International Immunology, Vol. 8, No. 9, pp. 1387–1394

Alteration of a polyclonal to an oligoclonal immune response to cecal aerobic bacterial antigens in TCRα mutant mice with inflammatory bowel disease

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Keywords: anaerobic bacteria, autoantibody, mutant mice, ulcerative colitis

Abstract

Since lumenal bacteria have been postulated to play an important role in the pathogenesis of inflammatory bowel disease (IBD), we investigated the humoral response to cecal aerobic bacterial antigens by Western blot analysis in TCRα$^{-/-}$ mice which spontaneously develop IBD. The sera from TCRα$^{-/-}$ mice revealed an alteration of the recognition pattern against aerobic bacterial antigens from polyclonal to oligoclonal with age. This alteration was not observed in TCRδ$^{-/-}$ and TCRα$^{+/-}$ mice. The alteration of the recognition pattern in TCRα$^{-/-}$ mice was associated with production of autoantibodies against tropomyosin and the development of IBD. The unique population of CD4$^+$ TCRα$^-$β$^+$ cells in TCRα$^{-/-}$ mice may be involved in the recognition of these bacterial antigens and the absence of the α chain may result in the alteration of immune response.

Introduction

Pathogenic or non-pathogenic microorganisms may participate in the development of organ-specific autoimmune diseases by possessing structural similarities with self-antigens (molecular mimicry) (1,2). The ternary TCR–antigen–MHC complex interactions that induce tolerance to self-antigens by elimination or functional inactivation of autoreactive cells may also play an important role in the development of autoimmune diseases (3,4).

The etiology of inflammatory bowel disease (IBD) [ulcerative colitis (UC) and Crohn's disease], a human disease of suspected autoimmune pathogenesis, is unknown (5). Recently, genetic animal models of IBD have been described. These include IBD in rats transgenic for human HLA-B27 (6) and in mice with a deletion in TCR, IL-2 or IL-10 genes among others (6–11). Although pathogenic organisms have not been isolated from animals with IBD, IL-2 mutant mice and HLA-B27 transgenic rats maintained in germ-free environments do not develop IBD, suggesting an important role of intestinal bacterial colonization in the pathogenesis of IBD (8,11,12). We have observed that IBD in TCRα$^{-/-}$ mice is more severe than in TCRβ$^{-/-}$ and TCRβ×δ$^{-/-}$ mice, whereas TCRδ$^{-/-}$ and RAG-1$^{-/-}$ mice do not develop IBD (7). In TCRα$^{-/-}$ mice, TCRγδ$^+$ T cells have been reported to increase with age (7,13,14) or following pathogenic infection (15). Interestingly, thymocytes in TCRα$^{-/-}$ mice express low levels of the TCRβ chain on the surface in the absence of TCRα chains (TCRα$^-$β$^+$). Small numbers of CD4$^+$ TCRα$^-$β$^+$ T cells are present in peripheral lymphoid tissue including the small intestine of TCRα$^{-/-}$ mice (13). These unique CD4$^+$ TCRα$^-$β$^+$ T cells are able to expand together with, but independently of, TCRγδ$^+$ T cells (13,15), and are capable of responding appropriately to stimulation with a viral superantigen, minor lymphocyte stimulating superantigen 1 (Mls-1) or via CD3 complex (16,17). It is not clear whether TCRα$^-$β$^+$ T cells can recognize microbial peptides presented in the context of the TCR–MHC complex.

Circulating autoantibodies have been detected in patients with UC. An autoantibody against tropomyosin, a 40 kDa cytoskeletal protein, has been detected in colonic tissues of patients with UC but not with Crohn's disease (18–20). The antigenicity of colonic epithelial tropomyosin may be related to molecular mimicry with proteins of enteric bacteria such as *Escherichia coli* (21,22).

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Transmitting editor: M. Taniguchi

Received 8 April 1996, *accepted* 30 May 1996

In the present study, we have investigated the ability of various TCR mutant mice, in particular TCRα$^{-/-}$ mice, to produce antibodies to normal bacterial flora and to tropomyosin and the correlation of the presence of these antibodies with the development of IBD.

Methods

Mice

Colonies of TCRα$^{-/-}$, TCRβ$^{-/-}$ (13), TCRδ$^{-/-}$ (23) and RAG-1$^{-/-}$ mutant mice (24) were developed at Massachusetts General Hospital from mice provided by Dr P. Mombaerts and Dr S. Tonegawa (Massachusetts Institute of Technology). These mice are derived from two (H-2^{b}) strains 129/sv and C57BL/6. TCRα heterozygous mice (TCRα$^{+/-}$) were generated by crossing TCRα$^{-/-}$ mice with C57BL/6 mice (purchased from The Jackson Laboratory, Bar Harbour, ME). The mice were housed in pathogen-free facilities at the Massachusetts Institute of Technology and Massachusetts General Hospital. Food, distilled water and cages were autoclaved at 250°F for 25 min and changed weekly.

