Alternative Methods for Assessing Experimental Colitis in vivo and ex vivo

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To develop and validate methods for the assessment of colonic damage in the rat that are rapid, simple to perform and quantitative. Rats (Sprague Dawley, male, N = 52) were administered 0.5 mL of 2,4,6-trinitrobenzenesulfonic acid (TNBS, 60 mg mL\(^{-1}\) in 50% ethanol) intracoloneally to induce reproducible experimental colitis. The rats were administered either orally or intracoloneally 1 g of the non-absorbable permeability marker phenol red in 0.5 mL of water 24-72 h post-colitis induction. Urine was collected for the next 24 h and analyzed for phenol red at 559 nm using a spectrophotometer. In separate studies, colonic tissue was excised 24-72 h post-colitis induction for mitochondrial DNA damage determination. Baseline permeability indicated that < 4% of phenol red dose was excreted in urine after oral or intracolonic administration to vehicle control rats. A ~3-4 fold increase in colonic permeability was apparent in colitic rats 48 h post TNBS, which correlated with gross macroscopic ulceration in the large bowel and colonic mitochondrial damage. Administration of dexamethasone 2 mg kg\(^{-1}\), or 5-aminosalicylic acid (5-ASA) 100 mg kg\(^{-1}\) resulted in significant gross macroscopic protection and reduction of colonic permeability and mitochondrial damage. In a group of rats TNBS was administered and then allowed to recover for 6 weeks. Subsequent tail vein administration of TNBS 5 mg kg\(^{-1}\) for 3 days reactivated the disease, which was also detected by an increase in colonic permeability of phenol red and oxidative mitochondrial damage. Several non-steroidal anti-inflammatory drugs administered in this model also appeared to exacerbate this disease and increase colonic permeability and mitochondrial damage. Oral or rectal administration of phenol red in experimental colitis can reproducibly detect colonic damage. This marker can also be used to non-invasively evaluate potential treatments for experimental colitis as well as its reactivation. Mitochondrial damage to colonic tissue parallels the increase in colonic permeability and appears to be a sensitive marker of disease activity in experimental colitis.

Key words: Colitis, permeability, phenol red, rat

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INTRODUCTION

The rat has become an accepted model for the study of colitis-induced adverse effects in the large bowel (Hoffman et al., 2002). The trinitrobenzene sulfonic acid (TNBS) model is being used extensively in this regard because of its simplicity and susceptibility to pharmacological treatment (Morris et al., 1989). In parallel with the clinical situation, TNBS colitis is susceptible to periods of alternating remission and active inflammation and non-steroidal anti-inflammatory drugs exacerbate TNBS colitis (Appleyard and Wallace, 1995; Sun et al., 2001; Reuter et al., 1996; Empney et al., 1992).

Inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are chronic inflammatory diseases of unknown etiology, which are difficult to treat pharmacologically. An inherent problem in the treatment of these diseases is that diagnosis is often difficult and it is usually symptomatic and often involves colonoscopy or a symptom-based activity index. In experimental models, such as the rat, the criteria described to quantify colonic damage and histological ulceration usually also involves a scoring system which varies considerably between investigators, often making comparison between laboratory findings difficult (Morris et al., 1989; Kruschewski et al., 2001; Rabau et al., 1996).

The degree of GI penetration by passively absorbed molecules is referred to as permeability. Impairment of epithelial barrier function causes increased permeability of the mucosa (Davies, 1998). Many investigators have demonstrated that an increase in colonic permeability of the large intestine occurs in experimental colitis in patients afflicted with this disease (Meddings and Gibbons, 1998; Gitter et al., 2001; Arslan et al., 2001; Jenkins et al., 1992).

