Bilirubin prevents acute DSS-induced colitis by inhibiting leukocyte infiltration and suppressing up-regulation of inducible nitric oxide synthase

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Abstract

Bilirubin is thought to exert anti-inflammatory effects by inhibiting vascular cell adhesion molecule-1 (VCAM-1)-dependent leukocyte migration and by suppressing the expression of inducible nitric oxide synthase (iNOS). As VCAM-1 and iNOS are important mediators of tissue injury in the dextran sodium sulfate (DSS) murine model of inflammatory colitis, we examined whether bilirubin prevents colonic injury in DSS-treated mice.

Methods: Male C57BL/6 mice were administered 2.5% DSS in the drinking water for 7 days, while simultaneously receiving i.p. injections of bilirubin (30 mg/kg) or potassium phosphate vehicle. Disease activity was monitored, peripheral blood counts and serum nitrate levels determined, and intestinal specimens analyzed for histological injury, leukocyte infiltration, and iNOS expression. The effect of bilirubin on IL-5 production by HSB-2 cells and on Jurkat cell transendothelial migration also was determined.

Results: DSS-treated mice that simultaneously received bilirubin lost less body weight, had lower serum nitrate levels, and exhibited reduced disease severity than vehicle-treated animals. Concordantly, histopathological analyses revealed that bilirubin-treated mice manifested significantly less colonic injury, including reduced infiltration of eosinophils, lymphocytes, and monocytes, and diminished iNOS expression. Bilirubin administration also was associated with decreased eosinophil and monocyte infiltration into the small intestine, with a corresponding increase in peripheral blood eosinophilia. Bilirubin prevented Jurkat migration, but did not alter IL-5 production.

Conclusion: Bilirubin prevents DSS-induced colitis by inhibiting the migration of leukocytes across the vascular endothelium and by suppressing iNOS expression.

Keywords: bilirubin, VCAM-1, eosinophil, iNOS, DSS colitis
Introduction

Bilirubin is generated during the physiological breakdown of heme, through the sequential activity of heme oxygenase and biliverdin reductase. Elevated serum levels of bilirubin can occur as a consequence of the accelerated release of heme from hemoglobin (e.g., hemolysis) or diminished hepatic conjugating activity (e.g., Gilbert’s syndrome). It was first postulated over 75 years ago that bilirubin exerts an anti-inflammatory effect when it was noted that patients with rheumatoid arthritis experienced a remission of symptoms after developing jaundice secondary to superimposed liver disease (28; 61). These observations led to small therapeutic trials in which intravenous bilirubin was found to variably induce sustained analgesia and diminished joint swelling in subjects with chronic arthritis (68), although technical hurdles resulted in the abandonment of this line of investigation (29). More recently, bilirubin has been shown to suppress inflammatory responses in animal models of autoimmune encephalomyelitis (40) and hind paw inflammation (74). With regard to inflammatory conditions of the intestine, indirect evidence supporting a potential protective effect of bilirubin are derived from the finding that individuals possessing the Gilbert’s polymorphism have a decreased risk of Crohn’s disease (20).

One mechanism whereby bilirubin has been postulated to exert an anti-inflammatory effect is through inhibition of Vascular Cell Adhesion Molecule-1 (VCAM-1)-mediated signaling. VCAM-1 promotes the binding and movement of leukocytes across activated vascular endothelium (43; 54; 72), and is believed to facilitate the immune-mediated tissue injury that occurs in inflammatory bowel disease (18; 27). Specifically, the infiltration of eosinophils into the intestinal mucosa, a VCAM-1-dependent process (13; 50), has been implicated in the pathogenesis of both ulcerative colitis and Crohn’s disease (1; 11; 75). In support of this
hypothesis, treatment with antibodies (10; 49; 65) or antisense oligonucleotides (56) directed against VCAM-1 suppresses intestinal inflammation in rodent colitis models, and blocking antibodies direct against leukocyte integrins that bind VCAM-1 have been shown to ameliorate Crohn’s disease in humans (25; 60). Bilirubin, a potent chain-breaking antioxidant (3; 66), inhibits VCAM-1-dependent migration of lymphocytes across endothelial monolayers by scavenging NADPH oxidase-generated superoxide (33), an early event in VCAM-1 signaling (15). Consistent with these in vitro findings, bilirubin has been demonstrated to attenuate allergen-induced pneumonitis in mice by blocking VCAM-1-mediated eosinophil infiltration into the lungs (33).

Bilirubin also may attenuate intestinal injury by preventing the up-regulation of inducible nitric oxide synthase (iNOS). Nitric oxide (NO) generated by the iNOS-catalyzed conversion of L-arginine to L-citrulline is thought to contribute to impaired intestinal mucosal integrity during sepsis (12), and also appears to play a key role in the pathogenesis of intestinal inflammation in animal models of colitis (30; 36; 37; 79). In humans, colonic iNOS expression and NO levels correlate with clinical and endoscopic indices of disease activity in patients with ulcerative colitis (17; 55; 62). While the influence of bilirubin on intestinal iNOS activity has not been directly examined, bilirubin has been shown to inhibit the up-regulation of iNOS in isolated murine macrophages, and in liver, renal, myocardial, and aortic tissues of endotoxin-treated rats (39; 74).