Antibodies

For Western blotting and ELISA, alkaline phosphatase (ALP)–goat anti-mouse IgG and horseradish peroxidase (HRP)–goat anti-mouse IgA and HRP–goat anti-IgM antibodies were purchased from Sera-Lab (Sussex, UK), ALP–goat anti-mouse Ig and IgG2a, and HRP–goat anti-mouse IgG1 and rat anti-mouse IgE were purchased from Southern Biotechnology (Birmingham, AL), and anti-tropomyosin (TM311) antibody was purchased from Sigma (St Louis, MO).

Histological analysis

For histological examination, specimens were obtained from rectum, distal and proximal colon, and cecum from every mouse tested. Specimens were fixed in 3% buffered formalin and embedded in paraffin. Multiple 4 μm sections were stained with hematoxylin & eosin. The severity of IBD was determined according to the diagnostic criteria previously described (7).

Extraction of bacterial proteins

Cecal lumenal contents were aspirated from the cecum by sterile needle and grown for 18 h in 20 ml LB medium (Difco, Detroit, MI) in aerobic conditions. To prevent contamination of proteins from other lumenal contents (food and epithelial cells), 100 μl of cultures was transferred in 30 ml of fresh LB medium and incubated at 37°C with shaking at 120 r.p.m. under aerobic conditions until 0.8 at OD_{650}. After culture, the bacteria was analyzed by the microbiology laboratory at the Massachusetts General Hospital. No anaerobic bacteria were detected in this culture. *E. coli* were cultured on Brucella blood agar with 5% horse blood and MacConkey II agar under aerobic conditions. In some experiments, the bacteria in log phase (OD_{650}: 0.8) were stressed by heat at 43°C for periods varying from 5 min to 3 h, or by 5% EtOH for periods varying from 5 min to 1 h. The cultures were centrifuged at 10,000 *g* for 10 min at 4°C and the pellet was washed in washing solution (cold PBS containing 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide and 10 μg/ml aprotinin). Two methods were used for lysis. (i) The pellet was diluted in washing solution to an optical density (650 nm) of 0.8–1.0 and the cells were disrupted by sonication on ice. After centrifugation and filtration by a 0.2 μm filter unit, the concentration of soluble proteins was determined by the Lowry assay (25). (ii) The pellet was directly homogenized in 1×sample buffer; 65 mM Tris (pH 6.8), 5% 2-mercaptoethanol, 3% SDS and 10% glycerol. The concentration of soluble proteins was determined by The Coomassie protein assay (26) (the Lowry assay could not be used due to the presence of 2-mercaptoethanol). There were no apparent differences between the two lysis methods. The latter method was used to extract bacterial antigens for Western blot analysis. In some experiments, bacterial proteins were extracted by sonication on ice in PBS without proteinase inhibitors and were treated with 200 μg/ml of proteinase K (Boehringer, Indianapolis, IN) at 37°C overnight.

Western blot analysis

Western blotting was carried out as previously described, with the following modifications (27). One microgram of tropomyosin, troponin (Sigma) or bacterial protein in 1×sample buffer containing 0.04% bromophenol blue was separated by SDS–PAGE on 8–12% gels and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). In some experiments, the gels were stained by the silver staining kit (BioRad, Hercules, CA) after SDS–PAGE. The transferred membranes were preincubated in TBS containing 1% skim milk overnight at 4°C. The blocked membranes were incubated with anti-tropomyosin antibody (Sigma) capable of recognizing two forms of tropomyosin (39 and 36 kDa) or various mice sera diluted 1:100 in TBS containing 1% BSA and 0.05% Tween 20, followed by incubation with various HRP- or ALP-conjugated anti-Ig subclass antibodies (described above) and developed with 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, WI) or with nitroblue tetrazolium and 5-bromo-4 chloro-3-indolyl phosphate (Promega Biotec, Madison, WI).

ELISA

ELISA was carried out as previously described, with the following modifications (20). A 96-well microtiter plate (Dynatech, Chantilly, VA) was coated with 2 μg of bacterial protein or 0.25 μg of tropomyosin (Sigma) per well in 100 μl of Dulbecco's PBS or carbonate buffer (pH 9.6), overnight at 4°C. After wash, the plates were blocked with 5% BSA and 1% goat serum for 2 h at 37°C, then 100 μl of several mouse sera diluted (1:100) was added per well in triplicate and the plates incubated for 2 h at 37°C. After washing, 100 μl of goat anti-mouse Ig was added per well and incubated for 1 h at room temperature followed by addition of 100 μl of *p*-nitrophenyl phosphate in 1 mM magnesium chloride and 50 mM sodium carbonate (pH 9.8). The plates were read at 405 nm using AUTO READER (BioTek, Winooski, UT). Results were statistically analyzed using the Mann–Whitney *U*-test.

