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Alteration of T cell maturation and proliferation in the mouse thymus induced by serum factors from patients with ulcerative colitis

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SUMMARY

Recently it has been reported that patients with ulcerative colitis (UC) often have thymus abnormalities, although the precise mechanisms which induce those abnormalities remain unclear. We have examined the effect of serum fractions from patients with UC and other colonic diseases on mouse thymus to clarify the possible existence of factors which have thymus growth activity. These fractions were separated from sera of patients with UC by gel filtration and anion exchange high performance liquid chromatography. In mice given UC serum fractions; (i) remarkable increases in weight and total cell number of the thymus were observed from day 4 to day 9; (ii) a significant increase in the number of peanut agglutinin $(PNA)^{+}$ thymus cells was demonstrated using flow cytometry on day 9; (iii) on quantitative analysis of surface antigens the percentage of Lyt-2^{+} thymus cells decreased and that of $L3T4^{+}$ thymus cells increased remarkably on day 13; the number of bright Thy-1·2^{+} cells and of dull Lyt-1^{+} cells increased. In contrast, the serum fractions from patients with other colonic diseases and from normal persons caused little change in mouse thymus throughout the study. The results suggest that factors fractionated from the serum of patients with UC disturb intra-thymic T cell maturation and enhance the proliferation of thymus cells.

Keywords ulcerative colitis thymus T cell maturation serum factor

INTRODUCTION

Abnormalities of cellular and humoral immune responses in patients with ulcerative colitis (UC) suggest that immune mechanisms may be involved in the pathogenesis of the disease. Analogies have been drawn with diseases for which autoimmune mechanisms are well established (Kirsner & Shorter, 1982). In the autoimmune process the immune apparatus including thymus may become altered so that abnormal responses are elicited to self antigens normally expressed on the cell surface (Rosai & Levine, 1976). The thymus is now recognized to perform a crucial role in the development of immunologically competent T lymphocytes (Cantor & Weissman, 1976). Thymus abnormalities, such as hyperplasia or the appearance of germinal centres, have been reported in patients with UC (Schmidt, 1970; Tsuchiya, 1984). However, the precise mechanisms which induce those thymus abnormalities remain to be elucidated.

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This series of studies has been conducted to analyse the mechanisms of thymus abnormalities which are frequently seen in UC. The effects of serum fractions from patients with active UC on mouse thymus were examined in terms of the alteration in lymphocyte maturation in the thymus.

MATERIALS AND METHODS

Patients' sera. Sera were obtained from seven patients with UC. The diagnosis was based on the presence of typical clinical, radiographic, and pathological features of UC. All patients were in the severely active stage extending up to the right colon. None of them were receiving corticosteroids or other immunosuppressants, nor had received them for 2 months before the study. All patients had thymus hyperplasia demonstrated by pneumomediastinography. Sera from five patients with Crohn's disease (CD) affecting ascending and transverse colon and six patients with other colonic diseases (three with colonic polyps, two with intestinal tuberculosis and one with colonic diverticulitis) were used for comparison. None of the eleven patients were treated with corticosteroids. Control sera were obtained from seven healthy laboratory workers.

After 0·45 μm millipore filtration (Millipore Filter Co., Bedford, MA), sera were frozen and stored in small aliquots at −80°C without any preservatives until further processing. Before use, the sera were inactivated by heating to 56°C for 30 min and centrifuged at 105,000 *g* for 20 min to remove complexes and aggregates.

Animals. Female mice of inbred C57BL/6 strains were purchased from the CLEA Japan Inc., Tokyo and used for experiments at 6 weeks of age.