The oral administration of dextran sodium sulfate (DSS) to mice induces a form of colitis that mimics many of the clinical and histological features of human ulcerative colitis (52),
including eosinophil infiltration. The fundamental role of eosinophils in inducing colonic injury in this murine model is highlighted by the finding that intestinal inflammation is markedly attenuated in mice lacking eosinophil peroxidase (an enzyme that produces oxidizing compounds implicated in inflammatory responses) or eotaxin (the principal eosinophil chemoattractant) (22). Previous investigations also support a contribution of iNOS activity to DSS-mediated intestinal damage (30; 36). Since bilirubin has been shown to modulate iNOS expression and inhibit VCAM-1-mediated eosinophil migration, we postulated that treatment with this bile pigment would prevent colitis induced by DSS. Hence, the aims of the present studies were to examine the influence of bilirubin on colonic inflammation, eosinophil accumulation, and iNOS activity in DSS-treated mice.
Glossary

DSS, dextran sodium sulfate; DAI, disease activity index; HUVEC, human umbilical vein endothelial cells; iNOS, inducible nitric oxide synthase; IL-5, interleukin-5; i.p., intraperitoneal;
PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1
Materials and Methods

Materials

Unconjugated bilirubin (bilirubin IXα) was obtained from Porphyrin Products (Logan, UT) and further purified according to the method of McDonagh and Assisi (46) in order to eliminate potential lipid contaminants. Bilirubin stock solutions were freshly prepared in 0.1 M potassium phosphate (pH 12), as previously described by our group (74). The addition of a small aliquot (≤ 0.4% v:v) of this stock solution had no effect on the pH of the cultured medium or on cell viability. Phorbol myristate acetate (PMA), ionomycin, collagen, fibronectin, tumor necrosis factor-α (TNF-α), Texas Red dextran 10,000 MW, and CellTrace Far Red were obtained from Life Technologies (Grand Island, NY).

Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from discarded umbilical cords using collagenase, as previously described (19). Cells were cultured in F-12K Medium supplemented with 10% fetal calf serum (FCS), endothelial cell growth supplement (Corning; Bedford, MA), 0.1 mg/mL heparin, 100 IU penicillin, and 100 µg/mL streptomycin. All experiments were performed using passages 3 to 7. The human acute T cell leukemia cell line, Jurkat, was purchased from ATCC and grown in RPMI 1640 supplemented with 10% FCS, 1 mM L-glutamine, 100 IU penicillin, and 100 µg/mL streptomycin. The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HSB-2 from Electro-Nucleonics, Inc (59). HSB-2 cells, a Human T-cell lymphoblastoid line, were cultured in RPMI 1640 medium with 10% FCS.
Induction of colitis by the administration of DSS

Animals were housed in the Laboratory Animal Medicine Services facility at the University of Cincinnati under controlled conditions (temperature 22 ± 2°C, relative humidity 50 ± 10%, 12 h light-dark cycle) and fed a standard chow diet. Experiments were performed on adult male C57BL/6/J mice (Jackson Laboratories, Bar Harbor, ME) because of the previously demonstrated susceptibility of this murine strain to DSS-induced colonic injury (42; 53). Colitis was induced by administering 2.5% DSS (w/v) in the drinking water for the entire course of the experiment, with control animals receiving filtered water that did not contain DSS. Twenty-four hours after commencing DSS treatment, animals received intraperitoneal (i.p.) injections of bilirubin (30 mg/kg), or an equivalent volume of the potassium phosphate vehicle, every 8 h for 7 days. All experiments were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Assessment of disease activity

Mice were weighed and examined for signs of colitis on a daily basis, using a standard clinical disease activity index (DAI) comprised of the following parameters graded on a 4 point scale (16; 69; 71): stool consistency, presence or absence of fecal blood, and weight loss. At the conclusion of the 7 day treatment period, animals were sacrificed by CO₂ inhalation. Blood was immediately obtained by cardiac puncture. The small and large intestines were resected and colon length was promptly measured.

Histological analyses of tissue specimens
Longitudinal sections of the entire colon and of the small intestine were fixed in 4% paraformaldehyde and stained with H&E, employing standard histological techniques. Each colon specimen was independently assessed for injury over serial low-power fields by three blinded observers, employing a well-defined quantitative scoring system (38; 71) that grades the severity of inflammation (on a scale of 0 - 3), the extent of injury (0 - 3), and crypt damage (0 - 4). The value assigned to each parameter is multiplied by a factor reflecting the percentage of tissue involvement and then summed, with a maximum achievable severity score of 40. A minimum of 22 independent fields were examined per specimen per observer. To determine whether treatment effects were site specific, a separate experiment was performed in which 2 cm sections of proximal (between the cecum and descending colon) and distal (between the descending colon and anus) colon were isolated, and contiguous low-power fields graded histologically in a blinded fashion by 3 independent observers.