Results

Immunoreactivity of TCRα$^{-/-}$ mice sera against antigens of aerobic bacteria from cecum

Cecal bacteria from a TCRα$^{-/-}$ mouse (20 weeks old) cultured under aerobic conditions were used as a source of antigens

for Western blot analysis with sera from TCRα$^{-/-}$ mice. Table 1 and Fig. 1 show the Western blot reaction patterns by sera from 180 TCRα$^{-/-}$ mice. Of 11 TCRα$^{-/-}$ mice <6 weeks old, all mice showed a reaction pattern composed of a few faint or undetectable bands, suggesting weak antibody responses against bacterial antigens. However, the sera of adult TCRα$^{-/-}$ mice showed a polyclonal recognition pattern that changed to an oligoclonal pattern with age (Table 1 and Fig. 1). Sera from 45 out of 58 TCRα$^{-/-}$ mice (8–20 weeks old) reacted with many bands (more than eight bands) from 50 to 120 kDa (polyclonal recognition pattern). These bands could be detected clearly up to 1:1000 dilution of sera (data not shown). The sera from 17 out of these 45 TCRα$^{-/-}$ mice (8–20 weeks old) showed strongly reactive bands within the polyclonal bands indicating increased antibody response to certain bacterial antigens. In 20- to 30-week-old TCRα$^{-/-}$ mice, sera from 26 out of 83 showed a polyclonal recognition pattern. However, the sera from the remaining 57 TCRα$^{-/-}$ older mice (20–30 weeks old) showed an oligoclonal pattern; only one or few restricted bands similar to the strong bands detected within the polyclonal pattern obtained with sera from 8- to 20-week-old TCRα$^{-/-}$ were present. Furthermore, the sera from 23 out of 28 TCRα$^{-/-}$ mice >30 weeks old showed an oligoclonal pattern, but no polyclonal pattern (Tables 1 and 2 and Fig. 1).

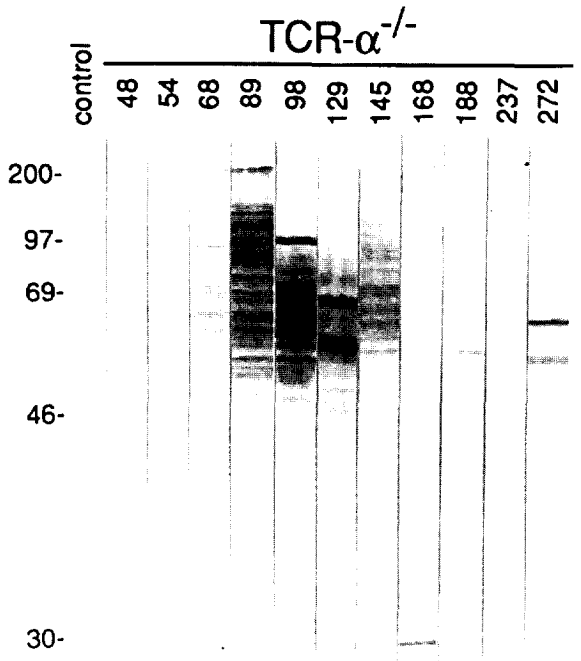

Fig. 1. The recognition pattern against cecal aerobic bacterial antigens from a TCRα$^{-/-}$ mouse (age: 150 day) with sera from TCRα$^{-/-}$ mice. Bacterial proteins were resolved by 10% SDS–PAGE, electrophoretically transferred to a membrane and treated with PBS (negative control) or sera (1:100) from different aged TCRα$^{-/-}$ mice, and reactive bands detected by the immunoperoxidase method. The numbers shown on the top indicate the age (day after birth) of mice whose sera was used. The pattern shown by the age group between 89 and 145 days (adult mice) was called the polyclonal pattern. In contrast, the restricted bands revealed by sera from older mice (168, 188, 237 and 272 days old) was called the oligoclonal recognition pattern. Mol. wt is shown on the left side in kDa.

To investigate if there were quantitative changes in antibody response to aerobic cecal bacterial antigens with age, antibody levels to bacterial antigens were detected by ELISA using 1:100 dilution of the sera from mice of different age groups. The sera from <6-week-old TCRα$^{-/-}$ mice showed weak reactivity (the average OD_{450} was 0.075 ± 0.003 from six mice). The reactivity of sera from TCRα$^{-/-}$ mice with a polyclonal pattern was higher than that with an oligoclonal pattern; the averages of OD_{450} from 20 TCRα$^{-/-}$ mice with a polyclonal pattern and 20 TCRα$^{-/-}$ mice with an oligoclonal pattern was 0.747 ± 0.095 and 0.449 ± 0.111 respectively. These results indicate that the antibody responses to aerobic bacterial antigens were less in TCRα$^{-/-}$ mice with an oligoclonal pattern as compared with those with a polyclonal pattern. However, when the sera of TCRα$^{-/-}$ mice revealing a polyclonal pattern was diluted to 1:1000 (OD_{450}: <0.4), the polyclonal bands were still clearly detected. These findings indicate that the alteration of the recognition pattern observed in TCRα$^{-/-}$ mice cannot be due to decreased synthesis of antibodies to all aerobic bacterial antigens.

