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Unconjugated Bilirubin Inhibits VCAM-1-Mediated Transendothelial Leukocyte Migration

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During lymphocyte migration, engagement of VCAM-1 stimulates the generation of endothelial cell-derived reactive oxygen species (ROS) and activation of matrix metalloproteinases, facilitating endothelial retraction. Because bilirubin is a potent antioxidant, we examined the hypothesis that this bile pigment inhibits VCAM-1-dependent cellular events. The migration of isolated murine splenic lymphocytes across monolayers of murine endothelial cell lines (which constitutively express VCAM-1) is significantly inhibited by physiological concentrations of bilirubin, in the absence of an effect on lymphocyte adhesion. Bilirubin administration also suppresses VCAM-1-stimulated ROS generation and reduces endothelial cell matrix metalloproteinase activity. In a murine asthma model characterized by VCAM-1-dependent airway inflammation, treatment of C57BL6/J mice with i.p. bilirubin decreases the total leukocyte count in the lung parenchyma and lavage fluid, through specific inhibition of eosinophil and lymphocyte infiltration. Blood eosinophil counts were increased in bilirubin-treated animals, while VCAM-1 expression in the capillary endothelium and cytokine levels in both lung lavage and supernatants from cultured lymph node lymphocytes were unchanged, suggesting that bilirubin inhibits leukocyte migration. Conclusion: bilirubin blocks VCAM-1-dependent lymphocyte migration in vitro and ameliorates VCAM-1-mediated airway inflammation in vivo, apparently through the suppression of cellular ROS production. These findings support a potential role for bilirubin as an endogenous immunomodulatory agent. The Journal of Immunology, 2005, 174: 3709–3718.

Adhesion molecules are transmembrane glycoproteins that mediate an array of cellular interactions and play an essential role in the process of inflammatory cell migration (1, 2). One such molecule, VCAM-1, is expressed on the surface of endothelial cells and promotes the binding and movement of lymphocytes and eosinophils across vascular endothelium (1–3). VCAM-1-mediated leukocyte recruitment and migration are implicated in the pathogenesis of several disease states, including inflammatory bowel disease (4), conjunctivitis (5), nephropathy (6, 7), and arthritis (8). The essential role of VCAM-1 in tissue injury has been corroborated in a number of animal models. For example, radiation-induced intestinal inflammation, which is characterized by increased endothelial VCAM-1 expression, is attenuated by treatment with anti-VCAM-1 Abs (9). Immunoneutralization of VCAM-1 has also been shown to ameliorate hepatic necrosis and neutrophil infiltration in response to endotoxin (4). Indeed, VCAM-1 appears to be integral to leukocyte recruitment in a variety of tissues, including the brain (10, 11), kidney (12), and intestine (13).

The molecular mechanisms involved in VCAM-1 signaling have recently begun to be elucidated. The binding of lymphocyte VLA-4 (α4β1 integrin) to endothelial cell VCAM-1 triggers the calcium- and Rac1-dependent activation of cellular NADPH oxidase (14), which generates low (micromolar) levels of reactive oxygen species (ROS)3 (3). This is in contrast with signaling by other endothelial adhesion molecules, such as selectins (15) and PECAM (16), which does not involve ROS production. The localized increase in ROS in response to VCAM-1 stimulation induces the activity of matrix metalloproteinase (MMP)-2 and -9. MMP-mediated digestion of the extracellular matrix and intercellular tight junction complexes facilitates transendothelial lymphocyte migration (17, 18). ROS also have been implicated in the induction of the morphologic changes necessary for cellular retraction, through modulation of the actin cytoskeleton (17). Because the generation of ROS appears to be critical to VCAM-1 signaling, oxygen radical scavengers would be expected to block this process. In this regard, antioxidants have been shown to inhibit leukocyte infiltration in murine nephritis (19) and asthma (20) models, and to decrease cytokine production (21) by cultured endothelial cells.