Eosinophils were identified by staining paraffin-embedded specimens with Sirius Red, according to the method of Meyerholz et al (47). Briefly, slides containing fixed tissue sections were immersed in Harris hematoxylin for 2 min followed by rinses in tap water and 100% ethanol. Slides were then immersed in an alkaline Sirius red solution (pH 8 - 9) for two hours and rinsed in tap water. Immunohistochemical staining for inducible nitric oxide synthase was performed by incubating frozen sections of colonic tissue in the presence of primary anti-mouse iNOS antibody (1:250; Transduction Laboratories) or the isotype control antibody (rat anti-mouse Ig; 1:500) at room temperature for 1 h, followed by an HRP-conjugated secondary antibody (67). Immunohistochemical staining of small intestine and colonic tissue for CD3
(prediluted, Ventana Medical Systems), CD68 (1:25; Abcam), and VCAM-1 (1:500; Biorbyt) was performed by the Cincinnati Children’s Hospital histology core.

Quantification of leukocytes in blood samples

Blood samples were collected in heparinized tubes and total white blood cell counts determined using a hemacytometer. Differential leukocyte counts were performed on blood smears treated with Wright’s stain, with a total of 200 cells counted per sample. Eosinophils were specifically identified by staining blood samples with Discombe’s solution (21; 41).

Serum assay for nitric oxide

Serum samples were filtered (10,000 mol wt) to exclude erythrocytes, and nitrate levels were measured using a Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical, Ann Arbor, MI), as previously described by our group (74).

Quantification of cellular interleukin-5 expression

HSB-2 cells were seeded in 12-well plates at a density of $2 \times 10^6$ per ml and grown for 24 h. Monolayers were induced to express interleukin-5 (IL-5) by the addition of 4 ng/mL PMA and 1 µM ionomycin to the culture medium (57). At the end of the incubation period, total RNA was extracted using a GeneJET PCR Purification Kit (ThermoScientific) and 5 µg of total RNA was reverse transcribed using a Thermo Scientific cDNA kit, according to the manufacturer's instructions. Quantification of IL-5 and GAPDH mRNA (the latter to control for amplification) was achieved using the ABsolute Blue QPCR SYBR Green detection system (ThermoScientific). Primers pairs were designed using Primer-blast NCBI: IL-5 (sense: 5’-gtttctgattgtgacttagct-
3’, antisense: 5’-tgccgtcaatgtatttctttattaag-3’) and GAPDH (sense: 5’-agaaggetgggetcatttg-3’,
antisense: 5’-aggggcacagtcagttc-3’).

226 _Transwell migration assay_

HUVEC were seeded in the upper chamber of 24-transwells with 8-µm pores (Costar,
Cambridge, MA) that were pre-coated with collagen (0.4 mg/cm²) and fibronectin (2 µg/cm²) at
a density of 4 x 10⁵ cells/insert and grown to confluence. Monolayer integrity was validated by
overlaying Texas Red dextran 10,000 MW (20 µg/mL) and measuring fluorescence intensity (ex:
595 nm, em: 625 nm) in the lower chamber. To induce adhesion molecule expression, HUVEC
monolayers were stimulated with TNF-α (5 ng/mL) for 16 h prior to performing migration
studies (14; 77). Jurkat cells were incubated in the presence of 25 µM CellTrace Far Red for 45
min at 37°C, washed, and migration initiated by overlaying these fluorescently-labeled cells (1 x
10⁵ per insert) onto the HUVEC monolayers in the upper chamber of the transwell. Studies were
performed in the presence of bilirubin (0 - 20 µM) or vehicle (0.1 M K₃PO₄), which was added
to the assay medium (F-12K plus 0.1% HSA) immediately prior to the start of the experiment.
Transendothelial migration was quantified by measuring CellTrace fluorescence intensity (ex:
625 nm, em: 670 nm) in the lower chamber.

240 _Statistical analysis_

Data were analyzed using a computer-based statistical program (SSI SigmaStat, San Jose,
CA). Mean values were evaluated by ANOVA with t-test to assess for statistical significance.
For data that were not normally distributed, a Kruskal-Wallis analysis of variance on ranks was
performed.
Results

Effect of bilirubin on DSS-induced disease activity, colon length, and intestinal histology

To determine whether bilirubin is able to suppress DSS-induced colitis, C57BL/6 mice were divided into each of four treatment groups comprised of 9 (vehicle treatments) or 10 (bilirubin treatments) animals: no DSS plus i.p. bilirubin vehicle (vehicle); DSS plus vehicle (DSS + vehicle); no DSS plus bilirubin (bilirubin); and DSS plus bilirubin (DSS + bilirubin). DSS-treated mice that received bilirubin exhibited less weight loss (Fig. 1A), diarrhea, and intestinal bleeding than did animals administered DSS plus vehicle, as reflected in the significantly lower DAI scores (Fig. 1B). Unexpectedly, and despite a normal appearance, mice treated with bilirubin alone failed to gain weight to the same degree as animals receiving vehicle alone. Consistent with the clinical findings, DSS-treated mice that were administered bilirubin demonstrated less colon shortening (Fig. 1C) and reduced mucosal inflammation (Fig. 2, upper panels) compared with animals that received DSS plus vehicle. The colons from DSS-treated mice that were administered vehicle exhibited dense cellular infiltrates, marked architectural destruction, and loss of epithelial lining. Mice receiving DSS plus bilirubin manifest less colonic inflammation and reduced morphologic injury, as evidenced by the significantly lower histologic injury scores (Fig. 2A). Administration of bilirubin alone was found to have no noticeable effect on colon histology as compared with vehicle treatment. Notably, bilirubin was found to exert a less pronounced effect in the distal (Fig. 2B) as compared with the proximal (Fig. 2C) colon.