The sera of the TCRα$^{-/-}$ mice were tested against aerobic bacterial antigens extracted from the cecum in TCRα$^{-/-}$ mice of different ages (51–230 days) to determine if the aerobic bacterial flora of the mice changed with aging and development of IBD. The reaction pattern in Western blotting remained the same regardless of the age of the mice (Fig. 2). Furthermore, the cultured bacterial strain from TCRα$^{-/-}$ mice of different ages (51–230 days) was analyzed after culture in LB medium under aerobic conditions (see Methods). *E. coli* was consistently detectable in the culture from all TCRα$^{-/-}$ mice tested at different ages (51–230 days). Anaerobic and other bacterial strains were not cultured under these aerobic conditions. These findings indicate that the transition of the recognition pattern in TCRα$^{-/-}$ mice was not caused by a change of cecal aerobic bacterial flora with aging and development of IBD. The reaction pattern of sera from TCRα$^{-/-}$ mice against aerobic bacterial antigens was identical to that

Table 1. Reactivity of sera from TCRα$^{-/-}$ mice against cecal aerobic bacterial antigens isolated from a TCRα$^{-/-}$ mouse[a]

Age (weeks)	No. of mice	Faint or undetectable bands	Polyclonal pattern	Oligoclonal pattern
<6	11	11	—	—
8–20	58	—	45	13
20–30	83	—	26	57
>30	28	—	5	23

[a]Western blot analysis with sera from TCRα$^{-/-}$ mice against cecal bacterial antigens from a TCRα$^{-/-}$ mouse (20 weeks).

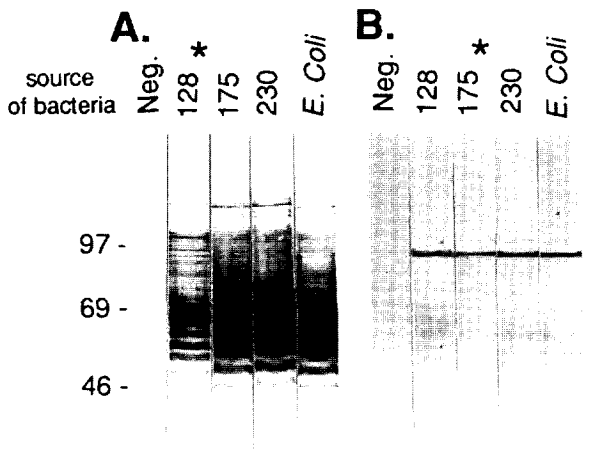

Fig. 2. Comparison of immunoreactivity of sera from TCRα$^{-/-}$ showing a polyclonal (A) and an oligoclonal (B) recognition pattern against aerobic bacterial antigens extracted from TCRα$^{-/-}$ of different ages (128, 175 and 230 days) and antigens extracted from *E. coli* cultured from cecal contents of TCRα$^{-/-}$ mice as analyzed by Western blot analysis. The numbers shown on the top indicate the age (day after birth) of the mouse from which aerobic bacteria was extracted. *Both serum and cecal aerobic bacterial antigens were obtained from the same mouse. Mol. wt is shown on the left side in kDa.

obtained against antigens extracted from *E. coli* cultured from cecum of TCRα$^{-/-}$ mice (Fig. 2). The treatment of bacterial proteins with proteinase showed only degraded bands (<20 kDa), indicating the reactivity against proteins, but not other components by sera. In addition, sera tested showed no reactivity against LB medium used for culture of bacteria (data not shown).

The analysis of the Ig subclass revealed that in the sera showing polyclonal patterns including restricted bands, the antibodies in the sera were mostly of IgG2a, and in some instances of IgG1 and IgM subclass (data not shown). Taken together, these findings suggest that (i) the polyclonal reaction pattern against cecal aerobic bacterial antigens becomes oligoclonal with age and (ii) antibodies reactive with these antigens undergo Ig switching to IgG in TCRα$^{-/-}$ mice.

The controls included 23 TCRα$^{+/-}$ and 18 C57BL/6 mice (Table 2). When the aerobic bacterial antigens extracted from their own cecum (TCRα$^{+/-}$ and C57BL/6 mice respectively) were used, all adult mice (8–20 weeks old) showed a polyclonal recognition pattern similar to that observed with sera from TCRα$^{-/-}$ mice. However, in contrast to TCRα$^{-/-}$ mice, the polyclonal pattern was preserved in the older wild-type mice (>30 weeks old) (Fig. 3), indicating that the change from a polyclonal to an oligoclonal recognition pattern against aerobic bacterial antigens is a distinct feature of TCRα$^{-/-}$ mice.

We also examined the reactivity of sera from 12 RAG-1$^{-/-}$ mice against aerobic bacterial antigens extracted from various mouse strains used in this experiment. No reactivity against aerobic bacterial proteins was detected (Fig. 4).

Immunoreactivity of TCRβ$^{-/-}$ and TCRδ$^{-/-}$ sera against cecal aerobic bacterial antigens

To investigate whether the unique T cell subset expressing TCRα^{-}β^{+} plays an important role in the change of bacterial recognition from polyclonal to oligoclonal, we compared reactivity of sera from two kinds of mice: (i) TCRβ$^{-/-}$ mice in which all T cells bear TCRγδ, but do not express TCRαβ (28), and (ii) TCRδ$^{-/-}$ mice that have only TCRαβ T cells, but no TCRγδ T cells (23).

