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Biochemical Pharmacology 65 (2003) 1529–1538
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Rabbit chronic ileitis leads to up-regulation of adenosine *A1/A3* gene products, oxidative stress, and immune modulation

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Received 26 April 2002; accepted 27 August 2002

Abstract

A rabbit model of chronic ileitis has helped decipher the mechanism of alteration of multiple electrolyte and nutrient malabsorptions in inflammatory bowel disease (IBD). This study examined alterations in the adenosine A1/A3 receptor, oxidant, antioxidant, and immune-inflammatory pathways in chronic ileitis. Chronic ileal inflammation was induced 13–15 days after infection with 10,000 *Eimeria magna* oocytes. Quantitative analysis in 16 rabbits was done for oxidants, antioxidants, A1 and A3 transcripts, transport, injury, and inflammatory mediators. Inflamed gut had villus blunting, crypt hyperplasia and fusion, and immune cell infiltration. Alkaline phosphatase and Na-glucose co-transport were reduced by 78% ($P = 0.001$) and 89% ($P = 0.001$), respectively. Real-time fluorescence monitoring (TaqMan)–polymerase chain reaction revealed a transcriptional up-regulation of 1.34-fold for A1 and 5.40-fold for A3 receptors in inflamed gut. Lipid peroxidation increased in the mucosa (78%, $P = 0.012$), longitudinal muscle-myenteric plexus (118%, $P = 0.042$), and plasma (104%, $P = 0.001$). Mucosal antioxidants were altered by inflammation: reductions occurred in superoxide dismutase (32%, $P = 0.001$) and catalase (43%, $P = 0.001$), whereas increases occurred in glutathione (75%, $P = 0.0271$) and glutathione reductase (86%, $P = 0.0007$). Oxidant enzyme activities were elevated by 21% for xanthine oxidase ($P = 0.004$), 172% for chloramine ($P = 0.022$), 47% for gelatinase ($P = 0.041$), and 190% for myeloperoxidase ($P = 0.002$). Mast cell tryptase increased by 79% ($P = 0.006$). Increases occurred in the plasma concentration of leukotriene B_4 (13-fold, $P = 0.003$), thromboxane B_2 (61-fold, $P = 0.018$), and tumor necrosis factor-α (9-fold, $P = 0.002$). In conclusion, chronic ileitis and tissue injury are associated with discrete alterations in complex multi-level oxidant, antioxidant, and immune inflammatory components. The rabbit ileitis model is a suitable model to gain further insight into chronic inflammation and IBD. We hypothesize that adenosine A3 and A1 receptors may provide a novel target for therapy in chronic ileitis and perhaps IBD.

Keywords: Rabbit ileitis; Chronic inflammation; Adenosine receptors; Enteric nervous system; Oxidative stress; Antioxidants

1. Introduction

IBD is a common, disabling, and lifelong gastrointestinal disorder. While more than 500,000 people are afflicted with IBD in the United States, we do not know its etiology, and there are no specific or adequate treatments for it. In gut inflammatory conditions including IBD, all aspects of the GI tract, the mucosa, the immune system, and the enteric nervous system are affected [1–4]. Chronic intestinal inflammation is associated with a variety of

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Abbreviations: CAT, catalase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSH-R, glutathione reductase; IBD, inflammatory bowel disease; iNOS, inducible nitric oxide synthase; LMMP, longitudinal muscle with attached myenteric plexus; LTB_4, leukotriene B_4; MPO, myeloperoxidase; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; TXB_2, thromboxane B_2.

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doi:10.1016/S0006-2952(03)00067-4

histochemical, neural, functional, transport, and biochemical abnormalities. In patients with IBD, nutrient absorption is inhibited in the intestine leading to the most common and disabling symptoms of this disorder: diarrhea, malnutrition, weight loss, abdominal pain, and eventually a failure to thrive.

In IBD, T lymphocytes, macrophages, and neutrophils are important in the production of immune-inflammatory mediators like TNF-α, lymphokines, and oxyradicals or ROS (superoxide anions, hydrogen peroxide, hydroxyl radicals, peroxynitrite) that are capable of affecting many effector tissue functions such as absorption, secretion, and motility and that induce tissue injury [5,6]. Generation of ROS in the chronically inflamed gut is likely to lead to cellular injury when the cellular antioxidant defense systems (which include SOD, CAT, GSH, GSH-R, and GSH-Px) are compromised [7,8].

Although there are no perfect animal models of chronic ileal inflammation comparable to the disease in humans, the rabbit model of chronic ileitis developed by Sundaram and co-workers [2–4,9] shares many similarities with IBD: (a) the *trigger*—an offending agent in a susceptible host triggers the immune system, which remains up-regulated even after the loss of the agent; (b) *clinical sequelae*—malabsorption, diarrhea, and weight loss; (c) *gross morphology*—the intestine is thickened, erythematous with a cobblestone appearance; (d) *histology*—microscopically no parasites are seen; there is villus atrophy, crypt hypertrophy, an increase in intraepithelial and lamina propria lymphocytes, histiocytes, and possibly mast cells and plasma cells; there is no increase in neutrophils or extensive destruction of the villus surface, all characteristics seen in this model; and (e) *activation of multiple immune-inflammatory mediator pathways*. In contrast, in acute inflammation there is extensive destruction of the villus surface and a marked increase in neutrophils and eosinophils but only a slight increase in lymphocytes, histiocytes, or plasma cells. We do not see this in the rabbit ileitis model. Mast cells can be present in either chronic or acute inflammation, and we do see them in rabbit *chronic ileitis*.

