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Characterization of Circulating Interleukin-1 Receptor Antagonist Expression in Children with Inflammatory Bowel Disease

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The cytokines IL-1β and IL-6 appear to be important in the pathogenesis of inflammatory bowel disease (IBD). Recently, a naturally occurring interleukin-1 receptor antagonist, designated IL-1ra, which inhibits IL-1β activity *in vitro* and *in vivo* has been described. The purpose of the present study was to assess the circulating levels and relative relationships of IL-1ra, IL-1β, and IL-6 in children with IBD of varying severity. Serum/plasma samples were obtained from 32 children with ulcerative colitis, 45 with Crohn's disease, and 24 control patients. Cytokine assays were performed by enzyme-linked immunoassay. IL-1ra levels were significantly elevated in children with ulcerative colitis or Crohn's disease of moderate/severe activity compared to patients with inactive/mild IBD or control subjects ($P < 0.001$). IL-1β was only detectable in the circulation of two subjects with severe colitis (one ulcerative colitis, one Crohn's disease), and both had extremely elevated IL-1ra levels. IL-1ra levels were significantly related to IL-6 levels for patients with IBD ($P < 0.00001$). Our results suggest that circulating IL-1ra appears in increasing concentrations in children with mounting degrees of disease severity as determined by clinical scoring methods as well as by the level of IL-6. Future work will need to address the clinical and prognostic value of measuring circulating IL-1ra in individuals with inflammatory bowel disease.

KEY WORDS: inflammatory bowel disease; interleukin-1 receptor antagonist; cytokines; inflammation.

Ulcerative colitis and Crohn's disease are chronic inflammatory disorders of the gastrointestinal tract of undetermined etiology. While the specific cause(s) of these diseases remains elusive, increasing evidence points to the important roles of inflammatory cytokines in disease pathogenesis (1–6). Two cytokines in particular, IL-1β and IL-6, have been described as major participants in the inflammatory response noted in intestinal tissue from patients with IBD. IL-1 promotes fever and anorexia and mediates inflammation via increased gene expression of multiple cytokines (eg, IL-2, IL-3, IL-6, IL-8), activation of endothelial cells, increased adhesion molecule expression, and synthesis of collagen and collagenases (7). Interleukin-1 may have a central role in the modulation of inflammation in the intestine (8–10). IL-6 has been shown to be a key

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factor in promoting hepatic acute-phase reactant synthesis (5, 6).

The interleukin-1 family is comprised of several structurally related polypeptides including interleukin-1α, interleukin-1-β, and IL-1ra (7). While IL-1β and IL-1α are the product of separate genes, they have approximately 26% amino acid homology, utilize the same surface receptors, and have similar biologic activities (7). Most IL-1α remains in the cytosol of cells with a portion being membrane-bound (7). IL-1β, in contrast, is released from the cell into the extracellular space and may circulate systemically (7). The systemic effects of IL-1β are profound and include (7, 11) fever, anorexia, hypozincemia, hypoferremia, hypoalbuminemia, and neutrophilia. Many of these clinical features and a marked acute-phase response are commonly observed in inflammatory bowel disease.

A naturally occurring inhibitor of IL-1 function designated interleukin-1 receptor antagonist (IL-1ra), is produced by the same cells which make IL-1. IL-1ra has 26% amino acid homology to IL-1β and 19% to IL-1α (7). IL-1ra binds to both IL-1 receptors (IL-1R I, IL-1R II) but does not activate them. Exogenously administered IL-1ra given to animals before the administration of endotoxin substantially abrogates the potent systemic effects of IL-1 (12). In a rabbit model of colitis induced by the intrarectal administration of formalin along with the systemic administration of immune complexes, pretreatment with IL-1ra decreases the severity of inflammation histologically and decreases the levels of inflammatory mediators such as prostaglandin E_2 and leukotriene B_4 in the tissue (13).

We hypothesized that if IL-1 has an important role in the pathogenesis of intestinal inflammation in IBD, then modification of IL-1 activity by IL-1ra might be important as well. The degree to which endogenous IL-1ra is generated in proportion to IL-1 might determine the magnitude of the local and systemic response to IL-1. Since procuring tissue cytokine levels at frequent intervals is logistically very difficult, and previous evidence has shown a significant relationship between disease activity and circulating cytokine levels (5, 6), we have measured the concentration of circulating IL-1ra in children with inflammatory bowel disease of varying severity, and compared IL-1ra levels with those of IL-1β and IL-6.

