Evaluation of intestinal permeability in patients with inflammatory bowel disease using lactulose and measuring antibodies to lipid A

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Abstract

This study looked at the intestinal permeability and the immune response to enteric bacterial antigens in patients with inflammatory bowel disease (IBD). They were evaluated by using a lactulose tolerance test and measuring blood anti-lipid A antibody concentrations, respectively. The lactulose tolerance tests were performed 22 times in 14 patients with Crohn's disease (CD), 19 times in 12 patients with ulcerative colitis (UC), and 12 times in 12 healthy controls. Blood lactulose concentrations were measured after oral administration every two hours for eight hours, also blood C reactive protein concentrations and anti-lipid A antibody concentrations were measured just before lactulose administration. Blood lactulose concentrations were significantly higher in patients with CD than in the controls from two to eight hours after administration, while in UC they were significantly higher than in the controls from six to eight hours. Maximum blood lactulose concentrations in each tolerance test in patients with the active phase significantly exceeded those in the inactive phase of either CD or UC. A significant correlation was also seen between the maximum blood lactulose concentrations and the C reactive protein concentrations. Blood anti-lipid A antibody concentrations in patients with CD were significantly higher than in the controls as well as in patients with UC in immunoglobulin (Ig) A class and IgG class. In UC they were significantly higher than in the controls in IgA class. But, they were not related to the severity of the disease of either CD or UC, and not correlated significantly with the maximum blood lactulose concentrations in either CD or UC. The intestinal permeability and the immune response to enteric bacterial antigens in patients with inactive CD were significantly increased over those in the controls as well as in patients with inactive UC. These findings suggest that an increase of the intestinal permeability and that of producing antibodies to enteric bacterial antigens are both important for the pathogenesis of IBD, and that the characteristics of CD and UC differ.

(Gut 1995; 36: 891-896)

Keywords: intestinal permeability, inflammatory bowel disease, lactulose, lipid A.

The causes of inflammatory bowel disease (IBD), that is Crohn's disease (CD) and ulcerative colitis (UC), and the mechanisms that lead to chronic disease are still unclear. The fundamental pathogenesis of IBD is that bacterial infection, dietary antigen or another exogenous antigen is triggered, and immune system abnormalities lead to IBD.¹⁻⁴ Although IBD has common characteristics such as chronic inflammation and resistance to treatment, CD has features differing from UC such as granuloma formation, involvement of all layers of the bowel wall, and involvement of entire digestive tract.⁵ It has been reported that in the studies of immunoglobulin (Ig) G subclass in patients with IBD, IgG 2 concentrations increased in patients with CD, while IgG 1 concentrations increased in UC, in both the involved tissue and the blood.⁶⁻⁸ These findings seem to suggest that the target and the immune response differ between CD and UC, thereby suggesting individual pathogenesis. Such differences between CD and UC present interesting suggestions about the causes of IBD.³ Among the many causative factors, the intestinal permeability, which indicates the ease of entry of exogenous agents, and the immune response to such agents are considered to be important for the pathogenesis of IBD.^{1-4 6-22} We therefore evaluated intestinal permeability in patients with IBD, simultaneously measuring blood C reactive protein and anti-lipid A antibody concentrations to enteric bacterial antigens, and assessed their clinical significance including differences between CD and UC. In this study, to evaluate the permeability in patients with CD and UC directly and simply, we used lactulose only and measured its blood concentrations.

Methods

Patients and controls

We studied 14 patients with CD (mean age 27.8 years, range 15-38, male/female ratio 7/7) and 12 patients with UC (mean age 25.9, range 15-47, male/female 7/5). Diagnosis of CD and UC included clinical, radiographic, endoscopic, and histological evaluation. There were 12 healthy controls (mean age 27.7, range 24-36, male/female 6/6). The lactulose tolerance test was performed 22 times in patients with CD (15 times during active phase and seven times during inactive phase), 19 times in UC (13 times during active phase and six times during inactive phase), and 12 times in the control subjects. The disease activity of CD

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Accepted for publication
11 October 1994

Figure 1: Reproducibility of the lactulose tolerance test in six control subjects. (A) Changes in the blood lactulose concentration (mean (SD)) measured at intervals of two hours showed a close agreement between the values obtained in the first and second studies. (B) The individual evaluation of blood lactulose concentration at two hours and four hours in the study showed no significant differences between the first and second studies (Wilcoxon's test).

was assessed in accordance with IOIBD assessment.²³ The active phase of CD was defined as a score of ≥ 2 and C reactive protein positive and the inactive phase as a score of ≤ 1 and C reactive protein normal. UC was considered to be inactive, when the symptoms were resolved and no inflammation was seen macroscopically. The severity of the mucosal damage in UC was categorised endoscopically into four grades in accordance with the Matts classification.²⁴ When categorised by the extent of the lesion, with CD 20 were the small-large intestine type and two were the large intestine type, while with UC 13 were the total colitis type and six were the left sided colitis type. None of the patients had a history of surgery. Informed consent was obtained from all patients and controls.

