International Immunology, Vol. 16, No. 5, pp. 737–745
doi: 10.1093/intimm/dxh075 Advance Access publication March 29, 2004

Vaccination with an immunodominant peptide of bovine type II collagen induces an anti-TCR response, and modulates the onset and severity of collagen-induced arthritis

Aki Honda[1,2,3,5], Akio Ametani[1,5], Takashi Matsumoto[1,4], Amane Iwaya[1], Hiroshi Kano[1], Satoshi Hachimura[1], Kensuke Ohkawa[1], Shucihi Kaminogawa[1], Koji Suzuki[2,3], Eli E. Sercarz[5] and **Vipin Kumar[5]**

[1]Applied Biological Chemistry, University of Tokyo, Bunkyo, Tokyo 113-8650, Japan
[2]Applied Chemistry, Keio University, Yokohama, Kanagawa 223-8582, Japan
[3]Japan Science and Technology Agency, CREST, Kawaguchi, Saitama 332-0012, Japan
[4]Research and Development Center, Nippon Meat Packers, Tsukuba, Ibaraki 300-2646, Japan
[5]Immune Regulation, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, USA

Keywords: anti-TCR response, collagen-induced arthritis, immune regulation

Abstract

T cell responses directed toward TCR-derived peptides have been shown to be an important regulatory mechanism of protection against autoimmunity. Here, we show that a naturally induced TCR-directed immune response can delay the onset of collagen-induced arthritis (CIA), an animal model of autoimmune rheumatoid arthritis in humans. DBA/1 mice were pretreated with an immunodominant peptide, p245–270, from bovine type II collagen (bCII) and were subsequently immunized with whole bCII for the induction of arthritis. The results showed that preactivation of p245–270-reactive cells delayed the onset and reduced the severity of CIA, compared with animals in the control group. Interestingly, the serum antibody response to bCII and the bCII-specific cytokine were not affected under these conditions. This result indicates that the observed protection was neither directly due to a lower antibody response nor due to the immune deviation of the anti-bCII T cell response. Furthermore, immunization with p245–270, but not bCII, induced a strong response to the B5 peptide, an immunodominant region of the TCR V_β8.2 (amino acids 76–101) that binds very strongly to I-A^q. These data suggest that at a critical phase in the loss of self-tolerance, an effective anti-TCR response, induced naturally, can regulate the pathogenic autoimmune response and thus may provide protection against autoimmunity.

Introduction

Collagen-induced arthritis (CIA) is a disease model of human autoimmune rheumatoid arthritis and can be induced by injection of susceptible animals with heterologous type II collagen (CII) in adjuvant. Both MHC and non-MHC genes are involved in the susceptibility to CIA, and H-2^q and H-2^r mice are the most susceptible haplotypes (1). As in most autoimmune disease models, autoreactive T cells are essential in CIA. CII-reactive $CD4^+$ T cells, derived from DBA/1 mice, were shown to develop attenuated CIA by passive transfer (2,3). Also, by immunohistological techniques, $CD4^+$ and IL-2 receptor-expressing T lymphocytes were regularly detected in the affected joints (4). Antibody to collagen molecules can also transfer attenuated disease (5), indicating that a humoral response to CII is also involved in this disease. Early studies of Osman *et al.* showed that CII-reactive T cell hybridomas in DBA/1LacJ mice preferentially use the TCR V_β8.2 gene

Correspondence to: A. Honda; E-mail: aki@educ.cc.keio.ac.jp

Transmitting editor: K. Okumura

Received 4 December 2003, *accepted* 22 February 2004

segment (6). Thus, anti-V_β8.2 antibody treatment also resulted in a significant reduction in the incidence of arthritis in DBA/1LacJ mice.

Self-reactive T cells bearing the V_β8.2 gene segment are often utilized in experimental autoimmune disease models, such as experimental autoimmune encephalomyelitis (EAE) (7) or diabetes in non-obese diabetic (NOD) mice (8). In earlier studies it was shown that natural remission of EAE was accompanied by an immune response against the TCR peptide from the V_β8.2 chain (9). Furthermore, we investigated the T cell proliferative response against various TCR V_β8.2-derived peptides and found that the immune response against the B5 peptide corresponding to residues 76–101 of the TCR V_β8.2 chain was spontaneously induced in EAE-susceptible mice (10). We found that $CD4^+$ regulatory T cells that recognize B5 and $CD8^+$ regulatory T cells that were reactive to a different V_β8.2 determinant from the CDR1/2 region (corresponding to 41–50) controlled the disease. Furthermore, we found that injection of B5 peptide has a suppressive effect against CIA (11) as well as EAE. In this study we show that CIA was significantly delayed and inhibited by the injection of p245–270, an immunodominant determinant of bovine CII (bCII) in DBA/1 mice, through activation of the B5-specific regulatory T cells. Notably, the B5 peptide binds to the $I\text{-}A^q$ molecule with a high binding affinity. These data suggest that during an autoimmune response to self-CII, feedback TCR–peptide-reactive regulatory T cell responses are also induced which are involved in the maintenance of peripheral self-tolerance.

Methods

Mice

Female DBA/1LacJ and DBA/1JNCrj (referred to as DBA/1J) mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and Charles River Japan (Yokohama, Japan) respectively.

Protein and peptide

bCII used for experiments with DBA/1LacJ mice was purchased from the Institute Jacques (Paris, France). bCII used for experiments with DBA/1J was purified by the salting-out technique after the digestion of bovine joint cartilage with pepsin. Its purity was confirmed by SDS–PAGE analysis. B1 (amino acid 1–30 with an additional C-terminal leucine) and B5 (amino acids 76–101) peptides from mouse TCR V_β8.2, chain, hen egg lysozyme (HEL) 74–90 and β-Lg119–133 were synthesized by Dr S. Horvath at the California Institute of Technology (Pasadena, CA) and purified by reversed-phase HPLC, as described earlier (12). Peptides of residues 245–270 (p245–270) and 316–333 (p316–333) from bCII were synthesized with a 430A machine (Applied Biosystems, Foster City, CA), and purified with reversed-phase HPLC.

The sequences of peptides used in this study were: p245–270 (bCII), ATGPLGPKGQTGEPGIAGFKGEQGPK; p316–333 (bCII), GFBGADGIAGPKGPBGER; B1 ($TCRV_\beta$8.2 1–30L), EAAVTQSPRNKVAVTGGKVTLSCNQTNNHNL; B5 ($TCRV_\beta$8.2 76–101), LILELATPSQTSVYFCASGDAGGGYE; HEL74–90, NLCNIPCSALLSSDITA; HEL93–113, NCAKKIVSDGNGMNAWVAWRN; β-Lg119–133, CQCLVRTPEVDDEAL.

CIA induction

Ten DBA/1J mice in each group were injected intradermally at the base of the tail with 100 μl of an emulsion containing (i) bCII p245–270 (15 μg/mouse) plus complete Freund's adjuvant (CFA; Difco, Detroit, MI), (ii) bCII p316–333 (10 μg/mouse) plus CFA, (iii) PBS/CFA or (iv) nothing, and 14 days later they were further injected with bCII (100 μg/mouse) plus incomplete Freund's adjuvant (IFA; Difco). The peptides and bCII were dissolved in PBS and 0.06% acetic acid respectively, and emulsified with the same volume of adjuvant. The clinical severity of the arthritis was assessed according to an arthritis scale for each limb, which was subjectively graded on a scale of 0–3: 0 = absence of arthritis, 1 = one finger swelling or mild swelling, 2 = two fingers swelling or swelling of tarsus and ankle, and 3 = hard swelling or bony deformity. A sum of the scale for four paws of a single mouse was calculated and a total of this sum in a group of mice was obtained. The arthritic index was defined by dividing this total by the number of mice in the group. Blood was taken every 10 days during the observation period to measure the antibody in the serum. Ten mice in each group were injected with bCII p245–270/CFA, bCII p316–333/CFA, PBS/CFA or nothing and then all the mice were s.c. immunized with bCII for the induction of the disease. We determined the severity of the arthritis by calculating the arthritic index for these four groups of mice (Fig. 1). We also compared the incidence of arthritis by calculating the percentages of arthritic mice and arthritic legs in each group. A mouse was regarded as arthritic when an individual had swelling in at least one leg. The percentage of arthritic mice was obtained by dividing the number of arthritic mice by the total number of mice in each group. The percentage of arthritic legs was obtained by dividing the number of arthritic legs by the number of all mice for each group; arthritic legs were those having swelling or deformity.

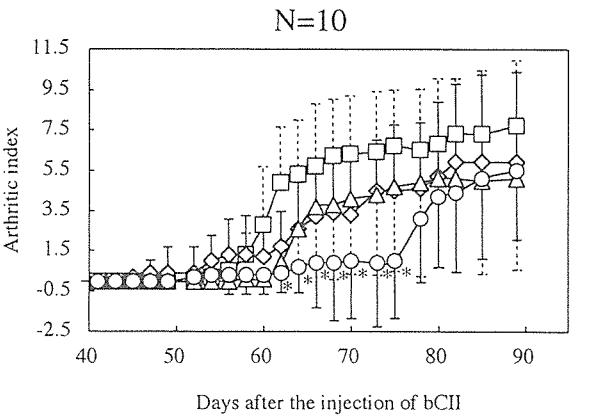

Fig. 1. The arthritic index of CIA after preimmunization with bCII-derived peptide. Groups of DBA/1J mice (10 in each) were preimmunized with p245–270 (circles) or p316–333 (inverted triangles) or PBS (diamonds) or were not preimmunized (squares) on day 0. CIA was induced on day 14 by injecting mice with bCII plus IFA, as described in the Methods. The data were analyzed by the Mann–Whitney *U*-test. *Significant difference at $P < 0.05$.

ELISA for detecting anti-bCII antibody in the serum

The titer of anti-bCII antibody in serum from mice treated for the CIA induction previously described was determined by ELISA. bCII was dissolved in PBS at a concentration of 100 μg/ml by heating; it was incubated in the wells of Maxisorp plates (Nunc, Roskilde, Denmark) overnight at 4°C. The solutions were removed and the wells were washed with PBS-Tween (0.05% Tween 20 in PBS). The serum samples were diluted with PBS-Tween and incubated in the wells for 2 h at room temperature. Bound antibody to the solid phase was detected by two sequential sets of incubation with alkaline phosphatase (ALP)-labeled anti-mouse subclass antibody for 2 h and its substrate of *p*-nitrophenylphosphate disodium salt for ~30 min after washing the wells with PBS-Tween. The conjugates used here were ALP-labeled anti-mouse IgG1, IgG2a and IgG2b (all from Zymed, South San Francisco, CA). Color development was measured at 405 nm.

Cell preparation and culture for proliferation and cytokine release assay

DBA/1J mice were s.c. injected with bCII p245–270 plus CFA H37Ra, PBS plus CFA or nothing. Seven days following the preinjection, the mice were immunized with 100 μg of bCII plus IFA in the base of the tail or treated with nothing. After 10 days, lymphocytes were removed from popliteal and inguinal lymph nodes and cultured at 5×10^5 in each well of a 96-well plate with 200 μl of RPMI 1640 containing 10% FBS with or without B5 (amino acids 76–101, $TCRV_\beta 8.2$) peptide or p245–270. IFN-γ in the supernatant was determined by sandwich ELISA using R4-6A2 (PharMingen, San Diego, CA) as the first antibody and XMG1.2 (PharMingen) as the second antibody. In other experiments, DBA/1LacJ mice were s.c. vaccinated with p245–270 (10 μg/mouse) plus CFA or PBS plus CFA as controls. Lymphocytes were taken and cultured in the protein-free medium X-vivo 20 (Biowhittaker, Walkersville, MD) with either B5 or B1 (amino acids 1–30, 31L, $V_\beta 8.2$), or without any antigenic peptide. Three days later, 1 μCi [^{3}H]thymidine was added to the culture and incubated overnight. The incorporation of thymidine into cultured cells was measured by liquid scintillation.

Competitive-binding assay of B5 (amino acids 76–101, $TCRV_\beta 8.2$) to I-A^q molecules

I-A^q molecules were purified from spleens of untreated DBA/1J mice as previously described (13). In brief, splenocytes from DBA/1J mice were solubilized and I-A^q molecules were purified with affinity column using anti-I-A^q antibody M5/114.15.2. Binding assay was performed as previously reported (14). The purified I-A^q molecule (14 nM), 2 μM or absence of biotinylated β-Lg119–133 with various concentrations of B1, B5, β-Lg119–133 or without competitor peptide were preincubated in 50 mM citrate/phosphate buffer, pH 5.0 containing 0.2% NP-40 and 2 mM EDTA at 37°C for 48 h. Meanwhile, Nunc Maxisorp plates were coated with anti-rat Ig at room temperature for 2 h and with M5/114.15.2 for another 2 h. Non-specific binding was blocked at 4°C for overnight with 50 mM Tris–HCl, pH 7.5 containing 0.3% BSA and 0.1% Tween 20. The incubated mixtures were transferred to the plate and incubated for 1.5 h at room temperature. Biotinylated peptide bound to the I-A^q molecule was detected with AP–streptavidin (Zymed) by development with its substrate, *p*-nitrophenylphosphate disodium. Color development was measured at 405 nm.