Preparation of serum fractions by a combination of gel filtration and high performance liquid chromatography. All serum fractionating manipulations were carried out at 4°C. Gel filtration columns were equilibrated in 50 mM Tris/100 mM NaCl (pH 8·0). Five millimetres of sera were applied to a 2·5 × 95 cm Sephacryl S-200 column (Pharmacia Fine Chemicals, Uppsala, Sweden) by reverse flow. This column was calibrated with the following markers: aldolase (ALD, mol.wt 158,000), bovine serum albumin (BSA, mol.wt 67,000), ovalbumin (OVA, mol.wt 45,000), chymotrypsinogen (CHYMO, mol.wt 25,000) and ribonuclease A (RNase, mol.wt 13,700). A flow rate of 20 ml/h was used and 5 ml fractions were collected. They were tested for bioactivity as described below and active fractions were pooled for further preparative steps.

High performance liquid chromatography of serum fractions from UC after gel filtration was performed with a Pharmacia fast protein liquid chromatography (FPLC) system (Pharmacia Fine Chemicals) (Tomono, Ikeda & Tokunago 1983). The separation was performed on a Mono Q HR 5/5 prepacked column (Pharmacia Fine Chemicals). The following buffers were used for the anion exchanger column. The starting buffer (buffer A) was 50 mM Tris-HCl, pH 8·6 and the final buffer (buffer B) was buffer A plus 0·5 M sodium chloride.

Bioactive fractions from active UC after gel filtration was injected into the Mono Q column. The column was initially washed with buffer A (20 min). The bound proteins were then eluted with a linear (sodium chloride concentration) gradient from 0 to 100% of buffer B in 10 min. The flow rate was adjusted to 2 ml/min and 1 ml fractions were collected. Each fraction was then dialysed against phosphate-buffered saline (PBS) and tested for bioassay.

Aliquots of the fractions were then concentrated on an Amicon YM2 membrane (nominal cut off molecular weight: 1,000, Amicon Corporation, Lexington, MA) to a final protein concentration of 100 μg/ml to avoid the nonspecific immunostimulation.

Administration into mice. Serum fractions (0·5 ml) were filter-sterilized (Millex-MA, 0·22 μm filter unit, Millipore Filter Co.) and then injected intravenously via the caudal vein into experimental mice as a single injection. Intraperitoneal injection was less effective and subcutaneous injection was not effective. Twenty-four hours after the injection, each mouse was given a single dose of cyclophosphamide (300 mg/kg) intraperitoneally to suppress the immune responses to the human proteins (Toyka *et al.*, 1977). Fractions positive for the biological activity were pooled and stored at −20°C.

Measurement of weight and cell counts. Mice were killed by decapitation after complete exsanguination at various intervals after injection of serum fractions. Thymus glands were

aseptically removed, weighed and squeezed with two sterile glass slides in PBS to obtain cell suspensions. After being passed through a stainless mesh, cell suspensions were depleted of erythrocytes by treatment with Tris-buffered 0·83% NH_4Cl solution and washed vigorously. Total and viable cells were counted by the trypan blue dye exclusion method.

Analysis of cell surface receptors for peanut agglutinin (PNA) using direct immunofluorescence. Thymus cells (2×10^6 viable cells) were incubated with 10 μl of fluorescein-conjugated PNA (Reisner, Linker-Israeli & Sharon, 1976) (FITC-PNA) (EY-Lab. Inc., San Mateo, CA) at room temperature for 30 min. After washing four times with medium, the percentage of cells bearing PNA receptors (PNA^+) was counted on a fluorescence activated cell sorter (FACS-II, Becton Dickinson Electronics Lab., Mountain View, CA). During analysis the cells were studied on a monitor presenting 'dot displays', i.e. 2-dimensional analysis of the scatter signals, that correlated to cell size vs the fluorescence intensity of each individual cell. The percentage of fluorescence positive cells was determined by counting the number of cells giving fluorescence signals above the autofluorescence and dividing it by the total number of viable cells examined. For each sample, a minimum of 5–10×10^4 individual cells were analysed.

