Th2 (B)-type cytokine production by splenic CD4\(^+\) T cells from mice fed OVA (filled squares) or PBS (open squares) prior to systemic challenge with OVA and CT. Purified CD4\(^+\) T cells were cultured with 1 mg/ml OVA in the presence of T cell-depleted and irradiated (3000 rad) splenic feeder cells from naive syngeneic mice in 24-well tissue culture plates for 5 days. The broken lines show detectable levels of each cytokine in this assay. The data represent the mean levels of cytokine ± SD of one experiment which is representative of four separate experiments.
Our results described thus far clearly suggest that lack of oral tolerance to protein antigen in aging is due to impairment of Peyer’s patch function in earlier stages and by T cell dysregulation in 2-year-old mice. To directly show that sequential loss of T cell responses in Peyer’s patches caused impairment of oral tolerance to protein antigen, we assessed Peyer’s patch CD4+ T cell proliferative and cytokine responses to oral OVA in 6- to 8-month-old mice, since our recent study

**Fig. 5.** The titers of IgG anti-TNP (A) and IgG subclass anti-TNP (B) responses in serum of young adult and aged mice fed TNBS (filled squares) or PBS (open squares) before systemic challenge. Mice were fed 0.25 ml PBS or 10 mg TNBS on days 0 and 7. On day 14 after intubation, these mice were challenged by applying 100 μl of 7% TNCB. TNP-specific antibody responses were determined 14 days after challenge. The results are expressed as the mean titer ± SD for nine to 12 mice in each experimental group. *P < 0.001 and **P < 0.01 when compared with control mice.
showed that mucosal immune responses are impaired in 12- to 14-month-old mice (13). We used our recently established protocol to evaluate oral tolerance induction in Peyer’s patch CD4+ T cells (38). Young adult or 6- to 8-month-old mice were given a high oral dose of OVA, and subsequently orally immunized with OVA and CT on three occasions at weekly intervals. Peyer’s patch CD4+ T cell proliferative responses specific for OVA were determined 7 days after the final immunization. For cytokine analysis, Peyer’s patch CD4+ T cells were isolated 3 days after OVA feeding and were then cultured with/without 1 mg of OVA in the presence of antigen-presenting cells (APC). Consistent with our recent study, CD4+ T cell proliferative responses to OVA were dramatically diminished in young adult mice fed OVA prior to oral challenge when compared with PBS-fed mice (Fig. 7, P < 0.001). In contrast, OVA-specific Peyer’s patch CD4+ T cell proliferative responses were comparable in 6- to 8-month-old mice fed OVA or PBS (Fig. 7). Interestingly, Peyer’s patch CD4+ T cells from tolerized, young adult mice showed dominant Th1-type cytokine responses when compared with those responses in 6- to 8-month-old mice (Table 1). Thus, non-tolerized 6- to 8-month-old mice showed higher IL-5 and IL-6 production, by Peyer’s patch CD4+ T cells, which are essential cytokines for B cell growth and differentiation into plasma cells (Table 1). These results show that T cell tolerance in Peyer’s patches is impaired in 6- to 8-month-old mice.

### Decreased dendritic cell (DC) activation in Peyer’s patches of aging mice

Mucosal immune responses are initiated following antigen uptake, processing and presentation by professional APC in the mucosal inductive tissues such as Peyer’s patches. This pathway is important both for induction of oral tolerance and mucosal immunity (43,44). Thus, we next examined changes in the DC (major APC in Peyer’s patches) with increased age by immunohistochemistry to determine if impaired T cell responses were due to decreases in DC function. In Peyer’s patches of 8-week-old mice, CD11c+ DC were observed in higher numbers in the subepithelial dome, scattered in the interfollicular regions and in the B cell follicles. In addition, similar numbers and distribution patterns of DC were seen in 6- to 8-month-old mice. In contrast, although CD11c+ DC were seen in Peyer’s patches of 14-month-old mice, their numbers were decreased and this reduced frequency was most obvious in the subepithelial dome (Fig. 8). We further assessed the changes in activation status of B cells and DC in Peyer’s patches by using PNA and FDC-M1 mAb as activation markers for germinal center (GC) B cells and follicular DC (FDC) respectively in the B cell follicles. In 6- to 8-month-old mice, diminished PNA+ GC B cells and reduced FDC-M1+ FDC were seen, although the numbers of DC in Peyer’s patches were comparable to those of young adult mice (Fig. 8). Furthermore, the numbers of both PNA+ GC B cells and FDC-M1+ FDC were remarkably diminished in 14-month-old mice (Fig. 8). This clearly suggests that decreased activation of FDC in Peyer’s patches seen in mice of advanced age may indirectly be associated with impaired T cell responses for oral tolerance induction in this tissue.