Bilirubin, one of the principal metabolic end-products of heme catabolism and a potent antioxidant (22), is generated by the sequential action of heme oxygenase (HO) and biliverdin reductase (BVR) (Fig. 1). There is a growing body of evidence supporting that bilirubin is cytotoxic, as this bile pigment has been shown to suppress postischemic myocardial dysfunction (23), protect against LPS-induced liver injury (24), and ameliorate local hind-paw inflammation induced by the injection of α-carrageenan. Because bilirubin is the most abundant endogenous antioxidant (22), with a potency comparable to α-tocopherol (25), we speculate that

3 Abbreviations used in this paper: ROS, reactive oxygen species; AM, acetoxyethyl ester; BDT, ditaurobilirubin; BVR, biliverdin reductase; DHR, dihydrorhodamine 123; HO, heme oxygenase; iNOS, inducible NO synthase; MBP, major basic protein; mHEV, murine high endothelial venule; mHEVas, axillary mHEV; mHEVc, cervical mHEV; MMP, matrix metalloproteinase; Rac, Ras-related C3 botulinum substrate; UGT1A1, UDP-glucuronosyltransferase.
FIGURE 1. Pathway of bilirubin metabolism. Heme is serially catabolized by the action of HO and BVR to form biliverdin and bilirubin, respectively. In the liver, bilirubin is conjugated with glucuronic acid by the action of UGT1A1 before secretion in the bile.

bilirubin may exert its cytoprotective effects by disrupting VCAM-1-mediated signaling. In support of this hypothesis, bilirubin has been shown to block oxidant-mediated leukocyte adhesion (26) and prevent oxidative injury to vascular smooth muscle cells (27). To determine whether bilirubin acts via a VCAM-1-dependent mechanism, we examined the effect of bilirubin on the migration of lymphocytes across isolated endothelial cell monolayers and on eosinophil migration across the pulmonary vascular endothelium in OVA-primed mice. Bilirubin administration inhibited the transendothelial migration of lymphocytes in both of these VCAM-1-dependent model systems, supporting a potentially important regulatory function of this endogenous bile pigment on inflammatory responses.

Materials and Methods

Materials

Unconjugated bilirubin (bilirubin IXα), biliverdin (biliverdin IXα), and ditaurobilirubin (BDT) were obtained from Porphyrin Products. Bilirubin IXα was further purified according to the method of McDonagh and Assisi (28) to eliminate potential lipid contaminants. Rat anti-mouse major basic protein-1 (MBP-1) was kindly provided by Drs. J. and N. Lee (Mayo Clinic, Scottsdale, AZ). Rat anti-mouse α4 integrin (VLA-4) was purchased from BioDesign International. Dihydrorhodamine 123 (DHR) was obtained from Molecular Probes.

Transwell migration assay

Murine high endothelial venule-like cells, derived from BALB/c mouse axillary murine high endothelial venule (mHEVα) or cervical mHEVα (mHEVβ) lymph nodes (29), were grown to confluence in the upper chamber of 12-μm transwells (Costar) in RPMI 1640 supplemented with 20% FCS, 2 mM glutamine, 1 mM HEPES, 10 mM sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin. Monolayers were preincubated for 1 h in the presence of bilirubin, biliverdin, BDT, or the corresponding vehicle, which were simultaneously added to layers were preincubated for 1 h in the presence of bilirubin, biliverdin, BDT, or vehicle. At specified time intervals, cells were lysed with 120 mM Tris buffer containing 0.25% Triton X-100, 0.010g NaCl, and 0.05% Triton X-100. MMP activity was measured at 0 min and 30 min after VCAM-1 stimulation using a Leica TCS4D confocal microscope equipped with an Omnivision krypton-argon laser. These time points were selected because they has previously been shown that maximal ROS generation occurs at 30 min (3). The field of observation was changed after each reading to avoid laser-induced photobleaching. Data are presented as the change in fluorescence intensity from 0 min and are corrected for background by subtracting the fluorescence intensity of identically treated cells that were not loaded with DHR.

Assay of MMP activity by gel zymography

Confluent endothelial cell monolayers were incubated with anti-VCAM-1 Ab-coated beads in the presence of bilirubin or the potassium phosphate vehicle. A set of time intervals, cells were lysed with 120 mM Tris buffer containing 0.25% Triton X-100, 0.010g NaCl, and 0.05% NaCl. Control gels were developed in the presence of EDTA (0.05 M Tris-HCl, 0.01 M EDTA (pH 8.0)) to demonstrate cation dependency. Gels were fixed and stained in 50% methanol, 10% acetic acid, and 0.25% Coo massie blue R250. MMP activity appears as clear bands, which were quantified by densitometry using Image J software (National Institutes of Health, http://rsb.info.nih.gov/ij/). MMP isoforms were identified based on the basis of molecular mobility and by comparison with purified standards (17).