TABLE 1. CHARACTERISTICS OF STUDY POPULATION

	Crohn's disease	*Ulcerative colitis*	*Control*
Patients (*N*)	45	32	24
F/M	21/24	14/18	9/15
Age (years)			
Mean	14.8	13.0	11.0
Range	6.2–21.5	4.7–20.0	5.5–17.2
Disease activity*			
Inactive	31%	28%	
Mild	41%	33%	
Mod/Sev	28%	39%	
Medications (% receiving)			
No medication	31	23	
Daily prednisone	20	31	
Alternate-day prednisone	24	8	
Sulfasalazine	27	56	
6-Mercaptopurine	27	0	
Metronidazole	14	0	

*Disease activity of Crohn's disease by physician global assessment (15) and of ulcerative colitis by criteria of Truelove and Witts (14).

MATERIALS AND METHODS

Patient Population. Seventy-seven children and adolescents with IBD (32 ulcerative colitis, 45 Crohn's disease) and 24 control patients were recruited from the clinical practice of the Division of Pediatric Gastroenterology and Nutrition at Hartford Hospital to participate in this study. The characteristics of the patient population are given in Table 1. Diagnoses of ulcerative colitis and Crohn's disease were made by conventional clinical, radiological, and histopathologic criteria. All patients with ulcerative colitis included in this study had either pancolitis or colitis extending beyond the rectosigmoid (left-sided colitis). Disease severity of ulcerative colitis was determined using the criteria of Truelove and Witts and classified as inactive, mild, moderate, or severe (14). Patients with Crohn's disease were classified as having isolated small bowel disease (25% of patients), ileocolitis (58%), or colitis (17%). Disease severity of Crohn's disease was determined using the pediatric Crohn's disease activity index (PCDAI) and was given a numerical score of 0–100 (15). A score of 0–10 denotes inactive disease, 11–30 mild disease, and a score of 31 or greater signifies moderate to severe disease. Disease severity of Crohn's disease was also classified as inactive, mild, moderate, or severe by global assessment by one of the investigators (J.S.H., W.R.T.) at the time of clinical assessment and before the results of any laboratory studies were known. Previous work has shown a significant correlation between the PCDAI and physician global assessment (15).

Control patients had a history, physical examination, and laboratory evaluation consistent with a noninflammatory condition of the gastrointestinal system (eg, irritable bowel syndrome, lactose malabsorption).

If either a control subject or a patient with IBD had signs or symptoms of an intercurrent viral or bacterial illness within 10 days prior to their visit, they were excluded from participation in this study. Likewise, if any subject developed an intercurrent illness within 48 hr of

having blood taken, they notified one of the study coordinators and their samples were not used for study purposes.

Informed consent was obtained in all cases, and the study was approved by the Investigational Review Committee at Hartford Hospital.

Patient Samples. Serum and heparinized plasma were separated from patient and control blood samples within 15 min of venipuncture by centrifugation at 1000*g* for 10 min at 4° C. The samples were then promptly aliquoted and frozen at −70° C until analysis. A total of 51 samples were obtained from the 45 patients with Crohn's disease (range 1–2 per patient), 39 from the 32 patients with ulcerative colitis (range 1–2 per patient), and 24 (1 per patient) from the control group. If more than a single sample was obtained from a patient with IBD, the interval between samples ranged between two and four months, and there was a change in global assessment category compared to the previous visit.

IL-1ra Assay. A commercially available ELISA (Quantikine, R & D Systems, Minneapolis, Minnesota) using a quantitative sandwich technique was employed. Briefly, a monoclonal antibody specific for IL-1ra was coated onto a microtiter plate. Standards and dilutions of patient serum were pipetted into the wells and any IL-1ra present bound to the immobilized antibody. After unbound proteins were washed away, an enzyme-linked polyclonal antibody specific for IL-1ra was added to the wells, which captured the IL-1ra immobilized during the primary incubation. A subsequent wash removed any unbound antibody-enzyme reagent, and a substrate solution was added to the wells. Color developed in proportion to the quantity of IL-1ra bound in the initial step. A curve was generated plotting the optical density versus IL-1ra concentration in the standard wells, and concentration of IL-1ra in the unknown samples was determined by reference to the standard curve. All samples were run in duplicate. According to the manufacturer's specifications, the median concentration of IL-1ra in plasma from healthy adult controls is 150 pg/ml, with 95% of samples <350 pg/ml.

IL-1β Assay. IL-1β was determined using two ELISA systems on both unextracted and extracted (chloroform) (16) plasma. Plasma samples were assayed with Quantikine IL-1β (R & D Systems) and High Sensitivity Interleukin-1β ELISA (Cistron, Pine Brook, New Jersey). Samples were run in duplicate. The lowest standards of the assays were 3.9 pg/ml with the ELISA kit from R & D Systems and 2 pg/ml with the Cistron ELISA.

