Increased bacterial urease activity in faeces in juvenile chronic arthritis: Evidence of altered intestinal microflora?

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PAEDIATRIC RHEUMATOLOGY

INCREASED BACTERIAL UREASE ACTIVITY IN FAECES IN JUVENILE CHRONIC ARTHRITIS: EVIDENCE OF ALTERED INTESTINAL MICROFLORA?

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SUMMARY

The intestinal microflora was indirectly evaluated in juvenile chronic arthritis (JCA) by analysing enzyme activities—urease, β-glucosidase and β-glucuronidase—in faeces. In 18 out of 26 JCA patients, the illness had been diagnosed during the past year. The control group was composed of eight age-matched control patients and 18 family members of JCA patients (3–36 yr). The mean [95% confidence interval (CI)] urease activity, but not the activities of β-glucosidase and β-glucuronidase, in faeces from the JCA group differed from that in the control group: 32.3 (26.6–38.1) nmol/min/mg protein *vs* 24.0 (16.8–31.6), $P = 0.07$. The difference was more marked in a comparison of JCA patients with family members ($P = 0.03$). In a subgroup of subjects, the effect of 10 days oral bacteriotherapy with *Lactobacillus* GG on faecal enzyme activities was then investigated ($n = 8$ JCA patients, $n = 8$ control patients). This short-term oral bacteriotherapy reduced the increased urease activity in faeces of JCA patients. Keeping in mind the small number of subjects, it may be inferred from the present results that the increased urease activity in JCA is specific for the disease, suggesting altered intestinal microflora in JCA.

KEY WORDS: Juvenile chronic arthritis, Intestinal microflora, Urease, β-glucosidase, β-glucuronidase.

CIRCUMSTANTIAL evidence has accrued suggesting a relationship between various gastrointestinal disorders and joint diseases. Impairment of the intestine's mucosal barrier function, allowing constant and aberrant antigen challenge, may be the key factor in the pathogenesis of rheumatoid arthritis in a genetically susceptible host [1–3].

The indigenous microflora is an important component in the intestine's defence barrier [4]. It limits colonization by enteric pathogens and absorption of harmful antigens from the gut lumen. There is some evidence that part of this defence barrier is abnormal in rheumatoid arthritis [5–7]. Investigation of the intestinal microflora may have been hampered by its very complexity. Furthermore, the classical bacterial culture techniques have proved insensitive and laborious [4, 8]. They are not able to show subtle changes in the composition and interactions of the intestinal microflora. Determination of intestinal bacterial metabolites, such as bacterial enzyme activities, may be more revealing. Although these activities detect particularly the metabolic activity of intestinal bacteria, they may also reflect the quantitative composition of the microflora, which is not seen by the classical culture techniques [9]. We therefore used indirect methods to evaluate the faecal flora in juvenile chronic arthritis (JCA). We analysed the enzyme activities (urease, β-glucosidase and β-glucuronidase) in faeces from patients with JCA and compared the results with those from control patients and close relatives of JCA patients.

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Lactobacilli constitute an important and active part of the intestinal microflora, and they are used in food supplements to improve the host's microbial balance [10]. Oral bacteriotherapy with specific strains of *Lactobacillus* has been shown to be successful in various gastrointestinal disorders [11, 12]. In our previous studies with viral gastroenteritis, *Lactobacillus* strain GG (*Lactobacillus* GG) was able to shorten the duration of the diarrhoeal phase and reduce elevated faecal urease activity [11]. We have also found by *in vitro* study that *Lactobacillus* GG is resistant to numerous pharmaceutical agents including anti-rheumatic drugs: sulphasalazine, hydroxychloroquine and auranofin [13]. Here, we analysed whether oral introduction of *Lactobacillus* GG could modify the enzyme activities in faeces in JCA.

METHODS

Study design

The study consisted of two parts. In the first part, the enzyme activities (urease, β-glucosidase and β-glucuronidase) in faeces were investigated in JCA patients as compared to controls, i.e. control patients and members of JCA patients' families. In the second part, a study was made of the effect of 10 days oral bacteriotherapy with *Lactobacillus* GG on the enzyme activities in faeces from JCA patients and age-matched control patients.