Results

Suppression of CIA by preimmunization with bCII p245–270

CIA can be induced in H-2^q DBA/1 mice by injection with bCII plus adjuvant. In our case, a single injection of 6- to 8-week-old female DBA/1J with bCII plus IFA could efficiently induce CIA with almost 100% incidence. Previous reports (15,16) and our results (data not shown) had indicated that p245–270 contained a dominant T cell determinant, judged from the significant proliferative response to p245–270 in lymph node cells from mice immunized with bCII. DBA/1J mice were preimmunized with p245–270 from bCII plus CFA in order to activate T cells specific for the immunodominant determinant in this region. We studied the effect of this preactivation of the dominant T cell population on the incidence of CIA. The data in Fig. 1 indicate that p245–270 preimmunization delayed the onset of arthritis and reduced the severity of the disease, compared with the other three groups of mice. The percentages of arthritic legs and arthritic mice for the four groups showed a similar pattern to that of the arthritic index. We also tested another schedule for studying the effect of p245–270 preimmunization. Before injection with bCII, the mice were treated twice with p245–270 or other peptides. Also, in this experimental set-up (data not shown), the onset of CIA was much delayed and the severity was reduced compared with mice in the other three groups. When we preinjected mice with p245–270, 35 days before injection with bCII (data not shown), the onset of CIA was also suppressed. However, the efficiency of the delay was less than with other protocols having shorter intervals between the p245–270 and bCII injections.

Suppression of CIA is independent of alterations in the antibody response or immune deviation of the anti-bCII response

We next examined the antibody response to bCII in the serum of individual mice from the four groups. The antibody titer versus the days after the first treatment of mice was plotted. After 21 days from the first treatment, the IgG1 and IgG2a titers to bCII increased. Although the onset and the severity of CIA were altered by bCII p245–270 preimmunization, the IgG1 antibody response to bCII was similar to that of the other three groups of mice (Fig. 2A). The response of IgG2a possessing complement-binding activity has been proposed to be more relevant to CIA than the IgG1 response (17). However, the mice preinjected with p245–270 showed an IgG2a titer specific for bCII that was similar to the other groups (Fig. 2B). These observations indicate that the suppression of CIA by p245–270 preimmunization was independent of the antibody responses to bCII.

Furthermore, we measured the amount of IFN-γ secreted by T cells specific for p245–270 (Fig. 3). Since the balance of the cytokines produced by T_h1/T_h2 subsets of the T_h cells plays an important role in the development of CIA (18), we considered that the regulation caused by preimmunization with p245–270

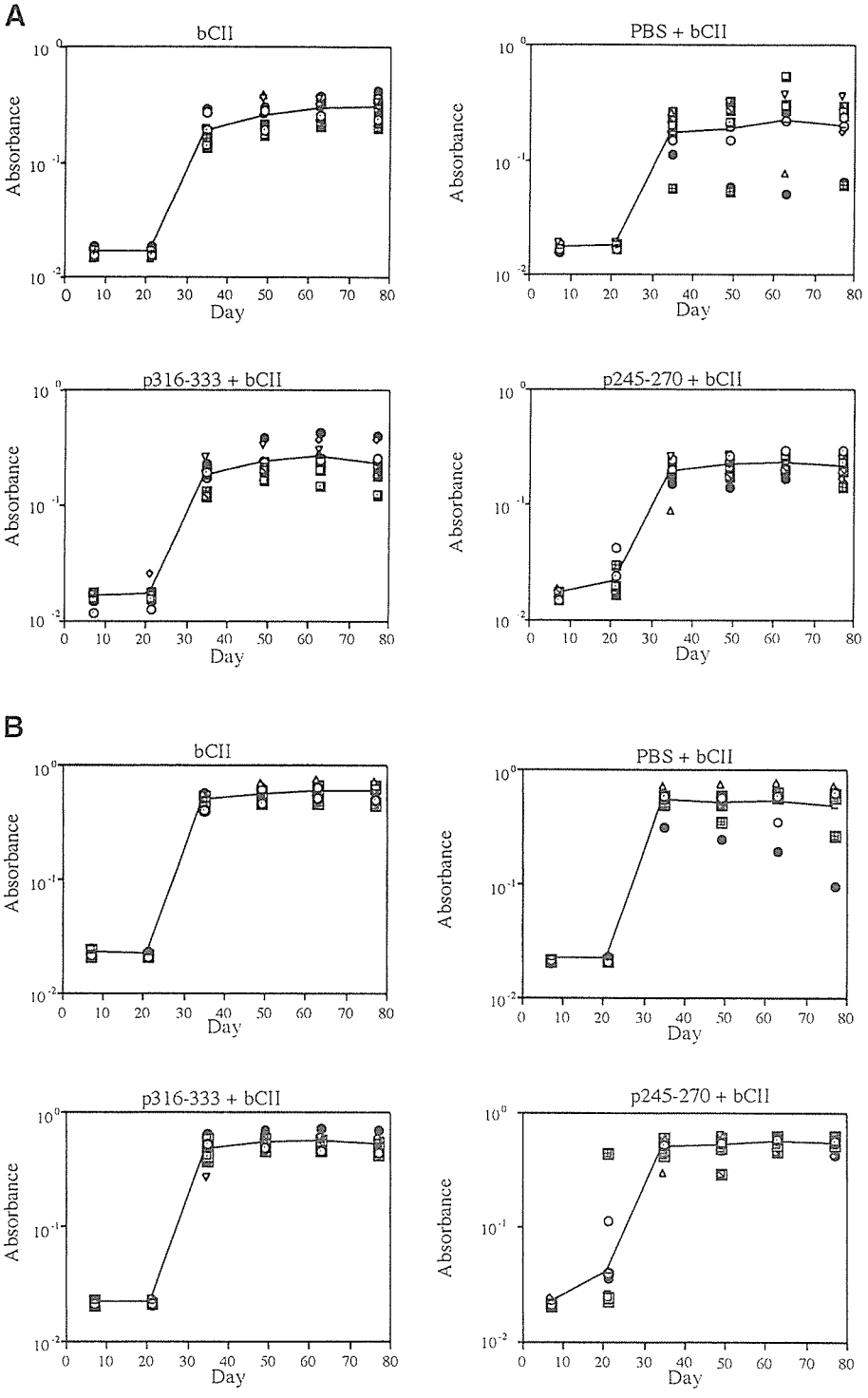

Fig. 2. Antibody responses against bCII. Anti-bCII IgG1 (A) and IgG2a (B) antibody amounts in the serum of individual mice were measured. Mice were the same as those shown in Fig. 1. Each symbol indicates absorbance for each individual mouse by ELISA and the average values for each day are linked with a straight line. Each panel indicates the following group: 'bCII' = mice immunized with bCII plus IFA without any preimmunization; 'PBS + bCII' = mice preimmunized with PBS plus CFA on day 0 and immunized with bCII on day 21; 'p316–333 + bCII' = mice preimmunized with p316–333 on day 0 and immunized with bCII on day 21; 'p245–270 + bCII' = mice preimmunized with p245–270 on day 0 and immunized with bCII on day 21.

might have been due to T_h1/T_h2 deviation. However, the IFN-γ response against p245–270 was clearly shown in mice preimmunized with p245–270 before the immunization with bCII in a dose-dependent manner. The IFN-γ response against

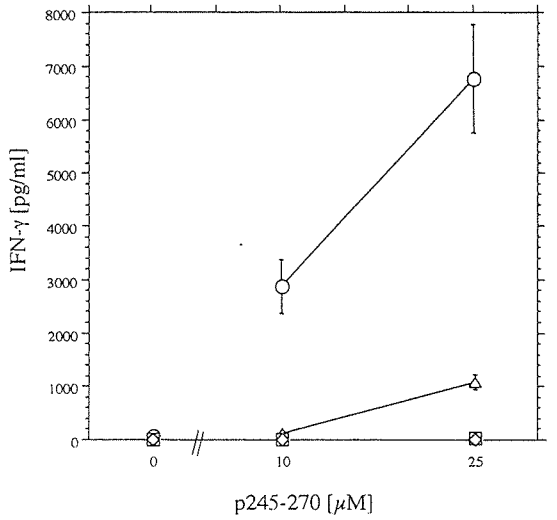

Fig. 3. The IFN-γ production of lymphocytes specific for p245–270. The triangles indicate the response of DBA/1J mice immunized with p245–270 on day 0 without further treatment; circles, preimmunized with p245–270 on day 0 and immunized with bCII on day 7; squares, immunized with bCII without preimmunization; diamonds, preimmunized with PBS on day 0 and immunized with bCII on day 7. Lymphocytes were taken on day 17 and cultured with different concentrations of p245–270 or without antigen.

p245–270 could not be detected in mice immunized with bCII without preimmunization with p245–270. A slight response was shown in mice immunized with p245–270 without further immunization. We also tested IL-2 and IL-4 included in the same supernatant with a bioassay. No significant difference between the two groups was apparent (data not shown). These results implied that regulation of CIA by preimmunization with p245–270 was not due to T_h1/T_h2 deviation.

We then considered the possibility that preimmunization with p245–270 regulated CIA via the activation of a natural immune regulatory system (11). We have shown earlier that a TCR-specific immune regulatory response inhibited CIA. We set out to examine whether immunization of DBA/1J mice with p245–270 could activate a TCR-specific response.

Immunization of DBA/1J mice with bCII p245–270 induces a T cell response against the dominant TCR peptide (B5: amino acids 76–101, V_β8.2)

Lymphocytes from individual DBA/1J mice immunized with p245–270 or control mice were examined for a TCR-specific proliferative response (Fig. 4A and B). Mice immunized with p245–270 showed proliferative responses to the B5 peptide, but not to the control peptide B1 (amino acids 1–30L, V_β8.2). We found similar data indicating a response to B5 in a repeated experiment using pooled lymphocytes from another set of three mice. Interestingly, lymphocytes from mice injected with PBS plus CFA also proliferated against B5, although the response was much weaker than those from mice immunized with p245–270. This result is consistent with the data in Fig. 1, which showed that PBS plus CFA or control peptide plus CFA delayed the onset of CIA. We also found that splenocytes from some, but not all, individuals showed T cell proliferative responses against B5 even without any treatment (unpublished data). These facts imply that the TCR-specific response represents a physiological immune regulatory system that is very easily triggered. Previous reports suggested that B5-specific $CD4^+$ regulatory T cells are required to produce a T_h1 response for effective regulation and prevention of autoimmune disease (19). We measured the B5-specific IFN-γ response of lymphocytes from the mice that were treated with the same schedule as the CIA induction (Fig. 4C). The result indicated that immunization only once with p245–270 induced a significant IFN-γ response to B5 and that preimmunization with p245–270 before immunization with bCII induced the strong IFN-γ response to the B5 peptide, suggesting that immunization with p245–270 before the induction of CIA induced an effective regulatory response. On the other hand, no IFN-γ was detected from other groups, those preinjected with PBS or nothing, before the immunization with bCII.

B5 (amino acids 76–101, V_β8.2) peptide binds to MHC class II I-A^q molecules

Next, we investigated whether TCR peptide B5 is able to bind to I-A^q molecules by a competitive-binding assay using the biotinylated β-Lg119–133 peptide (Fig. 5). In this experiment, increasing amounts of B1 peptide (V_β8.2, amino acids 1–30L), B5 or non-labeled β-Lg119–133 peptide were added as competitors to the mixture of I-A^q and biotinylated β-Lg119–133. The result indicated that peptides of B5 and β-Lg119–133 could bind to I-A^q molecules, while B1 did not (the slight inhibition by B1 peptide is a background effect). It could be shown that B5 and β-Lg119–133 competed for binding to I-A^q, suggesting that this TCR peptide binds well to I-A^q molecules and, thereby, can be presented to appropriate $CD4^+$ regulatory T cell populations, as has been shown in EAE (12).

Discussion

Regulation of immune responses using peptides derived from the determinant region of CII has been shown as an effective method to control CIA. Development of CIA likely requires responses of both T cells and antibodies. Although p245–270 contains the dominant determinant of CII in H-2^q mice (20), the peptide fragment that contains p245–270 cannot induce CIA (21) since it does not contain the appropriate B cell epitope of CII. However, i.v. or neonatal administration of peptide containing this determinant from chicken CII could inhibit CIA in DBA/1 mice (16). It has also been shown that oral administration of human CII or chicken CII determinant peptide inhibited CIA in DBA/1 mice (22). Previously we screened overlapping peptides derived from bCII by intranasal administration and found that the peptide corresponding to 253–272 of bCII could most effectively inhibit CIA (23). In these cases, the administration of peptides reduced either the T cell response or the antibody response against CII or the immunizing peptide due to tolerization of pathogenic lymphocytes.

