Intestinal inflammation-induced growth retardation acts through IL-6 in rats and depends on the −174 IL-6 G/C polymorphism in children

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Inflammatory diseases frequently impair linear growth. Crohn's disease inhibits growth in up to one third of affected children. In rats with trinitrobenzenesulphonic acid-induced colitis, 40% of growth impairment is attributable to inflammation, with the rest being due to undernutrition. In transgenic mice without inflammation, raised IL-6 retards growth, suppressing insulin-like growth factor (IGF)-I. We hypothesized that IL-6, induced by intestinal inflammation, suppresses growth and inhibits IGF-I expression. Therefore, an anti-IL-6 Ab was given to rats with trinitrobenzenesulphonic acid colitis. The Ab did not improve nutrient intake or decrease inflammation compared with untreated disease controls, but it significantly restored linear growth ($P = 0.023$) and increased IGF-I ($P = 0.05$). In humans, the IL-6 −174 G/C promoter polymorphism affects IL-6 transcription, with the GG genotype inducing the greatest IL-6 levels. Because IL-6 is increased in Crohn's disease, we further hypothesized that growth failure would vary with the IL-6 −174 genotype. At diagnosis, among 153 children with Crohn's disease, those with the IL-6 GG genotype were more growth-retarded than those with the GC or CC genotypes (height SD score, −0.51 vs. −0.10; $P = 0.031$). Also, the patients with the IL-6 GG genotype had higher circulating levels of C-reactive protein, an IL-6-induced product (36 vs. 18 mg/dl, $P = 0.028$). However, their risk of developing Crohn's disease was similar to other genotypes when compared with 351 healthy controls ($P = 0.7$). Thus, the IL-6 −174 genotype mediates growth failure in children with Crohn's disease.

Crohn's disease | height | insulin-like growth factor I | C-reactive protein | food intake

Linear growth retardation is a major complication of many childhood inflammatory diseases. For example, Crohn's disease, characterized by transmural inflammation of the gastrointestinal tract (1), severely inhibits growth in approximately one third of affected children (2–5). A significant proportion of children with Crohn's disease become short adults (6, 7). Despite repeated clinical reports over seven decades (8), the basis of this observation has not been fully explained. Also, it is not clear why only some children are affected. Growth failure in inflammatory bowel disease has traditionally been attributed chiefly to undernutrition (9). However, there is increasing evidence that the inflammatory process itself may directly inhibit linear growth (10, 11), possibly by the secretion of cytokines that suppress growth factors. Although insulin-like growth factor (IGF)-I is decreased in children with Crohn's disease (12), there is no evidence supporting an immune-based mechanism for growth failure.

IL-6 expression is increased in Crohn's disease (13, 14), and circulating levels parallel disease activity (15, 16). IL-6 induces acute-phase respondents, such as C-reactive protein (CRP), that are used clinically to monitor disease activity (17). IL-6 overexpression, induced by using transgenic techniques in mice without inflammation, results in growth faltering and reduced circulating IGF-I, in the presence of normal growth hormone (18). A neutralizing Ab to the IL-6 receptor reversed these observations. In a rat disease model, the hapten 2, 4, 6-trinitrobenzenesulphonic acid (TNBS), when instilled rectally, induces a chronic granulomatous colitis with many features analogous to human Crohn's disease, including anorexia and raised IL-6 (19). We have shown (20) that in prepubertal rats, TNBS-induced colitis also causes growth retardation. Comparison with healthy pair-fed controls demonstrated that ≈40% of the growth impairment could be attributed directly to inflammation, with undernutrition accounting for the remaining growth deficit.

The human IL-6 gene is located on chromosome 7p15–7p21 and consists of five exons and four introns (21–23). Because of its rapid clearance, IL-6 expression is primarily regulated by alterations in gene transcription (24). Fishman *et al.* (25) described a functional G→C single-nucleotide polymorphism at position −174 of the promoter. Data from *in vivo* and *in vitro* studies suggested that the GG allele was associated with greater induction of IL-6 compared with the GC or CC alleles. In this study, we hypothesized that increased IL-6 expression impaired growth in inflammatory bowel disease and that differences in IL-6 −174 genotype caused sufficient chronic variation in IL-6 levels to account for differences in growth. An *a priori* hypothesis was that patients with the GG genotype expressed greater levels of IL-6 and, thus, were more growth-impaired than other genotypes. We demonstrated that IL-6 caused growth suppression in rats with TNBS colitis. We also compared height with genotype in children at the time of diagnosis of Crohn's disease. Children with the −174 GG genotype were significantly more growth-retarded.