Study subjects

Part I. Faecal samples were obtained from 26 patients with JCA (the JCA group), aged 1–15 (mean 8) yr, and 26 controls (the control group), aged 3–46 (mean 18) yr. The control group comprised eight control patients, aged 4–15 (mean 9) yr, and 18

TABLE I
Clinical characteristic of JCA patients and controls

	JCA ($n = 26$)	Controls ($n = 26$)
Female/male	15/11	18/8
Age: mean (range) yr	8 (1–15)	18 (3–46)
JCA type (young onset)*		
Oligoarthritis	19 (14)	
Polyarthritis	6 (1)	
Spondyloarthropathy	1 (1)	
HLA-B-27 antigen		
Positive	8 (4)	
Negative	18 (12)	
Duration of disease: mean (range) months	10 (2–50)	
Active arthritis†	12	
Medical treatment†		
No	1	
Yes‡	25	
Anti-rheumatic	23	
Hydroxychloroquine	12	
Gold	5	
Sulphasalazine	5	
Prednisolone *per os*	1	
with NSAID	20	
NSAID alone	2	

*Young onset < 6 yr.
†Number of patients.
‡One patient may have one or more medical treatments.

members of JCA patients' families, aged 3–46 (mean 22) yr. In addition, a faecal sample was also obtained from 11 pairs of healthy siblings, aged 2–15 (mean 7) yr, to evaluate possible differences in enzyme activities in faeces between healthy siblings of the same households.

The patients with JCA participating in the study were making scheduled visits to Tampere University Hospital. The diagnosis of JCA, based on the European League Against Rheumatism criteria [14], had been made in 18 patients during the past year and in the others during the past 4 yr. The JCA patients were suffering from oligoarthritis (73%) or seronegative polyarthritis (23%), except for one who had spondyloarthropathy. The disease was considered active if one or more joints was currently inflamed. The criteria of joint inflammation were clinical swelling or limitation of motion with heat, pain or tenderness. Of the 26 patients with JCA, 18 had signs of active arthritis estimated by PV. The clinical data of JCA patients are given in Table I.

Of the eight control patients, six were examined in the out-patient clinic of Tampere University Hospital for abdominal pain, elevated blood pressure, short stature, repeated urinary tract infections or urinary incontinence. In addition, two control patients were admitted to the Division of Paediatric Surgery of the hospital for operation on minor malformations. The examinations or the clinical history of the study subjects in the control group and pairs of healthy siblings showed no evidence of joint or intestinal disease. Information was obtained on the diet and defaecation frequency of all study subjects by questionnaire or interview. Three JCA patients had some dietary restriction: one had a gluten-free diet, two did not eat fish, peanuts, strawberries, citrus fruits and chocolate. None of the study subjects in the control group or pairs of healthy siblings had any dietary restrictions, except for one who did not eat fish, peanuts, strawberries, citrus fruits and chocolate. None of the study subjects had received any antimicrobial treatment during the previous 2 months, except for one control who was on nitrofurantoin medication for the prevention of urinary tract infections. None of the study subjects in the control group or pairs of healthy siblings had received any NSAID therapy during the previous 2 months.

Part II. Eight patients with JCA, aged 2–14 (mean 8) yr, and their eight age-matched control patients were randomly chosen for 10 days oral bacteriotherapy with *Lactobacillus* GG. The clinical data of the study subjects are presented in Table II.

The strain chosen for the bacteriotherapy was *Lactobacillus* GG. It is a human strain and has a good safety record [15]. A freeze-dried *Lactobacillus* GG (ATCC 53103) powder (Valio Ltd, Helinski, Finland) was administered orally at a dose of 10^{10} colony-forming units twice daily for 10 days. The powder was mixed in liquid prior to ingestion.

Samples. All faecal samples were handled in the same way: immediately after spontaneous defaecation, the sample was cooled and stored at +6°C for transporting. Within a maximum of 12 h, the samples were delivered in a cold-transport box, frozen and stored (−50°C) until analysis. In Part II, a faecal sample was obtained before the introduction of *Lactobacillus* GG and within 0–2 days after cessation of the bacteriotherapy in all study subjects. One month after cessation of the bacteriotherapy, a faecal samples was obtained from four JCA and five control patients.