IL-6 Assay. A commercially available ELISA (Quantikine, R & D Systems) used a quantitative sandwich technique with patient serum samples. This assay recognized both natural and recombinant IL-6 with no cross-reactivity to other cytokines (manufacturer's specifications). All samples were run in duplicate. According to the manufacturer's specifications, 96% of healthy normal donor sera had IL-6 levels <3.13 pg/ml using this ELISA, and the remaining 4% range between 3.13 and 6.25 pg/ml. The lower limit of sensitivity for antigen detection by this assay was 0.3 pg/ml.

Statistical Analysis. All statistical calculations were performed on a Macintosh IIci personal computer using SYSTAT (SYSTAT, Evanston, Illinois) analytic software. Results are expressed as mean ± SEM. The effect of disease activity on group differences was assessed with an analysis of covariance model. All alpha levels were two-tailed. The Pearson product moment correlation coefficient (r) was calculated to assess the degree of linear correlation between interval or higher measures.

RESULTS

IL-1ra Levels. IL-1ra levels ranged from 120 to 672 pg/ml in the control population (mean 307 ± 27 pg/ml, median 267 pg/ml), from 176 to 5503 pg/ml in subjects with ulcerative colitis (mean 694 ± 148 pg/ml, median 451 pg/ml), and from 175 to 7990 pg/ml (mean 678 ± 158 pg/ml, median 450 pg/ml) in those with Crohn's disease. As can be seen in Figure 1, there was a strong association between

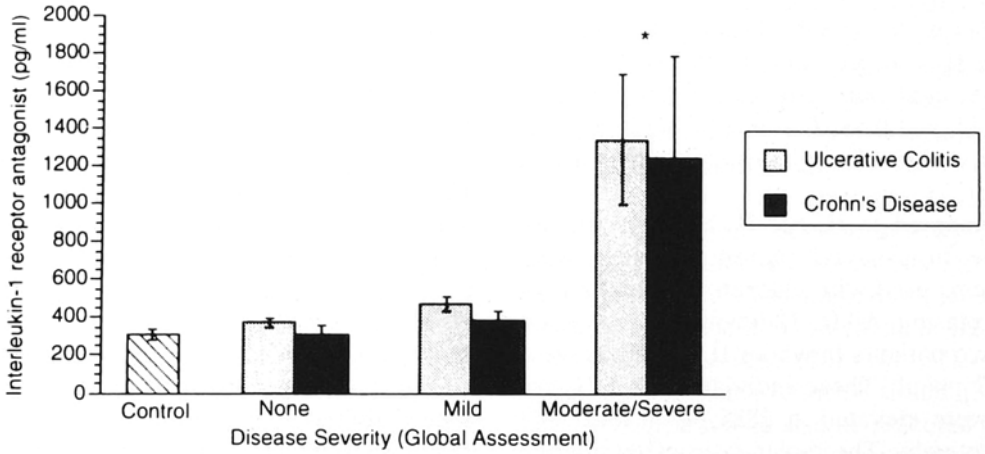

Fig 1. Relationship between circulating interleukin-1 receptor antagonist (IL-1ra) and disease severity in children with ulcerative colitis and Crohn's disease. *P < 0.001 compared to subjects with IBD of inactive/mild severity or control subjects.

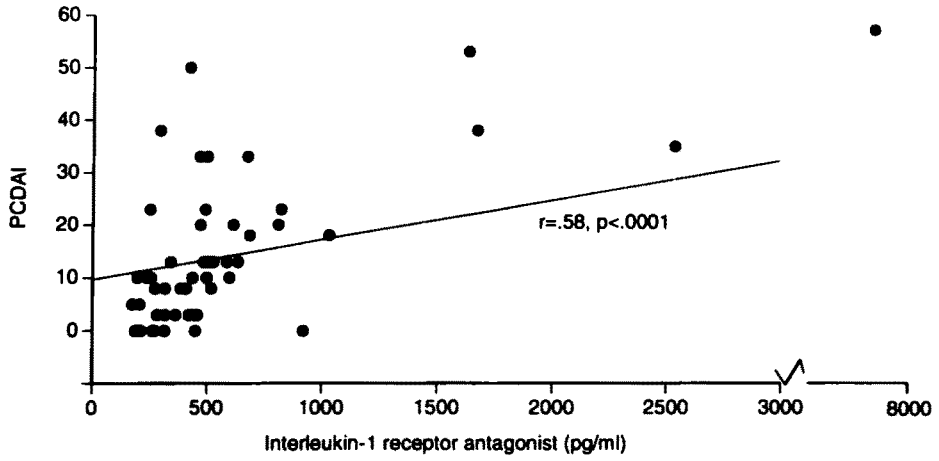

Fig 2. Relationship of circulating interleukin-1 receptor antagonist (IL-1ra) to the pediatric Crohn's disease activity index (PCDAI).