Administration of peptide with adjuvant is a general protocol for induction of antigen-specific T cells. In this report we induced activation of bCII-reactive T cells by immunization of DBA/1 mice with peptide plus adjuvant (Fig. 3). Thus, regulation of CIA in our case was caused by the activation of

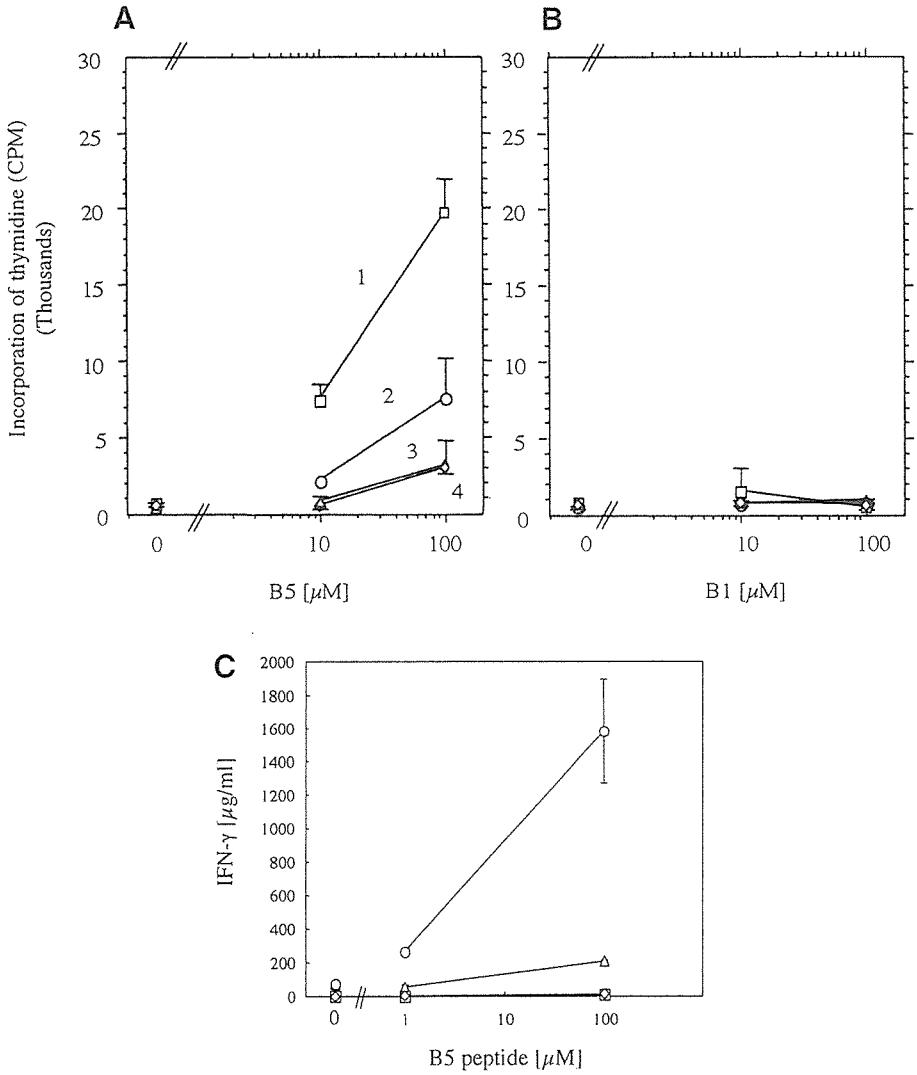

Fig. 4. B5-specific proliferative responses of lymphocytes. The assay was started 10 days after immunization of four individual mice with p245–270 (mouse 1: squares; mouse 2: circles) or PBS plus CFA (mouse 3: triangles; mouse 4: diamonds). The cells were incubated with various concentrations of B5 peptide V_β8.2 (amino acids 76–101, TCR V_β8.2) (A) or B1 (amino acids 1–30, L31, TCR V_β8.2) (B). B5-specific IFN-γ production of lymphocytes from DBA/1J mice immunized with p245–270 on day 0 without further treatment (triangles), preimmunized with p245–270 on day 0 and immunized with bCII on day 7 (circles), immunized with bCII without preimmunization (squares) or preimmunized with PBS on day 0 and immunized with bCII on day 7 (diamonds). Lymphocytes were taken on day 17 and cultured with graded concentrations of B5 peptide or without antigen (C).

regulatory T cells, but not by the induction of tolerance. Several reports showed that regulation of CIA due to tolerization accompanies reduction of the CII-specific IgG2a response (16,24). However, we have shown that bCII-specific IgG1 and IgG2a did not change by immunization with bCII p245–270 (Fig. 2), which also shows that our protocol induced the activation of T cells. $CD4^+$ T cells mainly differentiate into either T_h1- or T_h2-type effectors. T_h1-type cells produce IL-2, IFN-γ and tumor necrosis factor-β, and support cell-mediated immunity, whereas T_h2-type cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, and enhance humoral immune responses (25–27). The T_h1 response has been considered to play a major role in the pathogenesis of CIA (28). However, the role of IFN-γ may be different at different stages of the diseases, as the administration of IFN-γ can inhibit CIA (29). It is suggested from our data that regulation of CIA through activation of CII-reactive pathogenic T cells by immunization with the dominant CII peptide may not be due to the immune deviation of the anti-bCII response. Notably, the IFN-γ response raised by p245–270 was shown to be increased in the mice preimmunized with p245–270, and yet the disease was significantly delayed and inhibited.

Our data suggest that TCR peptide-reactive regulatory T cells that are naturally induced following expansion of bCII-reactive $V_\beta8.2^+$ cells are able to inhibit the disease. Osman *et al.* established T cell clones that were specific for p245–270

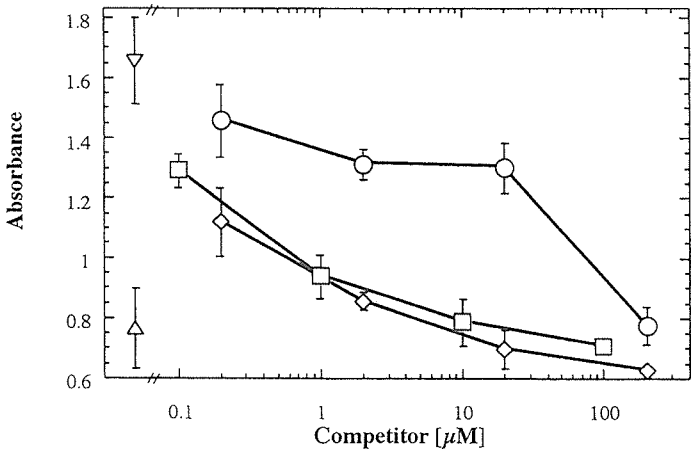

Fig. 5. Competitive-binding assay of I-A^q. The amount of a biotinylated peptide bound to I-A^q in the presence of B1 (circles), B5 (squares), β-Ig119–133 (diamonds), without biotinylated peptide (triangle) or competitor (inverted triangle).

from DBA/1 mice for the characterization of the pathogenic TCR repertoire and found that the V_β8.2 gene segment was preferentially utilized (58.3%) (6). It has also been demonstrated that the TCR V_β8.2 gene segment is preferentially utilized in the EAE of B10.PL mice (H-2^u) (7), in the uveoretinitis of B10.A (H-2^k) mice (30) as well as the EAE of Lewis rats (31). Other studies also implicate the role of $V_\beta8.2^+$ T cells in the pathogenesis of NOD mice, a model for insulin-dependent juvenile diabetes (32). Both in EAE and CIA, it has been shown that administration of antibodies specific for V_β8.2 significantly protects animals from disease (7,33,34). Therefore, we have examined the possibility that CIA was inhibited by an immune regulatory mechanism involving TCR V_β8.2 determinants.

We have already shown that immunization of DBA/1LacJ mice with B5 peptide, corresponding to amino acids 76–101 of the TCRV_β8.2 framework region, results in significant protection of the animals from CIA (11). Response to the B5 peptide was found to be naturally induced during the course of EAE in B10.PL mice. Both $CD4^+$ and $CD8^+$ regulatory T cells were necessary when remission of EAE was to occur. Furthermore, $CD4^+$ regulatory T cells were shown to be specific for the B5 peptide, whereas $CD8^+$ regulatory T cells were specific for a distinct peptide from the CDR1/2 region of the V_β8.2 chain. EAE was inhibited by priming a T_h1-type response against B5, whereas EAE was enhanced by T_h2-type priming (19). We have hypothesized that both $CD4^+$ and $CD8^+$ regulatory T cells recognize each of the TCR-derived peptides that were processed in professional antigen-presenting cells (APC) through the turnover of effector T cells. IFN-γ secreted by $CD4^+$ regulatory T cells was considered to be necessary for the recruitment/activation of $CD8^+$ regulatory T cells and the consequent inhibition of effector T cells. $CD8^+$ T cell activation probably occurs through a CD40 signal that enhances antigen processing and up-regulates co-stimulatory molecules on APC.

Our results show that immunization of DBA/1J mice with p245–270 induces an anti-B5 response accompanied by IFN-γ secretion. As shown earlier (11), the immunization of DBA/1J mice with B5 down-regulated the immune response against bCII and p245–270. We have also established B5-specific T cell lines from DBA/1 LacJ. Their responses to B5 were inhibited by anti-CD4 or anti-MHC class II antibodies (Koh, unpublished data). We propose that the expansion of $V_\beta8.2^+$ effectors and their turnover *in vivo* lead to processing and presentation of a B5-like peptide by APC. It is evident from our data that B5 is likely to be presented by APC, because it displays a strong binding affinity for the I-A^q molecule (Fig. 5). The expansion of effector T cells bearing V_β8.2 was not strong enough to induce effective regulation through bCII immunization; however, it becomes strong when the dominant determinant is used as the immunogen.

Jolly *et al.* reported that the expression of TCR V_β8.2 from the unrearranged gene in a murine lymphoid precursor cell line is higher than that of other V_β members of the repertoire at an early phase of T cell differentiation (35). It may be possible that V_β8.2 plays an unknown role in the body and the anti-B5 response may be a general regulatory system for the control of the immune response.

In EAE, a B5-specific T cell response was induced during the natural remission of the disease. We induced such a response through the activation of $V_\beta8.2^+$ T cells specific for the dominant determinant region of the antigen, bCII. Induction of the regulation by activating the dominant determinant-specific response has not been reported by others. Considering both of these cases with EAE and CIA, we can propose that the TCR peptide B5-specific T cell represents an important focus of the immune regulation system. Thus, B5-specific responses could be frequently induced by immune responses towards self-antigen, especially when the V_β8.2 T cells comprise a significant portion of the response.

The inflammation of limbs in mice suffering from CIA usually settles gradually, although they cannot be completely cured due to irreversible bony deformities. However, a reduction in the inflammation is the important aspect for therapy of rheumatoid arthritis. It is possible that eventual reduction in the inflammation is caused by a naturally induced anti-TCR response following immunization with bCII. Immunization with bCII may only have induced a weaker anti-TCR response

compared to immunization with p245–270. As a consequence, immunization with bCII induces strong CIA, whereas immunization with p245–270 leads to the regulation of the disease. These data further suggest that the production of the clinical manifestations of autoimmunity depend upon the balance between the pathogenic effector and regulatory populations. Each population undergoes its own development and then interaction will be decisive in determining the clinical outcome, as proposed earlier (36).

Autoimmune rheumatoid arthritis in humans and CIA have several similarities, e.g. susceptibility depends on the genetic background in both diseases. However, common T cell repertoires have not been identified in rheumatoid arthritis patients (37,38) except for a few reports showing a predominant TCR usage of $V_\alpha 11$, $V_\alpha 14$, $V_\alpha 28$ and $V_\beta 7$, $V_\beta 9$, $V_\beta 17$ in the early phase of rheumatoid arthritis or a common usage of the TCR $V_\beta 14$ in juvenile rheumatoid arthritis (39,40). Some reports have demonstrated successful vaccination with peptides derived from the TCR in rheumatoid arthritis patients (41,42). However, it is difficult to find an effective vaccine region for each patient, because one has to identify the dominant TCR repertoire for each patient. Our vaccination method, which is based upon vaccination with the dominant autoantigenic determinant, is superior in that we do not need to identify the dominant TCR repertoire for each patient. We assume that it is possible to find a functional TCR peptide region corresponding to the immunoregulatory B5 region commonly shared by different strains of mice.

Acknowledgements

This is manuscript #624 from the La Jolla Institute for Allergy and Immunology. We thank Dr Emanual Maverakis for the critical reading of the paper. V. K. is supported by the Arthritis Foundation, the National Multiple Sclerosis Society and the National Institutes of Health.