This model has helped decipher how multiple electrolyte and nutrient malabsorption mechanisms known to occur in IBD are altered [2–4,9]. Glucocorticoid therapy was shown to reduce the severity of symptoms and inflammation, and to reverse transport abnormalities occurring in the rabbit ileitis model [9]. Other than these transport studies [2–4,9], very little is known about the chronic ileitis model.

There is an increase in ROS-producing cells in patients with ulcerative colitis and Crohn's disease [10]. ROS-producing cells can also activate transcription factor NF-κB, leading to the expression of inflammatory cytokines and iNOS [11].

Epithelial hypoxia stimulates the release of TNF-α [12]. Endogenous adenosine is believed to play a key role in protecting against ischemic or hypoxic cell-injury, and to act as an antioxidant and anti-inflammatory mediator [13] at specific adenosine receptors. A1, A2a, A2b, and A3 receptors are widely and differentially distributed throughout the human small and large intestine in neural and non-neural cells of the gut [14].

The activation of A3 adenosine receptors (A3ARs) protects against ischemic cell injury by activating cellular antioxidant mechanisms [15]. Selective up-regulation of *A3* gene products has been suggested to be neuroprotective in hippocampal slices upon chemical preconditioning [16] and protective against airway inflammation [17]. A3 receptor activation may play an anti-inflammatory role in human neutrophil-mediated tissue injury [18]. An A3 agonist, IB-MECA, inhibits the release of TNF-α as well as eosinophil chemotaxic responses [18]. In fact, A3ARs in brain are very low or undetectable in non-pathologic tissues [19]. Adenosine A1 receptors (A1ARs) may afford protection similar to that of A3ARs and are up-regulated by a variety of conditions including glucocorticoid treatment, ethanol withdrawal, and oxidative stress [20–24].

To further characterize the recent rabbit ileitis model (which is produced by intragastric inoculation with *Eimeria magna* oocytes) as a suitable one in which to examine chronic intestinal inflammation, we conducted a detailed investigation of oxidant, antioxidant, immune-inflammatory, and adenosine receptor pathways. We sought to identify if adenosine receptor pathways are altered in chronic ileitis, since adenosine A1 and A3 receptors are suggested to have protective roles against oxidative stress, ischemia/hypoxia, and inflammation to combat tissue injury. An imbalance in the oxidant/antioxidant status in infected tissues could play an important role in the pathogenesis of mucosal injury. A better understanding of such an imbalance, together with what is known about alterations in transport mechanisms [2–4,9], may shed light in developing therapeutic strategies to improve the outcome of IBD. A novel finding is that adenosine receptor pathways are up-regulated in chronic ileitis and may offer a novel target for therapy in chronic intestinal inflammation or IBD.

2. Materials and methods

2.1. Rabbit model of chronic ileal inflammation

A rabbit model of chronic ileitis was produced by intragastric inoculation of pathogen-free 2- to 3-lb New Zealand White male rabbits with 10,000 *E. magna* oocytes in 2 mL of 0.9% NaCl. Control animals were sham-inoculated with 0.9% NaCl [2–4,9]. About 80% of the inoculations resulted in chronic ileal inflammation during days 13–15, and only cells from those animals that had confirmed chronic inflammation by histology were utilized for experimental analysis. For histological evaluation, tissue samples from the control and inflamed ileum were

fixed in formalin, embedded in paraffin, sectioned, and, after hematoxylin and eosin staining, examined by light microscopy. All inoculations were done in Dr. Uma Sundaram's laboratory.

2.2. 3-O-Methyl-D-glucose uptake

Isolated crypt cells (100 mg wet wt) were washed and resuspended in 4 mL of HEPES buffer containing 1 mM 3-*O*-methyl-D-glucose with 130 mM Na^+ or tetramethylammonium. ^{3}H-Labeled 3-*O*-methyl-D-glucose (40 μCi) was added, and 100-μL aliquots were removed at desired time intervals, added to 3 mL of ice-cold Na-HEPES buffer, and filtered through 0.45-μm Millipore (HAWP) filters. The filters were washed twice with 3 mL of ice-cold Na-HEPES buffer and dissolved in 3 mL of Optifluor scintillation fluid, and radioactivity was determined as previously described [2–4].

2.3. Villus-crypt cell separation

Rabbit ileal cells were separated according to a previously established set of criteria to ensure good villus-crypt cell separation [2].

2.4. Tissue preparation for biochemical analysis

Three feet of ileum was removed from each rabbit immediately after the animal was euthanized [2] and rinsed free of fecal material with an ice-cold solution containing 96 mM NaCl, 27 mM sodium citrate, 1.5 mM KCl, 0.8 mM KH_2PO_4, and 5.6 mM Na_2HPO_4, as well as 5000 U/L of penicillin, 5 mg/L of streptomycin, 10 mg/L of gentamicin, and 0.5 mM dithiothreitol (DTT), and gassed with 95% O_2–5% CO_2, pH 7.4, at 37° (solution A). The ileum was filled with 100 mL of solution A and incubated for 10 min at 37°. This solution (containing mucus, bacteria, and other luminal contents) was then discarded, and the ileum was further flushed with an ice-cold Kreb's solution [14]. A segment of the ileum was cut open along the mesenteric border, pinned flat on the bottom of a Sylgard dish, and the ileal mucosa scraped gently with a flat-edged forceps; approximately 0.3- to 0.5-g mucosa samples were frozen at −70° in liquid nitrogen and stored until later analysis. Mucosa was homogenized with a Brinkmann Polytron in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 20% homogenate for analysis of biochemical markers.