Assessment of cellular VCAM-1 expression

Confluent endothelial cell monolayers treated with bilirubin, biliverdin, BDT, or vehicle were harvested with PBS containing 0.03% EDTA, washed with PBS, and labeled with rat anti-mouse VCAM-1 (1/100 dilution) in PBS containing 0.3% BSA and 0.15% NaCl, for 30 min at 4°C. The cells were labeled with goat anti-rat FITC-conjugated IgG (1/500 dilution; Southern Biotechnology Associates), washed, and fluorescence intensity was quantified by flow cytometry (Coulter EPICS-XL). Unlabeled cells, which were incubated with secondary Ab alone, were used as a control for staining, and are comparable to isotype Ab-labeled cells (data not shown).
Murine model of OVA-induced asthma

Adult female C57BL6/J mice were sensitized with two serial i.p. injections of OVA (200 μl of 200-μg grade V OVA adsorbed to 180 mg of aluminum hydroxide in 4 ml of saline for 1 h at room temperature) 1 wk apart (days 0 and 7) (34, 35) (Fig. 2). Control animals received an equivalent volume of aluminum hydroxide in saline. Mice were challenged with 150 μg of OVA in saline intranasally on day 14, with control animals receiving intranasal saline. Rodents in each group were administered six doses of i.p. bilirubin (30 mg/kg) or the potassium phosphate vehicle at 8-h intervals (days 14 and 15), commencing immediately after OVA challenge. Animals were sacrificed by CO2 inhalation and the lungs were rapidly lavaged by injecting 0.5 ml of ice-cold PBS into the trachea using a 16-gauge needle. The lavage fluid was aspirated and the procedure repeated an additional time. Differential leukocyte counts were performed on H&E-stained cytospins, with a total of 200 cells counted from each of the pooled lavage samples. Frozen sections of mouse lung were stained with H&E to assess lung histology. Studies were reviewed and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Isolation and culture of bronchial lymph node-derived lymphocytes

Lymphocytes were obtained by gently pressing isolated bronchial lymph nodes through a 100-μm nylon mesh (3). Cells were counted, aliquoted to 96-well plates at a concentration of 5 × 10⁶ per ml, and grown in RPMI 1640, 1 mM HEPES, 10 mM NaHCO₃, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μM 2-ME, and 5% heat-inactivated FCS (pH 7.2) for 24 h. Samples obtained from each of the two saline control groups were pooled due to low cell yields. Cultured lymphocytes were stimulated with 500 μg/ml interferon (IFN)-γ, IL-10, IL-12, TNF-α, and MCP-1 levels in lung lavage fluid and in lymphocyte supernatants were measured using Cytometric Bead Array kits (BD Biosciences), according to the manufacturer’s instructions. Briefly, samples were incubated with cytokine-specific Ab-coated beads for 2 h, washed, and analyzed by flow cytometry.

Measurement of cytokine and chemokine concentrations

Cytokine (IL-2, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12, TNF-α, and MCP-1) levels were detected colorimetrically using the Mouse Eotaxin Immunoassay Kit (BioSource International), with data corrected for bilirubin absorbance, as previously described (30).

Immunohistochemical staining

Frozen tissue sections were prepared and incubated in the presence of the appropriate primary Ab (rat anti-mouse VCAM-1 1/200; rat anti-mouse MCP-1 1/500) or the isotype control Ab (rat anti-mouse Ig; 1/500) at room temperature for 1 h. For detection of VCAM-1, tissue sections were labeled with a FITC-conjugated secondary Ab (goat anti-rat IgG; 1/200) and analyzed by confocal microscopy (ex: 488 nm; em: 530 nm). Lung tissue sections were labeled with anti-MBP-1, a marker specific for eosinophils (36) and stained using an HRP-conjugated secondary Ab (goat anti-rat IgG; 1/500), followed by diaminobenzidine substrate and counterstaining with methyl green. The number of MBP-1-positive cells in 20 separate high-power fields was determined for each specimen.

Statistical analyses

Data were analyzed using a computer-based statistical package (Statistica 7; Analytical Software) with differences between mean values assessed by ANOVA with Scheffe comparison (30).