TABLE II
Clinical characteristic of eight JCA patients and controls selected for *Lactobacillus* GG treatment, study II

	JCA ($n = 8$)	Controls ($n = 8$)
Female/male	4/4	4/4
Age: mean (range) yr	8 (2–14)	9 (4–15)
JCA type (young onset)*		
Oligoarthritis	6 (6)	
Polyarthritis	1 (0)	
Spondyloarthropathy	1 (0)	
HLA-B-27 antigen (young onset)		
Positive	2 (2)	
Negative	6 (4)	
Duration of disease: mean (range) months	15 (5–50)	
Active arthritis†	6	
Medical treatment†‡	8	
Anti-rheumatic	8	
Hydroxychloroquine	3	
Gold	2	
Sulphasalazine	3	
with NSAID	7	

*Young onset < 6 yr.
†Number of patients.
‡One patient may have one or more medical treatments.

Determination of bacterial enzyme activities in faeces

Frozen faecal samples were thawed at +4°C. After weighing them in their plastic bags, 0.1 M potassium phosphate buffer (pH 7.0) was added (1:9) and the mixture was homogenized using a laboratory blender (Stomacher 400, Seward Medical, London). Thereafter, the samples were sonicated by ultrawave sonicator (4 × 15 s) and centrifuged at 500 *g* for 10 min at +4°C. The supernatant fraction was used for analysis.

In the assay for urease (EC 3.5.1.5) activity, the reaction mixture (1 ml) contained 0.02 M potassium phosphate buffer (pH 7.4), 10 mM urea and 0.2 ml of the faecal supernatant. The enzyme reaction was run at +37°C, and stopped at 10 and 20 min by adding 9 ml of 0.1 M sulphuric acid. Ammonia was determined with a specific ammonia electrode (Model No. 95-12, Orion, Helsinki, Finland) after addition of 1.0 ml of 10 M sodium hydroxide. Protein was measured in the faecal supernatant by the Lowry method [16] and using bovine serum albumin as standard. β-Glucosidase (EC 3.2.1.21) activity (substrate 2 mM *p*-nitrophenyl-β-D-glucopyranoside; Sigma Chemical, St Louis, MO, USA) and β-glucuronidase (EC 3.2.1.31) activity (substrate 1 mM phenolphthalein-β-glucuronic acid; Sigma Chemical) were determined at +37°C as described by Freeman [17].

Ethics

Informed consent was obtained from study subjects or their parents, and the study was approved by the ethical committee of Tampere University Hospital.

Statistics

The enzyme activities in faeces are expressed as means with 95% confidence interval (CI). A two-tailed Student's *t*-test and analysis of variance (ANOVA) with paired contrasts were used to determine differences in enzyme activities between the study groups. Spearman's rank correlation coefficient was used to determine the correlation between enzyme activities and the duration of the disease. The repeated observations were studied using ANOVA for repeated measures and a paired *t*-test.

RESULTS

Enzyme activities in faeces

The urease activity in faeces differed between the JCA group and the control group, i.e. the control patients and members of JCA patients' families (Fig. 1). The mean (95% CI) activity of urease was 32.3 (26.6–38.1) nmol/min/mg protein in the JCA group *vs* 24.0 (16.8–31.6) in the control group ($t = 1.80$, $P = 0.07$). By contrast, the activities of β-glucosidase and β-glucuronidase in faeces were comparable between the groups. The mean (95% CI) activities of β-glucosidase were 6.6 (5.3–7.8) nmol/min/mg protein in the JCA group *vs* 6.5 (5.3–7.8) in the control group ($t = 0.04$, $P = 0.97$) and that of β-glucuronidase 2.8 (2.0–3.7) *vs* 2.9 (2.1–3.7), respectively ($t = 0.05$, $P = 0.96$).