Since the 1980s, substantial efforts have been made to develop non-invasive methods of detecting gastrointestinal (GI) abnormalities. As the intracellular junctions of colonic epithelial cells appear to be susceptible to a variety of agents, they may be the first organelle to suffer when the energy production of the enterocyte is compromised. This disruption of intercellular integrity allows permeation of macromolecules into the GI mucosa. Several non-invasive probes have been previously utilized to examine colonic permeability clinically and in experimental models (Davies, 1998; Meddings and Gibbons, 1998; Gitter et al., 2001; Arslan et al., 2001; Jenkins et al., 1992). $^{1}$Cr-EDTA has been the most frequently employed probe, however, $^{99}$Tc-EDTA is a measure of total GI tract permeability (Davies, 1998; Arslan et al., 2001; Jenkins et al., 1992). To provide a simple method for assessment of intestinal colonic permeability, the permeation of inulin-fluorescein and it fluorescence intensity in plasma has been measured (Krimsky et al., 2000). In addition, the permeation of an isomolar water-soluble X-ray contrast medium CM ioxianol has been applied as an enema and the excretion in urine is quantified (Andersen et al., 1996, 1992). Differentiation of gastrointestinal damage sites can also be made with probes of similar molecular size, i.e., the use of sucrose, sacralose and lactulose carbohydrates (Meddings and Gibbons, 1998). However, all the present methodology for assessing colonic damage presents several problems and inherent drawbacks, including lack of site specificity in the direct ability to measure colonic damage and the need for specialized analytical equipment for measuring the excretion. $^{3}$Cr-EDTA measurement requires the analytical need of a gamma counter and exposes both the animals and researcher to radiation. Some oligosaccharides are found in diet and oligosaccharides analysis is often time-consuming and extraction and chromatographic procedures are often prohibitive for routine screening as high performance liquid chromatography (HPLC) with an electrochemical detector is necessary. In addition, measurement of ioxianol requires HPLC and measurement of inulin-fluorescein requires a fluorescence detector.

Oxidative stress is of substantial clinical importance not only because oxidants are common in inflammation, but also, because disruption of mitochondrial energy metabolism, which reduces ATP synthesis leading to a breach of the GI barrier function which can lead to mucosal barrier hyperpermeability and, in turn, lead to the initiation and/or perturbation of mucosal inflammation and injury (Seril et al., 2003; Tuzun et al., 2002; D’Odorico et al., 2001). Disturbance of enterocytes may also impair the ability to combat free oxygen radicals which are partly responsible for cell damage involved in colitis. Mitochondrial oxidative damage to intestinal enterocytes may occur in parallel with these permeability changes and be further perturbed by the inflammatory insult. This may be important for the transition from the inactive to active (flare up) phases of inflammation in which intestinal oxidants and pro-inflammatory molecules periodically create a vicious cycle that leads to sustained oxidative stress, increased permeability, inflammation and tissue damage and predisposition to colorectal carcinogenesis (Seril et al., 2003; Tuzun et al., 2002; D’Odorico et al., 2001).

We have previously reported the suitability of the rat as a model for non-invasive detection of NSAID induced gastroduodenal and small intestinal permeability (Davies, 1994; 1995). The use of phenol red as a marker of
intestinal permeability to indomethacin in rats has previously been suggested, however, it seems to require excessively large doses 200-100 mg kg⁻¹ (Nakamura et al., 1982). Permeability studies in the rat with phenol red have not been extended to colitis. Given the apparent parallels between the occurrence of colitis in rats and humans, it is of interest to examine novel ways of detecting this damage non-invasively in the colon. The aim of these studies was to examine the suitability of phenol red excretion and mitochondrial oxidative damage in the rat as simple experimental tools for screening and evaluating experimental colitis.

MATERIALS AND METHODS

Chemicals: Phenol red, dexamethasone, indomethacin, 5-acetylsalicylic acid and methylcellulose were purchased from Sigma Chemicals (St. Louis, MO, USA). DAEasy® Mini Kit 50 was obtained from Qiagen Inc (Stanford, CA, USA). 2,4,6-trinitrobenzenesulfonic acid (TNBS) stock solution (1 M in water) was purchased from Fluka Chemie GmbH (CH-9471 Buchs, Switzerland). Polyethylene glycol 600 (PEG 600), FCC grade, was purchased from Union Carbide Chemicals and Plastic Company Inc. (Danbury, CT, USA). ⁵¹Cr-EDTA was purchased from NEN Life Sciences Products (Boston, MA, USA).