Influence of bilirubin on eosinophil recruitment in response to DSS treatment
Since eosinophils are believed to play an important role in the pathogenesis of DSS colitis (22), and since bilirubin has previously been shown to inhibit eosinophil influx in a murine model of airway inflammation (33), we assessed whether bilirubin treatment prevents eosinophil recruitment to the colon of DSS-treated mice. Paraffin-embedded sections of distal colon were stained with the eosinophil marker, Sirius Red (Fig. 3, upper panels), and the average number of eosinophils per high power field was quantified (Fig. 3, lower panel). Eosinophil infiltration into the colon of animals that received DSS was significantly lower following bilirubin versus vehicle treatment, supporting an inhibitory effect of bilirubin on eosinophil infiltration.

Since VCAM-1 is up-regulated in the colon of mice administered DSS (65) and appears to contribute to tissue injury and eosinophil recruitment (6), we examined the effect of bilirubin on VCAM-1 expression by performing immunohistochemical staining of intestinal specimens. We found that the DSS-induced expression of VCAM-1 in the colon was markedly reduced in mice that were simultaneously administered bilirubin (Fig. 3, middle panels), approaching levels observed in animals that did not receive DSS (data not shown). These findings provide a potential explanation for the ameliorating effect of bilirubin. However, while the number of colonic eosinophils was not statistically different between the non-DSS control groups, there was a strong trend (p = 0.06) toward lower numbers of eosinophils/hpf in mice that were administered bilirubin versus vehicle, despite minimal detectable VCAM-1 in both groups. These data raise the possibility that bilirubin modulates eosinophil homing via mechanism(s) that are independent of VCAM-1 expression.
To determine whether the diminished number of eosinophils in the colon of bilirubin-treated animals might be due to fewer circulating cells available for recruitment, we measured total leukocyte and differential counts in the peripheral blood of bilirubin- and vehicle-treated mice, both in the presence and absence of DSS. While total peripheral blood leukocyte counts did not differ significantly between the various treatment groups (Fig. 4A), mice that received bilirubin exhibited a selective increase in the percentage of circulating eosinophils (Fig. 4B), regardless of whether the animals were administered DSS. An increase in the absolute number of eosinophils in the peripheral circulation of mice treated with bilirubin was confirmed by staining blood smears with Discombe’s solution in order to specifically label eosinophils (Fig. 4C). These data indicate that the observed reduction in the number of colonic eosinophils in mice treated with bilirubin does not result from fewer circulating eosinophils. On the contrary, the increased peripheral blood eosinophilia noted in bilirubin-treated animals suggests that eosinophil access to peripheral tissues is impeded.

The proliferation, differentiation, and release of eosinophils from the bone marrow into the systemic circulation is mediated primarily by interleukin-5 (IL-5) (34). To investigate whether the observed increase in peripheral blood eosinophils associated with bilirubin administration might be due to augmented production of IL-5, we examined the effect of bilirubin on cellular IL-5 expression by HSB-2 cells, a human T-cell leukemia line that has been shown to produce IL-5 in a manner similar to normal peripheral blood T-cells (57; 73). When stimulated with PMA and ionomycin, HSB-2 cells exhibited a marked (1000-fold) up-regulation in IL-5 mRNA that peaked at 6 h and was unaffected by co-treatment with bilirubin (Fig. 5A). When incubated in the presence of bilirubin alone, HSB-2 cells manifested a non-significant
(less than 2-fold) increase in IL-5 mRNA out to 24 h (Fig. 5B). In light of previous studies demonstrating no significant effect of bilirubin on IL-5 levels in isolated T-cells and lung lavage fluid obtained from mice with allergen-induced pulmonary inflammation (33), these data support that bilirubin does not induce peripheral eosinophilia through modulation of IL-5 production.

Bilirubin modulates the physiological homing of eosinophils to the intestinal tract

We have shown that bilirubin reduces eosinophil infiltration into the colon when inflammation is induced by treatment with DSS (Fig. 3). As eosinophils primarily reside in intestinal tissue under non-inflammatory states (34), we assessed whether bilirubin impairs the normal physiological homing of these cells to the gastrointestinal tract by quantifying the number of eosinophils in Sirius Red-stained sections of the mid-jejunum (Fig. 6, upper panels). Notably, mice that received bilirubin had significantly fewer tissue eosinophils than those administered vehicle, regardless of whether DSS was administered (Fig. 6, lower panel). These data, when taken in conjunction with our finding that the number of eosinophils in the peripheral blood increases in bilirubin-treated mice (Figs. 4B, 4C), suggest that bilirubin impedes the physiologic migration of eosinophils from the circulation to the gut.