2.5. Enzyme measurements

The determination of MPO activity in sonicated whole mucosa homogenates was carried out by using an assay kit (R&D Systems). The activities of SOD, CAT, GSH-R, and GSH-Px were measured spectrophotometrically as described previously [25]. The gelatinase content in mucosa homogenates was determined by a fluorometric assay (Molecular Probes). Xanthine oxidase activity was measured spectrophotometrically by evaluating the production of uric acid [26].

2.6. Determination of TNF-α, LTB_4, and TXB_2

TNF-α, LTB_4, and TXB_2 were measured using a specific enzyme-linked immunosorbent assay (R&D Systems).

2.7. Determination of mucosal lipid peroxidation content

The determination of lipid peroxidation was carried out by using an assay kit (R&D Systems). This procedure allows for the measurement of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations.

2.8. Determination of chloramine concentration

Chloramine concentrations in gut homogenates were determined by colorimetric measurement of the triiodide ion formed by the oxidation of potassium iodide [27].

2.9. Data analysis

Results are expressed as means ± SEM obtained from four animals for each marker/parameter. Comparisons among groups were evaluated by one-way ANOVA with a Newman–Keuls test of multiple comparisons. A P value of 0.05 or less was considered significant.

2.10. Quantitative analysis of A1 and A3 mRNAs using real-time (TaqMan) PCR

Real-time PCR was used to quantitate A1AR and A3AR mRNA in whole thickness ileum from chronically inflamed rabbits or age-matched controls. Tissues isolated from rabbits were immediately frozen in liquid nitrogen. The frozen tissues were pulverized in liquid N_2, and total cellular RNA was extracted using TRIzol reagent (Gibco-BRL). The cDNA was synthesized from RNA (5 μg) using Superscript II (Gibco-BRL). TaqMan probes and primers for adenosine A1 and adenosine A3 receptors based on the known A1R and A3R sequences in rabbits [28] were designed using Primer 3 software (MIT). TaqMan reactions were carried out using primers (800 nM) corresponding to individual adenosine receptors, fluorescence probe (200 nM), primers for an internal standard (18S-RNA, 100 nM), and 25 μL of TaqMan master mix for a final sample volume of 50 μL. The PCR was run at standard conditions in the AB1 Prism sequence detector system.

The sequence of the primers and probes used to detect A1 and A3 receptor transcripts was as follows: for A1AR, (forward) 5′-CGGGGCAGGAGTCTCAATCAC-3′, (reverse) 5′-TGTTTTTGCGATTGCCTTCTATT-

3′; fluorescent probe 5′-(FAM)-TGGGCCCACCACAGT-(TAMRA)-3′; for A3AR, (forward) 5′-TCTGATTTTCAA-AGCCCGTGT-3′, (reverse) 5′-TGCTCGCTGCTTGG G-TCT-3′; fluorescent probe 5′-(FAM)-CCTGCCAGCCCT-CCGATTCCC-(TAMRA)-3′.

3. Results

3.1. Histopathology and alterations in villus transport mechanisms

The total number of rabbits used in this study was 16. Injury to ileal mucosa in animals challenged with the *E. magna* oocytes was assessed first by histopathology to confirm chronic ileitis, before any biochemical analysis was done. During the chronic phase of inflammation (days 13–15), there was villus blunting, crypt hyperplasia and fusion, as well as immune cell (lymphocytes, plasma cells, neutrophils) infiltration of the mucosa, as shown previously [2–4,9]. Table 1 is a complete summary and statistical analysis of data for all immune-inflammatory and oxidant/antioxidant parameters measured in mucosa, LMMP, or plasma in normal and inflamed ileum.

The activity of alkaline phosphatase, a brush border membrane enzyme that is important for nutrient assimilation, was diminished markedly by 78% in the villus cell brush border membranes (membranes from isolated villus cells) during chronic ileitis (Table 1). Na-glucose co-transport also was diminished markedly (by 89%) in villus cells in the chronically inflamed intestine (Table 1), and these results were consistent with data reported previously [2].

3.2. Components of chronic mucosal inflammation

In the present study, infiltration and activation of neutrophils in the ileal mucosa of infected animals were assessed by measuring the activities of MPO and gelatinase, as well as the concentration of highly reactive chloramines [8,29,30]. As shown in Table 1, infection of animals resulted in a 190% increase in mucosal MPO activity, suggestive of extensive neutrophil infiltration. In contrast to an increase in MPO activity in the mucosa, its activity in plasma was reduced significantly in the inflamed gut compared with controls. Also, chronic infection resulted in a 47% increase in mucosal gelatinase activity and an ~172% increase in chloramine concentration, suggestive of extensive phagocyte activation.