Methods

Patients. All patients had Crohn's disease diagnosed before 16.0 years and did not have any other factor known to cause growth impairment. Their diagnoses were made by using standard criteria, including a colonoscopy. All patients were of Northern European, Caucasian origin because the IL-6 −174 polymorphism varies with ethnicity (25). Most patients attended the pediatric inflammatory bowel disease clinics at the Royal London and St Bartholomew's hospitals in London or the Astrid Lindgren Children's Hospital in Stockholm. Other patients were recruited from the Chelsea and Westminster, Northwick Park, Bristol Children's, Lewisham, and Bury St. Edmond's hospitals

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Abbreviations: CRP, C-reactive protein; IGF, insulin-like growth factor; IGFBP-3, IGF binding protein 3; SDS, SD score; TNBS, 2,4,6-trinitrobenzenesulphonic acid.

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in England. The human studies committee relevant to each hospital approved the study, allowing research samples to be obtained when phlebotomy was indicated clinically.

Case notes were reviewed, and data were abstracted. Height at diagnosis was defined as a height taken within 1 mo of the date of diagnosis and was expressed as height SD score (SDS) (26). Where parental (often paternal) height had not been recorded, parents were asked to attend to have their height measured. If attendance was not possible, an estimate of height was obtained, because estimates by family informants are known to be accurate (27). CRP levels at the time of diagnosis and within 6–10 weeks from the onset of the first therapeutic course (either enteral feeding or corticosteroids) were also recorded.

For ethical reasons, it was not possible to approach pediatric patients to act as controls. Therefore, we used DNA that had been collected previously from an ethnically matched population-based cohort of English teenagers (kindly donated after human studies approval by Robert Booy, Queen Mary University of London) and samples that were obtained from healthy anonymous adult Swedish blood donors.

DNA Extraction and Genotyping. DNA was extracted from fresh blood samples by using a salting-out process, from frozen samples by using the Quantikine kit (Amersham Biosciences), and from blood spots by using the Chelex technique. We whole-gene amplified 1 μl of each of the English teenage control samples by using the GenomiPhi method (Amersham Biosciences). High-throughput analysis of the IL-6 -174 polymorphism was undertaken by using TaqMan 5′ endonuclease assay on a 7900 HT sequence-detection system with SDS software (Applied Biosystems). We added the following primers to 1.5 μl of DNA solution: 5′-GCTGATTGGAAACCTTATTAAGATTGT-3′ (0.0225 μl; reverse) and 5′-GCTGCACTTTTCCCCCTAGT-3′ (0.0225 μl; forward); as well as 0.2 μl of FAM, 5′-TGTCTTGCCATGCTA-3′; 0.2 μl of VIC, 5′-TGTCTTGCGATGCTA-3′; 2.52 μl of TaqMan Universal PCR master mixture (part no. 4343202C; Applied Biosystems); and 0.555 μl of water to a total volume of 5 μl. PCR amplification was undertaken as follows in a Super Duncan thermal cycler (KBiosystems, Basildon, U.K.): 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 92°C for 15 s and 60°C for 60 s.

Animals and Induction of Colitis. We housed 25-day-old prepubertal Wistar rats (Charles River Laboratories) individually at an ambient temperature of 22°C with a 12:12-h light/dark cycle. The rats were given free access to standard laboratory chow (RMI cubed; Special Diet Services, Witham, U.K.) and tap water. All experiments were carried out in accordance with the United Kingdom Animal Scientific Procedures Act of 1986. We chose the rat TNBS colitis model of intestinal inflammation because, even in its early stages, it resembles human disease regarding T cell activation and cytokine profiles, including increased IL-6, TNF-α, IL-1, and IFN-γ (19, 28–31). Also, the disease inhibits linear growth, with a decrease in IGF-I (20) in a manner analogous to that observed in children (10). Rats were divided into the following three groups matched for sex, weight, and body length: healthy free-feeding controls ($n = 13$), a TNBS colitis group treated with anti-IL-6 Ab (TNBS/IL-6 Ab, $n = 11$), and a TNBS colitic group treated with nonspecific sheep IgG (TNBS/IgG, $n = 13$).

