Repeated Measurement of Intestinal Permeability as an Assessment of Colitis Severity in HLA-B27 Transgenic Rats

STEVEN W. KERR, WALTER W. WOLYNIEC, ZORAN FILIPOVIC, SUZANNE G. NODOP, FRANK BRAZA, RAYMOND J. WINQUIST, and THOMAS C. NOONAN

Department of Pharmacology, Research and Development Center, Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Connecticut

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ABSTRACT

We report on the development of a method for repeated monitoring of mucosal permeability that allows assessment of the severity of colitis and evaluation of treatment efficacy in HLA-B27 transgenic rats. We determined the extent to which intestinal permeability related to stool condition, colon weight, and histological pathology in precolitic and diseased rats up to 29 weeks old. Intestinal permeability was measured by the urinary excretion of iodixanol at 24 h after oral administration. Mean permeability values increased significantly with age in HLA-B27 rats but remained decreased in the background strain Fischer-344 (F-344) control animals. Macroscopic evaluation of HLA-B27 rat colons between 20 and 24 weeks old showed colonic thickening with colonic wet weights increased from 3.4 ± 0.13 mg/kg b.wt. in F-344 rats to 6.79 ± 0.73 mg/kg b.wt. (p < .05) in HLA-B27 rats. Histological examination of HLA-B27 rat colons confirmed the colonic inflammation as a chronic active mononuclear cell infiltrate. The increase in colon weight was associated with an increase in permeability: 1.16 ± 0.17 mg iodixanol versus 5.37 ± 1.3 mg of iodixanol in F-344 and HLA-B27 rats, respectively. Three weeks treatment of HLA-B27 rats with cyclosporin A, but not sulfasalazine, showed a dose-dependent decrease in mucosal permeability and colon weight. Neither treatment improved stool condition. We conclude that the measurement of intestinal permeability by iodixanol excretion is a useful biochemical marker that is associated with increases in colonic weight and histological evaluation of inflammation. These data indicate that this technique may be valuable for diagnostic and evaluation purposes in preclinical models of inflammatory bowel disease.

Altered intestinal permeability has been a feature reported in many clinical cases of inflammatory bowel disease (IBD) as well as experimental animal models. In most instances, intestinal permeability changes increase with the disease state and have been postulated to be responsible for the introduction of antigenic or infectious agents through the intestinal mucosa leading to an immune response and subsequent infiltration of inflammatory cells (Franchimont et al., 1994; Ma, 1997). Although many different methods of measuring intestinal permeability have been used (for reviews, see Travis and Menzies, 1992; Bjarnason et al., 1995), none have gained widespread clinical use because of poor reproducibility, difficulty of use, and the absence of an evaluation of permeability with disease progression.

Recently, water-soluble radiographic contrast media have been identified as valuable agents to measure intestinal permeability. These agents share many molecular characteristics that are optimal for use in the measurement of intestinal permeability including: hydrophilicity and lipophilicity, non-reactivity, absorption by passive diffusion, metabolic stability, and easy quantification after urine excretion (Andersen and Lerum, 1995). These radiopaque molecules have an added advantage in being useful for both the simultaneous radiological examination of the intestinal wall and the measurement of intestinal barrier function (Solheim et al., 1991). Quantification of the radiopaque agent iodixanol has been described in an induced model of colitis in rats. This study correlated changes in mucosal permeability with indices of disease severity such as gross and histochemical colonic injury (Andersen et al., 1992) when iodixanol was given by enema. A second preclinical model describing the excretion of iodixanol in a rat model of small bowel ischemia found a significant correlation between an increase in permeability and the level of ischemic damage (Andersen et al., 1995). Furthermore, urinary excretion of a related agent, iohexol, after oral administration has been shown to be elevated in patients with IBD and correlated with disease activity (Halme et al., 1997). Although each of these studies involved urinary excretion of a radiopaque agent as a measure of permeability, none were used to follow the progression of the disease or as a tool for evaluation of the efficacy of treatment.