3.3. Lipid peroxidation products

Lipid peroxidation is a possible mechanism of oxidative-stress-induced lethal injury [5,6]. Therefore, in this study, the levels of lipid peroxidation in ileal mucosal homogenates, in ileal homogenates of LMMP tissues, and in

Table 1
Alterations in immune-inflammatory or oxidative mechanisms in mucosa, LMMP, and plasma of chronically inflamed rabbit ileum

Parameter	Marker	Normal	Inflamed	*P* value
Changes in the mucosa				
Alkaline phosphatase (nmol/mg)	Brush-border enzyme	41.0 ± 4.0	9.0 ± 1.0	0.001
Na-dependent 3-OMG uptake (nmol/mg)	Na-glucose co-transport	5.40 ± 0.60	0.6 ± 0.1	0.001
MPO (ng/mg)	Neutrophils	11.61 ± 2.39	33.70 ± 3.67	0.0023
Lipid peroxidation (U/mg)	Oxidative stress	0.054 ± 0.002	0.096 ± 0.007	0.0121
Gelatinase (U/mg DNA)	Neutrophils	0.146 ± 0.011	0.215 ± 0.020	0.0418
Xanthine oxidase (mU/g)	O_2^- radicals	143.4 ± 5.48	173.1 ± 3.67	0.0040
Chloramine (abs/mg DNA/min)	Neutrophil oxidants	21.31 ± 5.43	57.89 ± 3.75	0.0227
Mast cell tryptase (abs/mg)	Mast cell enzyme	0.043 ± 0.005	0.077 ± 0.006	0.0060
Superoxide dismutase (U/mg)	Antioxidant	1.18 ± 0.03	0.80 ± 0.05	0.0010
GSH pathway in mucosa				
GSH (μg/mg DNA)	Antioxidant	46.0 ± 10.72	80.66 ± 11.75	0.0271
GSH-Px (nmol/min/mg)	Antioxidant	1.78 ± 0.04	1.71 ± 0.07	0.4769
GSH-R (abs/min/mg DNA)	Antioxidant	0.169 ± 0.019	0.314 ± 0.012	0.0007
Changes in LMMP				
Lipid peroxidation (U/mg DNA)	Oxidative stress	0.038 ± 0.005	0.083 ± 0.029	0.0427
Changes in plasma levels of inflammatory mediators				
TNF-α (pg/mL)	Pro-inflammatory mediator	5.7 ± 0.7	51.7 ± 8.0	0.0020
TXB_2 (pg/mL)	Eicosanoid pathway	<5.0 ± 2	306 ± 94	0.0175
LTB_4 (pg/mL)	Eicosanoid pathway	<5.0 ± 2	67.1 ± 15.2	0.0029
MPO (ng/mg)	Neutrophils	10.41 ± 0.82	4.23 ± 0.52	0.0002
Lipid peroxidation (U/mg)	Oxidative stress	2.52 ± 0.08	5.13 ± 0.58	0.0013

Abbreviations: TNF-α, tumor necrosis factor-alpha; TXB_2, thromboxane B_2, LTB_4, leukotriene B_4; MPO, myeloperoxidase; GSH, glutathione; GSH-Px, glutathione peroxidase; GSH-R, glutathione reductase; and LMMP, longitudinal muscle with attached myenteric plexus; ''abs'' = absorbance units. Values are means ± SEM, N = 4.