Rats were anesthetized by intramuscular injection of Hypnorm (Janssen), and a plastic cannula was inserted 5 cm proximal to the anus for administration of 8 mg per 100 g of body weight of TNBS (Sigma–Aldrich) in 40% ethanol (19) up to a final volume of 0.2 ml. In the healthy control group, a plastic catheter was also passed into the colon under anesthesia and removed after 1 min. Body weight and food and water intake were measured daily. Body length was measured as described in ref. 20 as the mean of two measurements of nose-to-tail base distance in anesthetized animals at induction of colitis (age, 26 days) and 5 days later (age, 31 days) immediately before the animals were killed. Although our studies have reported that colitic rats continue to remain growth-retarded for $\geq$14–21 days after TNBS installation (21), 5 days had been shown (20) to inhibit growth significantly. Intraobserver precision for measurement of linear length was 3.3%. At the end of the experimental period, animals were killed by overdose under anesthetic, and trunk blood was collected into EDTA tubes and centrifuged at 4°C for 10 min. Plasma was stored at -20°C until required. The liver was removed by means of a midline laparotomy and immediately stored at -70°C.

Assessment of Colitis. The colon was removed, and the severity of inflammation assessed macroscopically by using a previously validated scoring system that depends on the number and size of ulcers as well as the presence or absence of diarrhea and adhesions (33). The maximum score is 13, to which is added the thickness of the colonic wall measured in millimeters. A section of the colon 2 cm proximal to the anus was removed and stored at -20°C for later assessment of myeloperoxidase activity (34, 35). Wet and dry weights were obtained on the remaining left colon to determine intestinal tissue edema.

Ab Administration. We administered 5 ml/kg IgG polyclonal Abs to IL-6 (National Institute for Biological Standards and Control, Potters Bar, U.K.), equivalent to 10–15 mg/kg, to colitic rats to determine the effect *in vivo* of IL-6 on the IGF system and linear growth. IL-6 Ab was administered s.c. at induction and 2 days after induction of colitis. A vehicle-treated colitic group received nonspecific IgG (National Institute for Biological Standards and Control) in the same manner as a control.

Plasma and Hepatic Assays of IGF-I and IGF Binding Protein 3 (IGFBP-3). Plasma and liver concentrations of total IGF-I were measured by using an ELISA (Diagnostic Systems Laboratories, Webster, TX) after homogenization of liver tissue in PBS. IGFBP-3 was measured in plasma samples after 1:100 dilution by a highly specific two-site Ab ELISA (Diagnostic Systems Laboratories), with a minimum detection limit of 0.04 ng/ml, no cross-reactivity with other IGFBPs, and no interference from IGF-I. All plasma samples from one experiment were measured in duplicate in the same assay.

Hepatic IGF-I mRNA. RNA was extracted from liver by using TRIzol reagent (Invitrogen) and examined by RT-PCR. IGF-I mRNA was quantified as a proportion of β-actin mRNA in the sample. The following primers were used: IGF-I, 5′-AAGCCTACAAAGTCAGCTCG-3′ and 5′-GGTCTTGTTTCCTGCACTTC-3′; and β-actin, 5′-TGACGTTGACATCCGTAAAG-3′ and 5′-ACAGTGAGGCCAGGATAGAG-3′. The IGF-I primers amplified two differentially spliced mRNA species, IGF-IA and IGF-IB (36).

Statistical Analysis. Data were examined with the Kolmogorov–Smirnov test, and results are given as SEM or median (interquartile range). Normally distributed data were examined by using a two-tailed Student's t test, except for differences in children's height SDS (one-tailed). Differences in genotype frequency were analyzed by using the χ^2 test, and difference in CRP was determined by the Mann–Whitney U test and Wilcoxon rank test. Differences in macroscopic score of colitis were analyzed with the Mann–Whitney U test. Linear growth in rats is given as the change in body length during the 5-day experimental period.