Silver staining showed many obvious bands after SDS–PAGE of cecal aerobic bacterial antigens extracted from TCRβ$^{-/-}$ and TCRα$^{-/-}$, while none of the sera from 57 TCRβ$^{-/-}$ mice between 4 and 40 weeks old showed the polyclonal pattern against these antigens (Fig. 4). Furthermore, we examined the reactivity of sera from 46 TCRδ$^{-/-}$ mice between 4 and 60 weeks old against aerobic bacterial antigens extracted from them (TCRδ$^{-/-}$) and TCRα$^{-/-}$ mice. Sera from all adult TCRδ$^{-/-}$ mice, 10–20 weeks old, as well as older mice (20–40 weeks) showed the polyclonal recognition pattern. Even the sera from all TCRδ$^{-/-}$ mice >1 year old demonstrated this polyclonal pattern (Table 2 and Fig. 3).

Some TCRγδ^{+} T cells have been shown to be capable of reacting with heat-shock protein from bacteria and viruses (29). Heat-shock proteins have the ability to act as superantigens. Therefore, we examined the reactivity of sera from TCRα$^{-/-}$ mice, against various bacterial heat-shock proteins derived from bacteria exposed to heat and EtOH. There were no obvious differences in the reactivity of sera from TCRα$^{-/-}$ and TCRδ$^{-/-}$ mice of different ages with proteins from stressed or non-stressed bacteria (data not shown).

Production of autoantibody against tropomyosin

Since antibodies against tropomyosin have been detected in patients with UC and in experimental animals including TCRα$^{-/-}$ mice (17–20), we also investigated whether the change in immunoreactivity from a polyclonal to an oligoclonal pattern against cecal aerobic bacterial antigens also resulted in the development of antibody against tropomyosin, a cytoskeletal protein, in TCRα$^{-/-}$ mice.

The sera from TCRα$^{-/-}$ mice exhibiting a polyclonal recognition pattern as well as those from wild-type mice did not react with tropomyosin by ELISA and Western blot analysis. However, in TCRα$^{-/-}$ mice showing an oligoclonal recognition pattern, the reactivity of sera against tropomyosin was markedly increased in comparison with wild-type, TCRδ$^{-/-}$ and TCRα$^{-/-}$ mice expressing a polyclonal recognition pattern (Fig. 5). These findings suggest that the alteration of the recognition pattern from polyclonal to oligoclonal may be associated with the production of autoantibodies.

To confirm that the reactivity observed in ELISA was indeed due to tropomyosin, Western blot analysis of tropomyosin and troponin (a binding protein to actin and tropomyosin) was carried out with sera from TCRα$^{-/-}$ mice and with an anti-tropomyosin mAb (IgG1 subclass) (Fig. 5). Identical bands at 36 and 39 kDa were detected with the TCRα$^{-/-}$ sera and anti-tropomyosin mAb. However, these sera and the mAb showed no reactivity against troponin (data not shown). These findings suggest that the reactivity of sera from TCRα$^{-/-}$ mice against tropomyosin is specific.

To characterize the Ig subclass of autoantibody against tropomyosin, Western blot analysis using antibodies to total Ig, IgM, IgA, IgG, IgG1, IgG2a or IgE as second antibody was performed. The autoantibody produced by TCRα$^{-/-}$ mice was primarily of IgG2a subclass; faint reactivity was observed

Table 2. Recognition pattern of sera against aerobic bacterial antigens[a]

Age (weeks)	TCRα$^{-/-}$			TCRδ$^{-/-}$			Wild-type mice		
	No. of mice	Poly	Oligo	No. of mice	Poly	Oligo	No. of mice	Poly	Oligo
8–15	22	21 (95)[b]	1 (5)	6	6 (100)	0	5	5 (100)	0
15–20	36	24 (67)	12 (33)	5	5 (100)	0	7	7 (100)	0
20–25	34	12 (35)	22 (65)	6	6 (100)	0	14	14 (100)	0
25–30	49	15 (31)	34 (69)	11	11 (100)	0	8	8 (100)	0
>30	28	5 (18)	23 (82)	18	18 (100)	0	7	7 (100)	0

[a]Western blotting analysis with sera from TCRα$^{-/-}$, TCRδ$^{-/-}$ and wild-type (TCRα$^{-/-}$ and C57BL6) mice against cecal aerobic bacterial antigens, isolated from mice of the same strain as the source of sera.
[b]The numbers shown in parentheses indicate percentage showing the reacting pattern.