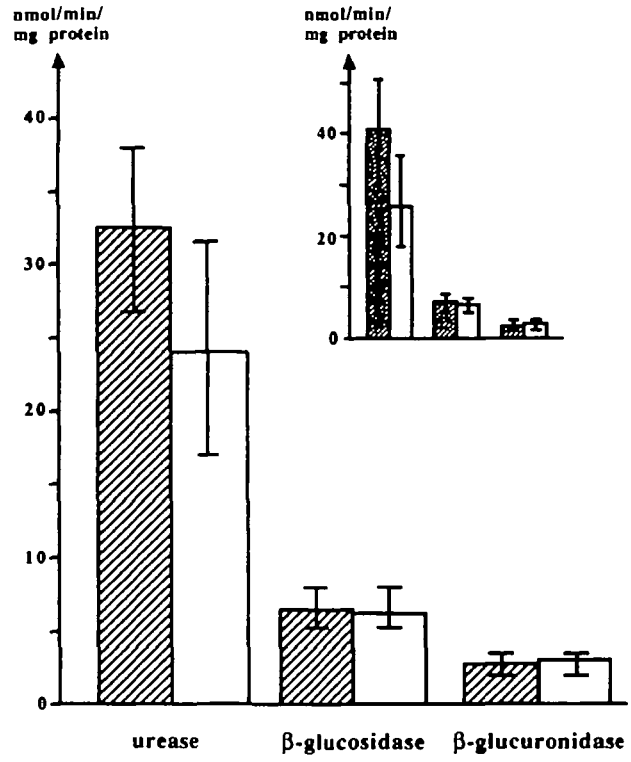

FIG. 1.—The mean (95% CI) activity of urease ($t = 1.80$, $P = 0.07$), β-glucosidase ($t = 0.04$, $P = 0.97$) and β-glucuronidase ($t = 0.05$, $P = 0.96$) in faeces in the JCA group, $n = 26$ (hatched column), and the control group, $n = 26$ (open column), in the large panel. The small panel shows separately faecal enzyme activities from JCA patients, $n = 18$ (hatched column), compared to family members, $n = 18$ (open column): urease ($t = 2.30$, $P = 0.03$).

In a separate comparison of the 18 JCA patients to family members, the faecal urease activity, but not the activities of β-glucosidase and β-glucuronidase, was seen to be significantly enhanced in the JCA patients (Fig. 1). The mean difference in urease activity between JCA patients and their relatives was 14.5 nmol/min/mg protein with 95% CI from 2.0 to 27.0 ($t = 2.30$, $P = 0.03$). No such difference was detected between the 11 healthy pairs of siblings in control families: the mean difference in urease activity was 3.3 nmol/min/mg protein with 95% CI from −3.6 to 10.3 ($t = 1.06$, $P = 0.31$).

The elevated urease activity in faeces from JCA patients compared to controls could not be explained by the activity of the disease, the duration of JCA or the type of JCA (oligoarthritis *vs* polyarthritis). Neither did the presence of HLA-B-27 antigen explain the elevated urease activity in JCA patients with young-onset disease ($F = 0.05$, $P = 0.80$) or older-onset disease ($F = 2.09$, $P = 0.20$). Twenty-two JCA patients received NSAID therapy (naproxen) and only four patients did not. The mean (95% CI) activities of urease, β-glucosidase and β-glucuronidase were significantly lower ($P < 0.05$) in JCA patients on NSAID therapy than in those without

TABLE III
The mean (95% CI) enzyme activities (nmol/min/mg protein) in faeces from 26 JCA patients with (+) or without (−) NSAID or anti-rheumatic medication. One patient may have one or more medications

Medication	Urease	β-Glucosidase	β-Glucuronidase
NSAID			
+(n = 22)†	30.0 (23.8–36.0)*	6.1 (4.9–7.3)*	2.4 (1.5–3.3)*
−(n = 4)	45.8 (25.1–66.5)	9.6 (2.8–16.3)	4.6 (2.2–6.9)
Hydroxychloroquine			
+(n = 12)	30.7 (21.2–40.3)	7.5 (5.7–9.2)	3.0 (1.5–4.5)
−(n = 14)	32.4 (23.5–41.2)	5.8 (3.9–7.7)	2.5 (1.5–3.4)
Gold			
+(n = 5)	38.2 (26.8–49.6)	5.7 (3.7–7.6)	18 (0.3–3.3)
−(n = 21)	30.1 (22.9–37.2)	6.8 (5.3–8.3)	2.9 (2.0–3.9)
Sulphasalazine			
+(n = 5)	31.6 (11.6–51.6)	4.6 (0.1–9.2)	3.3 (0.4–6.2)
−(n = 21)	31.6 (24.8–38.5)	7.0 (5.8–8.3)	2.6 (1.7–3.4)

*Significant difference compared to patients without therapy.
†Number of patients.