Results

Effect of bilirubin on transendothelial lymphocyte migration

To determine the effect of bilirubin on VCAM-1-mediated transendothelial lymphocyte migration, we used two separate endothelial cell lines, mHEVs (derived from murine axillary lymph nodes) and mHEVs (derived from murine cervical lymph nodes), which constitutively express VCAM-1. These cells have been shown to bind resting lymphocytes through VCAM-1 α₄ integrin interactions (29) and to promote transmonolayer migration. Because the viability of cultured mHEVs and mHEVc cells and isolated splenic lymphocytes (as determined by trypan blue exclusion) was unaffected by bilirubin levels up to 20 μM, concentrations within this range were used in our experiments. The effect of bilirubin, biliverdin, and BDT on the migration of freshly isolated splenic lymphocytes across confluent endothelial cell monolayers is depicted in Fig. 3. Treatment with either bilirubin or biliverdin was associated with a significant reduction in the number of lymphocytes in the lower chamber of the transwell at 24 h, while the bilirubin conjugate, BDT, had no effect on the transendothelial lymphocyte migration across endothelial cell monolayers. Confluent monolayers of mHEVa (left panel) and mHEVc (right panel) cells were preincubated for 1 h in the presence or absence of bilirubin, biliverdin, BDT, or the potassium phosphate vehicle, at the indicated concentrations. The number of lymphocytes in the lower chamber of the transwell at 24 h is plotted relative to untreated monolayers. Bars reflect the mean (± SEM) of four separate experiments, with three to four replicates performed per experiment. *, p < 0.05 vs vehicle and untreated controls.

FIGURE 3. Bilirubin and biliverdin inhibit lymphocyte migration across endothelial cell monolayers. Confluent monolayers of mHEVa (left panel) and mHEVc (right panel) cells were preincubated for 1 h in the presence or absence of bilirubin, biliverdin, BDT, or the potassium phosphate vehicle, at the indicated concentrations. The number of lymphocytes in the lower chamber of the transwell at 24 h is plotted relative to untreated monolayers. Bars reflect the mean (± SEM) of four separate experiments, with three to four replicates performed per experiment. *, p < 0.05 vs vehicle and untreated controls.

FIGURE 4. Expression of enzymes involved in bilirubin metabolism in endothelial cells. Monolayers of mHEVa and mHEVc cells were incubated in the presence or absence (N) of bilirubin (BR), biliverdin (BV), BDT, or the potassium phosphate vehicle (V) for 24 h and total cellular RNA extracted. Expression of HO-1 was assessed by Northern blotting (left panel), with SYBR Green II stained ribosomal RNA serving as a loading control. Message for BVR and UGT1A1 was detected by RT-PCR (right panel), with GAPDH serving as a control for amplification. Data are representative of two separate experiments. Total RNA isolated from mouse macrophage-like (RAW 264.7) cells incubated for 24 h in the presence of LPS was used as a hybridization control (24).
migration. Monolayer integrity, evidenced by the absence of erythrocyte translocation from the upper to the lower chamber of the transwell, was not altered by any of the treatments. These data suggest that physiologic concentrations of bilirubin, and its metabolic precursor, biliverdin, inhibit the migration of lymphocytes across endothelial cell monolayers. That this inhibitory effect is exerted at the level of the endothelial cell is supported by the finding that preincubation of lymphocytes for 24 h in the presence of bilirubin (20 μM), biliverdin (20 μM), or BDT (20 μM) had no effect on migration when treatments were removed before the addition of these pretreated cells to the transwell (data not shown).

As outlined in Fig. 2, the sequential activity of HO and BVR is required for the generation of bilirubin from heme, while the bilirubin-specific isofrom UGT1A1, catalyzes the main pathway of bilirubin metabolism. If bilirubin is an important modulator of inflammation, it follows that the expression of these enzymes in endothelial cells will be tightly regulated. As bilirubin has been shown to stimulate hepatic expression of the bilirubin-conjugating enzyme, UGT1A1 (37, 38), we sought to determine whether bilirubin, or its analogues, modulates endothelial cell expression of the enzymes involved in bilirubin production and metabolism. Northern blotting demonstrated expression of HO-1 mRNA in mHEVc cells, while message for BVR and UGT1A1 was detected by RT-PCR (Fig. 4), supporting the ability of this cell line to metabolize bilirubin. Similar results were obtained with the mHEVa cells (data not shown). The presence of message for BVR, the enzyme responsible for the conversion of biliverdin to bilirubin, also indicates that mHEV cells possess the enzymatic machinery necessary for the redox cycling of bilirubin. None of the bile pigments tested were found to alter cellular expression of the enzymes regulating bilirubin metabolism.