### Table 1. Peyer’s patch CD4+ T1 and T2 Cytokine Synthesis in Mice Given High Oral Doses of OVA

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<thead>
<tr>
<th>Age of mouse</th>
<th>T1-typeb</th>
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<td>IFN-γ</td>
<td>IL-2</td>
<td>IL-4</td>
<td>IL-5</td>
</tr>
<tr>
<td></td>
<td>(pg/ml)</td>
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<tr>
<td>6-8 wks</td>
<td>1 934 ± 142c</td>
<td>455 ± 90</td>
<td>24 ± 16</td>
<td>0</td>
</tr>
<tr>
<td>6-8 months</td>
<td>0</td>
<td>362 ± 82</td>
<td>14 ± 9</td>
<td>123 ± 8</td>
</tr>
</tbody>
</table>

- Each group of mice was fed 50 mg of OVA. Peyer’s patch CD4+ T cells (2×10⁶ cells/ml) were isolated after 3 days and were cultured with 1 mg/ml of OVA in the presence of T cell-depleted and -irradiated splenic feeder cells (4×10⁶ cells/well).
- Culture supernatants were harvested following 4 days of incubation and were analyzed by the respective cytokine-specific ELISA.
- Results represent the values (mean±SEM) from two separate experiments.

**Fig. 6.** The effects of prior TNBS feeding on DTH responses in young adult and aged mice systemically challenged with TNCB. The mice fed either TNBS (filled squares) or PBS (open squares) were sensitized by applying 20 μl of 0.8% TNCB in olive oil on both sides of the right ear and the same amount of olive oil on both sides of the left ear as a control. Ear swelling was measured 24 h later and the DTH response was expressed as the increase in ear swelling after sensitization with TNCB following subtraction of swelling in the control site. The results are expressed as the mean ± SD for nine to 12 mice in each experimental group. *P < 0.001, **P < 0.01 and ***P < 0.05 when compared with control mice.
Finally, we assessed tissues for the presence of DEC 205+ cells in the T cell zones of Peyer’s patches; however, no major differences were seen in numbers of DEC 205+ DC between young adult and aged mice (data not shown).

**Discussion**

In the present study, we addressed the ability to induce oral tolerance to OVA (a Peyer’s patch-dependent antigen) and TNBS (a Peyer’s patch-independent antigen) in young adult and aged mice at both the B and T cell levels. We also assessed the role of Peyer’s patches in regulation of oral tolerance during aging. Our results clearly show that oral tolerance to protein antigen is impaired with aging and this occurs as early as 6–8 months of age. This impairment is due to sequential loss of both DC and T cell responses in Peyer’s patches in the early stages of life, and by T cell dysregulation in later stages of life. OVA-specific serum IgG, and especially IgG1 subclass, antibody responses were diminished in OVA-fed young adult mice, whereas such reductions in IgG1 antibody responses were not seen in OVA-fed aged mice. Direct evidence that dysregulation of oral tolerance occurs in aged mice was obtained by the following experiments. When we assessed T cell responses in mice fed OVA prior to systemic challenge, significant reductions in OVA-specific DTH, splenic CD4+ T cell proliferative responses and cytokine...
production were seen in young adult mice fed OVA, while mice beyond 6 months of age were resistant to oral tolerance induction. Of interest, splenic CD4+ T cells from 2-year-old mice fed PBS were less responsive to OVA, while OVA-specific responses were enhanced in some mice when a high dose of OVA was fed prior to systemic challenge. These findings indicated that T cell immune responses markedly decline in this age group and high-dose feeding of protein antigen elicits positive responses rather than systemic unresponsiveness. On the other hand, TNP-specific IgG antibody responses were significantly diminished in young adult, 6- to 8-month-old and 12- to 14-month-old mice fed TNBS prior to systemic challenge, when compared with PBS-fed mice. However, such reductions in TNP-specific IgG antibody responses were not seen in 2-year-old mice fed TNBS before systemic challenge. In addition, TNP-specific T cell responses, as determined by DTH, were significantly reduced in young adult, 6- to 8-month-old and 12- to 14-month-old mice, but not in 20- to 24-month-old mice fed TNBS prior to systemic challenge. Further, direct evidence for dysregulation of anergic/suppressor functions of Peyer’s patches in aged mice was obtained by the assessment of Peyer’s patch CD4+ T cell proliferative responses specific for OVA in young adult and 6- to 8-month-old mice fed OVA or PBS prior to oral challenge with OVA and CT as adjuvant. In this regard, Peyer’s patch CD4+ T cells from 6- to 8-month-old mice, which cannot undergo oral tolerance, showed higher IL-5 and IL-6 production than was seen in young adult mice. In contrast, increased levels of IFN-γ production were induced by Peyer’s patch CD4+ T cells. A similar observation was seen in our previous studies in IFN-γ knockout mice (42). Furthermore, additional experiments showed that FDC in the B cell zone were affected in mice of advanced age and CD11c+ DC in the subepithelial dome were decreased in mice >1 year of age. These results suggest that dysregulation of both T and B cell responses in Peyer’s patches occurs in aging mice, and may be, in part, associated with senescence of immune function of both DC and FDC in Peyer’s patches.