Preparation of drugs: TNBS Stock solution (1 M) 2.047 mL of was added to a mixture of 2.953 mL water and 5 mL of alcohol to prepare 10 mL of TNBS working solution (60 mg mL⁻¹ TNBS in 50% ethanol). Dexamethasone was dissolved in PEG 600 to form a solution of 4 mg mL⁻¹. 5-ASA and indomethacin were dispersed in 2% methylcellulose.

Animals

Induction of colitis and phenol red excretion: Male Sprague-Dawley rats (350-450 g) were obtained from Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) were anesthetized with halothane. Colitis was induced by rectal intraluminal administration of 0.5 mL of (TNBS, 60 mg mL⁻¹ in 50% ethanol) using a Foley catheter. Two mm o.d., Bard Urological Division, C.R. Bard Inc., Covington, GA, USA). On the morning of the permeability study, rats were housed in individual metabolic cages (Techniplast, USA) with wire mesh floors allowing separate quantitative collection of urine and feces. Animals were fed a standard Purina rat chow and allowed free access to food and water for the duration of the experiment. Ethics approval for animal experiments was obtained from Washington State University Institutional Animal Care and Use Committee.

At 24, 48 or 72 h post-induction of colitis, 1 g of phenol red solution was administered orally or intrarectally to rats (n = 4-5 group) using an 18 gauge 5 cm curved feeding needle attached to a 1 mL syringe. Urine was collected 0 to 8 h and 8 to 24 h and phenol red excretion was examined 24 h post-dose. Following the test with phenol red, the rats were sacrificed with over-dosed halothane and dissected with the colon removed. The damage of the colon was assessed using a scoring scheme adopted from Appleyard and Wallace, (1995), with the total area of colon ulceration recorded.

Protective effect of dexamethasone: Rats were assessed in parallel in four experimental groups. For the negative control group (n = 5), saline was used instead of TNBS. For the positive control group (n = 5), colitis was induced using 0.5 mL of TNBS, (60 mg mL⁻¹ in 50% ethanol). For the dexamethasone treated group (n = 5), dexamethasone in PEG 600 was administered to rats (2 mg kg⁻¹) subcutaneously 12 and 1 h before induction of colitis based on previously reported effective doses (Kankuri et al., 2001; Holma et al., 2002). For the control group of dexamethasone, (N = 5) the vehicle PEG 600 without dexamethasone, was administered to rats subcutaneously 12 and 1 h before induction of colitis.

Protective effect of 5-ASA: For the 5-ASA treated group (n = 10), rats were orally administered 100 mg kg⁻¹ 5-ASA in 2% methylcellulose twice daily from 72 h before the induction of colitis for 3 days and then 1 h and 12, 24, 36 h post colitis induction (Nakamura et al., 1994; Galvez et al., 2000). Forty-eight hours after the induction of colitis, 5 of the rats were sacrificed and their injured colon tissues were taken and stored at -20°C for analysis of oxidative damage. The other 5 rats were dosed phenol red 1 g via oral gavage to investigate the phenol red excretion from urine.

Reactivation of colitis: Six weeks after the initial intracolonic administration of 0.5 mL (60 mg mL⁻¹ in 50% ethanol), rats were randomized into treatment and control groups and TNBS in 0.9% saline (5 mg kg⁻¹) or saline alone administered intravenously via a tail vein once daily for 3 days (Appleyard and Wallace, 1995, Sun et al., 2001). An hour after the last dose of TNBS, rats were administered 1 g of phenol red orally using an 18 gauge 5 cm curved feeding needle attached to a 1 mL syringe. Urine was collected from 0 to 8 h and from 8 to 24 h.