Because migration across mHEV monolayers is predicated upon the binding of endothelial cell VCAM-1 to leukocyte VLA-4, we assessed whether bilirubin disrupts the interaction between these adhesion molecules. For these studies, the adhesion of calcine AM-labeled splenic lymphocytes to cultured mHEV cells was assessed in the presence or absence of bilirubin, biliverdin, or BDT (Fig. 5). None of these bile pigments was found to alter lymphocyte binding to mHEV monolayers, while in contrast, Abs to VCAM-1 and VLA-4 significantly reduced the number of bound lymphocytes. Similarly, neither bilirubin, biliverdin, nor BDT treatment affected the expression of VCAM-1 by mHEV cells (Fig. 6). Taken together, these data support that bilirubin and its analogues do not block lymphocyte migration by disrupting the cellular expression or interaction of adhesion molecules.

Influence of bilirubin on VCAM-1-mediated cell signaling pathways

It has previously been demonstrated that lymphocyte migration across mHEV monolayers requires the activation of endothelial NADPH oxidase and the subsequent generation of extracellular superoxide radicals (3). Because bilirubin is a potent antioxidant (22), we speculate that bilirubin inhibits VCAM-1-mediated endothelial cell signaling by scavenging ROS produced by the activation of NADPH oxidase. To determine whether bilirubin inhibits NADPH oxidase-mediated ROS production, mHEVc cells were treated with either bilirubin or vehicle and then labeled with the redox-sensitive dye, DHR. Following stimulation with antivCAM-1-coated microbeads, the ROS-mediated conversion of DHR to the highly fluorescent rhodamine 123 was assessed by confocal microscopy (Fig. 7). Bilirubin attenuates the enhancement in rhodamine fluorescence in response to VCAM-1 stimulation, supporting that bilirubin blocks VCAM-1-induced ROS generation. Because increases in NADPH oxidase activity lead to the specific activation of MMP-2 and -9 in mHEV cells, we sought to determine whether bilirubin treatment alters this downstream event in VCAM-1 signaling. Following pretreatment with bilirubin (or vehicle), mHEV monolayers were stimulated with anti-VCAM-1-coated beads, and the MMP activity in cell lysates was assessed by
zymography. As shown in Fig. 8, treatment with bilirubin abolishes the time-dependent activation of MMP-2 and MMP-9, providing additional evidence that bilirubin impedes VCAM-1-mediated cellular events.

**Effect of bilirubin on allergen-induced airway inflammation**

To evaluate whether bilirubin is able to inhibit VCAM-1-mediated leukocyte migration in vivo, we used a murine model of OVA-induced airway inflammation (34, 35). C57BL/6J mice were administered OVA via i.p. injection. Subsequent exposure of sensitized animals to intranasal OVA produces an asthma-like Th2 immune response. The resultant pulmonary eosinophilia and lymphocytosis has previously been demonstrated to be VCAM-1-dependent (34, 35). Following intranasal OVA challenge, mice were administered a total of six doses of bilirubin (30 mg/kg), or potassium phosphate vehicle, i.p. at 8-h intervals before sacrifice. As expected, OVA-challenged mice exhibit a marked infiltration of inflammatory cells into the lung parenchyma as compared with saline-treated control animals (Fig. 9A, left panels). Notably, treatment with bilirubin was associated with a significant reduction in the number of inflammatory cells in the lung tissue, while bilirubin administration in the absence of OVA did not alter lung morphology. These findings were confirmed by measuring leukocyte accumulation in lung lavage fluid (Fig. 9B), which was markedly increased following OVA stimulation. Concomitant bilirubin administration significantly blunted this leukocyte response. When stratified by cell type, bilirubin specifically inhibited the infiltration of eosinophils and lymphocytes into the airways (Fig. 9C). These findings were corroborated by immunohistochemical staining of lung tissue sections for MBP-1, an eosinophil-specific marker (36), which demonstrates substantially lower numbers of eosinophils in lung tissue from bilirubin-treated vs vehicle-treated animals (Fig. 10). Because, in this model, both eosinophil and lymphocyte migration into the lungs have been shown to be VCAM-1-dependent (34, 35), our data are consistent with the hypothesis that bilirubin blocks pulmonary leukocyte infiltration by inhibiting VCAM-1 signaling.