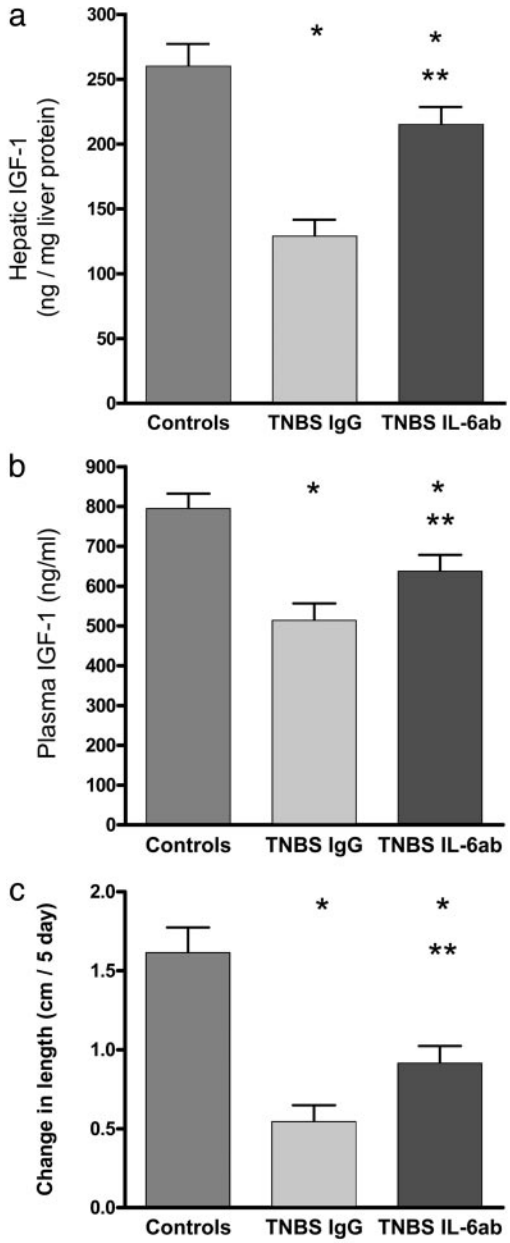

Fig. 1. IL-6 Ab increases IGF-I expression and linear growth in rats with colitis. Results are given for the end of the 5-day study period in healthy controls (n = 13) and TNBS colitis treated with control IgG (n = 11) or IL-6 Ab (n = 13). Values are given as mean ± SEM. (*a*) Hepatic IGF-I. *, $P < 0.05$ vs. controls; **, $P < 0.001$ for control IgG vs. IL-6 Ab. (*b*) Plasma IGF-I. *, $P < 0.001$ vs. controls; **, $P = 0.05$ for control IgG vs. IL-6 Ab. (*c*) Change in length over 5 days. *, $P < 0.001$ vs. controls; **, $P = 0.023$ for control IgG vs. IL-6 Ab.

Results

Anti-IL-6 Ab Enhanced Growth in TNBS Colitic Rats Without Improving Intestinal Inflammation or Increasing Nutrient Intake. Administration of TNBS/ethanol to prepubertal rats produced distal colitis extending from the splenic flexure to the anus. At the time at which the animals were killed, there was bowel wall thickening, edema, skip-lesions, and areas of ulceration. As in ref. 20, linear growth was reduced significantly in colitic rats compared with healthy controls (Fig. 1). Rats with colitis also ate significantly less than healthy controls.

Treatment with IL-6 Ab had no effect on the severity of colitis, (Table 1) as assessed by macroscopic inflammation, colon wall thickening, wall edema, or myeloperoxidase concentrations. Also, IL-6 Ab did not alleviate the suppression of feeding (Fig. 2). However, IL-6 Ab increased linear growth significantly in TNBS colitic rats ($P = 0.023$; Fig. 1). Because of the decreased nutrient intake, growth in the IL-6 Ab-treated colitic rats remained less than healthy controls.

Table 1. IL-6 Ab does not alter intestinal inflammation

Measurement of inflammation	Healthy controls	TNBS IgG	TNBS IL-6 Ab
Myeloperoxidase, units per g of tissue	0.03 (0.01)	0.52 (0.09)*	0.41 (0.08)*,**
Macroscopic score	0.0 (1.0)	8.0 (3.0)*	7.5 (4.0)*,**
Edema H_2O, ml/g	0.28 (0.04)	0.34 (0.07)	0.34 (0.06)**

The severity of colonic inflammation in healthy controls (n = 13) and rats with TNBS colitis treated with control IgG (n = 11) or IL-6 Ab (n = 12) is shown. See *Methods* for the calculation of the macroscopic score. Values are given as mean for myeloperoxidase and edema and as median (interquartile range) for macroscopic scores (with SEM shown in parentheses). *, $P < 0.001$ vs. healthy controls; **, $P > 0.05$ vs. control IgG.