To further examine this hypothesis, we investigated the effect of bilirubin on the transendothelial migration of leukocytes in vitro. Since VCAM-1 appears to play an essential role in the pathogenesis of DSS colitis (65) and in the recruitment of eosinophils to sites of inflammation (13; 26; 76), we elected to study Jurkat cells, a human acute T cell leukemia cell line that is known to express the VCAM-1-specific integrin VLA-4 (35). HUVEC monolayers were pre-incubated with TNF-α in order to induce the expression of VCAM-1 (14; 77), as
validated by measuring both cellular mRNA and protein levels (data not shown). Paralleling our in vivo findings and the results of our prior studies (33), bilirubin markedly inhibited the migration of Jurkat cells across TNF-α-stimulated HUVEC monolayers (Fig. 6C) without altering VCAM-1 expression, consistent with a direct modulatory effect on VCAM-1 signaling.

**Effect of bilirubin on the intestinal recruitment of T-lymphocytes and monocytes/macrophages**

To determine whether the inhibitory effect of bilirubin is specific to eosinophil recruitment, we stained sections of colon and small intestine for the pan-phenotypic T-lymphocyte marker, CD3, and for CD68, a glycoprotein that is expressed exclusively on monocytes and macrophages. Analogous to our findings for eosinophils (Fig. 3), bilirubin treatment was associated with a significant reduction in the DSS-induced infiltration of CD3 (Fig. 7) and CD68 (Fig. 8) positive cells into the colon. While there appears to be no effect of either DSS or bilirubin on the number of CD3 positive cells in the small intestine (Fig. 9), substantially fewer CD68 positive cells were present in small intestinal tissue from bilirubin-treated mice, irrespective of whether they received DSS (Fig. 10). These latter findings parallel our observation for eosinophils (Fig. 6), suggesting that bilirubin also impedes the migration of monocytes/macrophages from the peripheral circulation into the small intestine.

**Influence of bilirubin on DSS-induced colonic iNOS expression and systemic nitrate production**

Nitric oxide generated by iNOS is thought to contribute to the colonic injury induced by DSS (5; 30; 36). As bilirubin has been shown to inhibit the up-regulation of iNOS in response to
inflammatory stimuli (39; 63; 74), we sought to determine whether bilirubin treatment is associated with reduced iNOS expression in the colon of mice treated with DSS. Frozen sections of colon tissue from untreated and DSS-treated mice that were simultaneously administered bilirubin or vehicle were subjected to immunohistochemical staining for murine iNOS, employing isotype antibody as control. Consistent with prior reports (5; 67), the colonic expression of iNOS was markedly augmented by DSS. Additionally, we found that DSS-induced iNOS expression was substantially abrogated by bilirubin treatment (Fig. 11). Consistent with these histologic findings, mice treated with vehicle plus DSS manifested elevated serum nitrate levels (a marker of iNOS activity), which were significantly lower in bilirubin-treated animals (Fig. 5D). Taken together, these data support that bilirubin exerts an inhibitory effect on DSS-induced colonic expression and activity of iNOS.
Discussion

The present studies demonstrate that colonic inflammation induced by the oral administration of DSS to C57BL6/J mice is suppressed by bilirubin, as evidenced by reduced disease activity scores and by diminished histologic injury. The reason why bilirubin exerts a more pronounced protective effect in the proximal versus the distal colon is uncertain. We speculate that this could be due to the overall less vigorous inflammatory response in the former region, or possibly because levels of bilirubin in the colonic lumen decline caudally as a result of enterohepatic cycling (70). Our findings are consistent with previous reports that the bilirubin precursor, biliverdin, ameliorates DSS colitis in mice (7). Although the mechanism underlying this protective effect was not examined in this prior study, in light of our present findings, and since biliverdin is known to undergo rapid and quantitative conversion to bilirubin via the action of the ubiquitous biliverdin reductase enzyme (44; 45), we speculate that bilirubin is the principal physiologic mediator of the observed cytoprotection. This hypothesis is supported by the results of prior studies in which bilirubin, but not biliverdin, was found to attenuate endothelial cell activation (64).

Our data suggest two potential mechanisms by which bilirubin may exert a modulatory effect on DSS-induced colonic inflammation: 1) inhibition of eosinophil (and other leukocyte) infiltration into intestinal tissues, and 2) suppression of iNOS expression and activity. We previously have demonstrated that bilirubin blocks eosinophil migration into the lungs of mice with allergen-induced pneumonitis through a mechanism that involves the disruption of VCAM-1 signaling within vascular endothelial cells (33). Our present finding that bilirubin administration reduces eosinophil infiltration into the colon, while simultaneously increasing levels of circulating eosinophils, supports a similar mechanism of bilirubin action in the DSS
colitis model. Consistent with this proposition, we have directly shown that bilirubin, at
physiological concentrations (20 µM ≈ 1.2 mg/dL), effectively prevents the movement of Jurkat
cells across TNF-α activated HUVEC monolayers. Although TNF-α induces HUVEC to express
both VCAM-1 and ICAM-1, because Jurkat cells do not bind ICAM-1 (48), our findings imply
that bilirubin exerts its effects primarily through disruption of VCAM-1-dependent processes.
When taken in conjunction with our previous finding that bilirubin inhibits the movement of
lymphocytes across murine endothelial cell monolayers that constitutively express VCAM-1
(33), these data support that the effects of bilirubin are not eosinophil-specific but rather that it
interferes with any VCAM-1-mediated migration process. This hypothesis is supported by our
demonstration of reduced numbers of CD3 and CD68 positive cells in the colon of DSS-treated
mice that also received bilirubin, as both lymphocytes and monocytes/macrophages are known to
express VCAM-1-specific integrins (23; 24).