An examination of peripheral blood smears from OVA- and vehicle-treated mice demonstrates that bilirubin administration increases the number of circulating eosinophils (Fig. 11), indicating that the inhibition of pulmonary eosinophilia by bilirubin is not the result of diminished eosinophil production or release from the bone marrow. Immunohistochemical staining of lung tissue sections further demonstrates that bilirubin treatment does not significantly alter VCAM-1 expression in the pulmonary vasculature, suggesting that the associated decrease in leukocyte infiltration into the airway is not the result of impaired cellular adhesion (Fig. 12). To assess for the possibility that the bilirubin-induced reduction in eosinophil accumulation is due to altered chemotactic signaling, we measured the concentration of eotaxin, a chemokine responsible for eosinophil migration (39), in lung lavage fluid. Consistent with the findings of other investigators (40), eotaxin levels increase following OVA challenge, but remain unaffected by bilirubin administration (Fig. 13A). Concentrations of IL-5, a Th2 cytokine that acts as an eosinophil chemoattractant (41), are also elevated in response to OVA challenge (42) and are also not altered by bilirubin treatment (Fig. 13C). Indeed, bilirubin had no effect on any of the measured cytokine or chemokine levels in lung lavage fluid when compared with the corresponding vehicle-treated animals (Table I).

To determine the effect of bilirubin on cellular cytokine production, the response of activated bronchial lymph node-derived lymphocytes to OVA rechallenge was assessed in vitro. As seen in Fig. 13B, the production of eotaxin by isolated lymphocytes increases in response to OVA challenge, but remains unaffected by bilirubin
treatment. Lymphocytes derived from OVA-treated mice also exhibit specific responses to OVA exposure, with enhanced production of Th2 (e.g., IL-5) but not Th1 (e.g., IL-2) cytokines (Fig. 13, D and F). That bilirubin does not modulate levels of inflammatory stimuli is evidenced by the fact that an identical pattern of cytokine production is observed in lymphocytes derived from bilirubin- vs vehicle-treated animals. These data, along with similar findings for the Th2 cytokines IL-4, IL-6, and IL-10, the Th1 cytokines IL-2 and IFN-γ, and the chemokine MCP-1, are summarized in Table I. Taken together, our findings indicate that bilirubin prevents OVA-induced eosinophil infiltration into the lungs despite the presence of a sufficient circulating pool of available cells and abundant chemotactic stimuli, supporting the conclusion that bilirubin inhibits VCAM-1 signaling pathways responsible for leukocyte migration across the pulmonary vascular endothelium.

Discussion
The present studies demonstrate that both unconjugated bilirubin and the bilirubin precursor, biliverdin, inhibit transendothelial leukocyte migration in vitro. Because lymphocyte migration across mHEV monolayers is VCAM-1-dependent (3, 14, 17, 18), and we have shown that bilirubin suppresses cellular ROS generation and MMP activation, events which are downstream of VCAM-1 stimulation (3), our findings suggest that bilirubin disrupts endothelial VCAM-1 signaling. It is notable that these effects occur at bilirubin concentrations that are within the normal range present in human serum (1.4 mg/dL, ~24 μM) (43). Moreover, to ensure that bilirubin was adequately solubilized, experiments were conducted in the presence of 20% FCS, which is optimal for transendothelial lymphocyte migration (data not shown). This level of FCS corresponds to a BSA concentration of ~90 μM (44), and results in bilirubin:BSA molar ratios of between 0 and 0.2. As a result of the high affinity binding of bilirubin by BSA (45–47), even at the highest levels of bilirubin used (20 μM), the free bilirubin concentration in the medium is estimated to be very low (~3 nM), well within the reported solubility at physiologic pH (48). Because the free concentration dictates the cellular uptake and biological activity of bilirubin (49, 50), the observed effects can reasonably be extrapolated to in vivo processes.

The relevance of our in vitro data to physiological events is highlighted by the observation that the i.p. administration of bilirubin blocks the influx of leukocytes into the lungs of mice with OVA-induced asthma. It has previously been shown that Abs to α4 integrins or VCAM-1 inhibit the recruitment of eosinophils and lymphocytes into the airways of OVA-sensitized and OVA-challenged mice (34, 35). Our finding that bilirubin treatment has identical effects on OVA-induced leukocyte recruitment is consistent with a mechanism of action that involves the disruption of VCAM-1 signaling. This conclusion is bolstered by the observation that bilirubin does not alter VCAM-1 expression in the pulmonary vascular endothelium and that bilirubin-treated animals

![Figure 9. Bilirubin prevents leukocyte infiltration into the lungs of OVA-treated mice.](image)

![Figure 10. Effect of bilirubin on OVA-induced pulmonary eosinophilia.](image)
exhibit similar cytokine and chemokine responses as vehicle-treated controls. Moreover, a decrease in eosinophil infiltration into the airways occurs despite an enhanced pool of cells available for migration into the lungs, as evidenced by the significant increase in the number of circulating blood eosinophils following bilirubin administration. The mechanism underlying this bilirubin-associated blood eosinophilia, which was observed in both OVA-sensitized and -nonsensitized mice, is unclear. Although a stimulatory effect of bilirubin on eosinophil production cannot be excluded, because VCAM-1 has been shown to be involved in the trafficking of leukocytes to the intestine (51), bone marrow (52), and lymph nodes (53), we speculate that bilirubin enhances the number of eosinophils in the systemic circulation by blocking the normal migration into these tissues.