disease severity and circulating IL-1ra levels ($P < 0.00001$). While no difference was noted between the control population and those patients with ulcerative colitis or Crohn's disease of an inactive or mild nature, a significant difference was noted when either of these groups was compared to those with moderate to severe disease activity ($P < 0.001$). No differences were noted between mean IL-1ra levels in subjects with ulcerative colitis compared to those with Crohn's disease for any disease severity. For subjects with either Crohn's disease or ulcerative colitis, there was no statistical association between circulating IL-1ra levels and any medication or combination of medications. IL-1ra levels were similar in those patients with Crohn's disease affecting the small bowel only, colon only, and disease of both the small and large bowel.

For patients with Crohn's disease, the relationship between IL-1ra levels and the PCDAI was also examined. A significant correlation between the two was noted ($r = 0.58$, $P < 0.0001$) with increasing disease severity reflected by higher IL-1ra levels (Figure 2).

IL-1ra Compared to IL-1β. IL-1β was virtually undetectable in assayed patient samples with ELISA systems used with chloroform extracted or unextracted plasma. All IL-1β levels were <4 pg/ml except for two patients in whom IL-1β levels were 5.9 and 12.7 pg/ml. These individuals had IL-1ra levels that were elevated at 2885 pg/ml and 5503 pg/ml, respectively. The molar ratio of circulating IL-1ra to IL-1β was 345 in the first patient and 389 in the second. Both patients were severely ill with fulminant colitis (one ulcerative colitis, one Crohn's disease) and both were febrile.

IL-1ra Compared to IL-6. Since the relationship between IL-1ra levels and IL-6 levels was similar for patients with either ulcerative colitis or Crohn's disease, the results have been combined and are shown in Figure 3. A highly significant correlation between IL-1ra and IL-6 was noted ($r = 0.80$, $P < 0.00001$).

DISCUSSION

Control of proinflammatory cytokine activity may take several forms, including inhibition of cytokine production, decreased cytokine processing and release, binding by non-cell-bound receptors, and down-regulation or blocking of cell-bound receptors (7, 17–21). The best characterized of these antiinflammatory cytokine systems is that of IL-1. IL-4 decreases IL-1β production by monocytes and also increases production of IL-1ra (19). IL-10 has been shown to inhibit IL-1 production by activated macrophages (20). TGF-β appears to down-regulate IL-1 receptors (21) as well as to increase IL-1ra production in peripheral blood monocytes (18). Lastly, lipoproteins, lipids, and α_2-macroglobulin nonspecifically inhibit IL-1 as well as IL-2 and IL-6 (7, 11, 22). Therefore, IL-1 activity can be controlled at several points of IL-1 production or binding.

The balance of pro- and antiinflammatory cytokine activities is important in modulating the host response to infection and injury. IL-1ra circulates in extremely high levels in humans with septic shock as well as in volunteers receiving small doses of

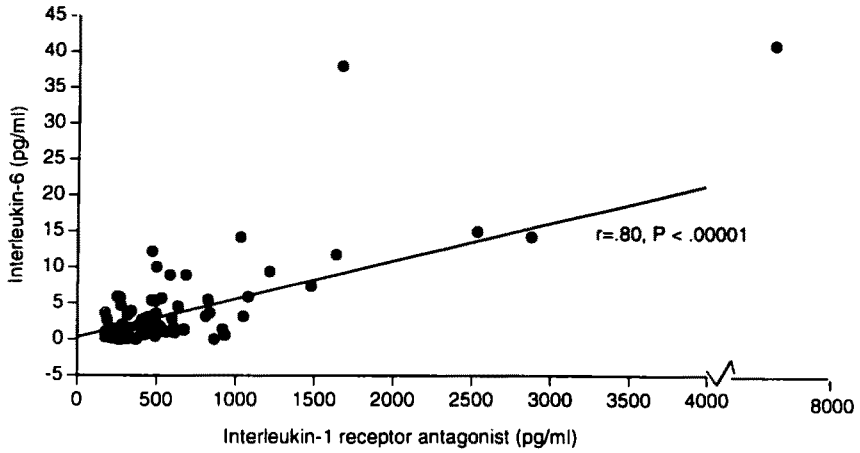

Fig 3. Relationship of circulating interleukin-1 receptor antagonist (IL-1ra) to circulating IL-6 in children with ulcerative colitis or Crohn's disease.

endotoxin when compared to healthy individuals (12). In subjects with Lyme arthritis, the ratio of IL-1ra to IL-1β in joint fluid appears to influence clinical outcome since those patients with higher amounts of IL-1ra compared to IL-1β have a shorter time to disease resolution (23).