Anti-IL-6 Ab Increased IGF-I Expression in TNBS Colitic Rats. Impaired linear growth in TNBS colitic rats was associated with a reduction in hepatic and plasma concentrations of IGF-I. (Fig. 1) Administration of IL-6 Ab significantly increased both liver IGF-IA and IGF-IB mRNA (data not shown) and significantly enhanced liver and plasma IGF-I protein concentration ($P < 0.001$ and $P = 0.05$, respectively) in rats with colitis (Fig. 1).

IGFBP-3 was measured to determine whether a decrease contributed to the low circulating concentration of IGF-I. However, IGFBP-3 was similar in colitic rats and healthy controls (data not shown), indicating that a decrease in IGFBP-3 (and, hence, IGF-I binding) did not cause a reduction in IGF-I in the colitic group.

Children with the IL-6 −174 GG Genotype Were More Severely Growth-Retarded at Diagnosis of Crohn's Disease. Because IL-6 caused growth faltering in rats with intestinal inflammation, we

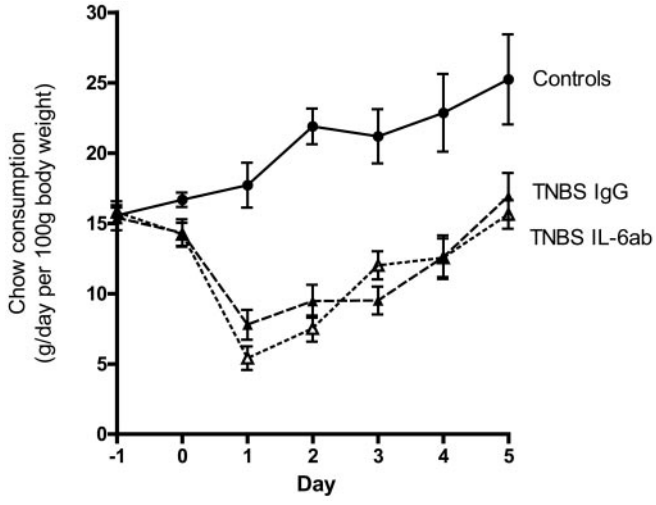

Fig. 2. IL-6 Ab does not alter food intake in inflammation-induced anorexia. Daily chow intake in healthy free-feeding controls (n = 13) and TNBS colitis treated with control IgG (n = 11) or IL-6 Ab (n = 13). Colitis was induced at day 0 (age, 26 days). After day 0, food intake was significantly less in both groups with TNBS colitis compared with healthy controls (all $P < 0.001$), but there were no differences between the control IgG- and IL-6 Ab-treated groups (all $P > 0.05$). Values are given as mean ± SEM.

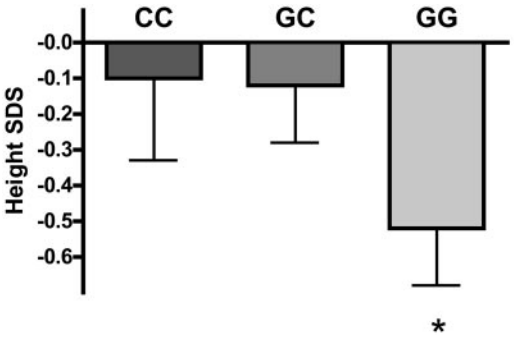

Fig. 3. Height at diagnosis varies by IL-6 −174 genotype in children with Crohn's disease. Height SDS was significantly more reduced in the children with the GG genotype at diagnosis ($n = 153$). *, $P = 0.031$ for GG vs. GC/CC. All children were of Northern European, Caucasian origin. Values are given as mean ± SEM.