Lactulose tolerance test

The lactulose tolerance test was performed in subjects who had been fasting for more than nine hours since the previous dinner. After collecting blood specimens in the morning, each subject drank 0.5 ml/kg of lactulose syrup containing 650 mg of lactulose per ml. Blood specimens were then collected every two hours for eight hours with the subject resting in bed and fasting. The obtained plasma was used as the sample.

Urine specimens were also collected every two hours in two patients with active CD. In three patients with active UC and six control subjects, urine was collected in three consecutive periods: 0–4, 4–8, and 8–12 hours. The

urine samples were assayed by the same method as the blood samples, and then the urinary excretion of lactulose was evaluated as a ratio to the dose given orally. Data were used to assess a correspondence between the blood concentration and the urinary concentration of lactulose.

To confirm the location of the lactulose in the intestine, we gave a small quantity of barium to drink with the lactulose syrup in three patients in inactive CD, two patients in inactive UC, and three control subjects. We then confirmed the location of the barium in the intestine by x ray every two hours.

Measurement of blood lactulose concentration

Lactulose concentrations in the samples were determined by the method previously described by Hinohara *et al.*²⁵ The sample (2 ml) was mixed with an equal volume of 0.015 M sodium borate, and loaded on a column filled with Dowex 1 $\times$ 8 resin (Borate type) (Dow Chemical, Midland, Michigan, USA). After flushing with 150 ml of 0.0075 M sodium borate and discarding the eluate, we loaded 80 ml of 0.05 M sodium borate and eluted lactulose. Cysteine, carbazole, and H₂SO₄ were added to this eluate to develop colour. Absorbance was measured at 540 nm, and the blood lactulose concentration was determined using a standard curve. We set the pre-administration value equal to 0, and assessed the blood lactulose as the increase in concentration after administration.

Reproducibility

In six of 12 control subjects, the reproducibility of lactulose tolerance test was checked by repeating the study on a second occasion, 8–12 weeks after the first study. There was close agreement with no significant difference at each time point between the values obtained in the first and second studies (Fig 1). The mean variability between the first and second studies at each time point was 37%, 35%, 50%, and 33%, respectively.

Measurement of blood concentration of C reactive protein and antibody to lipid A

C reactive protein concentrations and anti-lipid A antibody concentrations were measured in blood collected on the test day. C reactive protein concentrations were determined quantitatively using a laser nephelometer (Hoechst Japan, Tokyo, Japan). Anti-lipid A antibody concentrations were measured by enzyme linked immunosorbent assay (ELISA) according to the method of Fink *et al.*²⁶ An amount of 100-fold diluted serum was added to lipid A (List Biological Laboratory, Campbell, California, USA) solidified antigen obtained from *Salmonella minnesota* R 595. The mixture was then interacted with peroxidase conjugated sheep anti-human IgA, IgM, and IgG antibodies as secondary antibodies. We added *o*-phenylene diamine peroxide, and then added H₂SO₄ to

Figure 2: Changes in the blood lactulose concentrations (mean (SD)) after oral administration in patients with Crohn's disease ($n=22$) and ulcerative colitis ($n=19$) compared with the control subjects ($n=12$). Blood lactulose concentrations significantly increased at all time points in patients with Crohn's disease and at six and eight hours in ulcerative colitis v controls. * $p<0.001$ v controls (Mann-Whitney U test).

stop the enzyme reaction. We then measured an absorbance at 492 nm. Anti-lipid A antibody concentration was expressed as the cut off value of the absorbance based on 180 healthy subjects including the 11 control subjects having the tolerance tests. We set the cut off value equal to 1.0 in each immunoglobulin class as follows: IgA was mean (2 SD), IgM was mean (SD), and IgG was mean (2 SD).

Statistical analysis

Values are shown as the mean (SD). Statistical difference of the blood lactulose concentrations between the first and second studies were analysed by Wilcoxon's test.

Figure 3: Comparison of the maximum blood lactulose concentrations (mean (SD)) in patients with Crohn's disease and ulcerative colitis. Each point represents the maximum blood lactulose concentration in each tolerance test. * $p<0.01$ and † $p<0.001$ v controls (Mann-Whitney U test).