In the current study, we have demonstrated that IL-1ra circulates in high levels in children with inflammatory bowel disease of moderate to severe activity compared to patients with inactive disease or healthy control subjects. Concurrently, the levels of IL-1ra were found to parallel those of IL-6, which has previously been shown to bear a significant relationship to disease activity and the acute-phase response in children with IBD (6). A recent report (24) has demonstrated that acute-phase proteins may preferentially induce the synthesis of IL-1ra over IL-1β by human peripheral blood mononuclear cells, suggesting a possible mechanism for the antiinflammatory role of the acute phase response. Whether IL-1ra might prove to be useful in monitoring disease activity, as are acute-phase reactants or IL-6 levels, during various therapeutic maneuvers will require further study. Unfortunately, we did not have access to serum samples from children with bowel inflammation of other causes (eg, infection) to determine the specificity of the circulating IL-1ra elevation in children with IBD.

We were unable to demonstrate the presence of circulating IL-1β in our patient population except in two subjects who were febrile and severely ill with fulminant colitis and who had very high levels of IL-1ra. As controversy has arisen about the appropriate methods to detect IL-1β in plasma (25), it is possible that systemic levels of IL-β were elevated in our patients and we failed to detect them. We do not think that levels of IL-1β were low because of malnutrition, as most children were in good nutritional state despite their underlying disease.

A significant excess of IL-1ra to IL-1 is necessary to block the biological activity of IL-1 (12, 26). In *in vitro* systems, a 100- to 1000-fold molar excess of IL-1ra to IL-1 was required to inhibit IL-1-induced activity of T cells, synovial cells, and chondrocytes (27, 28). In rabbits, systemic injection of a 100-fold molar excess IL-1ra blocks IL-1-induced fever (29). In primates a 1000-fold excess of IL-1ra to IL-1 is required to block the effects of sublethal endotoxemia (30). In our two patients with elevated plasma IL-1β levels, the molar excess of circulating IL-1ra was approximately 300-fold. It has been suggested that the excessive concentration of IL-1ra to IL-1 reflects the fact that IL-1ra is more efficiently secreted than IL-1β (12, 29, 31). Upon IL-1β release, the body's normal homeostatic mechanisms would attempt to down-regulate the systemic response to inflammation, and IL-1ra production and release might be one mechanism.

The relationship of circulating IL-1β and IL-1ra levels to histologic disease activity in ulcerative colitis and Crohn's disease awaits further study. A preliminary report has suggested that an imbalance between IL-1ra and IL-1β exists in IBD tissue with a decrease in the ratio of IL-1ra to IL-1β compared to noninflammatory control tissue (32). Whether this imbalance would also be reflected in circulating levels is not known. Preliminary work in our laboratory has demonstrated elevated IL-1ra tissue levels in individuals with moderate/severe ulcerative

colitis compared to those with inactive disease or a control population (unpublished data). It would thus appear that circulating IL-1ra levels may reflect IL-1ra tissue levels.

The concept of a balance of pro- and antiinflammatory cytokines modulating inflammation in chronic inflammatory disorders may expand the horizons of possible therapeutic approaches. Corticosteroids and n-3 fatty acids, both of which are used in the treatment of IBD, reduce IL-1 production (11). Additional agents that target IL-1 production or processing might be of benefit. The administration of medications that promote the synthesis of IL-1ra, or the actual direct administration of IL-1 receptors or IL-1-receptor antagonists, either locally or systemically, might prove beneficial since previous observations in animals have shown that IL-1ra mitigates experimentally induced colitis (13). Of interest also is the potential effect of cytokine antagonists in the treatment of anorexia associated with IBD. Although the present evidence concerning a circulating factor (tumor necrosis factor-α) in patients with IBD, which depresses appetite, remains controversial (33, 34), the fact that cytokines have effects at concentrations much lower than can be measured with current assay systems still suggests this possibility. The ability to antagonize these effects through the induction or administration of cytokine antagonists is a possibility that awaits further study not only in IBD but in other chronic inflammatory disorders as well.

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