That cellular NADPH oxidase activity is integral to VCAM-1 signaling is supported by the observation that lymphocyte migration across mHEV monolayers is abrogated by specific inhibitors of NADPH oxidase, as well as by the ROS scavengers superoxide dismutase and catalase (3). As bilirubin is a potent antioxidant (22, 25), we postulate that this bile pigment inhibits VCAM-1-mediated leukocyte migration by scavenging the low (micromolar) concentrations of ROS generated by VCAM-1-dependent NADPH oxidase activation (18). Additionally, it has been shown that administration of an antioxidant mimetic, AEOL 10113, ameliorates OVA-induced pulmonary eosinophilia and lymphocytosis (20) and is further buttressed by our finding that bilirubin suppresses endothelial cell ROS production in response to VCAM-1 stimulation. Indeed, at 20-μM concentrations, bilirubin blocked lymphocyte migration to a comparable degree as previously shown for the ROS scavengers, superoxide dismutase (500 U/ml) and catalase (5000 U/ml), as well as an inhibitor of NADPH oxidase, apocynin (0.5 mM). However, these data do not preclude the possibility that bilirubin interferes with NADPH oxidase assembly or with upstream signaling events (14, 18).

Baranano et al. (25) recently proposed a cytoprotective mechanism in which bilirubin is oxidized to biliverdin and then recycled back to bilirubin by the action of cellular BVR. That redox cycling of bilirubin may be occurring in vascular endothelium receives support from our demonstration that mHEV cells express message for BVR and that treatment with biliverdin blocks lymphocyte migration to a comparable degree as bilirubin in vitro. In contrast, the ubiquitous nature of BVR activity in mammals, which results in the quantitative and rapid conversion of biliverdin to bilirubin under both physiological and pathophysiological conditions (54),

FIGURE 11. Bilirubin treatment increases blood eosinophil levels in mice. The average number of eosinophils (± SEM) in the peripheral blood of mice primed with saline or OVA and treated with bilirubin or vehicle is displayed. *, p < 0.001 vs saline + vehicle and OVA + vehicle cohorts.

FIGURE 12. Bilirubin administration does not affect VCAM-1 expression in the lungs of OVA-treated mice. A, Representative confocal images of VCAM-1 stained frozen tissue sections from the lungs of saline- and OVA-primed mice treated with bilirubin or vehicle are displayed. B, The results of quantitative analyses (± SEM) of tissue immunofluorescence (IF). Isotype Ab-stained sections serve as controls for background fluorescence.

FIGURE 13. Bilirubin treatment does not inhibit cytokine production. Levels of eotaxin (A and B), IL-5 (C and D), and IL-2 (E and F) in lung lavage fluid (A, C, and E), and in supernatants from OVA-stimulated lymphocytes isolated from bronchial lymph nodes (B, D, and F) are shown. *, p < 0.05 vs saline controls.
makes it likely that bilirubin is the more relevant inflammatory mediator in vivo. In contrast with biliverdin, treatment of cell monolayers with the stable bilirubin conjugate, BD, has no discernible effect on transendothelial lymphocyte migration, despite the fact that conjugated bilirubin also functions as an antioxidant (55). Although unconjugated bilirubin (and presumably biliverdin) is capable of diffusing rapidly through cellular membranes (56, 57), bilirubin conjugates, which are more hydrophilic, are membrane impermeant (58). Whether these differences in permeation are related to the inability of BD to modulate lymphocyte migration is unknown at this time, but remains an intriguing possibility. Alternatively, these findings may be explained by the fact that the potency of BD as an antioxidant is inferior to that of bilirubin or biliverdin (59).