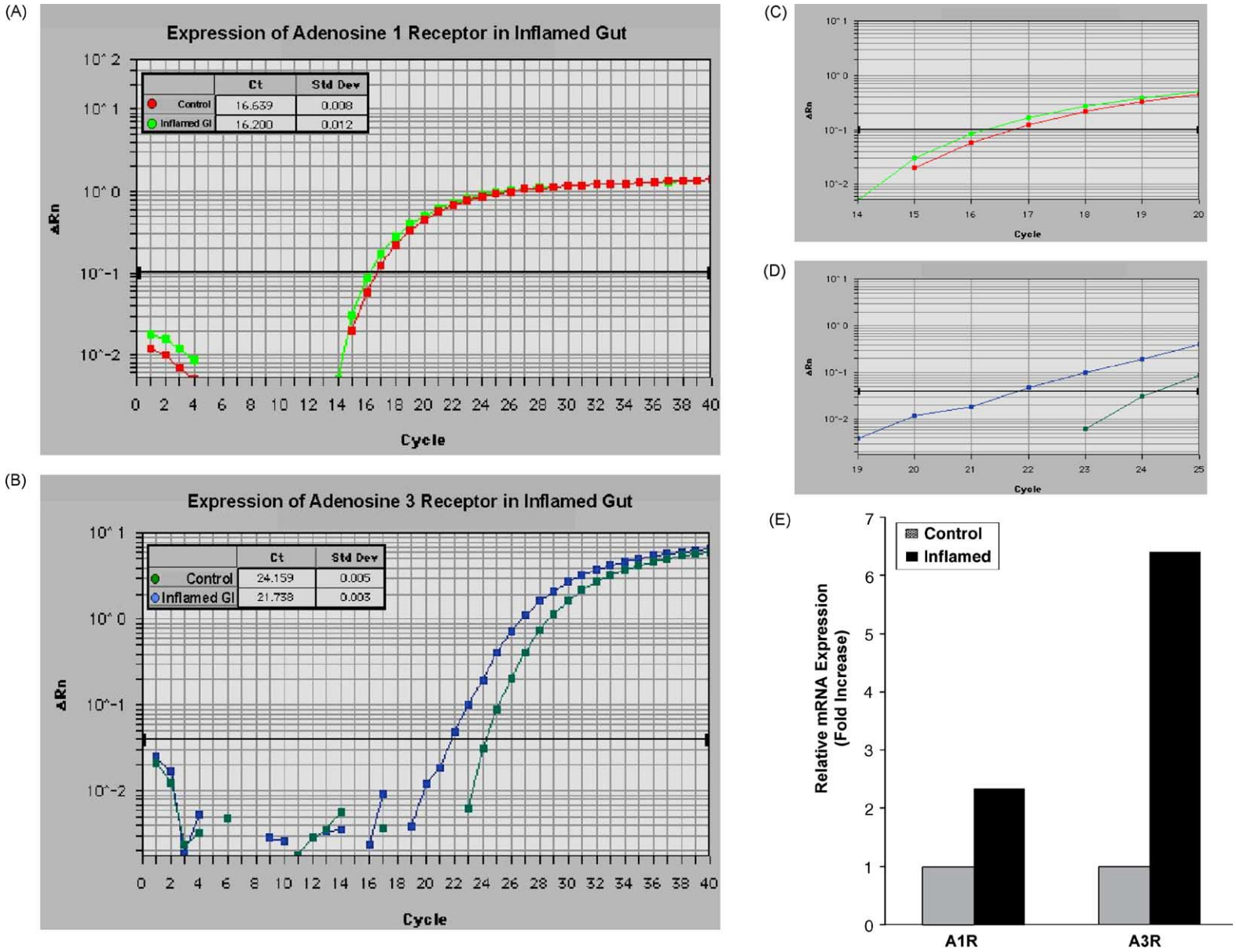

Fig. 1. Differential up-regulation of adenosine *A1* and *A3* receptor gene expression in chronic ileitis. Real-time TaqMan PCR was used to quantitate mRNA levels. (A, C) The amplification reaction revealed a parallel leftward shift in the A1 receptor gene expression curve in inflamed gut. (B, D) The amplification reaction revealed a much larger parallel leftward shift in the *A3* receptor gene expression curve in inflamed gut. Data presented for *A1* or *A3* receptor gene products are the pooled results for whole thickness ileum from each of four normal or inflamed animals. Tissues from each of four animals were pooled prior to the real-time TaqMan PCR. (E) The calculated data from the shifts in the curves are presented as fold increases in mRNA levels in normal and inflamed gut.

plasma of control and infected animals were also measured. As shown in Table 1, infection produced a 78% increase in lipid peroxidation products in mucosa, a 118% increase in LMMP, and a 104% increase in circulating plasma lipid peroxidation products.

3.4. Xanthine oxidase activity and mast cell tryptase in ileal mucosa

Since mucosal injury in intestinal inflammatory conditions may also be produced by the oxyradical O_2^-, xanthine oxidase activity was measured in mucosal homogenates. As shown in Table 1, the activity of xanthine oxidase was increased significantly (by 21% in the mucosa of *E. magna*-challenged animals).

The role of mast cells in the chronic inflammatory response was assessed by measuring mast cell tryptase. Mast cell tryptase was elevated by 79% in mucosa from infected animals (Table 1).

3.5. Circulating pro-inflammatory mediators

Inflammatory mediators of the eicosanoid (LTB_4 and TXB_2) or cytokine pathway (TNF-α) were assessed in plasma from age-matched control and infected rabbits. All mediators were elevated, stressing the multiplicity of signaling pathways contributing to chronic inflammation in the rabbit ileitis model. LTB_4 was elevated by 13-fold, TXB_2 by 61-fold, and TNF-α by 9-fold (Table 1).

3.6. Up-regulation of adenosine A1 and A3 receptor gene products

Western blot analysis with goat anti-A3 receptor antibodies raised against human or rat antigen (Santa Cruz) proved unsuitable for determining the expression of A1ARs and A3ARs (data not shown) because of species differences in the receptors; inter-species homology for the A3 receptor is the lowest of the four adenosine receptors cloned to date. We did find, however, that the human or rat specific antibody was suitable in identifying A3 receptor proteins in human and rat tissues [14]. Furthermore, adenosine receptor antibodies (Alpha Diagnostics) that we have successfully used to stain rodent gut tissues are all raised in rabbits and therefore are not suitable for staining rabbit gut tissue. Therefore, we used a real-time continuous fluorescence monitoring (TaqMan) PCR assay to quantitate the mRNA levels of the two rabbit adenosine receptor isoforms, A1AR and A3AR, in whole-thickness rabbit ileum. The transcription of these two receptors was differentially up-regulated in the chronically inflamed ileum (Fig. 1). When data were expressed as fold increase in mRNA level in inflamed ileum relative to age-matched control rabbits, the levels for A1 and A3 were 1.34- and 5.40-fold greater, respectively, in the inflamed ileum. The differences between age-matched controls and inflamed animals were estimated in two different ways: (a) random analysis of A1 or A3 mRNAs in individual control and inflamed animals; and (b) tissues were pooled from all four age-matched control animals and compared with tissues pooled from all four inflamed animals. Either method gave the same results for the up-regulation of each receptor, and the same relative difference between the two receptors.

3.7. Alterations in the antioxidant defense system

To assess the relative importance of the ileal mucosal antioxidant system in chronic ileitis, the activities of GSH content, GSH-R, GSH-Px, CAT, and SOD were determined in normal and inflamed tissues. In the chronically inflamed ileum, GSH content was elevated significantly by 75% in infected ileum, the GSH-Px remained unchanged, and GSH-R was elevated by 86% in infected ileum (Table 1). In contrast, there were significant reductions in SOD (Table 1) and CAT (data not shown) activities (32 and 43%, respectively).