ABBREVIATIONS: IBD, inflammatory bowel disease; mgl, milligrams iodixanol; CsA, cyclosporin A; F-344, Fischer-344.
We report the extension of a method for the assessment of mucosal permeability by measuring urinary iodixanol excretion. The technique involves oral administration and allows for assessment of disease severity, as well as evaluation of treatment efficacy, in an experimental animal model of IBD. We chose the HLA-B27 transgenic rat as a model of spontaneously developing IBD that shares many of the clinical manifestations of the human disease (Gough et al., 1994; Elson et al., 1995; Satoshi and Grisham, 1995). To determine whether mucosal permeability changes over time, urinary excretion of iodixanol was measured by HPLC in different age groups of HLA-B27 rats after oral administration to quantify the extent to which permeability changes correlated with the progression of disease symptoms. We compared these results with those using disease-free Fischer 344 (F-344) rats, which are the progenitor strain for the HLA-B27 transgenic rat. We also measured permeability changes during a 3-week course of treatment with either cyclosporin A (CsA) or sulfasalazine, agents used to treat Crohn’s disease and ulcerative colitis (Feagan et al., 1994; Lichtiger et al., 1994). We conclude that this measurement of permeability is a useful biochemical marker for evaluating disease severity, progression, and response to therapeutic compounds in an animal model of IBD and may also be of value for both diagnostic and evaluation purposes in the clinic.

Materials and Methods

Animals. Male transgenic rats expressing the human HLA-B27 and β2-microglobulin genes and their progenitor strain F-344 rats were obtained from Taconic Farms (Germantown, NY) at 4 to 9 weeks old. They were housed two to five per cage in our animal facility, fed a diet of standard rat chow, and administered water ad libitum. All experiments were carried out within the restrictions and guidelines of the Boehringer Ingelheim Animal Care and Use Committee.

Determination of Stool Condition. The stool consistency of the rats was observed three times per week and was scored and assigned a value of 1 for the following characteristics: pale color, visible mucus, softness, and fecal material adherent to the perianal area. A value of 2 was assigned for stool that did not have normal texture or pellet shape (unformed). A value of 3 was assigned for liquid stool, and a value of 4 was assigned for the presence of visible blood. The total score was summed for each animal.

Measurement of Mucosal Permeability. Iodixanol (Visipaque; Myodern Medical Supply, Norristown, PA) was orally administered to each rat at a dose of either 5 or 10 ml/kg (320 mg of iodixanol [mgI/ml] with an 18 gauge gavage needle. The chemical structure and isolation of iodixanol (5,5’-[(2-hydroxy-1,3-propanediyl)bis(acetylimino)]bis[2,3-diisoproxypropyl]-2,4,6-triiodo-1,3-benzenedicarboxamide) is reported elsewhere (Skjöld and Berg, 1986). The rats were housed individually in metabolic cages (Allentown Caging Co., Allentown, PA) containing a fine metal screen to separate the urine from feces; the urine was collected over 72 h. The time course of iodixanol excretion in the urine of six F-344 rats and six HLA-B27 transgenic rats was determined by collecting urine samples at 4, 24, 48, and 72 h for the determination of iodixanol concentration by HPLC. The results of the time course study indicated that a single urine sample after 24 h showed a statistically significant difference between F-344 rats and HLA-B27 rats, and 24-h urine collection was used for the remainder of the study. To determine the association of disease progression with permeability, measurements of iodixanol excretion were performed using a 24-h collection period on a weekly interval for 21 weeks to determine any changes over time.

Determination of Iodixanol Concentration in Urine. Iodixanol concentration in urine samples was determined by modification of a previously published method (Jacobsen et al., 1995). Briefly, a 1:20 dilution of urine in H2O was injected onto a C18 reversed phase column (250 × 4.6 mm, 5-μm pore size; Becton Dickinson, San Jose, CA) through a 712 Wisp Auto-Injector (Waters Corp., Milford, MA) using a step gradient of 100% buffer A (8% CH3CN/H2O) for 5 min, going to 100% buffer B (16% CH3CN/H2O) for 10 min, and then back to 100% buffer A for 5 min. UV absorbance was monitored at 250 nm using a model 684 UV spectrophotometric detector (Waters Corp., Milford, MA). Iodixanol eluted as dual peaks with retention times of 11.6 and 13.8 min for the exoisomers and endoisomers of iodixanol, respectively. Quantification of unknown samples was based on the peak area of the endoisomer of iodixanol standards. The detection limit of our standards was 0.64 μg/ml. Using a known iodixanol standard concentration, the intra-assay and interassay coefficients of variation were calculated to be 1 and 7.7%, respectively. At the end of each observation time, total excretion of iodixanol was determined based on the total volume of urine collected and expressed in mgI excreted. A quality control standard of iodixanol in H2O (0.032 mg/ml) was analyzed with each group of unknown samples and compared with the standard curve as a quantitative control.