Since VCAM-1 activation (65) and eosinophil infiltration (22) are important contributing
factors to the pathogenesis of DSS colitis, the ability of bilirubin to block VCAM-1-dependent
leukocyte migration, and thereby inhibit eosinophil recruitment, likely underlie its protective
effects. We show that bilirubin-treated animals manifest markedly attenuated colonic VCAM-1
expression in response to DSS, which may, at least in part, explain its ameliorating effect on
tissue injury. However, since the production of VCAM-1 by endothelial cells is stimulated by
pro-inflammatory cytokines, these data cannot differentiate as to whether this reflects an ability
of bilirubin to directly inhibit endothelial VCAM-1 expression or, rather, is a consequence of
reduced cytokine production. Our demonstration that bilirubin does not alter basal or TNF-α-
stimulated VCAM-1 mRNA or protein levels in cultured HUVEC supports the latter theory, with
the caveat that other investigators have reported an inhibitory effect of bilirubin on endothelial cell expression of VCAM-1 (32; 64). Our current findings conflict with a prior study by our group employing a murine model of allergic pneumonitis in which we observed that bilirubin did not alter pulmonary VCAM-1 expression. Notably, in this same study, bilirubin also did not influence the production of IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α or the main eosinophil chemoattractants IL-5 and eotaxin (33). While an important limitation of the present experiments is our inability to quantitate IL-5 or eotaxin levels in intestinal tissue, the disparate findings could possibly reflect the fact that allergic pneumonitis is a Th2 cytokine-dependent model (9) while DSS-induced colitis is Th1-mediated (2).

Because eosinophils are tissue resident leukocytes that localize primarily to the gastrointestinal tract (58), our observation that eosinophil levels in the jejunum are significantly reduced in bilirubin-treated animals (irrespective of whether they received DSS) supports that bilirubin also inhibits the normal physiological migration of eosinophils into the gut. This theory is bolstered by the concomitant increase in circulating eosinophils associated with bilirubin administration, a finding that corroborates previous observations by our group (33). Our finding that bilirubin does not alter IL-5 message in unstimulated or activated HSB-2 T-cell leukemia cells, which are a generally accepted in vitro model of IL-5 regulation (57; 73), indicates that the peripheral eosinophilia associated with bilirubin administration is unlikely to be the result of increased eosinophil production and/or release from the bone marrow. Notably, while the number of T-lymphocytes in the small intestine was unaffected by bilirubin treatment, the level of monocytes/macrophages was reduced, paralleling our eosinophil results. These data suggest that eosinophils and monocytes may share a similar trafficking mechanism to the small bowel.
As there was no detectable VCAM-1 expression in the small intestine in any of the treatment groups, it seems unlikely that bilirubin reduces leukocyte infiltration by modulating the expression of VCAM-1 in non-inflamed intestinal tissue. In previous studies, we have shown that physiological concentrations of bilirubin, a potent chain-breaking antioxidant (66), inhibits the transmigration of VLA-4-expressing leukocytes across murine endothelial monolayers by scavenging VCAM-1-dependent reactive oxygen species signaling intermediaries (33). We speculate that this same mechanism may underlie the inhibitory effect of bilirubin on the infiltration of eosinophils and monocytes into the small intestine.

We are perplexed by the observed difference in growth curves between vehicle and bilirubin-treated control animals, as we have not previously observed this phenomenon in mice receiving similar dosing of i.p. (33) or oral (63) bilirubin, which we have shown produces very modest 3- to 4-fold elevations in serum bilirubin levels (≤ 0.4 mg/dL) (74). Notably, Gunn rats, which are homozygous (j/j) for a mutation in the UGT1A gene locus leading to persistent, marked hyperbilirubinemia (~5 mg/dL) (63), do not manifest abnormal growth. Since bilirubin is generated as part of the physiologic degradation of heme, it is normally present in the circulation. In newborns, markedly elevated serum bilirubin concentrations (generally over 20 mg/dL ≈ 340 µM) can cause neurologic injury (kernicterus); however, toxicity in adults is negligible. Indeed, bilirubin has been administered intravenously to patients without overt sequelae (68), reaching serum levels as high as 22 mg/dL (normal ≤ 1.2 mg/dL ≈ 20 µM). More modest (< 3-fold) chronic elevations in serum bilirubin are commonly encountered in individuals with Gilbert's syndrome, a benign condition resulting from polymorphisms in the gene encoding UGT1A1 (8), the principal bilirubin conjugating enzyme. It is notable that our *in vitro* studies utilizing human...
cells support that bilirubin exerts substantial inhibitory effects on leukocyte migration at concentrations that are at the upper end of the normal physiologic range (20 µM).