4. Discussion

This study provided insights into the pathophysiological mechanisms involved in chronic ileitis. The findings clearly show that intragastric inoculation of pathogen-free rabbits with *E. magna* oocytes results in chronic ileitis associated with histopathology and mucosal injury that is similar to IBD, i.e. reduction in brush-border enzymes and transport mechanisms, elevation in certain pro-inflammatory enzymes and chemotactic pro-inflammatory mediators, and alterations in oxidant/antioxidant mechanisms. Novel data are provided for differential up-regulation of adenosine *A1* and *A3* gene transcripts in chronic ileitis. Overall, chronic ileitis is associated with a complex and diverse set of immune/inflammatory and transcriptional changes that deserve further consideration. Fig. 2 is our working hypothesis of chronic ileitis, detailing the complex signaling events believed to be involved in the development of the disease in accordance with our findings and/or supported by the literature. These signaling events will be the focus of the discussion.

4.1. Role of lipid peroxidation, mast cell tryptase, and gelatinase in cell injury

An increase in peroxidation of membrane lipids suggests the involvement of oxidative stress-mediated mechanisms of cell/tissue injury [15]. Lipid peroxidation is an irreversible chain reaction that occurs when polyunsaturated fatty acids react with ROS, resulting in changes in the structure and function of cellular membranes that may lead to increases in permeability and cell injury. During this process, the by-products generated (i.e. malondialdehyde

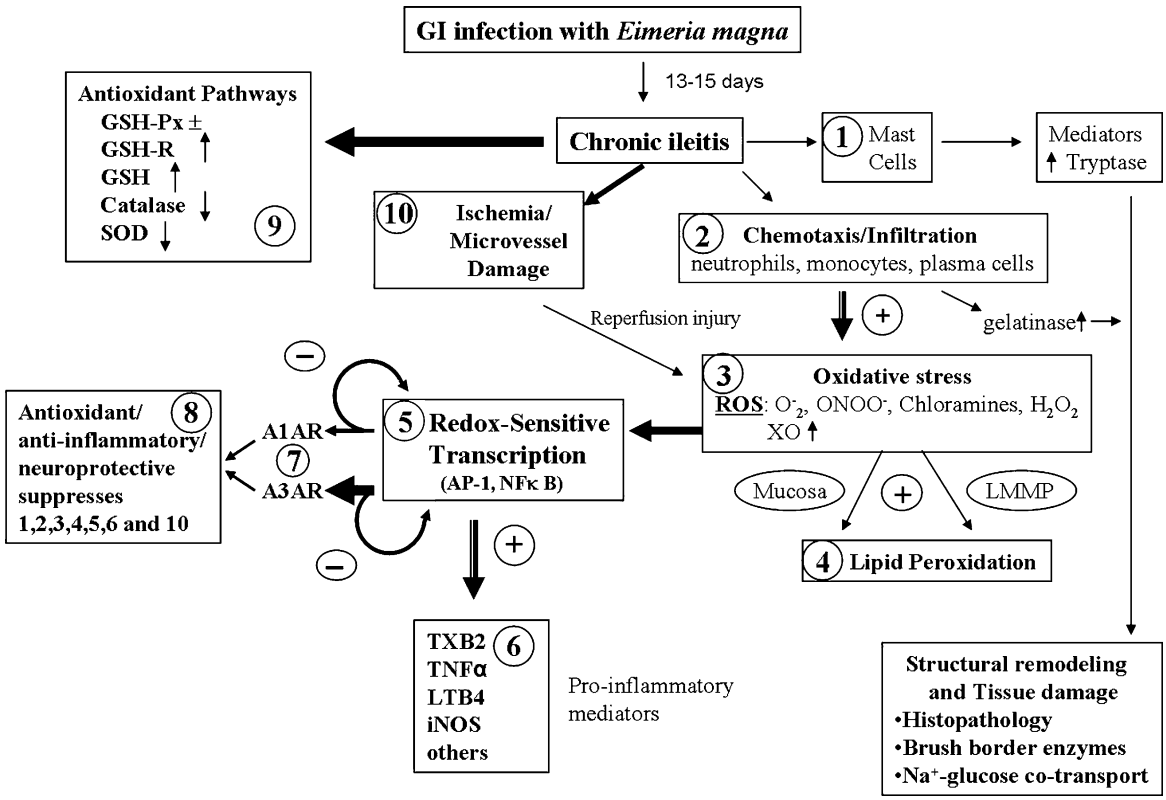

Fig. 2. Current hypothesis of cellular signaling mechanisms involved in the development of chronic ileitis induced in rabbits infected with *E. magna*. Chronic ileitis occurred at 13–15 days post-infection and was associated with the following events: (1) Mast cell release of mediators such as tryptase involved in structural remodeling. (2) Chemotaxis and gut infiltration of various immune cells; they can release harmful enzymes like gelatinase. (3) These immune cells release reactive oxygen species and cause oxidative stress, (4) leading to transmural lipid peroxidation and tissue injury. (5) Oxidative stress activates redox-sensitive transcription factors leading to production of (6) pro-inflammatory mediators. (7) Oxidative stress also differentially up-regulates adenosine A1 and A3 receptors that may have (8) antioxidant, anti-inflammatory, or neuroprotective properties and thus may be of therapeutic potential. (9) Other antioxidant pathways are differentially altered by chronic ileitis; some are compromised while others are up-regulated. (10) Ischemia/reperfusion can induce the ROS cascade of injury. The balance between all of these and other mechanisms determines the extent of tissue injury in chronic ileitis.

and lipid peroxides) may enhance the process of lipid peroxidation. Chronic ileitis is associated with transmural changes in the gut wall as evidenced by the increase in lipid peroxidation in both the mucosal and myenteric plexus tissues. Transmural damage would affect enteric neural reflexes and thus motility and secretion. The degree of lipid peroxidation was significant, but not as extensive as the several fold increase reported in other models of intestinal inflammation [5,31]. The lower levels of lipid peroxidation perhaps are attributed to compensatory elevations in antioxidant defense mechanisms that include *GSH/GSH-R*, *A1*, and *A3* gene products.