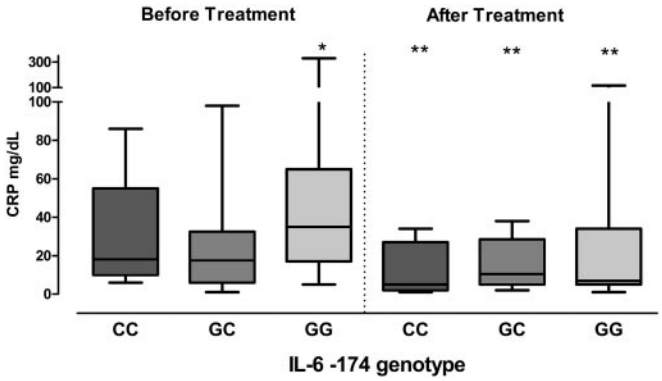

Fig. 4. Plasma CRP concentration at diagnosis varies by IL-6 −174 genotype in children with Crohn's disease. CRP level at diagnosis was greater in the children with the GG genotype. *, $P = 0.028$ for GG vs. GC/CC before treatment. Genotype has no effect on CRP level after the initial course of treatment to induce remission. **, $P < 0.001$ for effect of treatment on CRP. Values are given as the median (interquartile range). $n = 73$ pairs of observations.

hypothesized that the genetic regulation of IL-6 expression may mediate growth retardation in children with Crohn's disease. We examined the IL-6 −174 genotype in 153 patients whose Crohn's disease was diagnosed in childhood. Their mean age at diagnosis was 11.5 (SD, 2.7) years, and 99 (65%) were male. Their genotypes were CC, 34; GC, 60; and GG, 59, with no difference between the 110 English and 43 Swedish children ($P = 0.8$). The genotypes of the 351 controls were CC, 72; GC, 131; and GG, 148, with no difference between the 204 English and 147 Swedish control samples ($P = 0.8$). There was no difference in distribution of genotypes between the 153 children with Crohn's disease and the 351 controls ($P = 0.7$). All patients and controls were of Northern European, Caucasian origin.

The height at diagnosis of the 153 cases was reduced significantly, with a mean SDS of −0.26 (SEM, 0.10, $P = 0.012$). Their parents' heights were normal. The mean maternal height SDS was 0.09 (SD, 1.09), and the mean paternal height SDS was 0.08 (SD, 0.89), for 149 and 147 individuals, respectively, demonstrating that the impaired growth of the children in the study group was due to their untreated Crohn's disease. At diagnosis, children with the IL-6 −174 GG genotype were significantly more growth-retarded compared with the combined GC/CC group (mean height SDS, −0.51 vs. −0.1; SEM, 0.16 vs. 0.13; $P = 0.031$) (Fig. 3).

Children with the IL-6 −174 GG Genotype Had Higher CRP Levels During Active Disease. To test whether the IL-6 genotype is contributory only during active inflammation, we recorded CRP, which is an acute-phase respondent that is induced by circulating IL-6. There were 73 children who had CRP measured both before and after their initial therapeutic course. Before therapy, CRP was significantly higher in children with the −174 GG genotype than those with the GC/CC genotypes (median, 36 vs. 18 mg/dl, $P = 0.028$), (Fig. 4). After 6 weeks of treatment, CRP levels significantly reduced from a median of 21.5 to 9.5 mg/dl for all groups ($P < 0.001$). However, after treatment, cases with the −174 GG genotype no longer had higher CRP levels compared with the GC/CC group ($P = 0.68$; Fig. 4).

Discussion

Linear growth is central to human development, yet the pathological mechanisms that potentially disturb it are poorly understood. After infancy, growth hormone acting through IGF-I is the main determinant of linear growth velocity (37, 38). The actions of IGF-I, in turn, are modulated by a series of IGF binding proteins, principally, IGFBP-3 (39, 40). Although growth-hormone expression is normal in Crohn's disease, reports (41) repeatedly have demonstrated a decreased IGF-I, with concomitant growth retardation. Because undernutrition depresses IGF-I, growth failure in children with Crohn's disease has been ascribed to poor nutrient intake (11, 42–44). However, our previous animal work showed that the inflammatory process depressed growth and IGF-I independently of nutrition (20). Comparison with pair-fed healthy controls established that inflammation contributed 40% of the growth deficit observed in the TNBS colitis model, which like Crohn's disease, is characterized by raised IL-6 (20).