Abbreviations

ALP	alkaline phosphatase
APC	antigen presenting cell
bCII	bovine type II collagen
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CII	type II collagen
EAE	experimental autoimmune encephalomyelitis
HEL	hen egg lysozyme
IFA	incomplete Freund's adjuvant
NOD	non-obese diabetic

References

1 Holmdahl, R., Jansson, L., Andersson, M. and Larsson, E. 1988. *Immunogenetics* of type II collagen autoimmunity and susceptibility to collagen arthritis. *Immunology* 65:305.
2 Holmdahl, R., Klareskog, L., Rubin, K., Larsson, E. and Wigzell, H. 1985. T lymphocytes in collagen II-induced arthritis in mice. Characterization of arthritogenic collagen II-specific T cell lines and clones. *Scand. J. Immunol.* 22:295.
3 Kakimoto, K., Katsuki, M., Hirofuji, T., Iwata, H. and Koga, T. 1988. Isolation of T cell line capable of protecting mice against collagen-induced arthritis. *J. Immunol.* 140:78.
4 Holmdahl, R., Jonsson, R., Larsson, P. and Klareskog, L. 1988. Early appearance of activated $CD4^+$ T lymphocytes and class II antigen-expressing cells in joints of DBA/1 mice immunized with type II collagen. *Lab. Invest.* 58:53.
5 Trentham, D. E., Dynesius, R. A. and David, J. R. 1978. Passive transfer by cells of type II collagen-induced arthritis in rats. *J. Clin. Invest.* 62:359.
6 Osman, G. E., Toda, M., Kanagawa, O. and Hood, L. E. 1993. Characterization of the T cell receptor repertoire causing collagen arthritis in mice. *J. Exp. Med.* 177:387.
7 Urban, J. L., Kumar, V., Kono, D. H., Gomez, C., Horvath, S. J., Clayton, J., Ando, D. G., Sercarz, E. E. and Hood, L. 1988. Restricted use of T cell receptor V genes in murine autoimmune encephalomyelitis raises possibilities for antibody therapy. *Cell* 54:577.
8 Bacelj, A., Charlton, B. and Mandel, T. E. 1989. Prevention of cyclophosphamide-induced diabetes by anti-V beta 8 T-lymphocyte-receptor monoclonal antibody therapy in NOD/Wehi mice. *Diabetes* 38:1492.
9 Kumar, V. and Sercarz, E. 1993. T cell regulatory circuitry: antigen-specific and TCR-idiopeptide-specific T cell interactions in EAE. *Int. Rev. Immunol.* 9:287.
10 Kumar, V., Tabibiazar, R., Geysen, H. M. and Sercarz, E. 1995. Immunodominant framework region 3 peptide from TCR V beta 8.2 chain controls murine experimental autoimmune encephalomyelitis. *J. Immunol.* 154:1941.
11 Kumar, V., Aziz, F., Sercarz, E. and Miller, A. 1997. Regulatory T cells specific for the same framework 3 region of the Vbeta8.2 chain are involved in the control of collagen II-induced arthritis and experimental autoimmune encephalomyelitis. *J. Exp. Med.* 185:1725.
12 Kumar, V. and Sercarz, E. E. 1993. The involvement of T cell receptor peptide-specific regulatory $CD4^+$ T cells in recovery from antigen-induced autoimmune disease. *J. Exp. Med.* 178:909.
13 Gorga, J. C., Horejsi, V., Johnson, D. R., Raghupathy, R. and Strominger, J. L. 1987. Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line. *J. Biol. Chem.* 262:16087.
14 Jensen, P. E. 1992. Long-lived complexes between peptide and class II major histocompatibility complex are formed at low pH with no requirement for pH neutralization. *J. Exp. Med.* 176:793.
15 Tang, B., Brand, D. D., Chiang, T. M., Stuart, J. M., Kang, A. H. and Myers, L. K. 2000. Immunogenicity and arthritogenicity of recombinant CB10 in B10.RIII mice. *J. Immunol.* 164:481.
16 Myers, L. K., Stuart, J. M., Seyer, J. M. and Kang, A. H. 1989. Identification of an immunosuppressive epitope of type II collagen that confers protection against collagen-induced arthritis. *J. Exp. Med.* 170:1999.
17 Watson, W. C. and Townes, A. S. 1985. Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J. Exp. Med.* 162:1878.
18 Mauri, C., Williams, R. O., Walmsley, M. and Feldmann, M. 1996. Relationship between T_h1/T_h2 cytokine patterns and the arthritogenic response in collagen-induced arthritis. *Eur. J. Immunol.* 26:1511.
19 Kumar, V. and Sercarz, E. 1998. Induction or protection from experimental autoimmune encephalomyelitis depends on the cytokine secretion profile of TCR peptide-specific regulatory CD4 T cells. *J. Immunol.* 161:6585.
20 Brand, D. D., Myers, L. K., Terato, K., Whittington, K. B., Stuart, J. M., Kang, A. H. and Rosloniec, E. F. 1994. Characterization of the T cell determinants in the induction of autoimmune arthritis by bovine alpha 1(II)-CB11 in H-2q mice. *J. Immunol.* 152:3088.
21 Terato, K. 1994. Type II collagen-induced arthritis: the new approaches for the development of antigen-specific immunotherapy for treatment of autoimmune arthritis. *Clin. Immunol.* 26:1050.
22 Khare, S. D., Krco, C. J., Griffiths, M. M., Luthra, H. S. and David, C. S. 1995. Oral administration of an immunodominant human collagen peptide modulates collagen-induced arthritis. *J. Immunol.* 155:3653.
23 Matsumoto, T., Ametani, A., Hachimura, S., Iwaya, A., Taguchi, Y., Fujita, K., Shigehisa, T. and Kaminogawa, S. 1998. Intranasal

administration of denatured type II collagen and its fragments can delay the onset of collagen-induced arthritis. *Clin. Immunol. Immunopathol.* 88:70.

24 Cher, D. J. and Mosmann, T. R. 1987. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by T_H1 clones. *J. Immunol.* 138:3688.

25 Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.

26 Cherwinski, H. M., Schumacher, J. H., Brown, K. D. and Mosmann, T. R. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between T_h1 and T_h2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166:1229.

27 Fiorentino, D. F., Bond, M. W. and Mosmann, T. R. 1989. Two types of mouse T helper cell. IV. T_h2 clones secrete a factor that inhibits cytokine production by T_h1 clones. *J. Exp. Med.* 170:2081.

28 Myers, L. K., Seyer, J. M., Stuart, J. M., Terato, K., David, C. S. and Kang, A. H. 1993. T cell epitopes of type II collagen that regulate murine collagen-induced arthritis. *J. Immunol.* 151:500.

29 Boissier, M. C., Chiocchia, G., Bessis, N., Hajnal, J., Garotta, G., Nicoletti, F. and Fournier, C. 1995. Biphasic effect of interferon-gamma in murine collagen-induced arthritis. *Eur. J. Immunol.* 25:1184.

30 Rizzo, L. V., Silver, P., Wiggert, B., Hakim, F., Gazzinelli, R. T., Chan, C. C. and Caspi, R. R. 1996. Establishment and characterization of a murine $CD4^+$ T cell line and clone that induce experimental autoimmune uveoretinitis in B10.A mice. *J. Immunol.* 156:1654.

31 Burns, F. R., Li, X. B., Shen, N., Offner, H., Chou, Y. K., Vandenbark, A. A. and Heber-Katz, E. 1989. Both rat and mouse T cell receptors specific for the encephalitogenic determinant of myelin basic protein use similar V alpha and V beta chain genes even though the major histocompatibility complex and encephalitogenic determinants being recognized are different. *J. Exp. Med.* 169:27.

32 Bacelj, A., Charlton, B., Koulmanda, M. and Mandel, T. E. 1990. The role of V beta 8 cells in disease recurrence in isografts in diabetic NOD mice. *Transplant. Proc.* 22:2167.

33 Acha-Orbea, H., Mitchell, D. J., Timmermann, L., Wraith, D. C., Tausch, G. S., Waldor, M. K., Zamvil, S. S., McDevitt, H. O. and Steinman, L. 1988. Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell* 54:263.

34 Chiocchia, G., Boissier, M. C. and Fournier, C. 1991. Therapy against murine collagen-induced arthritis with T cell receptor V beta-specific antibodies. *Eur. J. Immunol.* 21:2899.

35 Jolly, C. J. and O'Neill, H. C. 1995. Expression of a TCR V beta 8.2 polypeptide from the unrearranged gene in a murine lymphoid precursor cell line. *Int. Immunol.* 7:1147.

36 Kumar, V. and Sercarz, E. 2001. An integrative model of regulation centered on recognition of TCR peptide/MHC complexes. *Immunol. Rev.* 182:113.

37 Uematsu, Y., Wege, H., Straus, A., Ott, M., Bannwarth, W., Lanchbury, J., Panayi, G. and Steinmetz, M. 1991. The T cell-receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal. *Proc. Natl Acad. Sci. USA* 88:8534.

38 Dedeoglu, F., Kaymaz, H., Seaver, N., Schluter, S. F., Yocum, D. E. and Marchalonis, J. J. 1993. Lack of preferential V beta usage in synovial T cells of rheumatoid arthritis patients. *Immunol. Res.* 12:12.

39 Ramanujam, T., Luchi, M., Schumacher, H. R., Zwillich, S., Chang, C. P., Callegari, P. E., Von Feldt, J. M., Fang, Q., Weiner, D. B. and Williams, W. V. 1995. Detection of T cell receptors in early rheumatoid arthritis synovial tissue. *Pathobiology* 63:100.

40 Grom, A. A., Thompson, S. D., Luyrink, L., Passo, M., Choi, E. and Glass, D. N. 1993. Dominant T cell-receptor beta chain variable region V beta 14^+ clones in juvenile rheumatoid arthritis. *Proc. Natl Acad. Sci. USA* 90:11104.

41 van Laar, J. M., Miltenburg, A. M., Verdonk, M. J., Leow, A., Elferink, B. G., Daha, M. R., Cohen, I. R., de Vries, R. R. and Breedveld, F. C. 1993. Effects of inoculation with attenuated autologous T cells in patients with rheumatoid arthritis. *J. Autoimmun.* 6:159.

42 Moreland, L. W., Morgan, E. E., Adamson, T. C., III, Fronek, Z., Calabrese, L. H., Cash, J. M., Markenson, J. A., Matsumoto, A. K., Bathon, J., Matteson, E. L., Uramoto, K. M., Weyand, C. M., Koopman, W. J., Heck, L. W., Strand, V., Diveley, J. P., Carlo, D. J., Nardo, C. J., Richieri, S. P. and Brostoff, S. W. 1998. T cell receptor peptide vaccination in rheumatoid arthritis: a placebo-controlled trial using a combination of Vbeta3, Vbeta14, and Vbeta17 peptides. *Arthritis Rheum.* 41:1919.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Mechanisms of Ageing and Development 125 (2004) 191–199
mechanisms of ageing and development
www.elsevier.com/locate/mechagedev

Senescence-associated decline of lymphocyte migration in gut-associated lymphoid tissues of rat small intestine

Takashi Ogino[a,1], Soichiro Miura[b,*], Shunsuke Komoto[a], Yuriko Hara[a], Ryota Hokari[b], Yoshikazu Tsuzuki[b], Chikako Watanabe[a], Seiichiro Koseki[a], Hiroshi Nagata[a], Satoshi Hachimura[c], Shuichi Kaminogawa[c], Hiromasa Ishii[a,2]

[a] *Department of Internal Medicine, School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[b] *Second Department of Internal Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan*
[c] *Department of Applied Biological Chemistry, University of Tokyo, Tokyo, Japan*

Received 29 May 2003; received in revised form 10 November 2003; accepted 14 November 2003

Abstract

Scenescence-induced changes in gut-associated lymphoid tissues may contribute largely to the impaired immune responses during aging. Age-related changes in lymphocyte recirculations were investigated in Peyer's patches of rat small intestine. Cell dynamics of labeled T lymphocytes were observed under an intravital fluorescence microscope and compared between young and aged rats. Lymphocyte transport through intestinal lymph was decreased in aged rats. The lymphocyte rolling and adherence in postcapillary venules (PCV) of Peyer's patches was also significantly impaired in aged rats, with decreased expression of L-selectin on lymphocyte surfaces. Immunohistochemical analysis revealed a significant decrease in the CD8-positive cell population in Peyer's patches of the aged group, although mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression in postcapillary venules was unaltered. Lymphocyte adoptive-transfer studies indicated that although both the donor and recipient factors influence the adherence of T cells, the former may play a predominant role in the age-related change. This study clearly demonstrated in situ that T cell migration into Peyer's patches is significantly decreased in the aged intestine, which may reflect the impaired immune responses in the aging process.