Analysis of thymus cell surface characteristics with monoclonal antibodies. Four kinds of monoclonal antibodies detecting external surface markers for functionally defined mouse T lymphocyte subsets, termed monoclonal anti-Thy-1·2, anti-Lyt-1, anti-Lyt-2 and anti-L3T4 (Becton Dickinson Monoclonal Antibody Center, Sunnyvale, CA) were used in the present study. Specificities of these monoclonal antibodies have been amply documented (Ledbetter & Herzenberg, 1979; Ledbetter *et al.*, 1980; 1981; Dialynas *et al.*, 1983).

Cell packs containing 2×10^6 viable thymus cells were incubated with 10 μl of directly fluorescein-conjugated anti-Thy-1·2, anti-Lyt-1, anti-Lyt-2 or anti-L3T4 at room temperature for 30 min. After being washed four times, cells were analysed on a FACS as described above.

Proliferation assays. Thymus cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). Cultures were established in microtitre flat-bottomed plates (Microtest II, Falcon Plastics, Oxnard, CA) in triplicate and consisted of 0·2 ml complete culture medium containing 2×10^5 cells/well. Concanavalin A (Con A, Difco Labo., Detroit, MI) was used as mitogen at the final concentration of 1 μg/ml. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 for 48 h. For the last 4 h of the culture period, 1 μCi of [methyl-^{3}H] thymidine ([^{3}H]TdR; specific activity 2Ci/mmol, The Radiochemical Centre, Amersham, England) was added to each culture well. At the end of incubation, the cells were collected on a microcell harvester (Titertek, Flow Laboratories, Irvine, Scotland) and the radioactivity was counted. Routine assays of spontaneous proliferation were performed during the first 12 h culture and in the absence of mitogen.

Statistical analysis. All values were expressed as mean $\pm$ standard deviation (s.d.). Statistical analysis was performed by two-tailed Student's paired and unpaired *t*-tests, and analysis of variance was determined using a Casio desktop programmable calculator. A probability (*P*) value of less than 0·05 was considered significant.

RESULTS

Changes of weight and cell counts in the thymus. In assessing the fractions for biological activity, a protein concentration range of 50 μg to 100 μg was used. Gel filtration of sera from active UC on Sephacryl S-200 column revealed that over 80% of the total thymus growth activity was present in fractions with molecular weight of approximately 12,000–16,000 (Fig. 1).

This bioactive fraction from active UC was concentrated by diafiltration and was applied to a Mono Q column, and eluted by a linear NaCl (0–0·5 M) gradient using a FPLC. Around 80% of thymus growth activity can be recovered as a distinct peak in fractions with NaCl concentration between 0·15 and 0·20 M (Fig. 2). These fractions from patients with UC, CD, other colonic diseases and normal controls were used for our thymus growth assay in the subsequent studies.

The weight and cell counts of the thymus were assessed on days 2, 4, 6, 9 and 13 after the administration of serum fractions (Fig. 3). The weight of the thymus in mice given serum fractions

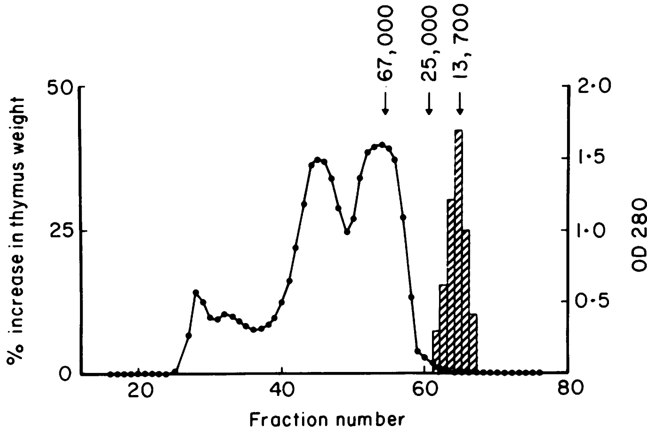

Fig. 1. Gel filtration of sera from patients with active UC on a 2·5 × 90 cm Sephacryl S-200 column equilibrated with 50 mM Tris/100 mM NaCl (pH 8·0). Fractions (5 ml) were collected and tested for thymus growth activity. The molecular weight standards were run separately over the same columns. Shaded bars represent the thymus growth activity. Over 80% of the total thymus growth activity was present in fractions that correspond to mol.wt 12,000–16,000.