Statistical difference of both the blood lactulose concentrations and the maximum blood lactulose concentrations among the various groups were analysed by the Mann-Whitney U test. A correlation between the maximum blood lactulose concentration and the Matts grade was analysed by Spearman's rank correlation. A correlation between the maximum blood lactulose concentration and the C reactive protein concentration was analysed by simple regression. A p value <0.05 was considered significant for all data analyses.

Results

Blood lactulose concentration in the tolerance test

Slight increases in the blood lactulose concentrations (mean (SD), $\mu\text{g/ml}$) were seen in the control subjects at two hours (8.4 (4.3)) and at four hours (7.0 (7.0)). Blood lactulose concentrations were significantly higher ($p<0.001$) in patients with CD than in the control subjects at all time points measured from two to eight hours (21.3 (6.1), 21.3 (11.4), 18.5 (11.2), and 9.0 (9.2), respectively) after administration. In patients with UC, the concentrations were significantly higher ($p<0.001$) than in the control subjects at six hours (18.6 (11.3)) and at eight hours (19.0 (14.5)) (Fig 2).

We compared the maximum blood lactulose concentrations in each tolerance test in patients with CD and UC. The lactulose concentrations (mean (SD), $\mu\text{g/ml}$) in patients with active CD (32.5 (8.5)) significantly exceeded those in the control subjects (10.5 (6.0), $p<0.001$) as well as those in patients with inactive CD (21.0 (6.1), $p<0.01$). The lactulose concentrations in patients with inactive CD also significantly exceeded those of the control subjects ($p<0.05$). The lactulose concentrations in patients with active UC (30.2 (12.1)) significantly exceeded those in the control subjects ($p<0.001$) as well as those in patients with inactive UC (16.3 (6.9), $p<0.05$). There were no significant differences in lactulose concentrations between the patients with inactive UC and the control subjects, nor were there any significant differences between the patients with active CD and active UC, or between the patients with inactive CD and inactive UC (Fig 3).

In patients with UC, the maximum blood lactulose concentrations were found to increase significantly as the Matts grade became higher ($r=0.836$, $p<0.001$) (Fig 4), suggesting an association with the severity of mucosal damage in this disease. Moreover, a significant correlation was found between the maximum blood lactulose concentration in each tolerance test and the C reactive protein concentration in patients with IBD and control subjects ($r=0.740$, $p=0.0001$, $n=53$, data not shown).

The urinary lactulose concentrations changed sequentially with the blood values, and the urinary excretion increased corresponding to the blood lactulose concentrations (Fig 5).

Figure 4: Maximum blood lactulose concentration (mean (SD)) in each tolerance test related to the macroscopic inflammatory grade assessed by Mats in patients with ulcerative colitis ($r=0.836$, $p<0.001$) (Spearman's rank correlation).

Blood concentration of antibody to lipid A

In IgA class, the concentrations of the anti-lipid A antibody in patients with CD were significantly higher than in healthy controls ($p<0.001$) as well as in patients with UC ($p<0.001$). The concentrations in patients with UC were also significantly higher ($p<0.001$) than in the controls.

In IgM class, there were no significant differences between the patients with either CD, UC, or the controls.

In IgG class, the concentrations in patients with CD were significantly higher than in the controls ($p<0.001$) as well as in patients with UC ($p<0.001$). There were no significant differences between the patients with UC and the controls.

There were no significant differences between the patients in the active phase and the inactive phase of either CD or UC in any immunoglobulin classes (Table), nor were there any significant differences according to the degree of mucosal damage in patients with UC. No significant correlation was found between the maximum lactulose concentrations and the anti-lipid A antibody concentrations in patients with either CD or UC in any immunoglobulin classes.

There were no significant differences in any parameters of this study between the patients with total colitis and left sided colitis in UC. As the large intestine type of CD involved only two cases, it was not possible to assess the differences in the extent of their lesions in CD.

Blood anti-lipid A antibody concentrations in IgA, IgM, and IgG class

	IgA	IgM	IgG
Control (n=180)	0.54 (0.23)	0.74 (0.26)	0.48 (0.26)
Crohn's disease (n=21)	1.28 (0.40)*†	0.78 (0.27)	1.39 (0.49)*†
Active (n=15)	1.27 (0.42)	0.77 (0.31)	1.37 (0.51)
Inactive (n=6)	1.29 (0.39)	0.81 (0.17)	1.43 (0.46)
Ulcerative colitis (n=17)	0.81 (0.24)*	0.86 (0.29)	0.62 (0.25)
Active (n=12)	0.83 (0.24)	0.87 (0.32)	0.63 (0.22)
Inactive (n=5)	0.74 (0.23)	0.84 (0.24)	0.59 (0.34)

Values are mean (SD).