Initial studies were done using a dose of 10 ml/kg iodixanol (320 mg/ml), but to lessen the chance of aspiration into the lungs, most studies were done using a 5 ml/kg dose. A comparison of each dosing volume was performed in both F-344 and HLA-B27 rats and indicated the 5 ml/kg dose of iodixanol resulted in one half the total excretion of the 10 ml/kg dose.

Effect of Test Agents on Iodixanol Excretion. Iodixanol excretion was determined on both HLA-B27 and F-344 rats over a 24-h collection period at a weekly interval. HLA-B27 rats were enrolled into treatment groups after they were observed to have pale, mucus-containing, and soft or unformed stools for 2 consecutive weeks in combination with elevated permeability. Elevated permeability was defined as iodixanol excretion above 1.75 mg/24 h (5 ml/kg/dose). This value was obtained by a power analysis considering the variability of intestinal permeability measurements in both the control and HLA-B27 groups. The analysis provided the value at which a 75% decrease in permeability would be statistically significant using six animals per group.

CsA (Sandimmune; Sandoz Pharmaceuticals, East Hanover, NJ) in olive oil was administered orally at doses of 1, 3, 10, and 30 mg/kg once a day for 3 weeks to HLA-B27 rats. CsA was also dosed orally to F-344 rats at 10 and 30 mg/kg/day. In general, the HLA-B27 rats were between 17 and 20 weeks old, and the F-344 control animals, although not age matched, were less than 29 weeks old when they were placed on study. Sulfasalazine in Methocel vehicle was administered orally at doses of 10, 30, and 100 mg/kg/day to HLA-B27 rats. The animals were individually placed in metabolism cages and dosed once with iodixanol each week and their urine was collected for 24 h for the determination of intestinal permeability. This procedure was repeated three times at 7-day intervals for 21 days.

Tissue Preparation. After the determination of mucosal permeability, animals were sacrificed with an overdose of sodium pentobarbital (Abbott Laboratories, Chicago, IL); their colons were excised at the cecal-colon junction, the luminal contents were removed, and the tissue was weighed. The tissues were then cut into three representative 5-mm sections for histological analysis. The sections were formalin fixed, embedded in paraffin, sectioned tangentially at 5-μm thick slices, and stained with hematoxylin and eosin (H&E). The inflammatory infiltrate and ultrastructure of the colonic sections were evaluated in a blinded fashion by a histopathologist.

Statistical Analysis. The results are reported as mean ± S.E. Data within each group were compared with the respective control value by a paired analysis based on test for normality. Either a paired t test or Wilcoxon sign rank test for matched pairs was used to test for significance from control. An adjustment for multiplicity of measurement was done for all multiple comparisons with control...
values. An unpaired Student’s *t* test was done for all comparisons between different treatment groups and control or vehicle-treated animals. Statistical significance was considered at the *p* < .05 level.

**Results**

**Intestinal Permeability in HLA-B27 Rats.** Figure 1 shows the cumulative time course for iodixanol excretion in the urine of six HLA-B27 rats 19 to 24 weeks old compared with six F-344 control rats over a 72-h collection period after a single oral administration of 10 ml/kg iodixanol. The amount of iodixanol excreted in urine reached a plateau by 48 h, with no significant additional accumulation between 48 and 72 h. The amount of iodixanol in the urine of HLA-B27 rats was significantly greater than that of the F-344 rats at the 24-, 48-, and 72-h time points. The amount of iodixanol at 24 h was approximately 75% of the total observed after 72 h. Therefore, we used 24-h collections for the remainder of the determinations in this report.

The mean value for intestinal permeability of HLA-B27 rats increased with age (Fig. 2). Mean excretion of iodixanol was 1.12 ± 0.29 mg at 8 weeks old compared with 2.94 ± 0.65 mg after 29 weeks old (*p* < .05, 5 ml/kg dose of iodixanol). This increase is in contrast to F-344 rats, which had a mean excretion of 0.55 ± 0.04 mg/l at 8 weeks and showed no significant change in permeability at any of the ages tested up to 29 weeks. Both groups of rats had significant increases in body weight over the 21-week period with the mean increase in weight for F-344 rats being 184 versus 111 g for HLA-B27 rats. Although the mean excretion of iodixanol increased over time, there was a subset of HLA-B27 rats (~10%) that maintained low permeability over the 21 weeks measured.