To date, several groups have addressed the decreased susceptibility of oral tolerance induction with advancing age (29,35-37). Previous work suggested that maturation into adulthood, from 8 to 24 weeks of age, significantly influenced the induction of oral tolerance in several strains of mice (35). That study assessed induction of oral tolerance to OVA in 20- to 38-week-old C3H/HeJ, A/J and B6D2F1 mice by reductions in OVA-specific serum antibody responses (35). Their results showed that oral tolerance was induced in all strains of young mice and in C3H/HeJ and B6D2F1 mice at 20 and 38 weeks of age respectively (35). On the other hand, oral tolerance was completely abolished in A/J mice at 38 weeks of age (35). Recently, this same group addressed the induction and maintenance of oral tolerance to OVA in young and aged B6D2F1 mice, since these mice were the most susceptible to oral tolerance induction at 8 weeks of age (36). It was shown that B6D2F1 mice become less susceptible at 25 weeks and were totally refractory at 70 weeks of age. Further, they showed that mice given oral OVA at 8 weeks remained tolerant to OVA at 70 weeks of age, indicating that aging affects the inductive, but not the effector, phase of oral tolerance. These results strongly suggested that susceptibility to oral tolerance induction to protein antigen is decreased at 25 weeks of age in mice; however, antigen-specific T cell responses were not characterized. The induction of oral tolerance to sheep red blood cells (SRBC) has also been addressed in young versus aged mice (37). In that study, diminished SRBC-specific IgM, but not IgG, antibody responses were seen in the spleen as determined by SRBC-specific plaque-forming cells in 3.5- to 4-month-old mice fed SRBC before systemic challenge, whereas oral tolerance was abrogated in 6.5-month-old mice. In addition, significant increases in SRBC-specific IgM and IgG antibody responses were observed in the spleen of mice 13.5 months of age. Of interest, T cells from SRBC-fed, young and aged mice did not abrogate the suppression of IgM antibody responses in B cell cultures. These results suggest that abrogation of oral tolerance in aged mice could be ascribed to B rather than T cells. To our knowledge, this is the only study which has addressed impaired T cell functions in oral tolerance with aging (37). However, their findings are in marked contrast to our results which directly show that impairment of oral tolerance induction with aging occurs at the level of T cells as determined by antigen-specific DTH, CD4+ T cell proliferative responses and by cytokine production. This difference may be due to differences in the immunogenicity of OVA and SRBC including T cell dependency for provision of help for B cell responses. The observations made in the previous studies that abrogation of oral tolerance induction begins to occur at 6 months of age are in complete agreement with our present study.

During this study, we observed that the Peyer’s patches became progressively smaller with advancing age, as has been reported by others (12). This suggested that a lack of Peyer’s patches may cause the dysregulation in oral tolerance which begins to occur at 6 months of age. It was recently shown that mice treated in utero with lymphotixin-β receptor Ig fusion protein lacked Peyer’s patches, but developed normal MLN (45,46). We have shown that organized Peyer’s patches are essential for induction of oral tolerance to OVA but not to hapten (31). Thus, in this study, we queried whether oral tolerance could be induced in the hapten TNBS, which does not require functioned Peyer’s patches for its induction (31). Our data showed that oral tolerance to TNBS could be induced in 6- to 8- and 12- to 14-month-old mice as well as in young adult mice, clearly indicating that sequential loss of T cell responses in Peyer’s patches lead to a lack of oral tolerance to protein antigen at earlier ages. This is the first direct evidence showing that diminished Peyer’s patches which occur with advanced age most likely explain the impairment of oral tolerance to protein antigen.

With regard to the mechanisms of impairment of oral tolerance to protein antigen with aging, one could suggest that aged mice would take up less antigen through Peyer’s patches and insufficient antigen uptake could then lead to a lack of oral tolerance. Previous studies have addressed this issue by quantifying the amounts of particles taken up into the Peyer’s patches and MLN after oral administration of microspheres in young adult and aged mice or rats (47,48). These studies showed that intestinal uptake of microspheres across the epithelium were either increased or unaffected in aged mice or rats respectively (47,48). These results clearly indicate
that antigen-uptake into the Peyer’s patches through M cells is not impaired by aging.