The administration of DSS to mice has been shown to induce iNOS expression in the colon (31; 37) and to increase nitrate concentrations in the serum (5; 37). Although conflicting data exist (4; 78), it is generally held that iNOS-derived NO contributes to the pathogenesis of DSS-induced colitis, as evidenced by the findings that iNOS-deficient mice (30; 36; 37) and animals administered iNOS inhibitors (37; 51) manifest reduced colonic injury. It is notable that bilirubin has previously been shown to suppress the endotoxin stimulated up-regulation of iNOS (39; 74). Consistent with these prior investigations, our experiments demonstrate that bilirubin treatment reduces colonic iNOS expression and serum nitrate levels in DSS-treated mice, suggesting that bilirubin may act, in part, by preventing peroxynitrite-induced tissue injury (79). Both iNOS (55; 62) and VCAM-1 (18; 27) are believed to contribute to the pathogenesis of inflammatory bowel disease in humans and, notably, natalizumab (an antibody directed against the α4 integrin subunit that mediates leukocyte binding to VCAM-1) has shown efficacy in inducing and maintaining clinical remission in Crohn’s disease (25; 60). Hence, our demonstration that bilirubin is able to disrupt both of these processes raises the specter of a potential therapeutic application.
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Figure Legends

**Figure 1:** Effect of bilirubin on body weight, disease activity, and colonic shortening induced by DSS

Mice were administered 2.5% DSS in the drinking water, followed by i.p. injections of bilirubin (grey squares) or the potassium phosphate vehicle (black squares). Control animals received bilirubin (grey circles) or vehicle (black circles) without DSS. Panel A depicts the average daily percent change in weight from baseline (± S.E.M.) following the initiation of DSS. Panel B displays the mean disease activity index for each group. The average length of resected colon specimens is depicted in panel C. In comparison with the other treatment groups, the colons from mice treated with DSS plus vehicle were significantly shorter and lacked formed stool. *p < 0.002 vs. all other groups, **p < 0.03 vs. bilirubin and bilirubin + DSS, ***p < 0.001 vs. vehicle + DSS

**Figure 2:** Bilirubin treatment reduces colon histologic injury following DSS administration

The four upper panels show representative low-power (200 x) images of H&E-stained sections of colon from mice treated with vehicle alone, DSS plus vehicle, bilirubin alone, or DSS plus bilirubin. The lower panels display histologic injury scores based on a blinded analysis of colon specimens using a standard 0 to 40 scoring system. Panel A depicts mean histologic scores (± SEM) assessed over the entire colon. Panels B and C show the results of a separate experiment in which histologic scoring of sections of distal and proximal colon were performed. *p < 0.002 vs. vehicle plus DSS
Figure 3: Bilirubin inhibits eosinophil infiltration and VCAM-1 expression in the colon of DSS-treated mice.

The upper panels display representative high-power (400 x) images of 2 cm sections of distal colon obtained from DSS-treated mice administered vehicle (left) or bilirubin (right) and stained with Sirius Red to highlight eosinophils (arrows). The middle panels show the results of immunohistochemical staining of proximal colon specimens for VCAM-1 (200 x). Graphed in the lower panel is the mean number of eosinophils (± S.E.M.) per high-power field (hpf) averaged over 10 separate fields (n = 9 - 10 per treatment group). * p < 0.001 vs. vehicle plus DSS.

Figure 4: Effect of bilirubin on peripheral blood leukocytes

Panel A depicts the average total leukocyte count (± S.E.M.) while panel B displays the mean percentage of neutrophils (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophils (Eos), and basophils (Baso) in the peripheral blood of mice treated with vehicle alone (grey bars), DSS plus vehicle (white bars), bilirubin alone (black bars), or DSS plus bilirubin (striped bars). In panel C, peripheral blood eosinophil counts were determined using Discombe’s staining, and are plotted as the average number of eosinophils/mL (x 10^6). # p < 0.05 vs. vehicle and vehicle plus DSS; * p < 0.01 vs. vehicle and vehicle plus DSS.

Figure 5: Influence of bilirubin on cellular IL-5 production, leukocyte transendothelial migration, and serum nitrate concentration

The effect of 10 µM bilirubin (grey symbols) on the time course for the expression of IL-5 mRNA in HSB-2 cells, as determined by qPCR, is displayed in Panel A. Cells were incubated...
in the presence (squares) or absence (circles) of 4 ng/mL PMA and 1 µM ionomycin with data reflecting IL-5 expression (± SEM) relative to that in untreated cells at time 0 (n = 4). In panel B, HSB-2 cells were incubated with 10 µM bilirubin for the indicated time intervals and IL-5 mRNA levels are plotted relative to expression at time 0. Panel C displays the time course for Jurkat cell migration across HUVEC monolayers pre-incubated for 16 h in the presence (squares) or absence (circles) of TNF-α (5 ng/mL). CellTrace Far Red-labeled Jurkat cells were overlaid onto the monolayers in the presence of 20 µM bilirubin (grey symbols) or vehicle (black symbols). Displayed is the percentage of overlaid cells that migrated to the lower chamber, as quantified by measuring fluorescence intensity (ex: 625 nm, em: 670 nm). Panel D shows the mean serum nitrate concentration for each treatment group. * p < 0.05 vs. all other groups

