Increased in vitro release of soluble interleukin 2 receptor by colonic lamina propria mononuclear cells in inflammatory bowel disease

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Abstract
Increased concentrations of the soluble form of the interleukin 2 receptor have been observed in the sera of Crohn's disease and ulcerative colitis patients. In this study we have observed the spontaneous release of soluble interleukin 2 receptor by unstimulated, isolated normal and inflammatory bowel disease colonic lamina propria mononuclear cells. Lamina propria mononuclear cells from Crohn's disease patients (median = 204 U/ml, range = 126–396, n = 17) secreted significantly more soluble interleukin 2 receptor than normal controls (median = 124.5 U/ml, range = 108–131, n = 12). No statistically significant differences were seen between ulcerative colitis (median = 135 U/ml, n = 20) and normal controls. Moreover, significantly increased amounts of soluble interleukin 2 receptor were secreted by colonic diverticulitis lamina propria mononuclear cells (median = 259 U/ml, range = 149–282, n = 15) which were used as disease specificity controls. Time course experiments showed that the majority of soluble interleukin 2 receptor was released by isolated lamina propria mononuclear cells in the first six days of culture. Upon stimulation with pokeweed mitogen, Crohn's disease (median = 2258 U/ml, range = 1435–3584, n = 14), normal control (median = 2622 U/ml, range = 2030–3180, n = 14) and diverticulitis lamina propria mononuclear cells (median = 2745 U/ml, range = 1733–3192, n = 10) reached similar maximal soluble interleukin 2 receptor secretion levels, while ulcerative colitis lamina propria mononuclear cells secreted significantly less soluble interleukin 2 receptor (median = 912 U/ml, range = 494–1259, n = 17). These results suggest that enhanced shedding/secretion of soluble interleukin 2 receptor by intestinal lymphocytes may account in part for increased serum soluble interleukin 2 receptor concentrations during chronic intestinal inflammatory reactions.

Recent studies have described the activation of peripheral blood T cells in Crohn's disease and ulcerative colitis, which results in the increased expression of lymphocyte activation antigens. These include transferrin receptor, interleukin 2 receptor, HLA-DR antigens and an activated calcium channel recognised by the 4F2 monoclonal antibody. The expression of activation antigens on intestinal lymphocytes, however, is controversial: an increased state of intestinal lymphocyte activation has been observed in both Crohn's disease and ulcerative colitis, whereas other studies found intestinal T cell activation only in Crohn's disease, or have not seen any significant differences in the degree of intestinal lymphocyte activation between inflammatory bowel disease and normal controls.

Mueller et al. and Crabtree et al. have recently described increased concentrations of soluble interleukin 2 receptor in sera from active Crohn's disease and ulcerative colitis patients, which, in Crohn's disease, were found to be related to the clinical activity. Overall concentrations in serum soluble interleukin 2 receptor were higher in Crohn's disease than in ulcerative colitis. The authors also showed an enhanced capacity of peripheral blood mononuclear cells to secrete soluble interleukin 2 receptor in vitro. This was regarded as being partly because of in vivo activation of peripheral blood T lymphocytes, which in a resting state do not express cell surface interleukin 2 receptor and do not secrete soluble interleukin 2 receptor, but upon in vivo or in vitro activation can express membrane bound interleukin 2 receptor and can then shed a soluble form of this receptor. Soluble interleukin 2 receptor, which corresponds to the 'Tac' antigen (α-chain), can bind interleukin 2 and thus may participate in events regulating interleukin 2 mediated lymphocyte activation. In addition to activated T lymphocytes, both activated macrophages and B cells can release soluble interleukin 2 receptor as well. Moreover, Mahida and coworkers recently...
observed that soluble interleukin 2 receptor is actively released in the venous blood from inflamed inflammatory bowel disease tissue, thus indicating that substantial amounts of soluble interleukin 2 receptor may be secreted by intestinal lymphocytes.\(^2\)

This study evaluated intestinal lamina propria mononuclear cells as a possible source for the release of soluble interleukin 2 receptor in inflammatory bowel disease. Local release of soluble interleukin 2 receptor within the involved intestinal tissue could have relevance for the control of intestinal lymphocyte activation in ulcerative colitis and Crohn’s disease. Moreover, the disease specificity of soluble interleukin 2 receptor release by lamina propria mononuclear cells was investigated by the use of colonic specimens from chronic diverticulitis patients, a bacterial inflammatory disease distinctly different from inflammatory bowel disease.