In the present study, we directly addressed the suppressive or anergic functions of Peyer’s patch CD4+ T cells which were induced in young versus aged mice fed OVA or PBS before oral challenge. Our results showed that the suppressive or anergic functions of Peyer’s patches were already impaired in 6- to 8-month-old mice although Peyer’s patch CD4+ T cells from these mice fed PBS were comparable in function with those taken from Peyer’s patches of young adult mice. Our recent study showed that mucosal immune responses, including Peyer’s patch CD4+ T cell proliferative responses induced by oral immunization with OVA and CT as mucosal adjuvant, had markedly declined in 12- to 14-month-old mice (13). From these observations, we conclude that impairment of negative responses (oral tolerance) occurs prior to positive responses (IgA antibody responses) in mucosal compartments with advancing age.

Recent studies have extensively characterized DC in terms of both lineage and function. The importance of DC in homeostasis of lymphoid cells and tissues for initiation of immune responses is well documented (49–51). The DC subsets present in various mucosal compartments have also been elucidated and their unique functions for initiation of mucosal immune responses have been described (43,44,52–62). The importance of DC in the induction of oral tolerance has also been investigated (43,63–66). However, functional changes in DC in aging have only been addressed in the systemic immune compartment (64) and DC from old individuals have been shown to be as effective as DC from young people for T cell stimulation when highly immunogenic proteins were used (67–69). However, to our knowledge, no studies have yet assessed the effects of aging on DC in mucosal immune compartments and their responses to either tolerogens or immunogens. In the present study, we addressed this issue by analysis of DC subsets and FDC in B cell zones. Our studies clearly showed that CD11c+ DC in the subepithelial dome were decreased in mice >1 year of age. Furthermore, fewer DFC and germinal center B cells were noted in the B cell zone during aging. Our results suggest that, in addition to impaired T cell responses, lack of oral tolerance induction seen in aged mice may be associated with decreased DC and FDC functions in Peyer’s patches. Further analyses are ongoing to characterize the alterations in DC functions which occur with advancing age.

Senescence of immune function is well documented (1,2,5). Our study showed that OVA-specific splenic CD4+ T cell responses from 2-year-old mice fed PBS prior to systemic challenge with OVA and CT as adjuvant were poorly responsive, indicating that T cell responses were essentially non-functional at this age. Of interest, we also observed that a high oral dose of OVA prior to systemic challenge with OVA and CT as adjuvant significantly enhanced splenic CD4+ T cell responses specific for OVA. This result indicated that a high dose of OVA feeding can elicit positive responses rather than immune unresponsiveness in senescent mice. This finding should be taken into consideration when oral tolerance is clinically used for treatment of autoimmune diseases in the elderly.

Impairment of oral tolerance induction has been assessed in several studies; however, most were done at the level of the B cell-derived antibody responses and the mechanisms for impairment of oral tolerance were not pursued. In the present study, we addressed the ability to induce oral tolerance to OVA (a Peyer’s patch-dependent antigen) or TNBS (a Peyer’s patch-independent antigen) in young adult and aged mice at both B and T cell levels, and assessed the role of Peyer’s patches in regulation of oral tolerance with aging. The results clearly indicated that oral tolerance to protein antigen was impaired as early as 6–8 months of age, and this was caused by a sequential loss of T cell responses and decreased numbers of DC and FDC in the Peyer’s patches of 12- to 14-month-old mice and by T cell dysregulation in aged, 2-year-old mice. Further, immunosenescence affects negative (tolerance) responses prior to effects on positive (Tc cell-mediated antibody) responses in the mucosal immune compartment. Peyer’s patches have long been considered as mucosal inductive sites for positive immune responses in the gastrointestinal tract. However, our present study on the dysregulation of oral tolerance with aging provides additional insight into the roles of Peyer’s patches as a regulatory tissue for oral tolerance. In addition, our results indicate that high-dose oral tolerance will be less effective for treatment of immune-mediated disorders such as autoimmune diseases. Further, we should also recognize that high-dose feeding of protein antigens may actively enhance immune responses rather than unresponsiveness in the elderly.

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Abbreviations

| APC | antigen-presenting cell |
| CT | cholera toxin |
| DC | dendritic cell |
| DTH | delayed-type hypersensitivity |
| FDC | follicular dendritic cell |
| GC | germinal center |
| HRP | horseradish peroxidase |
| MLN | mesenteric lymph node |
| OVA | ovalbumin |
| PNA | peanut agglutinin |
| SRBC | sheep red blood cells |
| TNBS | 2,4,6-trinitrobenzene sulfonic acid |
| TNCB | 2,4,6-trinitrochloro-1-benzene |
| TNP | trinitrophenyl |

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