Keywords: Peyer's patches; Adhesion molecules; L-Selectin; MAdCAM-1; Lymphocyte subset; Adoptive-transfer

1. Introduction

It is well established that aging is associated with systemic immunodeficiency. Advancing age is associated with B and T lymphocyte dysfunctions and the T cells in the elderly are often characterized by altered phenotypes, reduced responses to mitogens and impaired cytokine production (Ernst et al., 1993; Flurkey et al., 1992; Okumura et al., 1993). It is likely that these age-related T cell deficits contribute to diminished B cell and antibody responses (Zharhary, 1986). The gastrointestinal tract in the elderly is particularly susceptible to infectious and inflammatory diseases, suggesting that mucosal immune defenses are compromised (Schmucker et al., 1996, 2001). For example, the IgA titer in the intestinal lumen declines 15–20% between maturity and senescence in mice (Lim et al., 1981). Furthermore, Schmucker et al. (1988) demonstrated significant declines in the intestinal IgA antibody response to cholera holotoxin in rats. They also suggested that the decline in the number of specific antibody-containing cells in the intestinal mucosa of old immunized rats in comparison to that in young animals suggested that ageing compromises the migration of putative IgA plasma cells to the intestinal mucosa (Schmucker et al., 1988; Van der Heijden et al., 1988). Recently, Koga et al. (2000) demonstrated that age-associated alterations in antigen-induced cytokine responses occur earlier in the mucosal immune system than in the systemic immune compartment. Despite reports of

* Corresponding author. Tel.: +81-429-95-1211x2367; fax: +81-429-96-5201.
E-mail addresses: t-ogino@nx.catv.ne.jp (T. Ogino), miura@me.ndmc.ac.jp (S. Miura), hishii@sc.itc.keio.ac.jp (H. Ishii).
[1] Tel.: +81-3-3498-8898; fax: +81-3-3498-8898.
[2] Co-corresponding author. Tel.: +81-3-3353-1211x2260; fax: +81-3-3356-9654.

0047-6374/$ – see front matter © 2003 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mad.2003.11.014

age-associated changes in the mucosal immune system, the mechanisms underlying mucosal immunosenescence remain poorly understood.

During immune surveillance, naive lymphocytes preferentially enter secondary lymphoid organs where they sample sequestered antigens before returning to the circulation. Naive lymphocytes usually migrate very efficiently from the blood into secondary lymphoid tissues, such as lymph nodes and Peyer's patches, by extravasating through the endothelium of specialized postcapillary or high endothelial venules (HEV) (Butcher and Picker, 1996; Girard and Springer, 1995). Lymphocytes are thought to enter secondary lymphoid organs through a cascade of steps (Springer, 1994). In mice, the α4/β7 heterodimer is the principal homing receptor that mediates the tissue-specific binding of lymphocytes to venules in the gut-associated lymphoid system by interacting with the endothelial mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (Berlin et al., 1993; Briskin et al., 1993; Fujimori et al., 2002). Furthermore, studies have shown that there are several adhesion molecules with overlapping functions (Bargatze et al., 1995). Recently Schmucker et al. (2000) reported the effect of ageing on the expression of lymphocyte homing molecules in the rat intestine. However, we are unaware of any data concerning ageing affects on lymphocyte–endothelial interactions in Peyer's patches.

Recently, we developed a method for monitoring the dynamic in vivo process of lymphocyte migration into rat small intestinal Peyer's patches using intravital microscopy and fluorochrome carboxyfluorescein diacetate succinimidyl ester (CFSE) (Miura et al., 1995). In the present report, this procedure was employed to investigate how ageing modifies the initial interaction of T lymphocytes with postcapillary venules (PCV) endothelium and to investigate the possible contributions of changes in the expressions of α4-integrin, L-selectin, and endothelial MAdCAM-1 expression to the age-related changes of T lymphocyte–endothelial cell adhesive interactions.

2. Methods

2.1. Collection and labeling of T lymphocytes

Male Fischer 344 rats from the National Institute on Aging colony (Harlan–Sprague–Dawley, Inc., Indianapolis, IN) were used in this study. The animals were segregated into young adult (12 weeks) and older (>77 weeks) age groups and maintained on standard laboratory chow (Oriental Yeast Mfg., Ltd., Tokyo) and water ad lib. All animals were handled according to the guidelines of the Keio University, School of Medicine, Animal Research Committee. The rats were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and the main mesenteric lymphatic duct was cannulated as described by Bollman et al. (1948). After cannulation, the animals were maintained in a Bollman's cage and isotonic saline was infused via the jugular vein (2.4 ml/h) to replace the fluid and electrolytes lost due to lymphatic drainage. Lymph samples were collected in ice-cold vials containing heparin (6 units/ml), 5% fetal bovine serum, and RPMI 1640 medium (pH 7.4; GIBCO, Grand Island, NY). The lymphocytes were washed three times with RPMI 1640, containing 1% penicillin, streptomycin, and 0.1% bovine serum albumin before separation and labeling.

A T cell-rich fraction of lymphocytes from mesenteric lymph was obtained using a nylon-wool column. Lymphocytes (1×10^8) in 20 ml of RPMI medium with 1% fetal bovine serum were incubated at 37 °C for 1 h and subsequently passed over a nylon-wool column (Kanto Kagaku, Japan) and the pass-through fraction was designated the T cell-rich fraction. Lymphocyte viability was unaffected as assessed by trypan blue exclusion.

In some experiments, T lymphocytes were separated into CD4-positive and CD8-positive subpopulations using magnetic cell sorting (MACS). T lymphocytes (1×10^7 cells) were suspended in 90 μl of phosphate buffered saline containing 0.5% bovine serum albumin and 5 mM ethylenediaminetetraacetic acid (EDTA), and incubated with 10 μl of anti-rat CD4-labeled or anti-rat CD8-labeled MACS microbeads (Miltenyi Biotech, Bergish Gladbach, Germany) for 15 min at 6 °C. The treated T cells were passed through a separation column (type LS, Miltenyi Biotech), which was placed in the magnetic field of a MACS separator (MidiMACS, Miltenyi Biotech). The magnetically labeled CD4- or CD8-positive cells were retained in the column, whereas the unlabeled CD4- or CD8-negative cells passed through. The CD4- or CD8-negative cell populations were used for migration studies. The purity of each lymphocyte subpopulation was confirmed by flowcytometry, and the cells were resuspended in RPMI containing 5% fetal calf serum on ice until used. Immediately prior to transfer, suspended cells (1×10^8 in 20 ml RPMI) were incubated in carboxyfluorescein diacetate succinimidyl ester (CFDASE, Molecular Probes, Eugene, OR) for 30 min at 37 °C.

2.2. Intravital observation of lymphocyte migration in rat Peyer's patches

The abdomen was opened via a midline incision and 12 cm segment of the ileum near the ileocecal valve was placed gently on a plastic plate for observation. The intestine was kept warm and moist by continuous superfusion with physiologic saline at 37 °C. Two small proximal and distal incisions were made and warm Krebs–Ringer solution (pH 7.4) was instilled via a tube in the proximal end in order to maintain luminal pressure at 15 cm H_2O. Areas of the microcirculation in Peyer's patches were observed from the serosal side using an intravital microscope (Diaphot, TMD-2S (Nikon, Tokyo)) equipped with a TV-video tape recording system. The fate of the fluorescence-labeled lymphocytes was observed on a TV-monitor via a fluorescence microscope equipped with a silicon intensified target image

tube camera (SIT) with contrast enhancement (C-2400-08, Hamamatsu Photonics Co., Shizuoka, Japan) following excitation at 470–490 nm and emission at 520 nm (Miura et al., 1995).

Lymphocytes (3×10^7 cells in 1 ml RPMI) were injected into the jugular vein of recipient rats over 3 min. The migration of CFSE-labeled T lymphocytes through the Peyer's patch microvasculature was recorded on S-VHS video tapes during the first 20 min and at 10 min intervals for the subsequent 60 min. Lymphocyte behavior in the PCV's can be classified into two different types according to the pattern of their interaction with the vascular walls as assessed by frame analysis. Lymphocytes that adhered to the wall but exhibited movement along the vessel wall were classified as "rolling" lymphocytes. The percentage of rolling lymphocytes per total number of entered cells was calculated. Lymphocytes that adhered to the vessel wall without movement following transient rolling for more than 30 s were classified as "sticking" lymphocytes. The number of sticking lymphocytes was determined in 1 mm^2 fields containing PCVs 25–50 μm in diameter.

2.3. Immunohistochemical study

The small intestine was removed, fixed in periodate–lysine–paraformaldehyde (PLP) and used for immunohistochemical study using labeled streptavidin biotin method. Segments of ileal mucosa containing Peyer's patches were vertically embedded in OCT (Sakura Fineteck Inc., Tokyo, Japan) and 7 μm cryostat sections were used for immunohistochemistry. The primary antibodies were directed against MAdCAM-1 (OST-2) (Hokari et al., 2001), rat macrophage (ED-1), CD4 and CD8. They were visualized by streptavidin-FITC and examined by fluorescence microscopy. Vessels positive for MAdCAM-1 in the lamina propria and submucosa were calculated using an image analyzer and expressed as the positively stained area per unit area. The infiltrated cells were expressed as the number of CD4-, CD8- or ED-1-positive cells per mm muscularis mucosa.

2.4. Fluorescence-activated cell sorter analysis

T lymphocytes (1×10^6) were washed in Hank's balanced salt solution containing 0.2% bovine serum albumin and 0.1% NaN_3. This medium was used throughout the staining procedure and all incubations with antibodies were performed at 4 °C for 30 min. Antibodies against rat L-selectin (HRL3, IgG1) (Seikagaku Co., Tokyo), α4-integrin (MRα4-1) (PharMingen, San Diego, CA) and CD18 (WT.3) (PharMingen) were used. Lymphocytes (2×10^5) were incubated in antibodies directed against α4-integrin or CD18, washed in 400 μl Hank's balanced salt solution, centrifuged at $1500 \times g$ for 30 s (3×), incubated with 1 ml FITC-labeled anti-mice IgG, washed twice, and resuspended for analysis. L-selectin was visualized by using a primary antibody (HRL3) and a secondary antibody, FITC-conjugated anti-hamster IgG (Cappel, West Chester, PA). Flow cytometry was performed using FACSort (Becton Dickinson, Mountain View, CA), and dead cells identified their failure to exclude propidium iodide dye, were excluded from the data pool.

2.5. Statistics

All results were expressed as the mean ± S.E.M. The differences among groups were evaluated by one-way ANOVA and Fisher's post hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Age-related changes in lymphoid cell subsets and endothelial adhesion molecules in Peyer's patches

The total number of Peyer's patches in the small intestine was 15.6 ± 2.4 in young adult rats and 11.8 ± 2.5 in aged animals, but the decline was not statistically significant. However, there was a significant increase in the total area of Peyer's patches in the aged group (11.2 ± 2.1 mm^2) in comparison to that in young adults (5.35 ± 2.9 mm^2).

Fig. 1 shows mesenteric lymph flow in cannulated rats and lymphocyte flux (number of collected lymphocytes per hour) in the mesenteric lymphatics. Although there was no difference in lymph flow between the young adult and the aged groups, a significantly lower lymphocyte flux was observed in the mesenteric lymph in the aged rats in comparison to the young adults.

We compared the distribution of lymphocyte subsets and ED-1-positive cells in the Peyer's patches by immunohistochemistry. Fig. 2 shows a representative picture of CD4- and CD8-positive lymphocytes in Peyer's patches of young adult and aged animals. There was a marked decrease in the

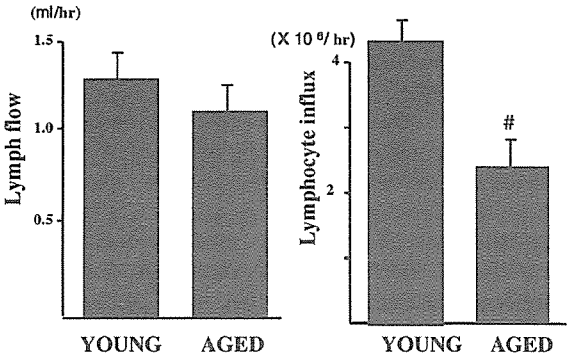

Fig. 1. Mesenteric lymph flow and lymphocyte flux in mesenteric lymphatics of young adult and aged rats. (#) $P < 0.05$ vs. young adults; data expressed as the mean ± S.E.M. for six animals.