In chronic inflammation, in addition to the potential deleterious actions of ROS in causing lipid peroxidation, other mediators may also contribute to tissue injury such as tryptase and gelatinase enzymes that are elevated in chronic ileitis. Mast cell tryptase is known to act on myenteric neurons via protease-1 and -2 receptors [32] and therefore directly affects/alters neuronal and synaptic behavior of the neurons leading to chronic changes and abnormalities in gut behavior and gut reflexes that are known to occur in IBD and Crohn's patients.

4.2. Free radical damage

Cell tissue damage in IBD may involve oxidative stress and the production of ROS [1]. There is an increase in ROS-producing cells in the inflamed gut of patients with ulcerative colitis or Crohn's disease compared with controls. A small fraction of these cells were CD15-positive cells, suggesting that they were monocytes/granulocytes [10]. Our study in the rabbit ileitis model has not delineated the sources of ROS that contribute to injury, but it has identified some of the different oxyradical species. Activated neutrophils and other phagocytes release ROS, O_2^-, and chloramines, known to exert deleterious effects directly on intestinal mucosal structures and function [29,30,33], or indirectly on mucosal reflexes involved in hypersecretion and diarrhea. In chronic ileitis, chloramines [29] were elevated in the mucosa where they likely contribute to oxidative stress and lipid peroxidation. Xanthine oxidase (XO) catalyzes the formation of O_2^-, and the observed increase in XO activity in chronic ileitis implies that O_2^- plays a significant role in mucosal injury and lipid peroxidation. The possibility also exists that peroxynitrite

($ONOO^-$) contributes to mucosal injury and lipid peroxidation [34].

4.3. Antioxidant defense mechanisms

Cytoprotection from free radicals is provided by antioxidants (vitamins A, C, and E), reduced GSH, as well as antioxidant enzymes that include SOD, CAT, and GSH-Px. GSH-R complements the actions of antioxidant enzymes by converting oxidized GSH to its reduced form. All of these enzymes work together to scavenge free radicals and maintain a high level of reduced GSH. GSH-R and GSH were increased in chronic ileitis which would provide antioxidant protection, because GSH serves as a reductant in the metabolism of various hydroperoxides (i.e. H_2O_2) that are also generated following the peroxidation of membrane lipids, a reaction catalyzed by GSH-Px [5,31]. However, the antioxidant-buffering capacity of the mucosa is compromised due to reductions in both SOD, an enzyme that inactivates O_2^- and prevents free radical tissue injury, and catalase. In chronic ileitis, a reduction in the antioxidant-buffering capacity may increase the susceptibility of tissues to oxidative stress, since tissue injury via oxidative stress is believed to occur only when there is an imbalance between the oxidant/antioxidant status [7,8].

4.4. Adenosine and up-regulation of A1 and A3 receptors

Endogenous adenosine release under conditions of oxidative stress is believed to play a cytoprotective role in the cardiovascular system and in the central and enteric nervous systems [13,18,24,35] by acting at specific cell surface receptors. A recent study in mice [35] showed that the adenosine kinase inhibitor, which elevates endogenous adenosine levels, improves experimental colitis and reduces the inflammatory response. Furthermore, activation of A3ARs stimulates the antioxidant defense mechanisms and leads to a 2- to 3-fold increase in SOD, CAT, and GSH-Px activities, and increases in GSH-R activity in diverse cell types [15]. Our current study is the first to provide evidence that chronic ileitis causes up-regulation of *A3* and *A1* gene transcripts. The remarkable A3 up-regulation observed (5.40-fold increase) was several fold higher than that of A1 receptors (1.34-fold increase), and is the highest level of A3AR up-regulation reported for any pathophysiological condition. In fact, A3 up-regulation in ileitis is several fold higher than that reported for CNS neuroprotection [16] or that observed in association with airway inflammation [17]. A3 up-regulation has been suggested to occur transiently in an acute manner, and to return to baseline levels within 24 hr [23]. Our study identifies a prominent role for *A3* adenosine receptor gene products in chronic inflammation in addition to its known anti-inflammatory role in human neutrophil-mediated tissue injury [18] and in chemotaxis. Similarly, up-regulation of *A1* adenosine receptor gene transcripts is much higher than that observed in a variety of other patho-physiological conditions [20–24]. A1 receptors are up-regulated in response to oxidative stress [20], and they are likely to serve a cytoprotective role in ileitis as they do in the central nervous system [13].