**Determination of Physical Properties of Disease.** The F-344 rats exhibited normal stool characteristics; the stools were dark, firm, dry, and pellet shaped and had a mean stool score of 0 in our stool assessment criteria. HLA-B27 rats were generally between 19 and 25 weeks old when they presented with altered stool, at which time their stool was consistently pale, contained mucus, and was typically softer than that of F-344 rats. The mean stool score for HLA-B27 rats at 19 to 25 weeks old was 3.50 ± 0.56. The incidence of completely unformed stool or diarrhea increased with the age of HLA-B27 rats.

The mean colon wet weight for HLA-B27 rats was significantly higher than that for F-344 rats (6.79 ± 0.73 versus 3.45 ± 0.13 mg/g b.wt., *n* = 7, respectively) when they presented with colitis based on elevated stool score for 3 consecutive weeks. In general, these animals were between 15 and 20 weeks old, and removal of their colons showed thickening of the colon wall throughout and dilation of the colon that was most prominent at the cecal-colon junction compared with F-344 rats. Histopathological evidence of colitis was confirmed in these animals by examination of H&E sections of colon (Fig. 3). The colons of HLA-B27 rats presenting with 3 weeks of unformed stool showed a mononuclear cell infiltrate into the lamina propria and submucosal layers, as well as disruption of crypt morphology. In contrast, F-344 rats showed no significant histopathological changes.

**Comparison of Permeability with Physical Properties of Disease.** The mean stool score of HLA-B27 rats that presented with colitis based on 3 weeks of unformed stool was associated with an increase in mucosal permeability. Furthermore, F-344 rats had a mean stool score of 0 and consistently maintained low permeability. However, when a direct correlation plot was done within the group of HLA-B27 rats, there was no statistically significant correlation between stool score and permeability (data not shown).

The increase in HLA-B27 colon wet weight and associated histopathological inflammation was accompanied by a higher value for intestinal permeability compared with F-344 rats of similar age (5.37 ± 1.30 versus 1.16 ± 0.17 mg/l, respectively; *p* < .05; 10 ml/kg dose of iodixanol; Fig. 4A). Therefore, in addition to the lower permeability values of F-344 rats, the...
rats displayed lower colon wet weights. However, we observed that HLA-B27 rats sacrificed using stool score alone for the determination of colitis showed a wide range of permeability measurements (Fig. 4B). HLA-B27 rats with high permeability (2.5–9 mgI; using a 10 ml/kg dose of iodixanol) had elevated colon weights, and animals with low permeability (0–1 mgI) had lower colon weights. There was a subgroup of animals that, although they showed an increase in colon wet weight, maintained only a moderately elevated permeability measurement (1–2.5 mgI). Therefore, entering HLA-B27 rats into treatment groups on presentation of elevated permeability ensured assessment of a colitic condition.

Effect of CsA. The effect of 3 weeks of CsA treatment at 1, 3, 10, and 30 mg/kg doses once a day on iodixanol excretion (5 ml/kg dose iodixanol) was tested in HLA-B27 rats and compared with F-344 control animals. CsA decreased iodixanol excretion in a dose- and time-dependent fashion (Fig. 5A). The reduction in iodixanol excretion was significant at both the 10 and 30 mg/kg doses. The 1.0 and 3.0 mg/kg doses did not significantly change iodixanol excretion compared with vehicle. After 3 weeks of treatment, 30 mg/kg CsA/day decreased iodixanol excretion by 75 ± 6%, but the level was still significantly higher compared with F-344 rats. Neither olive oil nor CsA, at 10 and 30 mg/kg, had an effect on iodixanol excretion in F-344 rats (Fig. 5B).