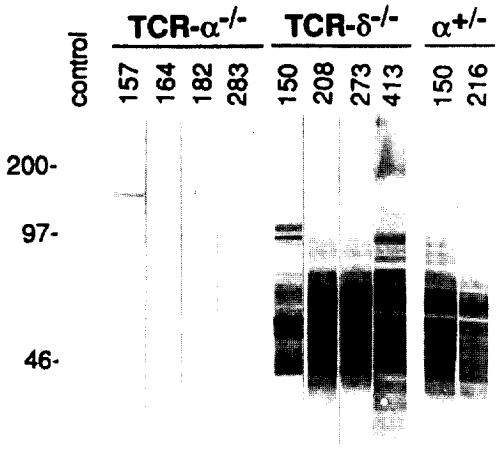

Fig. 3. Comparison of immunoreactivity of sera from TCRα$^{-/-}$, TCRδ$^{-/-}$ and TCRα$^{+/-}$ mice against aerobic bacterial antigens extracted from their own cecum (TCRα$^{-/-}$, TCRδ$^{-/-}$ and TCRα$^{-/-}$ mice respectively) by Western blotting analysis. The numbers shown on the top indicate the age (day after birth) of the mouse whose sera was used. The recognition pattern changed from polyclonal to oligoclonal at ~20 weeks old in the TCRα$^{-/-}$ mice. In contrast, the polyclonal pattern was preserved in the TCRδ$^{-/-}$ and TCRα$^{+/-}$ mice. Mol. wt is shown on the left side in kDa.

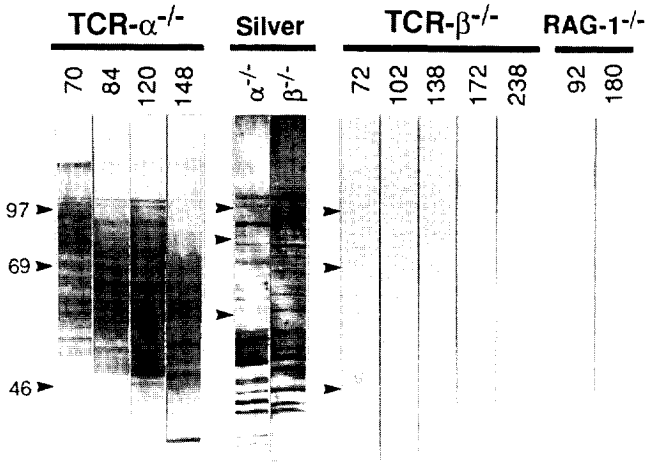

Fig. 4. Comparison of immunoreactivity of sera from TCRα$^{-/-}$, TCRβ$^{-/-}$ and RAG-1$^{-/-}$ mice against aerobic bacterial antigens extracted from their own cecum (TCRα$^{-/-}$, TCRβ$^{-/-}$ and RAG-1$^{-/-}$ mice respectively) by Western blot analysis. The age of the mouse is indicated by the number on the top. The TCRα$^{-/-}$ mice, but not TCRβ$^{-/-}$ and RAG-1$^{-/-}$ mice, show a polyclonal pattern. Silver staining of aerobic bacterial proteins, extracted from TCRα$^{-/-}$ and TCRβ$^{-/-}$ mice, is shown. Arrows indicate mol. wt (kDa).

for IgM antibody. IgA, IgG1 (Fig. 6) and IgE (data not shown) antibodies were not detected.

Correlation between the alteration of the recognition pattern to oligoclonal and the development of IBD

The severity of IBD was determined by histological analysis of colon. All TCRα$^{+/-}$ and TCRδ$^{-/-}$ mice showed no IBD. Among 62 TCRα$^{-/-}$ mice revealing a polyclonal recognition pattern against cecal bacteria, 26 mice (42%) showed IBD; severe disease was found only in 11 mice (18%). On the contrary, 71 mice out of 79 (90%) TCRα$^{-/-}$ mice revealing an oligoclonal recognition pattern exhibited IBD. Furthermore, 48 of these mice (61%) manifest severe disease (Table 3).

These results indicate that the alteration of the recognition pattern against cecal aerobic bacterial antigens such as *E. coli* from polyclonal to oligoclonal is strongly associated with the development of IBD.

Discussion

Although the etiology of human IBD (UC and Crohn's disease) is unknown, it is generally believed that alteration of mucosal immune responses to lumenal antigens is involved in the pathogenesis of these inflammatory disorders. Recently, spontaneous development of IBD in experimental animals has been described in mice with T cell-deficient states (TCR mutant mice) or absence of certain cytokines (IL-2 and IL-10 mutant mice) and in rats transgenic for HLA-B27 (6–10). The

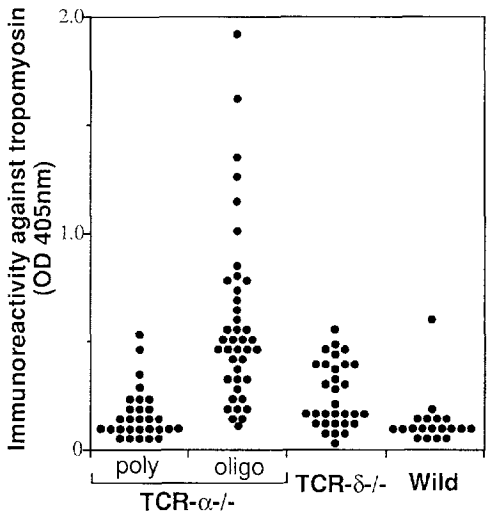

Fig. 5. Scatterogram showing reactivity of sera (1:100 dilution) against tropomyosin by ELISA. TCRα$^{-/-}$ mice were separated into two groups: mice revealing a polyclonal recognition pattern (poly) and mice revealing an oligoclonal recognition pattern (oligo). The immunoreactivity of sera from TCRα$^{-/-}$ mice showing an oligoclonal recognition pattern against aerobic bacterial antigens was significantly ($P < 0.001$) higher than that from other groups.