Exacerbation of colitis: Diclofenac was administered 10 mg kg⁻¹ orally to rats 3 h and every 12 h after induction of colitis (Reuter et al., 1996). An hour after the last dose
Table 1: Criteria for macroscopic scoring of colonic damage

<table>
<thead>
<tr>
<th>Feature</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>Normal appearance</td>
<td>0</td>
</tr>
<tr>
<td>No adhesions</td>
<td>0</td>
</tr>
<tr>
<td>No diarrhea</td>
<td>0</td>
</tr>
<tr>
<td>Focal hyperemia, no ulcers</td>
<td>1</td>
</tr>
<tr>
<td>Minor adhesions</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>1</td>
</tr>
<tr>
<td>Ulceration without hyperemia or bowel wall thickening</td>
<td>2</td>
</tr>
<tr>
<td>Ulceration with inflammation at one site</td>
<td>3</td>
</tr>
<tr>
<td>Ulcerations/inflammation at two or more sites</td>
<td>4</td>
</tr>
<tr>
<td>Major sites of damage extending &gt;1 cm along the length of the colon</td>
<td>5</td>
</tr>
<tr>
<td>When an area of damage extended &gt;2 cm along the length of the colon, and the score was increased by 1</td>
<td>6-10</td>
</tr>
<tr>
<td>for each additional cm of involvement</td>
<td></td>
</tr>
<tr>
<td>Plus Maximal bowel wall thickness (x), in mm</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Primer sequences for rat QPCR

<table>
<thead>
<tr>
<th>Gene Cluster (TRPM-2)</th>
<th>Sequence (5' to 3')</th>
<th>Direction</th>
<th>Amplicon Length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'81</td>
<td>AGACGGGCTAGACACGCTGAC</td>
<td>Sense</td>
<td>12.5 kb</td>
</tr>
<tr>
<td>18314</td>
<td>CGACCAGCTTCTGGAGTAGC</td>
<td>Anti-sense</td>
<td>12.5 kb</td>
</tr>
<tr>
<td>13559</td>
<td>AATACTTCCGCAACGTTTATGC</td>
<td>Sense</td>
<td>13.4 kb</td>
</tr>
<tr>
<td>10633</td>
<td>GGAAATGAGGTGTGAGGCTA</td>
<td>Anti-sense</td>
<td>13.4 kb</td>
</tr>
<tr>
<td>14678</td>
<td>GCTCTGATATATTGAACTT</td>
<td>Sense</td>
<td>235 bp</td>
</tr>
<tr>
<td>14885</td>
<td>GTCTGGGTCTCCTCTAGTAGATCG</td>
<td>Anti-sense</td>
<td>225 bp</td>
</tr>
</tbody>
</table>

Fig. 1: Typical calibration curve of phenol red in urine test sample was mixed and then centrifuged again at 15,000 g for 3 min. The absorbance at 559 nm was measured using a Shimadzu spectrophotometer UV 2100U (Shimadzu, Kyoto, Japan) with 0.375 mL of NaOH 2N in 1.5 mL of H₂O as a reference. When the absorbance exceeds 2, further dilution of sample is required. Phenol red concentration was directly determined from the standard curve and the dilution factor. A typical standard curve is presented in Fig. 1.

Indomethacin³⁵Cr-EDTA and phenol red: Indomethacin in 2% methylcellulose and 2% methylcellulose alone were administered orally to rats (n = 5/group) using an 18 gauge 5 cm curved feeding needle attached to a 1 mL syringe. Phenol red 1 g was administered orally and its excretion was examined 24 h post-dose. ³⁵Cr-EDTA in urine was measured by relative permeability determined by calculating the radioactivity present in urine samples as a percentage of the administered dose as previously described (Davies, 1995).