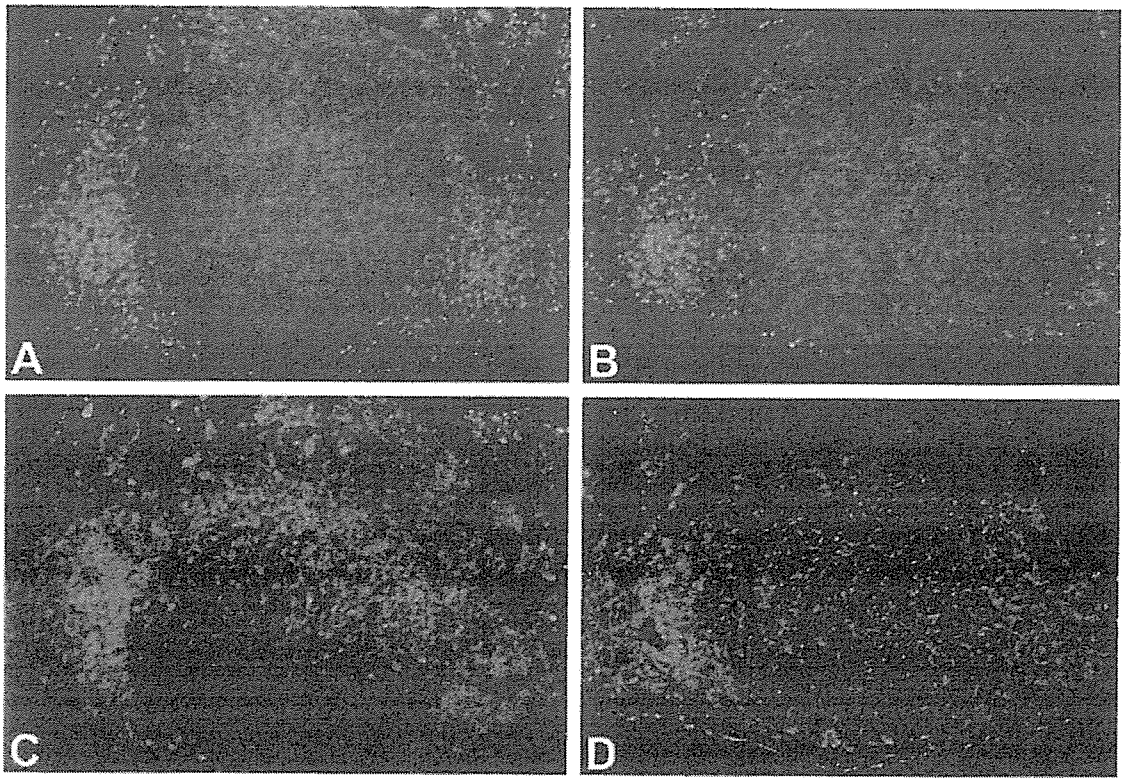

Fig. 2. Immunohistochemical staining of CD8- and CD4-positive cells in the Peyer's patches of young adult and aged rats. (A) CD8-positive cells in young adults (100×). (B) CD8-positive cells in aged rats, showing a marked decline, especially in the interfollicular zones of the Peyer's patches in the aged group. (C) CD4-positive cells in young adults (100×). (D) CD4-positive cells in aged rats (100×). There was also a decline in the number of CD4 cells in the Peyer's patches of aged rats, although these declines were not statistically significant vs. values in the CD8 cells.

number of CD8 cells, especially in the interfollicular zones of Peyer's patches in the aged rats. There were also fewer CD4 cells in the Peyer's patches of the aged rats, but the decline was not statistically significant in comparison to the decline in the CD8 cells. There was also a slight decline in the number of B cells in the aged rats, but there was no difference between the number of ED-1 cells in the young adult group in comparison to that in the aged group (data not shown).

We examined MAdCAM-1 expression in the venular endothelium of Peyer's patches using immunohistochemistry. Fig. 3A shows the immunohistochemical staining of MAdCAM-1 in young adult and aged rat small intestines. Fig. 3B includes the quantitative data. There was significant constitutive expression of MAdCAM-1, primarily in the postcapillary venules of Peyer's patches in both young adult and aged rats. Furthermore, there was no quantitative difference in MAdCAM-1 expression between these two groups.

3.2. Age-related changes in T lymphocyte migration into Peyer's patches

Some fluorescence-labeled and transferred lymphocytes exhibited a characteristic rolling behavior on the surface of PCV endothelium in Peyer's patches. However, more than 70% of the labeled cells passed through the Peyer's patches without rolling or adhesion. The total number of lymphocytes entering the PCVs within 10 min of transfer into the jugular vein did not differ significantly between young and aged animals, 1.3 ml/h versus 1.05 ml/h, respectively. However, at 10 min after transfer, the percentage of rolling cells from young donors in young recipients (28.1 ± 4.8) was significantly greater than that of rolling cells from aged donors in aged recipients (12.1 ± 4.3, $P < 0.05$), suggesting diminished lymphocyte–endothelial interaction in the old animals.

Some lymphocytes adhered to PCV endothelium (Fig. 4A). On the one hand T cells from young donors adhered selectively to the PCVs of Peyer's patches in young recipients. On the other hand, in old recipients fewer sticking lymphocytes from old donors were observed at 20 min postinfusion. The number of sticking lymphocytes gradually increased during the 40 min post transfer observation period. Fig. 4B compares the time-course of changes in the number of cells sticking to PCVs of Peyer's patches. The data for lymphocytes inside the microvascular walls and the data for lymphocytes located along the microvascular walls are included. The number of young donor adherent cells transferred into young rats increased, especially during the

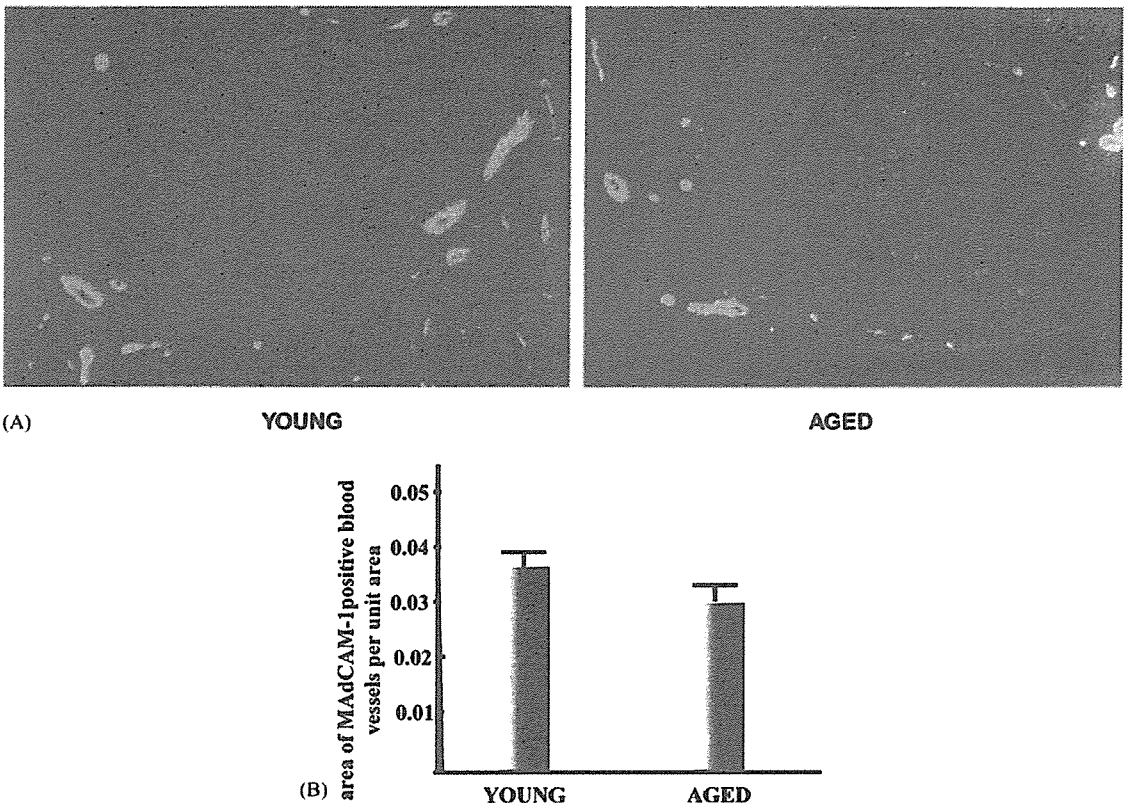

Fig. 3. (A) Representative images of MAdCAM-1 expression in Peyer's patches. The primary antibody used in the immunostaining was a monoclonal antibody directed against MAdCAM-1 (MECA367) (left panel, young adults; right panel, aged rats). MAdCAM-1 is expressed in the endothelial cells of postcapillary venules in Peyer's patches. (B) MAdCAM-1-positive vessels in the lamina propria and in the submucosa were determined as areas of positively stained vessels per unit area. Results are expressed as the mean ± S.E.M. of six animals; there were no significant differences between the young adult and the aged groups.

initial 10 min after transfer, whereas only a few of the T cells from aged donors transferred into aged rats adhered to PCVs during the same post transfer-interval. Nevertheless, the number of these cells gradually increased during the observation period.

We also determined whether or not there was an age-related shift in the migration of CD4 and CD8 T lymphocytes to Peyer's patches. Fig. 5 illustrates the relative numbers of cells sticking to the PCVs of Peyer's patches at 20 and 40 min after the transfer of 3×10^7 CD4- or CD8-negative lymphocytes (a) from young donors into young recipients or (b) from aged donors into aged recipients. In young animals, the numbers of either CD4- or CD8-negative adherent cells were always greater than that found in aged animals. Furthermore, CD8-negative cells always adhered more efficiently to the PCVs than did CD4-negative cells in either young and aged recipients. However, the inhibition of cell adhesion at 40 min post transfer was not statistically significant (for the CD4-negative or the CD8-negative populations, i.e. 86 and 89%, respectively). These data suggest that there is no apparent age-related shift in the adhesion of specific T lymphocyte subpopulations.

3.3. Migration of T lymphocytes after cross transfer

Fluorescent-labeled T lymphocytes from young or old donors were transferred into young and old recipient animals and lymphocyte kinetics were monitored. T cells from young donors transferred into young syngeneic rats exhibited the most efficient adherence to Peyer's patch PCVs (Fig. 6). The adherence of young donor cells to PCVs in old recipients was significantly lower, but was still greater than that found with cells from old donors transferred into old recipients. T cells from old donors did not adhere efficiently to PCVs in Peyer's patches of young syngeneic recipients, suggesting that the age-related decline is mainly due to changes in the donor lymphocytes. Nevertheless, changes in recipients are also involved because cells from old donors adhered more efficiently to PCV endothelium in young recipients in comparison to that in old rats.

3.4. Analysis of surface adhesion molecules on T lymphocytes

Expression of adhesion molecules (α4-integrin, β2-integrin and L-selectin) on the surface of T lymphocytes was

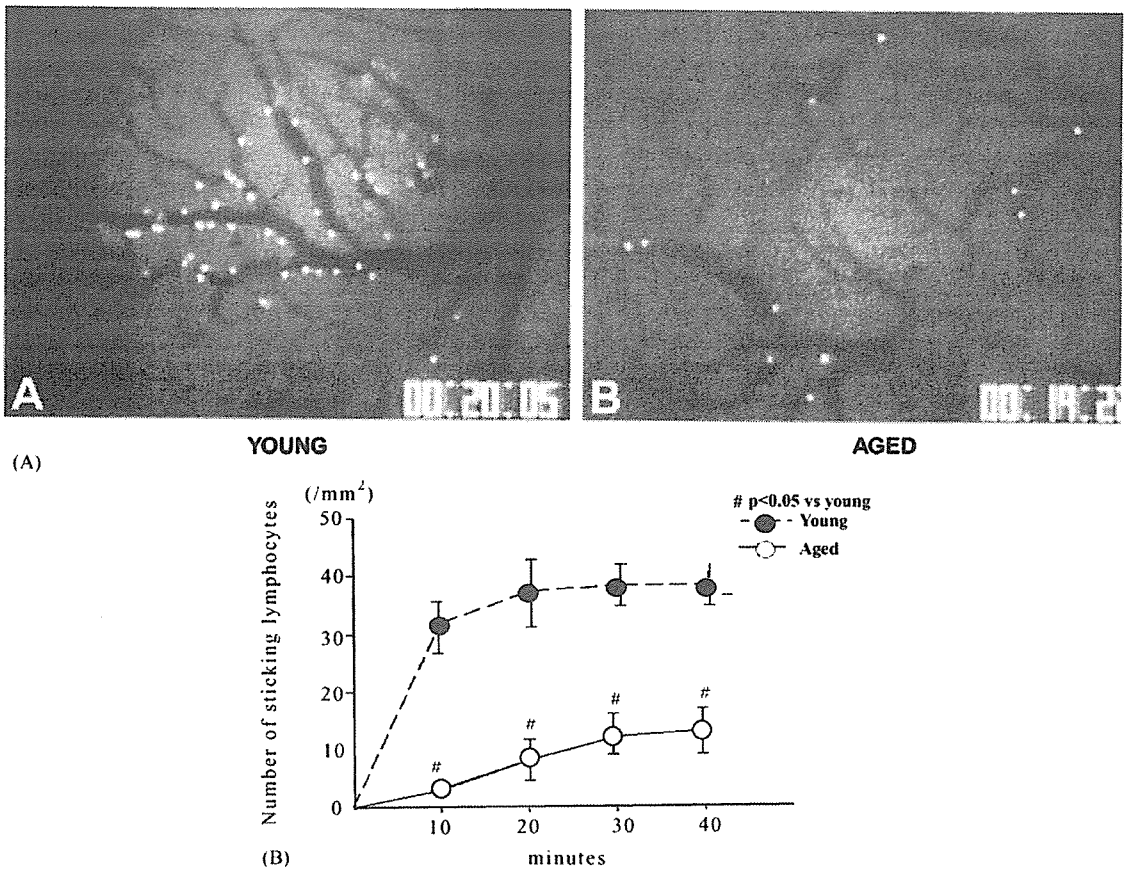

Fig. 4. (A) Representative photomicrographs showing the distribution of CFSE-labeled T lymphocytes in the postcapillary venules of Peyer's patches 20 min after infusion. (Left) Lymphocytes (3×10^7) from young adults were transferred into young animals. (Right) lymphocytes from aged rats were transferred into aged animals. (B) Time-course of changes in the number of T lymphocytes sticking to postcapillary venules in rat Peyer's patches and the difference between young adult and aged rats. Lymphocytes located inside or along venules were counted in the 1 mm^2 observation field. (#) $P < 0.05$ vs. young adult; data expressed as the mean ± S.E.M. for six separate animals.

determined using monoclonal antibodies. The populations of CD4- and CD8-positive lymphocytes in the intestinal lymph from both age groups were similar, i.e. ~55% CD4 and ~31.5% CD8 cells (data not shown). The cell surface expression of L-selectin was greater in young T cells in comparison to aged T cells, whereas the expressions of α4-integrin and CD18 did not differ significantly between young and aged T cells (Fig. 7).