(Table III). By contrast, anti-rheumatic medication, i.e. hydroxychloroquine, orally administered gold and sulphasalazine, had no statistically significant effect on the enzyme activities in faeces (Table III).

The effect of Lactobacillus GG on enzyme activities in faeces

Judging from information obtained in interviews, oral administration of *Lactobacillus* GG had no clinical effects on control patients. It did not cause any changes in the clinical status of the JCA patients, but the consistency of stools changed during the therapy from watery to normal in two cases and from normal to watery in one.

As shown in Fig. 2, 10 days oral bacteriotherapy with *Lactobacillus* GG had an effect on the activity of urease, but not on the activities of β-glucosidase and β-glucuronidase, in faeces. The effect was specific for JCA: the mean (95% CI) urease activity decreased from 32.5 (18.6–46.4) nmol/min/mg protein to 18.5 (5.6–31.4) in patients with JCA. The ANOVA for repeated measures confirmed the concept that JCA patients and their controls had different outcomes during *Lactobacillus* GG therapy: the faecal urease activity in JCA patients decreased, while in control patients it remained unaltered ($F = 6.14$, $P = 0.02$). One month after cessation of oral bacteriotherapy, the faecal urease activity in JCA patients remained at a reduced level (Fig. 2) and the changes in faecal urease activity after the cessation of therapy were not statistically significant in either group ($F = 0.49$, $P = 0.51$).

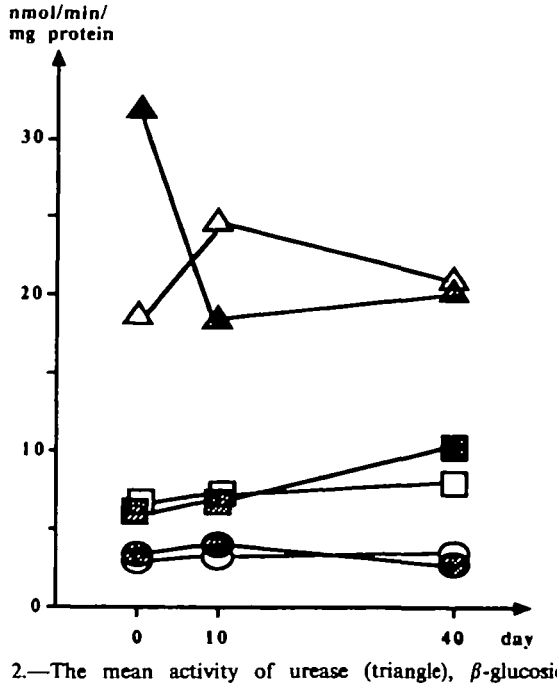

FIG. 2.—The mean activity of urease (triangle), β-glucosidase (square) and β-glucuronidase (circle) in faeces from patients with JCA (hatched symbol) and controls (open symbol) before (day 0), immediately after (day 10) the *Lactobacillus* GG therapy and 1 month later (day 40).

DISCUSSION

Dysfunction of the intestine's mucosal barrier as a consequence of abnormal intestinal microflora and/or intestinal inflammation may be the interconnecting link between the gut and joint in inflammatory joint diseases [1–3]. A persistently impaired mucosal barrier function may also be responsible for perpetuation of the arthritis. This concept is supported by observations that patients with rheumatoid arthritis gain relief of clinical symptoms by fasting or specific dietary regimens [18–20]. Sulphasalazine therapy also diminishes the signs of active joint disease, possibly by altering the intestinal microflora [21, 22].