Intestinal epithelial hypoxia in chronically inflamed intestine (microvessel breakdown) stimulates the release of TNF-α [12] and adenosine, a neuroprotective agent [36]. TNF-α is elevated in chronic ileitis as it is in Crohn's disease. In Crohn's disease, TNF-α is produced by macrophages, monocytes, and activated T cells and recruits circulating inflammatory cells to local tissue sites in the mucosa [37]. TNF-α stimulates endothelial production of adhesion molecules, compromises barrier function, and induces collagen production involved in tissue remodeling [37]. Anti-TNF-α is the first therapy to show efficacy in the treatment of active, moderate Crohn's disease and Crohn's fistulae [38]. Intestinal ischemia/hypoxia in the chronically inflamed gut also leads to microvessel damage, and reperfusion injury occurs via oxidative stress from free radicals that include O_2^-, $ONOO^-$, chloramines, and H_2O_2, or up-regulation of xanthine oxidase. Adenosine co-release during intestinal ischemia is expected to act against the deleterious effects of reperfusion injury [13,15,16,18,24,35] by acting at adenosine A1 or A3 receptors. In addition, adenosine can suppress TNFα production from immune cells [39] by acting at A3 receptors. Expression of pro-inflammatory interleukins is not affected by A3 activation [40].

4.5. Transcriptional regulation of A1/A3ARs and pro-inflammatory mediators

Up-regulation of both antioxidant (A3ARs) and inflammatory mediators (TNF-α) occurs via redox-sensitive AP-1 transcription. This would provide an efficient mechanism for suppressing TNF-α production (or other mediators, see Fig. 2) and prevent or reduce cell damage. Activation of both A3AR and A1AR is expected to suppress redox-sensitive transcription via either AP-1 or NF-κB [40]. Other pro-inflammatory mediators produced via redox-sensitive transcription through AP-1 or NF-κB are LTB_4, TXB_2, iNOS, and cyclooxygenase and lipoxygenase enzymes. The circulating leukotriene LTB_4 in chronic ileitis is synthesized in neutrophils or monocytes [41,42], and is known to exert a variety of biological effects including chemokinesis, chemotaxis, stimulation of O_2^- production and release of lysosomal enzymes [41], leading to further tissue injury. Induction of iNOS and production of nitric oxide may contribute to highly reactive peroxynitrite species ($ONOO^-$) to further perpetuate the damage. In rabbit ileitis, nitric oxide is elevated in mucosa and plasma, and iNOS immunoreactivity is significantly up-regulated in the mucosal, myenteric, and submucous neurons [34]. Therefore, it is possible that peroxynitrite radicals contribute to transmural damage by acting on

mucosal, submucous, and myenteric neurons. It should be pointed out that another link between other oxyradicals and $ONOO^-$ is that ROS can activate the transcription factor NF-κB leading to the expression of iNOS (and inflammatory cytokines) [11]. Therefore, the potential exists for a reverberating pathway resulting in further cell injury. Another mediator that is elevated in chronic ileitis through activation of redox-sensitive transcription is TXB_2. Thromboxane plays an active role in the development of chronic inflammatory lesions in the bowel [32,43]. All the pro-inflammatory mediators likely work synergistically to cause cell and tissue injury.

4.6. Therapeutic potential of the chronic ileitis model

Chronic ileitis is associated with histopathology, mucosal injury, malnutrition, and an inflammatory—oxidant/antioxidant profile that is similar to IBD, suggesting that it is a useful model not only to study the disease, but also to test new strategies for therapy. The observed imbalance in the oxidant/antioxidant defense mechanisms of the host would increase the susceptibility of the intestine to inflammation and implies that supplementation with antioxidants may be of therapeutic value. Whether oxidative stress-induced injury is a primary or secondary phenomenon in the development of inflammation is unknown. However, it contributes to the symptomatology and perpetuation of the disease.

Clearly, the endogenous purine/adenosinergic pathway is unable to cope with the complex oxidant/antioxidant and pro-inflammatory signaling pathways leading to chronic ileitis, and up-regulation of adenosine A1 and A3 receptors is insufficient to prevent the damage. However, this knowledge is useful in providing us with a novel target for therapy in chronic ileitis and perhaps IBD. Adenosine is believed to be neuroprotective, anti-inflammatory, antioxidant, and anti-chemotactic. In the enteric nervous system, it gates excitability of intrinsic primary afferent and motor neurons (via A1/A3 receptors) involved in gut mucosal and distension reflexes [14,36,44] that govern luminal secretion and peristaltic movements of the gut. Therefore, a potential treatment strategy might involve a drug intervention that elevates endogenous levels of adenosine in chronic inflammation to activate these up-regulated receptors. The multiple actions of adenosine in the gut and at the up-regulated A3 and A1 receptors are expected to reduce abdominal pain, protect the enteric nervous system, provide antioxidant activity, reduce or prevent diarrhea, and reverse malnutrition by suppressing the neuro-immune response, and prevent/or reverse weight loss. In fact, a recent study in mice [35] showed that the adenosine kinase inhibitor GP515 improves experimental colitis. Adenosine kinase inhibitors elevate endogenous adenosine levels to protect the gut from colitis. Selective antagonists/agonists for adenosine A1/A3 receptors may also prove useful. It also remains to be proven whether combination therapy with antioxidants and adenosine drugs may be of additional benefit in chronic inflammation.

Acknowledgments

This work was supported by NIH R01 DK44179 to F.L.C.; R01 DK 45062 and DK 58034 to U.S.; and R01 DK 57016 to H.J.C. We are grateful to Michele Walsh and Iveta Grants for assistance in preparing the manuscript. Jorge Guzman is an M.D./Ph.D. student in the medical scientist program on an NIH fellowship (DK 44179-07S1) to study adenosine A3 regulation in the gut.

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