**Methods**

**ISOLATION OF HUMAN INTESTINAL LAMINA PROPIA MONONUCLEAR CELLS**

Unless indicated otherwise, all chemicals were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum was obtained from GIBCO (Grand Island, NY, USA) and heat inactivated before use. Colonic tissue was obtained as previously described by Mac Dermott et al\(^1\) at the time of surgery from patients with active inflammatory bowel disease (ulcerative colitis, n = 21 and Crohn’s disease, n = 22), from disease specificity controls (chronic diverticulitis, n = 15) or from normal individuals from whom organs were harvested for transplantation (n = 15). Intestines were obtained from the latter group of individuals immediately after the removal of other organs while still being perfused and oxygenated.\(^1\) Tissues obtained from inflammatory bowel disease specimens were histologically involved with active disease. All patients with inflammatory bowel disease had undergone surgical resections because of refractory disease or complications.

Mononuclear cells from intestinal lamina propria were isolated as previously described by Bull and Bookman\(^1\) and Mac Dermott et al\(^1\). In brief, the mucosa was dissected free from underlying musculature and minced into small pieces. Epithelial cells were subsequently removed by washing repeatedly in Hank’s balanced salt solution (without Ca\(^{++}\) or Mg\(^{++}\)) containing EDTA (0.75 mM) for 45 minutes at 20°C. Mononuclear cells were freed from tissue by overnight collagenase digestion (16 U/ml, Worthington, Freehold, NJ, USA, 37°C) and subsequently isolated by serial density centrifugations using Ficoll-Hypaque (Pharmacia, Piscataway, NJ, SG 1.077) and Percoll-HBSS (Pharmacia, SG 1.040) as described previously.\(^1\) Contamination by epithelial cells was evaluated microscopically and found to be <1%. Viability was determined using either trypan blue or propidium iodide exclusion. Only lamina propria mononuclear cells preparations with ≥95% viable cells were used in this study. In parallel, peripheral blood mononuclear cells, exposed to enzymatic procedures similar to the intestinal lamina propria mononuclear cells, were used as controls. Those peripheral blood mononuclear cells spontaneously secreted less than 30 U/ml soluble interleukin 2 receptor.

All study protocols were reviewed and approved by medical ethics committees at Washington University, St. Louis, MO, USA and University of Pennsylvania, Philadelphia, PA, USA. Blood and tissue donations were voluntary and obtained after informed consent. Organ donor tissue was obtained in accordance with our human studies protocols, after agreement by the transplant service coordinator and after informed consent of the donor relatives.

**FLUORESCENCE ACTIVATED CELL SORTER ANALYSIS**

Isolated human lymphocytes at a concentration of 20×10\(^6\)/ml were added to wells of a V-bottom microtitre plate (final volume 0.05 ml) and washed twice (400 g, 10 min) in fluorescence activated cell sorter buffer (phosphate buffered saline with 1% BSA and 0.1% sodium azide). The pellet was resuspended in 0.05 ml of unlabelled anti-CD25 antibody (Becton-Dickinson, Mountain View, CA, USA, 1:2.5 dilution) and incubated for 30 minutes. After two washes, incubation in Texas Red\(^a\) coupled goat anti mouse IgG (0.05 ml, 30 minutes, Organon-Technika-Cappell, West Chester, PA, USA) was performed. After two more washes directly labelled (phycoerythrin) antibodies against T cell (CD3-leu 4 (1:10); CD4-leu 3a (1:5); CD8-leu 2a (1:2.5), all Becton-Dickinson) or B cell specific determinants (CD19-leu 12 (1:2.5), Becton-Dickinson) were added (0.05 ml total volume). After incubation (30 minutes) cells were washed twice, fixed in paraformaldehyde (2% (w/v), 10 min), resuspended and analysed on an Epics 753 flow cytometer (Coulter, Hialeah, FL). The entire procedure was carried out at 4°C and dilutions used are given in parentheses with the antibodies. A total of five normal control specimens from organ donor intestines were used in addition to two specimens from a patient with atomic colon and a patient with caecal haemangioma, respectively. Eight ulcerative colitis, five Crohn’s disease and six diverticulitis specimens were analysed. In addition, normal peripheral blood mononuclear cells were exposed to similar conditions as those used for enzymatic digestion of lamina propria without seeing significant expression of interleukin 2 receptor induced by the procedure.

**ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SOLUBLE INTERLEUKIN 2 RECEPTOR**

Supernatants from 14 day cultures with or without pokeweed mitogen (final concentration, 1% v/v, Gibco) or serum samples from patients were analysed for concentration of soluble interleukin 2 receptors by a sandwich ELISA (T cell Sciences, Cambridge, MA, USA). In brief, 96 well plates were coated with anti interleukin 2 receptor antibody, blocked, and soluble interleukin 2 receptor from samples (in duplicate)
absorbed. After extensive washing, a murine, horseradish peroxidase coupled antibody directed against a different soluble interleukin 2 receptor epitope was added. After washing, substrate was added for 30 minutes at room temperature, the reaction stopped with H₂SO₄, and absorbance read at 490 nm on a Dynatech MR-7000 ELISA reader. The assay was calibrated by standards (from stimulated human lymphocyte supernatants), which were supplied by the manufacturer and comprised a range from 200 U/ml to 1000 U/ml.

STATISTICS AND SAMPLE SELECTION
Only specimens from Crohn’s disease patients with a Crohn’s disease activity index > 150 were used in the study. Ulcerative colitis specimens were obtained from clinically active patients. In addition, the fresh specimens obtained at operation were ranked on a scale of 1 (normal) to 10 (most inflamed) by macroscopic appearance and only those with a score > 5 were used in this study. Diverticulitis specimens were obtained from patients being operated on because of chronic, active disease. All tissue samples were from macroscopically involved sites. All diagnoses were confirmed by a pathologist. Most inflammatory bowel disease patients were on steroids (18/22 Crohn’s disease (mean 12 mg prednisone daily (8-1) (SD)) and 16/21 ulcerative colitis patients (9-1 mg (8-3)) and/or azulphidine/5-ASA therapy (21/22 and 14/21, resp) at the time of surgery. No correlation, however, between levels of in vitro soluble interleukin 2 receptor secretion and preoperative steroid treatment was observed. Exclusion criteria were treatment with cytotoxic agents or an age younger than 18 or over 75 years. Statistics were carried out using the Wilcoxon’s rank-sum test for groups > 10. Data from pokeweed mitogen stimulated samples were analysed by a modification of the Wilcoxon’s rank-sum test for multiple groups based on a modified χ² test. Membrane bound interleukin 2 receptor expression on lymphocytes as analysed by flow cytometry was evaluated using the Student’s t test. Statistical significance for differences was assumed with a p value < 0.05. If not stated differently, data are given as median (interquartile range).

Results

SERUM CONCENTRATIONS OF SOLUBLE INTERLEUKIN 2 RECEPTOR
Serum concentrations of soluble interleukin 2 receptor in inflammatory bowel disease patients were evaluated. In both active Crohn’s disease (738 U/ml (412-1097), mean (SEM) 802 (95) n = 20) and active ulcerative colitis (694 U/ml (395-1152), 809 (109), n = 17) raised concentrations of soluble interleukin 2 receptor were detected in serum compared with controls (487 U/ml (362-612), 501 (33), n = 17). These differences were statistically significant (p = 0.02 and p = 0.01, respectively) and confirmed the previous results reported by Mueller et al. and by Crabtree et al.

TIME COURSE OF SOLUBLE INTERLEUKIN 2 RECEPTOR RELEASE BY ISOLATED COLONIC LAMINA PROPRIA MONONUCLEAR CELLS DURING 14 DAY CULTURES
The time course of spontaneous release of soluble interleukin 2 receptor was investigated (Fig 1). No differences in the kinetics of soluble interleukin 2 receptor release were seen between normal controls, Crohn’s disease, ulcerative colitis, and colonic diverticulitis. The increase in spontaneous soluble interleukin 2 receptor concentrations in the supernatants reached a plateau between days 2 and 4 and then stayed constant to day 14. In contrast interleukin 2 receptor secretion by lamina propria mononuclear cells, which were stimulated with pokeweed mitogen, reached a plateau in supernatant concentrations by day 11, although much higher absolute concentrations were reached (Figs 2, 3).

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Figure 2: Spontaneous secretion of soluble interleukin 2 receptor by isolated colonic lamina propria mononuclear cells during 14 day culture. Lamina propria mononuclear cells isolated from specimens of patients with colonic Crohn’s disease (n = 17) and with diverticulitis (n = 15) secreted significantly (p < 0.01) more soluble interleukin 2 receptor than lamina propria mononuclear cells from normal controls (n = 12). No statistically significant difference between ulcerative colitis (n = 20) and normal controls was seen. All data are given as median (interquartile range). * = p < 0.01.