Mitochondrial oxidative damage: A quantitative polymerase chain reaction (QPCR) technique was developed based on the work of (Torres et al., 2000; Santos et al., 2002; Ogiso et al., 2001; Wright et al., 1997). DNA was isolated using Qiagen® genomic tip and genomic DNA buffer set kit for mammalian DNA extractions (Qiagen Inc. Valencia, CA, USA). DNA quantitation was performed utilizing the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA). QPCR involved the use of GeneAmp XL PCR kit (Applied Biosystems, Branchburg, NJ, USA) and dNTPs (Pharmacia, Peapack, NJ, USA). Primers were based on sequences already optimized by Van Houten (Torres et al., 2000; Santos et al., 2002; Ogiso et al., 2001; Wright et al., 1997) (Table 2). PicoGreen® was used to quantify dsDNA fragment. Fluorescence was measured using a CytoFluor® 4000 fluorescence multi-well plate reader (Applied Biosystems, Foster City, California, USA). After fluorescent readings for all samples were subtracted from the no template controls, relative amplification was calculated in paired samples.
**Statistical analysis:** Differences between two means were determined by Students unpaired t-test. Differences between more than two means were determined by one-way ANOVA followed by Duncan's multiple range test at α = 0.05.

**RESULTS**

After colitis induction, macroscopic inflammation was assessed using previously defined criteria (Table 1), as shown in Fig. 2a. Inflammatory mucosal lesions were found 24 h after TNBS enema. The size of ulcerative lesions reached its maximum at 48 h and remained increased from baseline thereafter until the end of the 72 h follow-up. The increase in macroscopic damage showed similar time dependency as the increase in permeability and excretion of phenol red (Fig. 2b). The intracolonic administration of phenol red 48 h post-colitis demonstrated a significant increase in colonic permeability which was similar to that observed after oral administration (Fig. 2c). Based on these data, 48 h post-colitis induction was chosen as the assessment time point for the studies of drug effects on TNBS induced colitis assessed by phenol red excretion and colonic mitochondrial damage.

The single dose treatment with indomethacin 10 mg kg⁻¹ orally caused significant macroscopic ulceration and increased intestinal permeability as measured by ⁵¹Cr-EDTA excretion in urine 24 h post-indomethacin compared to control levels (Fig. 3a). The urinary excretion of phenol red 24 h post-indomethacin was not significantly elevated compared to the control group (Fig. 3b).
Fig. 3b: Excretion of phenol red in urine of rats treated with indomethacin 10 mg kg\(^{-1}\) 24 h post-dose (N = 5, Mean±SEM)

Fig. 4a: Effects of 5-ASA and Dexamethasone on macroscopic damage score after acute colitis (N = 5, Mean±SEM)

Fig. 4b: Effects of 5-ASA and Dexamethasone on phenol red excretion in Acute Colitis (N = 5, Mean±SEM)

Fig. 5a: Macroscopic colonic damage score of colitis in rats (N = 5, Mean±SEM)

Fig. 5b: Effects of reactivation of experimental colitis on Urinary Excretion of oral phenol red excretions (N = 5, Mean±SEM)

Treatment with dexamethasone and 5-ASA attenuated macroscopic damage in the 48 h model of the acute experimental colitis (Fig. 4). In a parallel fashion, there was a significant reduction in the urinary excretion of phenol red in the TNBS-colitis group treated with these agents compared to positive TNBS treated controls (Fig. 4b).

Six weeks after intracolonic administration of TNBS (30 mg in 50% ethanol) to rats, some macroscopically visible inflammation and damage was still evident (Fig. 5). Intravenous administration of saline to these TNBS treated rats did not significantly affect the colonic damage score. When, TNBS was given intravenously to these rats that had been treated intracolonically with TNBS 6 weeks earlier, the colonic damage scores increased significantly (p<0.001) and the colons from these rats exhibited extensive ulcerations and marked thickening and
**DISCUSSION**