**Figure 6: Bilirubin reduces eosinophil infiltration into the small intestine**

Displayed in the upper panels are representative high-power (400 x) images of sections of small intestine stained with Sirius Red to identify eosinophils (arrows). Treatment groups are as previously described in Figure 2. The mean number of eosinophils per hpf (± S.E.M.) for each group is graphed in the lower panel. An average of 39 separate high power fields was examined per specimen (n = 9 – 10 per group). * p < 0.001 vs. all other groups

**Figure 7: Treatment with bilirubin decreases T-lymphocyte infiltration into the colon of DSS-treated mice**

Shown are representative low-power (200 x) images of colon tissue stained for the T-lymphocyte marker CD3. Immunohistochemical staining was performed on sections of colon obtained from mice treated with vehicle alone (upper left panel), vehicle plus DSS (upper right panel), and vehicle plus DSS plus bilirubin (lower left panel).
panel), bilirubin alone (middle left panel), or bilirubin plus DSS (middle right panel). The lower panel displays the mean number of CD3 positive cells per hpf (± S.E.M.) for each treatment group. An average of 29 separate high power fields was examined per specimen (n = 3 - 4 per group). * p < 0.02 vs. all other groups

Figure 8: Bilirubin inhibits the recruitment of monocytes/macrophages to the colon in response to DSS

Representative colon specimens stained for CD68, a marker for monocytes and macrophages, are displayed (200 x). Conditions are as described under Figure 7. The marked infiltration of CD68 positive cells in animals receiving DSS plus vehicle precludes accurate quantitation.

Figure 9: DSS and bilirubin do not alter T-lymphocyte levels in the small intestine

Histologic sections of small intestine (200 x) were stained for CD3, as detailed in Figure 7. Quantification of CD3 positive cells is represented as the number (± S.E.M.) per intact villus (lower panel). An average of 32 villi was examined per specimen (n = 3 per treatment group).

Figure 10: Monocyte infiltration into the small intestine is reduced following bilirubin treatment

Representative small intestinal specimens stained for CD68 positive cells (arrows) are shown (200 x), as described under Figure 8. The lower panel displays the mean number of CD68 positive cells per hpf (± S.E.M.) for each treatment group. An average of 32 separate high power fields was examined per specimen (n = 3 per group). * p < 0.01 vs. both vehicle treatment groups
Figure 11: Bilirubin inhibits DSS-induced colonic iNOS expression

Immunohistochemical staining for iNOS was performed on frozen sections of colon tissue obtained from mice treated with vehicle alone (upper left panel), vehicle plus DSS (upper middle panel), bilirubin alone (lower left panel), or bilirubin plus DSS (lower middle panel). Representative low-power (200 x) images are shown. The right panels display specimens stained with an isotype antibody as control.
References


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Figure 1

A

Change in weight (%)

Day #

0 1 2 3 4 5 6 7

B

Disease Activity Index

Vehicle Vehicle + DSS Bilirubin Bilirubin + DSS

Vehicle Vehicle + DSS Bilirubin Bilirubin + DSS

C

Colon length (cm)

Vehicle Vehicle + DSS Bilirubin Bilirubin + DSS
Figure 3
Figure 5

A

Fold induction

Time (h)

B

Fold induction

Time (h)

C

% Migration

Time (min)

D

Serum nitrate (µM)

Vehicle
Vehicle + DSS
Bilirubin
Bilirubin + DSS
Figure 6

Comparison of eosinophils/HPF in different treatment groups:
- Vehicle
- Vehicle + DSS
- Bilirubin
- Bilirubin + DSS

Graph showing the number of eosinophils/HPF:
- Vehicle: 10 ± 2
- Vehicle + DSS: 20 ± 3
- Bilirubin: 2 ± 1
- Bilirubin + DSS: 12 ± 2

Significance indicated by asterisks (*)
Figure 7

Vehicle

Vehicle + DSS

Bilirubin

Bilirubin + DSS

CD3+ cells / hpf
Figure 9

[Images of histological sections showing CD3+ cells per hpf for different groups: Vehicle, Vehicle + DSS, Bilirubin, and Bilirubin + DSS.]

The bar graph shows the CD3+ cells per hpf for each group, with Vehicle + DSS having the highest number, followed by Bilirubin + DSS, Vehicle, and Bilirubin.

Legend:
- Vehicle
- Vehicle + DSS
- Bilirubin
- Bilirubin + DSS

CD3+ cells / hpf

0 10 20 30
Figure 10

CD68+ cells / hpf

Vehicle

Vehicle + DSS

Bilirubin

Bilirubin + DSS

Vehicle

Vehicle + DSS

Bilirubin

Bilirubin + DSS

*
Figure 11