It is notable that HO, the rate-limiting enzyme in bilirubin synthesis, is up-regulated at sites of inflammation and that the stimulated activity of this enzyme is associated with diminished tissue injury in a host of model systems (60–63), including allergen-induced airway disorders (64, 65). That bilirubin is a key mediator of the cytoprotective effects of HO is supported by a number of in vivo studies demonstrating that bilirubin ameliorates cellular oxidative damage. One of the ways in which bilirubin has been shown to inhibit tissue injury is by preventing the induction of NO synthase and consequent increases in NO levels (24). However, data are conflicting as to whether inducible NO synthase (iNOS) contributes to lung inflammation in the OVA asthma model. For example, it has been reported that pretreatment of mice with specific iNOS inhibitors decreases airway eosinophilia in response to allergen stimulation, either by blocking chemokine expression (71) or by delaying the efflux of eosinophils from the bone marrow (72). In contrast, other investigators have been unable to demonstrate a protective effect of iNOS inhibition on pulmonary leukocytosis (73–75), and iNOS-deficient mice paradoxically exhibit enhanced lung injury in response to OVA challenge (76). Our demonstration that bilirubin treatment does not alter chemokine levels and is associated with an increase (as opposed to a decrease) in the number of circulating eosinophils makes it unlikely that the effects of this bile pigment on airway inflammation are related to alterations in iNOS expression. Furthermore, mHEV cells do not express iNOS (data not shown), rendering it doubtful that the mechanism underlying bilirubin-mediated inhibition of transendothelial lymphocyte migration is iNOS-dependent.

Our finding that both endothelial and inflammatory cells (24) express all of the necessary enzymes involved in bilirubin synthesis and degradation, implies a high degree of regulation of cellular bilirubin levels and supports a potentially broad role for this endogenous bile pigment as a physiological regulator of inflammation. VCAM-1-mediated leukocyte recruitment is implicated in the pathogenesis of a number of inflammatory conditions, including atherosclerosis (77, 78) and inflammatory bowel disease (4, 9, 13). With regard to the former, VCAM-1 is detectable in atherosclerotic plaques (79) and endothelial expression of this adhesion molecule has been shown to be an early event at sites predisposed to atherosclerosis (80). Moreover, disruption of VCAM-1 expression in the atherosclerosis-prone low density lipoprotein receptor knockout mouse is associated with a significant decrease in the number of vascular lesions (78). Consistent with the hypothesis that bilirubin may modulate the process of atherogenesis by inhibiting VCAM-1 signaling, a host of epidemiological analyses have identified an inverse correlation between serum bilirubin levels and both the risk and severity of cardiovascular disease (81–84). In summary, our data demonstrate that bilirubin suppresses Th2 cytokine-mediated airway inflammation in response to an allergen challenge by inhibiting endothelial cell VCAM-1 signaling, and that the mechanism underlying these effects appears to involve the scavenging of NADPH-oxidase-generated ROS. As the essential role of low concentrations of ROS as cellular second messengers becomes more clearly elucidated (85, 86), our data raise the intriguing possibility that bilirubin may regulate a variety of fundamental cellular processes through BVR-catalyzed redox cycling.

**Disclosures**

The authors have no financial conflict of interest.

**References**


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### Table I. Effect of ovalbumin and bilirubin on cytokine secretion in lung lavage fluid and activated lymphocytes (lymph node) in picograms per milliliter (± SEM)

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Lymph Node</th>
<th>Lung Lavage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline + Vehicle</td>
<td>Ovalbumin + Vehicle</td>
</tr>
<tr>
<td>Th1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>6.2</td>
<td>4.2 (± 0.6)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>6.0</td>
<td>19.0 (± 8.3)</td>
</tr>
<tr>
<td>Th2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>0</td>
<td>7.1 (± 1.9)</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.6</td>
<td>191.4 (± 79.6)</td>
</tr>
<tr>
<td>IL-6</td>
<td>98.1</td>
<td>661.9 (± 224.8)</td>
</tr>
<tr>
<td>IL-10</td>
<td>0</td>
<td>157.6 (± 37.3)</td>
</tr>
<tr>
<td>Chemokine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td>673.9</td>
<td>2939.1 (± 1479.5)</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>7.5</td>
<td>16.6 (± 1.4)</td>
</tr>
<tr>
<td>Other cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>122.7</td>
<td>256.6 (± 64.1)</td>
</tr>
<tr>
<td>IL-12</td>
<td>31.0</td>
<td>41.9 (± 3.2)</td>
</tr>
</tbody>
</table>

* Data obtained from pooled samples.

* p < 0.05 versus saline controls.