Phenol red is a non-absorbable molecule almost completely ionized at pH above 1. Its excretion probably reflects both the small intestinal and colonic permeability. However, because this probe reaches the colon within 3 h similar to sucrase, most phenol red absorption occurs within the colon after oral administration (Meddings and Gibbons, 1998; Ogiso et al., 2001). It appears to be more suitable for assessing colonic permeability. Nevertheless, this probe can also be administered directly into the colon, with similar findings to oral administration.
Similar to our previous findings in the rat with NSAIDs (Davies et al., 1994; 1995), the rat appears to be a suitable model for examining colonic permeability after experimental colitis induced with TNBS. Data from the present study demonstrated that the urinary recovery of phenol red is significantly increased when acute colonic damage was produced (Fig. 2). In addition, in parallel with the observations in the rat using the methods of scoring the damage (Fig. 3), TNBS induced colitis was reduced by 5-ASA and dexamethasone as measured by damage score and a reduction in phenol red excretion (Kankuri et al., 2001; Holm et al., 2002; Nakamura et al., 1994; Galvez et al., 2000). Therefore, this method can be used as a simple screening test for drugs used in the treatment of colitis.

As shown in Fig. 5, the healing period of 6 weeks was enough to restore the colonic damage to near baseline. This is correlated well with the data of the urinary recovery of phenol red. From the results described above, this method may be utilized as a simple, useful, a non-invasive screening test for assessment of colonic damage in vivo.

It is well known that administration of NSAIDs cause gastrointestinal ulceration. In our previous reports, indomethacin-induced enteropathy is maximal at 24 h post-dose as measured by 51Cr-EDTA excretion, or faecal bleeding and intestinal ulceration after a 10 mg kg⁻¹ dose (Davies et al., 1994; Wright et al., 1997; Dillon et al., 2003). Previous findings also suggest that indomethacin-induced ulceration requires a dose of 20-100 mg kg⁻¹ to detect appreciable intestinal damage using phenol red (Nakamura et al., 1982). Our present findings are consistent with these previous findings.

Phenol red is an attractive alternative to 51Cr-EDTA for assessing colonic disease because the latter radioactive probe possesses restrictions for routine or serial tests. It is also an attractive alternative to carbohydrates as it possesses a UV chromophore and multiple samples can be analyzed quickly, accurately and reliably. Such a probe could also conceivably be instilled in patients during a colonoscopy procedure to evaluate colonic permeability.

The results shown in Fig. 6 confirm that mitochondrial DNA damage measured in lesions is an event in the pathogenesis of colonic toxicity due to TNBS. These results appear to parallel the findings of urinary excretion of 8-hydroxydeoxyguanosine excretion in TNBS colitis (Dukers and Naginski, 1998). This technique allows us to determine that this damage is specific to the mitochondria rather than nuclear cellular DNA and parallels the results obtained with colonic permeability and gross damage scores. It also parallels previously demonstrated results with cisplatin in leukemia cells (Kallinowski et al., 1992). Mitochondrial DNA damage may also be a useful marker technique in other disease state studies that involve oxidative damage.

Damage to mitochondrial DNA (mtDNA) can be assessed without the need for isolation of mitochondria or mitochondrial DNA. Gene specific DNA damage provides more insight into the role played by oxidative stress in disease and mitochondrial DNA damage appear to be an excellent biomarker of oxidative stress in epithelial cells (Kallinowski et al., 1992).

Measurement of the phenol red concentration excreted into urine after an oral or a rectal dose is a sensitive index of colonic damage. It has the advantages over a scoring method of being quantitative and allowing the time course of development to be studied non-invasively. Permeability tests are safe, reproducible and easy to perform. The non-invasive nature of this test can be easily applied to diagnostic screening and research and could replace the need for invasive investigations of colonic disease such as radiology and colonoscopy. An implicit advantage of this permeability test is that it reflects functional integrity over a major area of the colon. Given the well documented changes in gastrointestinal permeability in various diseases, parallel with an increasing body of knowledge of a mechanistic understanding of gastrointestinal disease process and the importance of oxidative damage in the colon, these techniques may help us in further understanding the pathogenesis of colitis. Further studies are ongoing in our laboratory to examine various novel flavonoids and stilbenes with utility in experimental colitis (unpublished observations) and to examine in detail the pathogenic sequences involved in the process of colonic damage and pharmacokinetic/pharmacodynamic relationships between drug, dose and concentration required to elicit these effects.

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