Figure 3: Pokeweed mitogen stimulated secretion of soluble interleukin 2 receptor by isolated colonic lamina propria mononuclear cells during 14 day culture. No statistical differences were seen between maximally stimulated colonic lamina propria mononuclear cells from normal controls (n = 14), Crohn’s disease (n = 17) and diverticulitis (n = 10). Ulcerative colitis lamina propria mononuclear cells (n = 20) raised significantly less soluble interleukin 2 receptor than normal controls or colonic diverticulitis (p < 0.005). All data are given as median (interquartile range). ** = p < 0.005.
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SPONTANEOUS RELEASE OF SOLUBLE INTERLEUKIN 2 RECEPTOR BY ISOLATED COLONIC INTESTINAL LAMINA PROPRIA MONONUCLEAR CELLS DURING 14 DAY CULTURES

As seen in Figure 2, significantly (p<0.01) more soluble interleukin 2 receptor was spontaneously released by Crohn's disease lamina propria mononuclear cells (204 U/ml (126-396), n 17) than by normal controls (124-5 U/ml (108-131), n 12). Moderately increased secretion of soluble interleukin 2 receptor by ulcerative colitis intestinal lamina propria mononuclear cells was observed (135 U/ml (92-196), n 20), but was not statistically different from normal control lamina propria mononuclear cells (Fig 2). No correlation between spontaneous soluble interleukin 2 receptor release and the macroscopic degree of mucosal inflammation or preoperative steroid dosage was seen. Colonic tissue from diverticulitis patients was examined as a disease specificity control. Importantly, diverticulitis lamina propria mononuclear cells also showed significantly (p<0-01) increased concentrations of spontaneous soluble interleukin 2 receptor release when compared with normal controls (259 U/ml (149-282), n 15). This result indicates that the findings observed for inflammatory bowel disease lamina propria mononuclear cells are not uniquely specific for Crohn's disease or ulcerative colitis.

POKEWEED MITOGEN STIMULATED RELEASE OF SOLUBLE INTERLEUKIN 2 RECEPTOR BY ISOLATED COLONIC LAMINA PROPRIA MONONUCLEAR CELLS DURING 14 DAY CULTURES

When sufficient lamina propria mononuclear cells were available, cells were cultured in the presence of pokeweed mitogen, a mitogen which activates B cells as well as T lymphocytes and macrophages. No significant differences between maximally stimulated soluble interleukin 2 receptor concentrations in supernatants from normal control lamina propria mononuclear cells (2622 U/ml (2030-3180), n 14), Crohn's disease lamina propria mononuclear cells (2258 U/ml (1435-3584), n 14), or colonic diverticulitis lamina propria mononuclear cells (2745 U/ml (1733-3192), n 10) were seen (Fig 3). Ulcerative colitis lamina propria mononuclear cells exhibited a significantly (p<0.005) diminished release of soluble interleukin 2 receptor in response to pokeweed mitogen stimulation (912 U/ml (494-1259), n 17), compared with pokeweed mitogen stimulated normal, diverticulitis, or Crohn's disease lamina propria mononuclear cells.

PHENOTYPIC ANALYSIS OF ISOLATED LAMINA PROPRIA MONONUCLEAR CELLS

We were interested to know whether increased soluble interleukin 2 receptor secretion could be attributed to differences in the distribution of lamina propria mononuclear cells subpopulations rather than the state of activation. In addition to subpopulation specific determinants, membrane interleukin 2 receptor expression of freshly isolated lamina propria mononuclear cells was assessed with fluorescent labelled monoclonal antibodies. Three colour flow cytometry using monoclonal antibodies against T lymphocyte (CD3), B lymphocyte (CD19), T cell subpopulation (CD4, CD8) and interleukin 2 receptor specific determinants (CD25, anti-α) were used. No significant differences between ulcerative colitis (eight), Crohn's disease (five), diverticulitis (six) and normal control intestines (seven) were observed with regard to the relative representation of lamina propria mononuclear cells subpopulations. T lymphocytes accounted for 45 (15)%/43 (11)%/41 (14)%/36 (14)% (mean SD) of total lamina propria mononuclear cells, respectively, and 19 (14)%/17 (9)%/16 (12)% (21) (7)% of lamina propria mononuclear cells were identified as B cells. CD4/CD8 ratios ranged between 1-6 and 2-5. In both Crohn's disease and ulcerative colitis an increase in interleukin 2 receptor expressing cells was observed in the T cell (8 (5)%/31 (12)%/20 (10)% for normal controls, ulcerative colitis and Crohn's disease, respectively) and as in the cell population (4)%/49 (8)%/26 (12%) (p<0.05 for all Crohn's disease and p<0.005 for all ulcerative colitis lamina propria mononuclear cells subpopulations compared with normal controls). Similar findings were seen in both the CD4+ and the CD8+ T cell subpopulations (10 (6)%/29 (8)%/20 (8)% and 9 (5)%/37 (9)%/20 (9%), respectively).