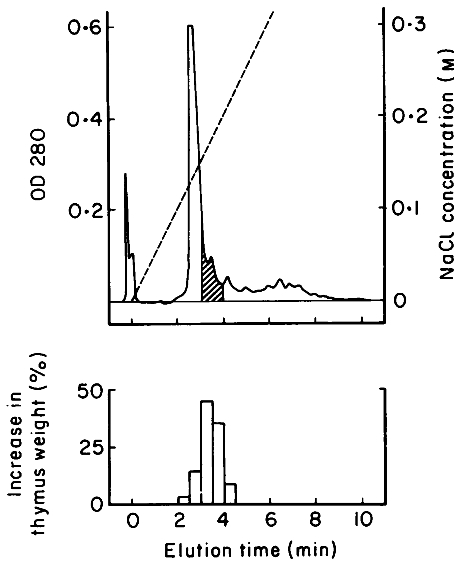

Fig. 2. High performance anion exchange chromatography of serum fractions from active UC after gel filtration using a FPLC system. Bioactive fractions from Fig. 1 were pooled, applied on the Mono Q column equilibrated in 50 mM Tris-HCl buffer, pH 8·6 and eluted with a linear NaCl gradient (- - -) at a flow rate of 2 ml/min. The absorbance of the effluent at 280 nm is shown in the upper panel (——). Fractions were collected at 0·5 min intervals and assayed for our thymus growth activity (lower panel). The hatched area in the upper panel represents those fractions in consecutive runs that were pooled and concentrated.

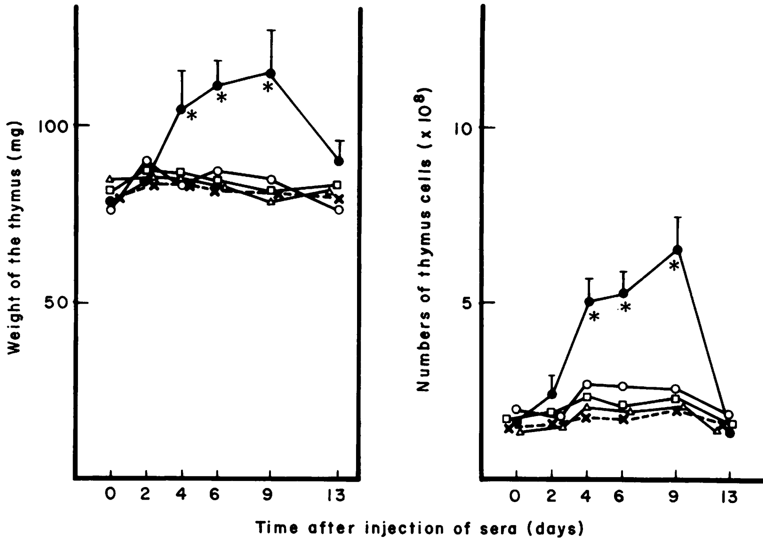

Fig. 3. The effect of serum fractions from UC (●), CD (○), other colonic diseases (△), and normal controls (□), on mouse thymus weight and cell counts. Significant* ($P < 0{\cdot}001$) increases of weight and total cell counts of the thymus were revealed in mice treated with UC serum fractions, compared with those treated with normal serum fractions or untreated (×).

from patients with UC was greater than in untreated controls or mice given normal serum fractions. The thymus weight increased apparently on day 4 (101·8 ± 11·2 mg, mean ± SD) in mice given UC serum fractions and then gradually reached to the peak level on day 9 (110·4 ± 13·6 mg). It then declined nearly to the control level on day 13 (87·6 ± 7·0 mg). The total number of thymus cells in mice given UC serum fractions increased on days 4 ($4{\cdot}9 \pm 0{\cdot}6 \times 10^8$) and 9 ($6{\cdot}5 \pm 1{\cdot}0 \times 10^8$). On day 13 cell counts returned to the same level as on day 0. The administration of normal human serum fractions and those from patients with CD and other colonic diseases did not affect the weight or cell counts of mouse thymus throughout the entire 13 observation days.