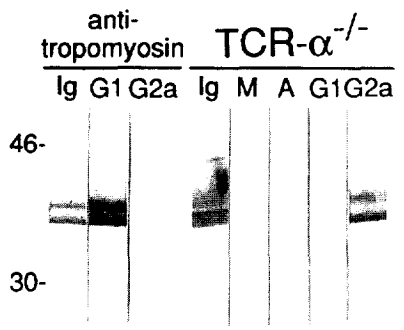

Fig. 6. Ig isotyping of autoantibody produced in TCRα$^{-/-}$mice, against tropomyosin by Western blotting. Anti-tropomyosin (IgG1 subclass) mAb shows two bands (36 and 39 kDa) following incubation with anti-mouse Ig and IgG1, but not IgG2a as secondary antibody in the staining method. Sera from TCRα$^{-/-}$ mice showing an oligoclonal recognition pattern revealed the same mol. wt bands when anti-mouse Ig, IgM, and IgG2a, but not IgA and IgG1 were used as secondary antibody in the staining method.

absence of disease in IL-2-deficient mice and HLA-B27 transgenic rats under germ-free conditions supports the contention that enteric bacteria are important in the development of IBD (8,11,12).

The present study examined the humoral response to bacterial antigens extracted from the cecum and the production of autoantibody in TCRα$^{-/-}$ mice that consistently develop a UC-like disease. For comparison purposes, we also tested the humoral responses in wild-type mice and mice with a mutation in the TCRδ or TCRβ genes. The sera from 8- to 20-week-old TCRα$^{-/-}$ mice and all TCRδ$^{-/-}$ and wild-type mice showed a polyclonal pattern of reactivity against aerobic bacterial antigens extracted from their own cecum. In contrast, the sera from TCRβ$^{-/-}$ mice did not exhibit reactivity with their cecal bacterial antigens suggesting that humoral response to cecal aerobic bacterial antigens may not be directly involved in the development of colitis in TCRβ$^{-/-}$ mice. Since both TCRα$^{-/-}$ mice and TCRβ$^{-/-}$ mice contain TCRγδ T cells, it is unlikely that the immune response to bacterial antigens is due to TCRγδ T cells. Furthermore, the presence of normal numbers of TCRγδ^{-} intestinal intraepithelial lymphocytes in germ-free mice suggests that TCRγδ^{+} cells are not primarily involved in the recognition of bacterial antigens (30). The TCRβ chains on the surface of TCRα β^{-} cells in thymus are associated with CD3ε, γ and δ chains and CD3ζζ dimers (31), and disulfide-linked with a pre-T cell receptor α (pTα). gp33, that may play a role in early T cell development (32,33) and in thymocyte expansion (34). However, the pTα gene product is undetectable in peripheral CD4^{+} TCRα^{-}β^{-} T cells of TCRα$^{-/-}$ mice, indicating the presence of another mechanism for TCRβ surface expression by these cells (35). The immune response to the T cell-dependent antigen, ovalbumin, is notably absent in TCRβ$^{-/-}$ mice and weakly present in TCRα$^{-/-}$ mice (14). It is therefore possible that the TCRβ chain in peripheral T cells of TCRα$^{-/-}$ mice may be associated with an unknown molecule and may be capable of recognizing enteric bacterial antigens. In a recent study of CD4^{+} TCRβ^{+} T cells isolated from bronchoalveolar fluids from influenza virus-infected TCRα$^{-/-}$ mice, these cells were shown to be reactive with endogenous (Mls-1) and bacterial (staphylococcal enterotoxin B) superantigens (16). Therefore, it is also possible that the recognition process for normal enteric bacterial antigens by TCRα^{-}β^{+} T cells may be similar to the response to superantigens as recognized by the TCRβ chain alone (36). We have recently shown that CD4^{+} TCRα^{-}β^{+} T cells are capable of secreting IL-4 when activated with an anti-TCRβ antibody *in vitro* (17). Taken together, these findings indicate that the TCRβ chain

Table 3. Severity of IBD and immune response to cecal bacteria[a]

Recognition pattern	No. of mice	Severity of IBD (no. of mice)			Reactivity to tropomyosin (OD_{405})
		Normal	Mild	Severe	
Polyclonal	62	36	15	11	0.156 ± 0.013
Oligoclonal	79	8	23	48	0.553 ± 0.089

[a]Severity of IBD in TCRα$^{-/-}$ mice showing a polyclonal and an oligoclonal recognition pattern with sera against cecal aerobic bacterial antigens from TCRα$^{-/-}$ mice was evaluated by histological examination of the colon.