To our knowledge, glycosidase (β-glucosidase and β-glucuronidase) or urease activities in faeces from patients with rheumatological diseases have not previously been reported. The intestinal microflora is considered to be a major source of β-glucosidase and β-glucuronidase [9] and urease [23, 24] in the intestine. Analysis of enzyme activity does not cover specific bacteria, but the activity levels of constitutive bacterial enzymes in the intestine can be relied on as indicators of changes in the quantitative composition of the intestinal microflora [9]. β-Glucosidase and urease of certain bacterial species are constitutive enzymes reflecting the amount of bacteria producing them [9, 24]. By contrast, β-glucuronidase is an enzyme inducible by its substrate, reflecting particularly the metabolic state of the intestinal bacteria [9].

Our study indicates that in JCA, specifically the activity of urease, but not the activities of β-glucosidase and β-glucuronidase, in faeces is increased. In the gastrointestinal tract, urease is produced by numerous species of bacteria, mainly anaerobes [23–25]. Urease catalyses the hydrolysis of urea, a major nitrogenous waste product of mammals, to yield ammonia and carbon dioxide [24]. Urease and ammonia enable pathogenic bacteria to survive in the gastrointestinal tract and contribute to mucosal tissue damage [24, 25].

Our finding of increased urease activity in faeces from JCA patients may reflect a disturbance in the population of anaerobic microflora. Such a concept is parallel with recent observations made by Eerola *et al.* [26] using analysis of bacterial cellular fatty acids in faeces by direct gas–liquid chromatography.

There is continuing debate as to whether the disturbed gut mucosal barrier in rheumatoid arthritis is a specific triggering agent or secondary to environmental factors, e.g. diet or medical therapy [2, 27–29]. Investigation of the intestine's mucosal barrier in rheumatoid arthritis or JCA is hampered by the difficulty of obtaining a study population who have not ingested even NSAID. Also, in our study, all of the JCA patients except one were receiving medication (NSAID alone or with one or more anti-rheumatic drugs) and only four patients were not on NSAID therapy. It is therefore difficult to separate the effects of disease and therapy as a cause of increased urease activity in faeces. Thus, the results of medication effect on faecal enzyme activities have to be interpreted with caution. Regular long-term use of NSAID in patients with rheumatoid arthritis has been associated with intestinal inflammation, increased intestinal permeability and altered intestinal microflora [27–29]. In our study, in JCA patients with NSAID therapy the mean enzyme activities in faeces were lower than in those without therapy. Thus, NSAID therapy in JCA patients appeared to counteract increased urease activity in faeces, although the mean urease activity in JCA patients on NSAID therapy was still higher than in controls. NSAID therapy may alter the metabolic milieu in the intestine. This may in turn alter the composition of the intestinal microflora or reduce the amount of substrates for enzymes, which are detected as decreased enzyme activities in faeces. Moreover, there is evidence that diminished prostaglandin synthesis in the intestinal mucosa as a consequence of NSAID therapy may diminish glycoprotein secretion [30, 31]. This may result in a reduced amount of substrates for enzymes or enzyme-producing bacteria. In JCA, the faecal enzyme-decreasing effect of NSAID may be host protective by strengthening the gut mucosal barrier.

Increased urease activity in faeces may reflect imbalanced microflora in JCA. It is of interest to observe that short-term oral bacteriotherapy with *Lactobacillus* GG was able to counteract this alteration. The beneficial effects of *Lactobacillus* GG have been related to the production of antimicrobial substances and interference with the survival of pathogens [10]. *Lactobacillus* GG is also able to strengthen the immunological barrier of the intestine in gastrointestinal disorders by promoting IgA immune response [12, 13]. The urease-reducing effect of *Lactobacillus* GG in JCA was also noticeable 1 month after cessation of oral bacteriotherapy, although this must be regarded as a preliminary result due to the small number of samples we were able to analyse at this point. We suggest that increased faecal urease activity in JCA may be specific for the disease and not due to external factors such as medication. It is further suggested that the results may indicate altered mucosal barrier function in JCA. A final note of caution has to be added because of the small numbers of patients studied. However, further investigations are under way to confirm the present preliminary results in a larger group of subjects.

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