* $p<0.001$ v controls. † $p<0.001$ v the patients with ulcerative colitis. (Mann-Whitney U test).

When confirming intestinal sites with barium, most of the barium had arrived in the large intestine after six hours in two patients with UC and three control subjects, and in three other patients with CD after four hours.

Discussion

Blood lactulose concentration in patients with IBD significantly increased over the controls, representing an increase of intestinal permeability in patients with IBD. The pattern of the sequential increases after administration differed in patients with CD and UC, perhaps reflecting differences in the sites of intestinal damage. An increase of blood lactulose concentration may represent a disturbance of the intestinal barrier function entailing mucosal changes such as erosion, ulceration, and oedema.¹⁸⁻²⁰

Maximum blood lactulose concentrations in patients in the active phase significantly exceeded those in the inactive phase of either CD or UC. The maximum lactulose concentration significantly correlated with the C reactive protein value as well as with the severity of the mucosal damage in UC. These findings suggest a relation between intestinal permeability and disease severity. In patients with inactive CD, the maximum blood lactulose concentrations significantly exceeded those in the control subjects, which differed from inactive UC. This seemed to result from differences in the pathophysiology between CD and UC. Even during the inactive phase of CD in which both a circulating inflammatory response became negative and an improvement in symptoms was seen, a few erosions sometimes remained in the intestine. Recovery of the mucosal barrier function may have been inadequate, and this may explain why the patients with CD have repetitive relapses for shorter periods than the patients with UC.²⁷⁻²⁹

We investigated the influence of enteric bacterial antigens in this hyperpermeable state using an anti-lipid A antibody, which represents an immune response to the antigens of Gram negative rods.³⁰ Blood anti-lipid A antibody concentrations were significantly higher in patients with CD than in UC in this study. Similar results have been reported by Kruis *et al*²¹ and Kamoi *et al*.²² Although there were no differences between CD and UC in the maximum lactulose concentrations, a difference in the production of antibodies to enteric bacterial antigens was found, suggesting that differences exist in the immune response to CD and UC. The studies of the IgG subclass in IBD may point to differences in the corresponding antigens between CD and UC,⁶⁻⁸ as well. Because IgG2 antibody responds to polysaccharides,³¹ it may suggest that the participation of bacterial antigens is greater in CD than in UC, including hypersensitive response. This does not seem to conflict with the results of the anti-lipid A antibodies in this study. Anti-lipid A antibody concentrations in patients in the inactive phase of CD were the same or higher than in the active phase. This finding may be related to the intestinal

Figure 5: Correlation between the blood concentrations and urinary concentrations of lactulose. (A) Simultaneous changes of the blood concentration and the urinary concentration were seen in a patient in the active phase of Crohn's disease. (B) We showed the urinary excretion and the blood lactulose concentration of six control subjects and three patients in active phase of ulcerative colitis using the same test. The urinary excretion, as well as the blood lactulose concentration, in the patients greatly increased over the controls in a four to eight hour period. In control subjects the values of the blood concentrations and the urinary excretion represent mean and mean (SD), respectively.

hyperpermeability and immunological hypersensitivity, and seems to be a specific feature of CD.

Intestinal permeability is usually evaluated by measuring urinary excretion of orally administrated markers such as lactulose, ^{51}Cr -EDTA, polyethyleneglycols, L-rhamnose and mannitol, or by a combination of those markers.^{9-15 18-20} Many studies in patients with CD have shown an increase in the intestinal permeability,^{9-12 15} while in UC results have not shown an agreement.^{11-13 15} The studies using ^{51}Cr -EDTA or polyethyleneglycol 600 by enema or colonoscopy reported an increase of the permeability of the colon in patients with UC.^{16 17} The method measuring blood lactulose concentration seems to represent the permeability of the intestine directly, including that of the colon. This method of measuring lactulose requires

neither special technique nor special instruments except for an absorbent machine. Although five to eight hours is required to elute lactulose, this method may be invaluable in studying intestinal permeability.

Our studies for the intestinal permeability and the immune response to enteric bacterial antigens seem to reflect the individual pathophysiology of CD and UC. Additional studies for treatments associated with these hyperpermeable states are necessary, because patients who have not recovered adequate permeability may easily have a relapse. Future research on the permeating routes of lactulose, using tracers, is required to elucidate the routes of bacterial and other exogenous antigens in developing IBD.

We are grateful to Mr Yoshikazu Hinohara (Chugai Pharmaceutical Co Ltd, Tokyo, Japan) who instructed us in the measurement of blood lactulose concentrations, and to our assistants, Hitoshi Nakano, Hideo Ikeda, and Keiichi Mitsuyama, for their guidance and advice.

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