Cell surface receptors for PNA. Surface receptors for PNA on thymus cells were examined on days 2, 4, 6, 9 and 13. Sixty-eight to seventy-four percent of thymus cells were PNA^+ in untreated and normal serum fractions-treated mice during the study. Thymus cells from mice given UC serum fractions showed a higher percentage of PNA^+ cells (80–84%) than those from untreated mice on day 9, while no clear-cut changes in the percentage of PNA^+ cells were seen in thymus cells from mice given serum fractions from CD or other colonic diseases.

We also revealed the increase of rather small cells with the analysis of light scatter signals that correlated to cell size (data not shown).

Analysis of thymus cell surface antigens. Cell surface antigens of thymus cells were examined by direct immunofluorescence with monoclonal antibodies on days 2, 4, 6, 9 and 13. Percentages of Thy-1·2^+, Lyt-1^+, Lyt-2^+ or L3T4^+ cells in UC serum fractions-treated mice did not differ from those in untreated controls on days 2, 4 and 6 (data not shown). There were no difference of thymus cell populations among the four groups. On day 13, however, UC serum fractions-treated mice showed a slight reduction of Lyt-1^+ cells (86 ± 6%), a marked reduction of Lyt-2^+ cells (53 ± 5%) and an increase of L3T4^+ cells (90 ± 3%), compared with the percentages of Lyt-1^+ cells (92 ± 2%), Lyt-2^+ cells (80 ± 5%) and L3T4^+ cells (80 ± 2%) in untreated mice.

The profiles obtained from UC or normal human serum fractions-treated mice on day 13 are presented as representative in Fig. 4. The percentage of Lyt-2^+ cells was significantly decreased and L3T4^+ cells was increased in UC serum fractions-treated mice. The number of Thy-1·2^+ cells was slightly increased, and the profile of Thy-1·2^+ cells shifted to the bright side of fluorescence intensity

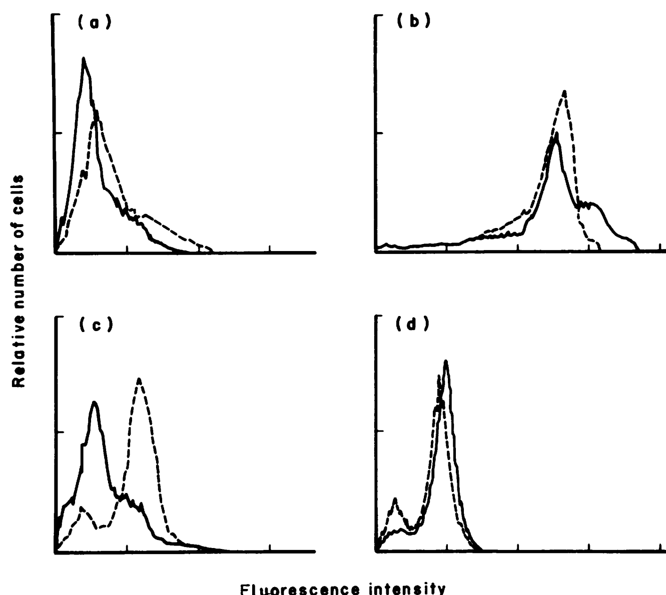

Fig. 4. Fluorescence profiles of mouse thymus cells on day 13 stained with fluorescein-conjugated Lyt-1 (a), anti-Thy-1·2 (b), Lyt-2 (c) or L3T4 (d). In the mice treated with UC serum fractions (——), the fluorescence profile of Thy-1·2$^+$ cells shifted to the brighter side and that of Lyt-1$^+$ cells shifted to the dull side of fluorescence intensity, compared with those treated with normal serum fractions. (- - - -). The percentage of Lyt-2$^+$ cells was remarkably decreased and of L3T4$^+$ cells was increased in mice treated with UC serum fractions.