This study demonstrates that blocking the effect of IL-6 enhances growth in the TNBS colitis model. IL-6 Ab did not reduce intestinal inflammation or improve nutrient intake. Thus, the enhanced growth was due to the decrease in circulating IL-6. If IL-6 regulates growth in inflammatory diseases, we hypothesized that genetic variations in IL-6 expression would vary the consequences of disease activity on growth retardation. We chose to study the well characterized IL-6 −174 polymorphism (45–49) because variations in this region of the IL-6 promoter are associated with variations in the IL-6 response. This study shows that the IL-6 −174 genotype determines growth retardation at diagnosis in childhood-onset Crohn's disease. However, there is no association of this genotype with height in healthy people, in whom the promoter would not have been active (50).

Because height was measured accurately at diagnosis, we excluded a confounding effect of treatment, particularly from corticosteroids, which can suppress growth. Also, we demonstrate that the genetic basis for the variation in height is distinct from that causing a susceptibility to Crohn's disease. The IL-6 –174 genotype conferred no propensity toward developing the disease, confirming other work describing a lack of association of the IL-6 gene with Crohn's disease (51). This effect of the IL-6 promoter is in marked contrast to polymorphisms in the *NOD-2* gene which are increased in Crohn's disease (52, 53), yet do not affect growth (54).

We did not measure IL-6 levels in children at diagnosis, because routine clinical practice does not require this measurement. Also, human research studies approval was not obtained to measure IL-6 routinely in children. However, IL-6 directly induces CRP, which is widely used clinically. By using CRP as a measure of IL-6 activity, we showed that children with the GG genotype also had the highest concentrations during active inflammation. Our results support the suggestion of De Benedetti *et al.* (18) that an IL-6-mediated decrease in plasma IGF-I inhibits growth. In the TNBS colitis model, we have shown (20) that exogenous administration of IGF-I increases plasma

IGF-I concentrations. Injecting IGF-I resulted in an increase in linear growth, indicating that a reduction in circulating IGF-I does indeed contribute to the growth deficit. Nevertheless, IL-6 could, in theory, also affect the growth plate, because it may alter bone metabolism (55).

The conclusions drawn from the animal experiments depend on demonstrating that the IL-6 Ab used did not suppress intestinal inflammation. However, this lack of suppression is in contrast to a number of other blocking Abs, the actions of which are not limited to inhibiting circulating cytokines; in other experiments using an anti-TNF-α Ab, we observed decreased intestinal inflammation (data not shown). In clinical practice, when used as therapy for Crohn's disease, anti-TNF-α Ab (infliximab) reduces intestinal inflammation.

Researchers have debated why the IL-6 −174 genotype is important in controlling IL-6 transcription. Fishman *et al.* (25) speculated that lower transcription by the IL-6 −174 CC genotype was due to the creation of a binding site for transcription factor nuclear factor 1, a known suppressor in HeLa cells (56). This conclusion was subsequently challenged by Terry *et al.* (57), who reported that transcription of IL-6 differed depending on the type of transfected cell line. Certainly, many factors control IL-6 transcription (58), and the effect of a particular polymorphism probably depends on the specific milieu and tissue that are involved. The IL-6 −174 genotype is inherited as a haplotype in association with two other polymorphisms and a variable-nucleotide tandem repeat. Although it has been speculated that there may be a potential glucocorticoid receptor at position −557 to −552, the sequence around the −572 site does not have a strong homology to any known transcriptional factor binding site. Thus, these observations, together with the wealth of IL-6 −174 genotype-association studies (45–50), indicate that, in Northern Europeans, the IL-6 −174 genotype is probably the main promoter polymorphism determining variation in IL-6 induction.

Our results demonstrate that IL-6 regulates growth during intestinal inflammation. In children of Northern European background with Crohn's disease, the IL-6 −174 polymorphism mediates growth failure but it is not associated with susceptibility to the disease. The study indicates that the use of growth-sparing therapies should be a priority in children with the IL-6 −174 GG genotype. For example, enteral feeding induces remission without inhibiting growth (12, 59). Such treatment, now used as a primary therapy in many pediatric gastroenterology units, results in a rapid fall in IL-6, followed by significant increases in IGF-I (16). Although enteral feeding is the only growth-sparing treatment that has been used in randomized trials with height as an end point, anti-IL-6 receptor Ab therapy has been used in adults (60), although is not yet available for children with Crohn's disease. We speculate that these findings could be relevant to other inflammatory conditions in childhood associated with growth impairment.

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