Discussion

In this study we have shown a marked increase in the spontaneous release of soluble interleukin 2 receptor by isolated intestinal lamina propria mononuclear cells from patients with Crohn's disease as well as lamina propria mononuclear cells from patients with colonic diverticulitis, who served as a disease specificity control. Intestinal lamina propria mononuclear cells from patients with ulcerative colitis showed a moderately increased spontaneous release of soluble interleukin 2 receptor. The release of soluble interleukin 2 receptors could be further stimulated by addition of pokeweed mitogen, a polyclonal activator of B and T lymphocytes as well as monocytes/macrophages. In vitro soluble interleukin 2 receptor release by lamina propria mononuclear cells stimulated with pokeweed mitogen reached similar concentrations in normal controls, Crohn's disease, and colonic diverticulitis but was significantly lower in ulcerative colitis. Phenotypic analysis of isolated colonic lamina propria mononuclear cells showed increased percentages of B cells, T lymphocytes, CD4+ T cells and CD8+ T cells expressing interleukin 2 receptor in both Crohn's disease and ulcerative colitis, indicating activation of these lymphocyte populations. No differences in the percentages of B or T cells in the total inflammatory bowel disease lamina propria mononuclear cells populations were seen, thus ruling out the possibility that the increase in soluble interleukin 2 receptor secretion in inflammatory bowel disease reflects differences in the distribution of lamina propria mononuclear cells subpopulations rather than in the state of activation.

The expression of interleukin 2 receptor on T cells, B lymphocytes or macrophages is regarded

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as mirroring early processes in cellular activation. After surface expression, a soluble form of the receptor can be released by all these cell types. In the peripheral blood, the population of activated T cells is regarded as the main source for soluble interleukin 2 receptor release by activated peripheral blood mononuclear cells, a finding which might in part be explained by the fact that T lymphocytes comprise the largest proportion of cells in peripheral blood in comparison with other mononuclear cell subpopulations.

Serum concentrations of soluble interleukin 2 receptor were significantly increased in patients with Crohn's disease and ulcerative colitis as compared with healthy volunteers, similar to previous studies. Mueller et al as well as Crabtree and coworkers showed increased concentrations of soluble interleukin 2 receptor in the serum of patients with active Crohn's disease as well as active ulcerative colitis but not patients with chronic longstanding ulcerative colitis. The authors concluded that increased soluble interleukin 2 receptor serum concentrations in inflammatory bowel disease may mirror peripheral blood T cell activation.

Our findings suggest that intestinal lamina propria mononuclear cells could represent another possible source for increased serum soluble interleukin 2 receptor. This suggestion is underscored by the in vivo findings of Mahida et al showing secretion of soluble interleukin 2 receptor into effluent venous blood from inflamed inflammatory bowel disease intestine. One possible lamina propria mononuclear cell source of soluble interleukin 2 receptor might be intestinal T cells, which show increased expression of early lymphocyte activation antigens in both diseases. Our present study confirmed increased T cell and B lymphocyte interleukin 2 receptor expression and thus activation in both diseases without differences between CD4+ and CD8+ T cell lymphocytes. Previous studies by our group and others have also shown increased intestinal B cell activation as well as increased activation of intestinal macrophages. Thus, intestinal T cells, B lymphocytes and macrophages showing an enhanced expression of membrane interleukin 2 receptors might be potential sources of soluble interleukin 2 receptor as well. Future studies, therefore, will be important to functionally evaluate isolated intestinal lamina propria T cells, B cells and macrophages.

It is interesting that intestinal lamina propria mononuclear cells from ulcerative colitis patients in our study exhibited less spontaneous and less pokeweed mitogen stimulated soluble interleukin 2 receptor secretion than those from Crohn's disease patients although such differences were not seen in interleukin 2 receptor expression of freshly isolated cells. Thus, in vitro soluble interleukin 2 receptor secretion may not necessarily reflect the in vivo state of activation, but may be indicative of the in vivo completion of activation processes. In addition, our studies confirmed very recent similar results to be reported by Matsuura and coworkers, also indicating lower concentrations of soluble interleukin 2 receptor secretion in ulcerative colitis than in Crohn's disease. Mueller et al observed...
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27 Matsusara T, West G, Klein J, Ferraris L, Foschi C. Comparison of soluble interleukin 2 receptor (sIL2R) levels in peripheral blood and intestinal mucosa in inflammatory bowel disease. [Abstract]. Gastroenterology Vol 100 #5 part 2, A729.