Table 1. Spontaneous and Con A-induced [^{3}H]TdR incorporation in thymus cells

Mice	Number of sera	Spontaneous* (ct/min/well)	Con A-induced† (ct/min/well)
Untreated	7	1,302 ± 56‡	8,450 ± 255
UC serum fractions-treated	7	3,268 ± 337§	22,580 ± 6,820§)
Normal serum fractions treated	7	1,454 ± 184	7,690 ± 232

* Thymus cells (2×10^5/well) were cultured for 12 h in the absence of mitogens and [^{3}H]TdR incorporation was measured during the last 4 h of culture.

† Thymus cells (2×10^5/well) were cultured for 48 h in the presence of Con A (1 μg/ml) and [^{3}H]TdR incorporation was measured during the last 4 h of culture.

‡ Results were expressed as mean ± s.d. of five separate experiments.

§ Significantly different from the untreated group ($P < 0{\cdot}01$).

in UC serum fractions-treated mice. The fluorescence profile of Lyt-1$^+$ cells showed a shift to the dull side of fluorescence intensity in UC serum fractions-treated mice, although the percentage of whole Lyt-1$^+$ cells of UC serum fractions-treated mice was not so different from that of untreated mice. Reactivities of thymus cells with these monoclonal antibodies and fluorescence profiles were virtually unaffected by CD, other colonic diseases or normal serum fractions-treatment.

Spontaneous and Con A-induced proliferation. As shown in Table 1, thymus cells from UC serum fractions-treated mice spontaneously incorporated [^{3}H]TdR. This activity was significantly more pronounced ($P < 0{\cdot}01$) than in thymus cells obtained from untreated mice. Moreover, thymus cells from UC serum fractions-treated mice exhibited proliferative responses to Con A higher than those in untreated or normal human serum fractions-treated mice.

DISCUSSION

More than 20 years have elapsed since the central role of the thymus in immunity was proposed (Miller & Osoba, 1963). This topic has already elicited an extraordinary number of studies dealing with the function of the organ itself as well as its cellular products in autoimmune diseases. In patients with myasthenia gravis, the thymus glands are often morphologically abnormal (tissue hyperplasia or thymoma), and thymectomy has been reported to be effective in improving the clinical state in most of the patients (Papatestas *et al.*, 1971; Lebrigand *et al.*, 1972). We have found that the factors from patients with myasthenia gravis are able to induce intra-thymic T cell maturation and proliferation in mice (unpublished). Recently it has been reported (Schmidt, 1970; Mizuno *et al.*, 1976; Tsuchiya, 1984) that patients with UC also have thymus abnormalities such as enlarged and persistent thymus, higher incidence of thymic lymphoid follicles and increased B cells in thymus.

The present study is designed to clarify the mechanisms of thymic contribution to immunological abnormalities in UC. Our results showed that serum fractions from patients with UC, approximate molecular weight of 12–16 kD, exerted a variety of effects on mouse thymus. A remarkable increase in the weight and total cell counts of the thymus was revealed. This was peculiar to UC sera since the administration of normal human serum fractions and those from patients with other colonic diseases including CD at the same concentration did not affect the size or cell counts of mouse thymus. These results suggest that the hyperplastic thymus frequently seen in patients with UC (Schmidt, 1970; Mizuno *et al.*, 1976; Tsuchiya, 1984) may be induced partly by serum factors which may not be present in normal human sera.