4. Discussion

We demonstrated an age-associated decline in the migration of T lymphocytes to the PCVs in Peyer's patches. We also showed a significant decline in lymphocyte flux in the intestinal lymph of aged-rats, suggesting that the kinetics of T lymphocyte movement within Peyer's patches is inhibited with increasing age. Lymphocyte recirculation does not occur randomly, but is regulated by mechanisms inherent in lymphocyte–endothelial cell recognition (Butcher and Picker, 1996). Naive lymphocytes can migrate very efficiently from the blood into secondary lymphoid tissues (e.g. lymph nodes and Peyer's patches) by extravasating through the endothelium of specialized postcapillary or high endothelial venules (Girard and Springer, 1995). In contrast, memory and effector lymphocytes primarily access the extralymphoid immune effector sites (Butcher and Picker, 1996; Hokari et al., 1999; Mackey, 1993). Previously we reported that both CD4 and CD8 naive lymphocytes from the intestinal lymph recirculate through the intestine under physiological conditions, entering Peyer's patch PCVs and subsequently flowing out to the interfollicular zones (Hokari et al., 2001; Miura et al., 1995). This process enables naive cells to increase the frequency with which they encounter their cognate antigens. We suggest that there is an age-related impairment in the recirculation of naive T lymphocytes within Peyer's patches and that this may be related to the compromised gastrointestinal mucosal immune defense in the elderly.

We observed significant age-related impairments in both CD4 and CD8 lymphocyte migrations. The distribution of the CD8 population appears to be more readily suppressed

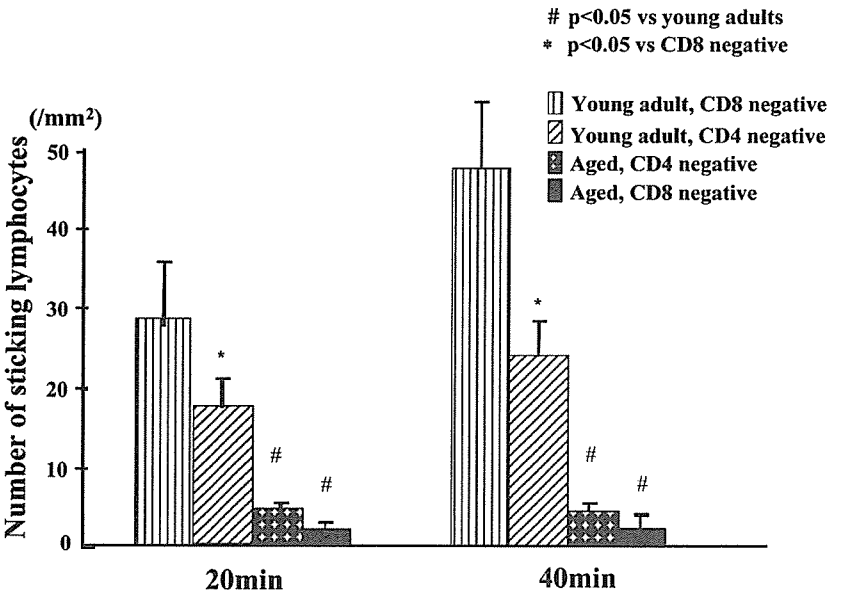

Fig. 5. The number of sticking lymphocytes to the postcapillary venules of Peyer's patches at 20 and 40 min post transfer of CD4- or CD8-negative cells into young and aged animals. Lymphocytes (3×10^7) from young adults were injected into different young animals and lymphocytes from aged rats were injected into the aged animals. (*) $P < 0.05$ vs. CD8-negative; (#) $P < 0.05$ vs. young adults; data expressed as the mean ± S.E.M. for six animals.

by ageing in comparison to the CD4 subpopulation. This finding is in agreement with the report by Kawanishi and Kiely (1987, 1989), who observed a decline in the T suppressor/cytotoxic cell subpopulation in the Peyer's patches of old mice. Several research groups have used flow cytometry to demonstrate the absence of age-related changes in the CD4 or CD8 subsets in Peyer's patches (Daniels et al., 1993; Fló and Massouh, 1997). The latter group also reported immunohistochemical evidence suggesting that ageing is accompanied by a shift in the distribution of CD8 cells from the interfollicular zone throughout the Peyer's patches (Daniels et al., 1993). The suppressor/cytotoxic T cell population may be more susceptible to age-related shifts in comparison to helper/inducer T cells in GALT. However, our in vivo data did not demonstrate a preferential decline in the CD8 lymphocyte–endothelial cell interaction in comparison

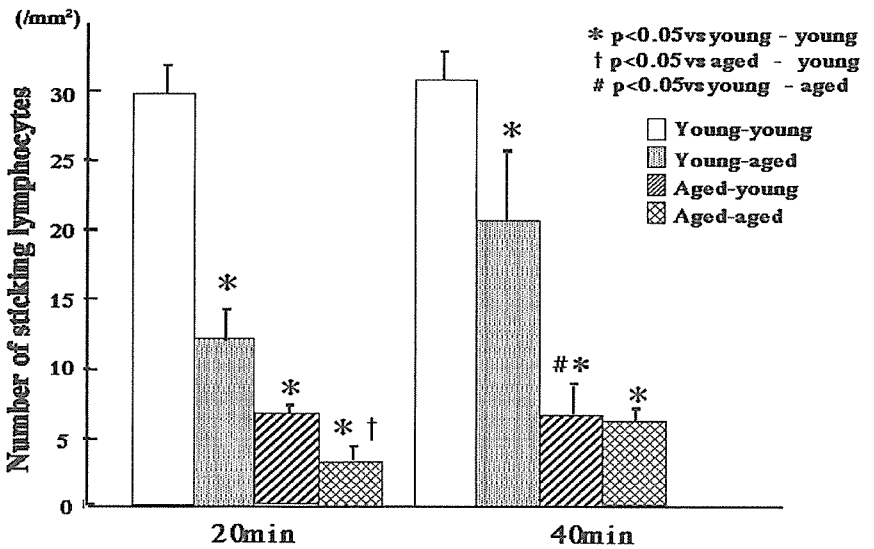

Fig. 6. The number of lymphocytes sticking to the postcapillary venules in Peyer's patches at 20 and 40 min after adoptive-transfer. The labeled T lymphocytes from young donors were transferred into old recipients, whereas the cells from old donors were transferred into young recipients. The kinetics of labeled T lymphocytes was compared among four different combinations, namely young–young, young–aged, aged–young, and aged–aged. (*) $P < 0.05$ vs. young–young; (#) $P < 0.05$ vs. young–aged; (†) $P < 0.05$ vs. aged–young; data expressed as the mean ± S.E.M. for four animals.

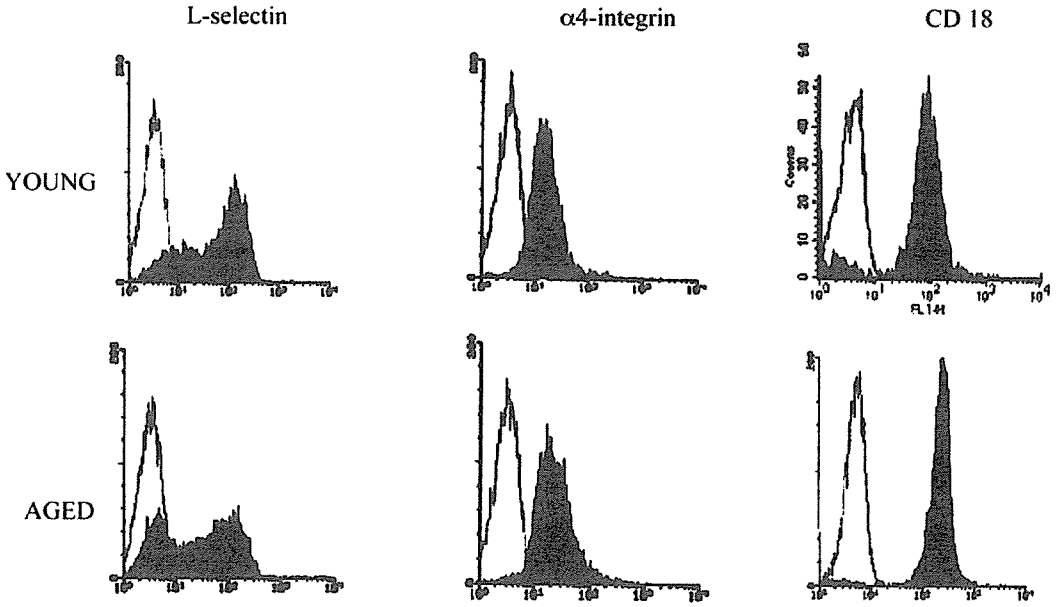

Fig. 7. The expression of adhesion molecules (L-selectin, α4-integrin, CD18) on T lymphocytes determined by flow cytometric analysis. Lymphocytes (2×10^5) were incubated with anti-rat monoclonal antibodies against L-selectin (HRL3, IgG1), α4-integrin (MRα4-1), and CD18 (WT.1). They were then incubated with 1 ml FITC-labeled anti-mouse IgG or anti-hamster IgG. Flow cytometric analysis was performed using FACSort (Becton Dickinson). Representative data from at least four individual measurements are shown.

to CD4 subsets. The subsequent migration processes into the follicular zones of Peyer's patches should be examined in the future investigations.

The impaired migration may reflect the altered expressions of homing receptors on lymphocytes or of their respective ligands on the vascular endothelial cells. We determined the expression of adhesion molecules on lymphocytes by flow cytometry and MAdCAM-1 expression on vascular endothelial cells by immunohistochemistry. We also performed adoptive-transfer studies using T lymphocytes isolated from young and old donors and young and old recipients. In mice, the α4/β7 heterodimer is the principal homing receptor that mediates lymphocyte binding to venules in the gut-associated lymphoid system via interaction with the endothelial addressin, MAdCAM-1 (Berlin et al., 1993; Briskin et al., 1993; Fujimori et al., 2002). Rather than one adhesion molecule with an exclusive function, studies have described several adhesion molecules with overlapping functions. For example, L-selectin and CD11a/CD18 also play a role in the interaction of lymphocytes and high endothelial venules in the gut wall (Arbones et al., 1994; Bargatze et al., 1995; Steeber et al., 1996). We provide evidence that ageing does not diminish the expression of either the lymphocyte integrin α4 or the vascular endothelial addressin, MAdCAM-1. Our data also suggest that ageing impairs the expression of L-selectin on lymphocytes. This is consistent with data reported by Steeber et al. (1996) suggesting that, (a) the expression of L-selectin is impaired by ageing and (b) this interferes with the migration of lymphocytes to secondary lymphoid tissues. Our results also demonstrated that T cells from old donors do not adhere to the endothelium of PCVs in Peyer's patches in young syngeneic recipients to the extent they did to the endothelium in old recipients. Furthermore, the number of adherent cells was significantly greater when T cells from young donors were used. These results strongly suggest the possibility that the age-related decline in T lymphocyte adhesion in Peyer's patches is due to changes in lymphocyte adhesion molecule expression, including decreased expression of L-selectin. Recently, Schmucker et al. (2000) reported that the proportion of α4β7-positive mononuclear cells in young rats was significantly higher than that measured in the blood of senescent animals. Therefore, a decline in the α4β7–MAdCAM-1 interaction in aged animals cannot be excluded, because (a) we did not test antibodies against α4β7 heterodimer epitope, and (b) L-selectin is known to facilitate the subsequent α4β7-MAdCAM-1 interaction in Peyer's patches (von Andrian and Mackay, 2000). Furthermore, age-related changes in recipient rats are not excluded because the adherence of T cells from young donors to PCV endothelium in old recipients is significantly lower in comparison to that in young recipients.