(TCRα$^-$β$^+$) alone may be involved in the recognition of non-pathogenic aerobic bacterial antigens.

Interestingly, we found that the immune response to aerobic cecal bacterial antigens changed with age in TCRα$^{-/-}$ mice, but not in other mice tested. The polyclonal pattern recognized by the sera from younger mice (8–20 weeks old) changed to an oligoclonal pattern in older mice (>20 weeks old). We have also found that there is transition of recognition pattern from polyclonal to oligoclonal against the anaerobic bacteria *Bacteriodes* (unpublished results). This alteration may reflect the presence of dominant bacterial antigens that are recognized by CD4$^+$ TCRα$^-$β$^-$ T cells. In some organ-specific autoimmune disease models, a restricted usage of the V_β region has been described (37). It has recently been reported that the recognition of superantigens by the TCRβ chain might also be modulated by the TCRα chain (38). Therefore, the lack of TCRα chains may induce a skewed usage of TCRβ chains, resulting in the transition of the recognition pattern against bacterial antigens to oligoclonal in TCRα$^{-/-}$ mice. In addition, the expression of TCRα, but not TCRβ mRNA is required for expression of antigen-specific suppressor factor bioactivity (39,40). Therefore, the absence of the TCRα chain may lead to an unregulated immune response to bacterial antigens after the recognition by CD4$^+$ TCRα$^-$β$^+$ T cells. Alternatively, TCRγδ$^+$ T cells can play immunoregulatory functions; TCRγδ$^+$ T cells have been shown to have unique regulatory functions during certain immune responses (41), are capable of activating B cells producing autoantibodies (42), are involved in the development of autoimmune inflammation (43), and have an immunoregulating role in oral tolerance (44,45). It has been reported that TCRγδ$^+$ T cells are able to cross-regulate TCRαβ T cells responses (45). Therefore, we cannot rule out the possibility that TCRγδ$^+$ T cells that are expanded in TCRα$^{-/-}$ mice may also be involved in the alteration of immune responses to bacteria after the recognition of bacterial antigens by TCRα$^-$β$^-$ T cells.

Patients with UC have been shown to have detectable antibodies to tropomyosin, a cytoskeletal protein present in colonic epithelial cells (18–20). Since tropomyosin shares immunogenic epitopes with group A streptococcal M proteins, it is quite possible that aerobic bacterial proteins in the intestinal lumen can generate autoantibody formation to colonic tropomyosin. We detected antibodies to tropomyosin in TCRα$^{-/-}$ mice. Interestingly, there was a correlation between the titer of anti-tropomyosin antibodies and the presence of an oligoclonal reaction pattern against aerobic cecal bacterial antigens. It has been suggested that many autoantibodies are produced as a result of bacterial and viral antigen recognition, which have certain structural similarities with self-antigens in not only individuals with autoimmune disease, but also healthy individuals (1,46). These antibodies could help in removing proteins eluted from dead cells (1,46). We propose that the polyclonal immune response, preserved in normal conditions, against enteric bacterial flora associates with the production of autoantibodies which remove dead cells. On the other hand, the transition into an oligoclonal pattern of recognition results in overproduction of restricted types of autoantibodies such as anti-tropomyosin that may be involved in tissue damage. A possible role of microorganisms in the development of autoimmune diseases has been proposed (4,21).

Although TCRα$^{-/-}$ mice maintained in pathogen-free conditions showed severe colonic inflammation, there was no evidence of systemic autoimmune diseases (7). In another strain of TCRα$^{-/-}$ mice, autoantibodies against nuclear antigens such as small nuclear ribonucleoproteins have been detected (47). It is possible that TCRα$^{-/-}$ mice may develop other autoantibodies when exposed to conventional housing conditions, in which the mice may be exposed to large varieties of microorganisms in comparison with pathogen-free conditions.

The findings in the present study suggest that the absence of the TCRα chain is associated with an oligoclonal response to enteric bacteria and the development of autoantibodies and IBD. Whether the altered immune response is directly involved in the pathogenesis of IBD in TCRα$^{-/-}$ mice needs to be determined in future studies.

Acknowledgements

We are grateful to Dr P. Mombaerts for providing help in developing colonies of TCR mutant mice. We thank Drs R. S. Blumberg and J. L. MacLean for the critical reading of the manuscript. We also thank M. Levy for technical assistance, J. Holden for bacterial culture analysis and C. A. Nason for preparation of the manuscript. This work was supported by NIH grant DK47677 (A. K. B.), the Massachusetts General Hospital/New England Regional Primate Center for the Study of Inflammatory Bowel Disease (DK43551) and by a grant from Yakult Honsha Co, Ltd (S. T.).

Abbreviations

APL	alkaline phosphatase
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
UC	ulcerative colitis

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