In an attempt to ascertain if populations of the thymus cells were altered, we used FITC-PNA as cell marker. It has been established that hydrocortisone-sensitive, immunologically immature cortical thymocytes are able to bind PNA whereas the more mature thymocytes of the medulla can not (Reisner *et al.*, 1976; 1978; London, Berrin & Bach, 1978). PNA$^+$ cells are assumed to undergo a thyumus-dependent maturation step and PNA receptors disappear from the surface membrance of thymocytes as these cells differentiate into T lymphocytes. The experiments presented in this paper showed a significant increase in the number of PNA$^+$, rather small cells in the thymus from mice given UC serum fractions.

Moreover, we analysed the expression of Thy-1·2, Lyt-1, Lyt-2 and L3T4 antigens on mouse thymus cells. Mouse Thy-1·2, Lyt-1, Lyt-2 and L3T4 lymphocyte surface antigens have been used as immunogenetic markers with selective expression which defines and separates maturational and functional T cell subpopulations (Cantor & Boyse, 1975; 1977; Mathieson *et al.*, 1979). Recently, quantitative examination of these antigens by monoclonal antibodies showed characteristic changes in surface-density expression as T cells mature in the thymus (Ledbetter & Herzenberg, 1979; Ledbetter *et al.*, 1980; 1981; Micklem *et al.*, 1981; Van Ewijk, van Soest & van den Engh, 1981; Dialynas *et al.*, 1983; Scollay & Shortman, 1985). The widely accepted view is that the main thymus cell populations are stained brightly with Thy-1·2. The dull Thy-1·2$^+$ cells are the mature thymocytes which correspond in brightness to the peripheral Thy-1·2$^+$ cells. The majority of thymus cells are also stained dully with Lyt-1. The small number (approximately 10%) of bright Lyt-1$^+$ cells are the mature thymocytes. Both Lyt-2 and L3T4 antigens are expressed in cortical thymocytes, although neither are found in the precursor thymocytes in the subcapsular region. Mature, medullary cells express either Lyt-2 or L3T4 in a mutually exclusive manner. In the peripheral blood, Lyt-2$^+$ L3T4$^-$ T cells are thought to be suppressor cells, and Lyt-2$^-$ L3T4$^+$ T cells are thought to be helper cells. In this paper, it was demonstrated that the number of bright Lyt-1$^+$ cells was less, whereas that of bright Thy-1·2$^+$ cells was greater, in mice treated with UC serum

fractions than in untreated mice. These results indicate that the proportion of immature cells may increase in the thymus of mice given UC serum fractions. Moreover, the decrease of Lyt-2^+ cells and the increase of L3T4^+ cells in treated mice suggests that the intrathymic cells which are destined to become suppressor cells in the periphery may decrease and those destined to become helper T cells may increase in these mice. Although the precise mechanisms of the effect by serum factors have not been clarified yet, one possibility is that the serum factors have duplicate effects on the intra-thymic T cell maturation process; (1) they promote proliferation of the cortical immature thymocytes with bright Thy-1^+, dull Lyt-1^+, Lyt-2^+, L3T4^+ surface antigens, (2) they prevent Lyt-2^+ L3T4^+ cells from differentiating into Lyt-2^+ L3T4^- (suppressor) T cells.

Our previous studies (Aiso *et al.*, 1982; Hibi *et al.*, 1982) have shown that the number of circulating suppressor T cells was revealed to decrease in patients with UC. Then, the abnormalities of circulating T cell populations in UC patients may derive from the disturbance of intra-thymic T cell maturation which was caused by serum factors. Recent findings suggest that the disturbance of immunoregulatory T cells may contribute to the pathogenesis of some autoimmune disorders including UC. Our findings concerning serum factors from patients with UC modifying the intra-thymic T cell maturation in mice raise the possibility that a similar mechanism may be responsible for the disturbance in immunoregulatory T cells in the patients. Detailed characterization of these factors should be warranted.

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