Thoreux et al. (2000) recently hypothesized that an age-related delay in the migration of IgA immunoblasts to the effector site, i.e. the intestinal mucosa, contributes to mucosal immunosenescence. Using lymphocyte adoptive-transfer, they demonstrated that factors intrinsic to both the donor cells and the recipients influence the migration of immunoblasts from the mesenteric lymph nodes to the effector site. Since we focused our studies on the altered migration of naive T cells in nonimmunized animals,

age-related changes in antigen-specific T cell migration remain to be elucidated. While the exact mechanism is unclear, our study is the first to report direct evidence of an age-related decline in the migration of naive T cells to the Peyer's patches in situ.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture of Japan and by grants from Keio University, School of Medicine, and form National Defense Medical College.

References

Arbones, M.L., Ord, D.C., Ley, K., Ratech, H., Maynard-Curry, C., Otten, G., Capon, D.J., Tedder, T.F., 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin (CD62L) deficient mice. Immunity 1, 247–260.

Bargatze, R.F., Jutila, M.A., Butcher, E.C., 1995. Distinct roles of L-selectin and integrins α4β7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. Immunity 3, 99–108.

Berlin, C., Berg, E.L., Briskin, M.J., Andrew, D.P., Kilshaw, P.J., Holzmann, B., Weissman, I.L., Hamann, A., Butcher, E.C., 1993. α4β7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1. Cell 74, 185–195.

Bollman, J.L., Cain, J.C., Grindlay, J.H., 1948. Techniques for collection of lymph from liver, small intestine or thoracic duct of the rat. J. Lab. Clin. Med. 33, 1349–1352.

Briskin, M.J., McEvoy, L.M., Butcher, E.C., 1993. MAdCAM-1 has homology to immunoglobulin and mucin like adhesion receptors and IgA1. Nature (London) 363, 461–464.

Butcher, E.C., Picker, L.J., 1996. Lymphocyte homing and homeostasis. Science 272, 60–66.

Daniels, C.K., Perez, P., Schmucker, D.L., 1993. Alterations in CD8+ cell distribution in gut-associated lymphoid tissues (GALT) of the aging Fischer 344 rat: a correlated immunohistochemical and flow cytometric analysis. Exp. Gerontol. 28, 549–555.

Ernst, D.N., Weigle, W.O., Noonan, D.J., McQuitty, D.N., Hobbs, M.V., 1993. The age-associated increase in IFN-gamma synthesis by mouse CD8+ T cells correlates with shifts in the frequencies of cell subsets defined by membrane CD44, CD45RB, 3G11, and MEL-14 expression. J. Immunol. 151, 575–587.

Fló, J., Massouh, E., 1997. Age-related changes of naive and memory CD4 rat lymphocyte subsets in mucosal and systemic lymphoid organs. Dev. Comp. Immunol. 21, 443–453.

Flurkey, K., Miller, R.A., Harrison, D.E., 1992. Cellular determinants of age-related decrements in the T cell mitogen response of B6CBAF1 mice. J. Gerontol. 47, B115–B120.

Fujimori, H., Miura, S., Koseki, S., Hokari, R., Komoto, S., Hara, Y., Hachimura, S., Kaminogawa, S., Ishii, H., 2002. Intravital observation of adhesion of lamina propria lymphocytes to microvessels of small intestine in mice. Gastroenterology 122, 734–744.

Girard, J.-P., Springer, T.A., 1995. High endothelial venules (HEVs): specialized endothelium for lymphocyte migration. Immunol. Today 16, 449–457.

Hokari, R., Kato, S., Matsuzaki, K., Iwai, A., Kawaguchi, A., Nagao, S., Miyahara, T., Itoh, K., Sekizuka, E., Nagata, H., Ishii, H., Iizuka, T., Miyasaka, M., Miura, S., 2001. Involvement of mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the pathogenesis of granulomatous colitis in rats. Clin. Exp. Immunol. 126, 259–265.

Hokari, R., Miura, S., Fujimori, H., Koseki, S., Tsuzuki, Y., Kimura, H., Higuchi, H., Serizawa, H., Granger, D.N., Ishii, H., 1999. Altered migration of gut-derived T lymphocytes after activation with concanavalin A. Am. J. Physiol. 277, G763–G772.

Hokari, R., Miura, S., Nagata, H., Fujimori, H., Koseki, S., Kato, S., Kurose, I., Sekizuka, E., Granger, D.N., Ishii, H., 2001. Intercellular cell adhesion molecule-1 regulates lymphocyte movement into intestinal microlymphatics of rat Peyer's patches. J. Leukoc. Biol. 70, 896–902.

Kawanishi, H., Kiely, J., 1989. Immune-related alterations in aged gut-associated lymphoid tissues in mice. Dign. Dis. Sci. 34, 175–184.

Kawanishi, H., Kiely, J., 1987. Immunoregulatory defects in murine aged Peyer's patches. Eur. J. Immunol. 17, 1223–1228.

Koga, T., McGhee, J.R., Kato, H., Kato, R., Kiyono, H., Fujihashi, K., 2000. Evidence for early aging in the mucosal immune system. J. Immunol. 165, 5352–5359.

Lim, T.S., Messiha, N., Watson, R.R., 1981. Immune components of the intestinal mucosae of ageing and protein deficient mice. Immunology 43, 401–407.

Mackey, C.R., 1993. Homing of naïve, memory and effector lymphocytes. Curr. Opin. Immunol. 5, 423–427.

Miura, S., Tsuzuki, Y., Fukumura, D., Serizawa, H., Suematsu, M., Kurose, I., Imaeda, H., Kimura, H., Nagata, H., Tsuchiya, M., Ishii, H., 1995. Intravital demonstration of sequential migration process of lymphocyte subpopulations in rat Peyer's patches. Gastroenterology 109, 1113–1123.

Okumura, M., Fujii, Y., Takeuchi, Y., Inada, K., Nakahara, K., Matsuda, H., 1993. Age-related accumulation of LFA-1 high cells in a CD8+CD45RA high T cell population. Eur. J. Immunol. 23, 1057–1063.

Schmucker, D.L., Heyworth, M.F., Owen, R.L., Daniels, C.K., 1996. Impact of aging on gastrointestinal mucosal immunity. Dig. Dis. Sci. 41, 1183–1193.

Schmucker, D.L., Owen, T.M., Issekutz, T.B., Gonzales, L., Wang, R.K., 2000. Expression of lymphocyte homing receptors alpha 4 beta 7 and MAdCAM-1 in young and old rats. Exp. Gerontol. 37, 1089–1095.

Schmucker, D.L., Thoreux, K., Owen, R.L., 2001. Aging impairs intestinal immunity. Mech. Ageing Dev. 122, 1397–1411.

Schmucker, D.L., Daniels, C.K., Wang, R.K., Smith, K., 1988. Mucosal immune response to cholera toxin in ageing rats. I. Antibody and antibody-containing cell response. Immunology 64, 691–695.

Springer, T.A., 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell 76, 301–314.

Steeber, D.A., Green, N.E., Sato, S., Tedder, T.F., 1996. Lymphocyte migration in L-selectin-deficient mice. Altered subset migration and aging of the immune system. J. Immunol. 157, 1096–1106.

Thoreux, K., Owen, R.L., Schmucker, D.L., 2000. Intestinal lymphocyte number, migration and antibody secretion in young and old rats. Immunology 101, 161–167.

Van der Heijden, P.J., Bianchi, A.T., Stok, W., Bokhout, B.A., 1988. Background (spontaneous) immunoglobulin production in the murine small intestine as a function of age. Immunology 65, 243–248.

von Andrian, U.H., Mackay, C.R., 2000. T cell function and migration. Two sides of the same coin. New Engl. J. Med. 343, 1020–1034.

Zharhary, D., 1986. T cell involvement in the decrease of antigen-responsive B cells in aged mice. Eur. J. Immunol. 16, 1175–1178.

ESTABLISHMENT AND ANALYSIS OF GERMFREE T CELL RECEPTOR TRANSGENIC MICE

MIRAN FUJIOKA[1], SATOSHI HACHIMURA[2], AKIRA HOSONO[1], RYO NAKAMURA[1], KAZUHIRO HIRAYAMA[3], KIKUJI ITOH[3], AND SHUICHI KAMINOGAWA[2]

[1] Department of Food Science and Technology, Nihon University, Fujisawa-shi, Kanagawa 252-8510, Japan, and Departments of [2]Applied Biological Chemistry and [3] Veterinary Public Health, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Abstract

Recent studies have shown that intestinal bacteria affect the intestinal immune system. In order to elucidate the effects of intestinal microflora on T-cell mediated immune responses of the intestine, we established germfree (GF) T cell receptor transgenic (TCR-Tg) mice. GF ovalbumin (OVA)-specific TCR-Tg mice were obtained from conventional (CV) TCR-Tg mice by hysterectomy. Cells from spleen, Peyer's patch (PP) and lamina propria (LP) were isolated from GF TCR-Tg mice, and the ratio of $CD4^+$ T cells was assessed by flow cytometry and the production of cytokines, in response to in vitro OVA stimulation, was measured by ELISA. The numbers of PPs were significantly lower in GF TCR-Tg mice compared with CV TCR-Tg mice and the ratios of $CD4^+$ T cells in PP and LP cells from GF TCR-Tg mice were decreased. When stimulated with OVA, PP cells from GF TCR-Tg mice secreted higher levels of interferon (IFN)-γ, interleukin (IL)-5 and IL-6, and LP cells from GF mice secreted higher levels of IFN-γ and IL-6 compared with CV mice. These results suggested that although the gut-associated lymphoid tissue is poorly developed in germfree mice, intestinal T cells developing under these conditions possessed an enhanced ability to secrete cytokines in response to antigenic stimulation. Our TCR-Tg system should be an informative system to evaluate the effect of intestinal microflora on antigen-specific T cell responses.

1. Introduction

Recent advances in intestinal flora research has demonstrated that intestinal bacteria affect the intestinal immune system. Of particular note are comparative studies between conventional and germfree animals that have shown that indigenous bacteria play crucial roles in the development of the gut-associated lymphoid tissue (GALT), the exclusion of microbial pathogens and the induction of oral tolerance. In germfree mice, B cell follicles of Peyer's patches (PPs) lack germinal centers

K. Yagasaki et al. (eds.), Animal Cell Technology: Basic & Applied Aspects, Volume13, 243-247.

and gut lumen lamina propria (LP) contain few IgA plasma cells (1, 2). The oral dose of *Listeria monocytogenes* required to kill germfree mice was $1\text{-}5\times10^2$ (3), which is far lower than that of conventional mice, and, in addition, oral tolerance was not induced in germfree mice (4). However, details of the effects upon intestinal immune responses that are caused by intestinal bacteria are not fully understood. Thus, we established germfree T cell receptor transgenic (GF TCR-Tg) mice in order to investigate the effect of intestinal microflora on T-cell mediated intestinal immune responses.

2. Materials and Methods

2.1 Mice

Conventional OVA23-3 TCR-Tg mice (CV TCR-Tg mice), transgenic for OVA 323-339 specific and I-A^d restricted TCR-$\alpha\beta$, on a BALB/c genetic background were originated from Professor Sonoko Habu of Tokai University. GF TCR-Tg mice were obtained from conventional OVA 23-3 mice by hysterectomy carried out under germfree condition and fosternursed by lactating germfree BALB/c mice in an isolator.

2.2 Preparation of spleen (SP), PP and LP cells

To obtain LP cells, murine small intestines were excised and the PPs were removed carefully. The intestine was reversed by using polyethylene tubing, and wiped carefully a few times with a paper towel. The intestine was cut into 4 pieces and placed in a 50 ml tube containing HBSS (–) (Hanks' balanced salt solution, Ca, Mg free, containing 5% FCS). This was incubated at 37°C with shaking for 30 min at 150 rpm (×3), the supernatant discarded by filtration with gauze, minced into 10 mm pieces and treated with collagenase (45 mg/45 ml HBSS (–) containing 5 U/ml DNase 1) in a 100 ml-flask with gentle stirring at 37°C for 20-30 min. After stirring, the preparation was filtrated with gauze, washed, suspended in HBSS (+) (containing Ca and Mg, and 5% FCS), filtrated by glass wool column and then washed by centrifugation at 4°C, 1,300 rpm for 5 min. The upper supernatant was removed and 3 ml of 100% Percoll added to the cells and filled to 10 ml with HBSS (30% Percoll), mixed, then centrifuged at 20°C, 1,800 rpm for 20 min and upper supernatant removed. Subsequently, 4.1 ml of 100% Percoll was added to 1 ml of the cell suspension, and filled to 10 ml with RPMI containing FCS (44% Percoll), then mixed. Two ml of 70% Percoll was injected into the bottom of the tube and was centrifuged at 20°C, 1,800 rpm for 20 min. The cells located at the interface of the 70% and 44% Percoll fractions were collected and washed with RPMI. Single cell suspensions of SP and PP cells were obtained by crushing with the organ with the end of a syringe.