Furthermore, 3 weeks of CsA treatment of HLA-B27 rats decreased mean colon wet weight in a dose-dependent manner (Fig. 6). Colon wet weight decreased significantly at both the 10.0 and 30.0 mg/kg doses compared with vehicle. There was no significant difference at the 1.0 and 3.0 mg/kg doses. The maximum decrease in colon wet weight was 34 ± 4.0%, effected by 30 mg/kg CsA/day. Although CsA treatment reduced colon weight, all CsA-treated mean colon weights were significantly greater than the mean colon weights for F-344 rats. Histological examination of colons from animals treated with CsA at both the 10 and 30 mg/kg doses showed a reduction in both the number of infiltrating leukocytes and the extent of tissue layer penetration (Fig. 7). Although the extent of tissue inflammation was reduced, it was not completely resolved. The histopathological findings of colonic inflammation at the 1.0 and 3.0 mg/kg doses were not different from vehicle-treated control animals. However, despite the significant decrease in permeability and colon wet weight observed after CsA treatment, the quantification of mean stool score was not significantly changed (data not shown).

Effect of Sulfasalazine. We tested the general anti-inflammatory agent sulfasalazine, commonly used in the treatment of IBD, for its effect on intestinal inflammation in HLA-B27 rats. Three weeks of sulfasalazine treatment once a day at 10.0, 30.0, and 100.0 mg/kg doses did not signifi-
cantly change iodixanol excretion (data not shown). F-344 rats were not studied due to the lack of effect in HLA-B27 rats. In a similar fashion to the results for intestinal permeability, sulfasalazine treatment did not significantly affect colon wet weight, mean stool score, or the histopathological findings in the colon of HLA-B27 rats (data not shown).

**Discussion**

We report the development of a method to measure intestinal permeability in an animal model of IBD using HLA-B27 transgenic rats. The method is quantitative and reproducible and allows for repeated measurements in each animal. In addition, we showed that permeability increases with age in HLA-B27 rats to nearly a 5-fold difference compared with disease-free control animals. The increase in permeability we measured was associated with an increase in colon wet weight, although there was a cohort of animals that did not become colitic or increase in permeability. We also demonstrated the efficacy of CsA treatment in decreasing both permeability and colon wet weight but not stool condition.
Sulfasalazine treatment did not improve these same parameters. Much of the concern regarding the use of intestinal permeability as a measure of the progression of IBD in either preclinical animal models or clinically arises from the lack of a reliable method that is associated with indices of disease severity. Our results show that iodixanol can be orally administered to rats and easily measured in urine with an excretion time course of 48 to 72 h (Fig. 1). Quantification of iodixanol by HPLC is rapid, reliable, and consistent with other reports in the literature (Svaland et al., 1992; Jacobsen et al., 1995). Our method for oral administration and collection of urine allowed for repeated measures of permeability in the same animal, permitting the measurement of disease progression and the effect of therapeutic agents.

In addition, we were able to show that animals presenting with elevated permeability possessed thick and inflamed colons as determined by wet weight, macroscopic appearance, and histology. In contrast, control F-344 rats and young (8–10 weeks) HLA-B27 rats not presenting with altered stool had low permeability and decreased colon weights and were histologically normal. Interestingly, there was not a significant linear correlation between permeability and colon weight within the HLA-B27 rats. HLA-B27 rats with moderately increased permeability (1–2 mgI) had colon wet weights not significantly different from the high permeability group (2–5 mgI; Fig. 3). There also was a subgroup of animals that, although they had elevated stool score, maintained low permeability (1.0 mgI) and low colon weight. These data suggest that the increased colon weight of HLA-B27 rats preceded permeability changes. The increase in colonic weight we observed is also supported by a recent study that suggests young 10- to 15-week-old HLA-B27 rats develop histological and physiological manifestations of colonic inflammation before mucosal permeability changes as measured by the absorption of $^{51}$Cr-EDTA or 8-deamino- D-arginine vasopressin (Lunden et al., 1998). By incorporating the combination of stool condition and intestinal permeability as entry criteria for study, we were able to ensure enrolled animals showed both physical and functional symptoms of disease. This method also allows selection of rats that present with similar levels of disease severity. Because increased colon weights precede increases in permeability, this enrollment method selects animals with a uniform and relatively severe degree of colitis.

By measuring changes in colonic wet weight, intestinal permeability, and histology, we were able to show treatment efficacy in this experimental animal model using an agent that has been used in the clinic for the treatment of human IBD. Three weeks of CsA treatment significantly decreased permeability in a dose-dependent fashion to near control levels. The decrease in permeability observed after CsA treatment was associated with a significant decrease in colon wet weight, although the weights remained above normal.
control, indicating the inflammation was improved but not resolved. Further evidence for improvement, but not resolution, in colonic inflammation was shown by the decrease in the number of infiltrating inflammatory cells as observed histologically. However, it is interesting to note that even though CsA treatment improved the above-mentioned measures of disease, it did not significantly improve stool condition. The reason for a lack of improvement in stool condition is not clear, but considering the fact that colonic inflammation is not totally resolved, the possibility exists that stool consistency is the last measure of disease to improve. The decrease in permeability and colon weight observed with CsA treatment suggests that the inflammation and permeability changes in the HLA-B27 rat are T-lymphocyte-dependent events, as has been shown by others (Hammer et al., 1990; Breban et al., 1993). Thus, this animal model of IBD may be useful for testing new agents that modulate T-cell function for the possible treatment of IBD.

The increase in permeability observed in HLA-B27 rats is consistent with reports in the literature using other animal models of IBD (Hollander et al., 1986; Yamada et al., 1993). We believe the mentioned aspects of this method provide many advantages over others reported in the literature, such as the use of radioactive compounds (e.g., Cr\textsuperscript{51}-labeled EDTA; Pironi et al., 1990; Teahan et al., 1991; Lunden et al., 1998) or the administration of indicators by enema (Andersen et al., 1992), which hinder the ability for repeated measurements in the same animal.

Although we observed CsA to be effective in this model of IBD, we found that treatment with sulfasalazine, a drug commonly used to treat colitis patients (Hirschfeld and Clearfield, 1995; Bonner, 1996), was ineffective in ameliorating the symptoms of colitis in this model. Neither intestinal permeability, colon weight, histology, nor stool condition was improved after treatment with sulfasalazine. Our failure to observe a decrease in intestinal permeability is not consistent with a previously published finding (Satoshi et al., 1996), which showed sulfasalazine treatment attenuates the increase in mucosal permeability in HLA-B27 rats as performed by a blood-to-lumen technique. Differences in the permeability measurements, which were done using blood-to-lumen clearance of \textsuperscript{51}Cr-EDTA, involving endothelial permeability as opposed to epithelial permeability, and a higher dose of sulfasalazine administered (131 versus 100 mg/kg/day) may be reasons for this discrepancy. In addition, there are differences in the enrollment of animals between the studies; the present study uses permeability changes as a criterion, possibly indicating a more advanced stage of colitis for the study group. One other consideration is that sulfasalazine may be more effective when coincident with surgery-induced trauma associated with the blood-to-lumen technique used by Satoshi et al (1996). Because the method

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**Fig. 7.** A, an H & E cross section from the proximal colon of a typical HLA-B27 rat that was treated 3 weeks with olive oil vehicle. The section showed marked mononuclear cell infiltration (heavy arrows) throughout the lamina propria (LP). The luminal space is also indicated (L). B, similar colon section from an HLA-B27 rat that was treated 3 weeks with 10 mg/kg CsA once a day. CsA treatment partially resolved the inflammation of mononuclear cells.
described here is noninvasive, it has the advantage of measuring intestinal inflammation without surgical trauma. However, these investigators also found no decrease in colon weight or improvement in stool condition, which was in agreement with the present study. The results with sulfasalazine indicate that the treatment of colitis in these HLA-B27 rats with an anti-inflammatory agent that is useful in keeping colitis patients in remission may not be efficacious in treating fulminate inflammation in the intestines. The lack of efficacy using sulfasalazine treatment is further evidence that HLA-B27 rats present with a severe state of colitis.

In summary, we report the use of a technique to calculate intestinal permeability that allows for repeated measurements, determination of disease severity, and evaluation of treatment efficacy in a model of IBD in HLA-B27 rats. We were able to show an association between the measurement of permeability and the physical properties of disease, such as colon weight and histological evaluation of inflammation. In addition, our data demonstrate the effectiveness of a 3-week therapeutic treatment with CsA, but not sulfasalazine, in decreasing intestinal permeability and colonic inflammation. This method of measuring permeability may be useful in evaluating the severity of disease and monitoring treatment efficacy clinically as well as in preclinical models.

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References

Send reprint requests to: Dr. Steven W. Kerr, Department of Pharmacology, Boehringer Ingelheim Pharmaceuticals Inc., 900 Ridgebury Rd., Ridgefield, CT 06877.