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Therapeutic Effects of Interleukin-4 Gene Transfer in Experimental Inflammatory Bowel Disease

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Abstract

Inflammatory bowel disease (IBD) is characterized by altered immunoregulation and augmented intestinal synthesis of nitric oxide. The purpose of this study was to determine the effects of exogenous IL-4, introduced by a recombinant human type 5 adenovirus (Ad5) vector, on the tissue injury associated with an experimental model of colonic immune activation and inflammation. Colitis was induced in rats by the intrarectal administration of trinitrobenzene sulfonic acid (TNB) dissolved in 50% ethanol, and control rats received saline via the same route. 1 h later, all rats were randomized into two groups. The first group was injected intraperitoneally (ip) with 3.0 × 10⁶ plaque forming units (PFUs) of Ad5 transfected with murine interleukin-4 (Ad5IL-4) and the second group was injected ip with the same amount of Ad5 expressing the Escherichia coli Lac Z gene (Ad5LacZ). One-half of the colitic and control rats were injected again with 3.0 × 10⁶ PFUs of Ad5IL-4 or Ad5LacZ on day 3 of the 6-d study. When introduced once or twice via the peritoneal route into control rats, Ad5LacZ was localized to the serosal lining of the peritoneal cavity, the diaphragm and the liver on day 6. One or two injections of Ad5IL-4 into rats also produced measurable infection in the liver. We have also previously shown that Ad5 transfected with murine interleukin-6 cDNA (1) inserted into lipid vehicles (2, 3) or cDNA incorporated into a virus (see reference 4 for review). Of the types of viral vectors (e.g., retrovirus, vaccinia, herpes virus, etc.) used for in vivo gene delivery, the replication-deficient recombinant human type 5 adenovirus (Ad5) has gained the greatest prominence because Ad5 vectors can be grown in very high density and, unlike retrovirus, Ad5 can insert foreign genes into nondividing mammalian cells (5). Many investigators have shown that Ad5 vectors can successfully deliver mammalian cytokine genes to many different tissues and organs, including the gastrointestinal tract. For example, Braciak et al. (6) showed that Ad5 transfected with murine interleukin-6 cDNA induces elevated synthesis of this cytokine, particularly in the liver. We have also previously shown that Ad5 transfected with murine IL-4 cDNA (Ad5IL-4) can be delivered directly to the rodent intestine, resulting in measurable infection and pronounced changes in circulating IL-4 and intestinal function (7). Another advantage of this method of gene delivery is that Ad5 does not stably integrate into the genome of the host. Therefore, gene delivery through replication-deficient Ad5 provides an ideal method for studying the consequences of transitory increased synthesis of a single cytokine under in vivo conditions.

During what in vivo conditions would it be advantageous to augment levels of a particular cytokine through gene transfer? Using an Ad5-based gene delivery system, it has been possible to explore the transient role of various cytokines in pathological processes in the lung (6, 8) and the intestine (7), and to test cytokine efficacy in the treatment of cancer (9, 10) and cardiovascular disease (11). In addition, the therapeutic implications of this approach are only now being realized in light of emerg-

1. Abbreviations used in this paper: Ad5IL-4, human ad5 adenovirus transfected with murine IL-4 cDNA; Ad5LacZ, human ad5 adenovirus transfected with β-galactosidase cDNA; IBD, inflammatory bowel disease; MPO, myeloperoxidase; NOS II, inducible nitric oxide synthase type II; PFU, plaque forming unit; TNB, trinitrobenzene sulfonic acid.

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In the present study, we documented that increased synthesis of IL-4 after AdSIL-4 injection through the peritoneal route had a therapeutic effect in rats experiencing TNB-colitis due, in part, to a reduction in NOS II expression and nitric oxide synthesis. On day 6 postinduction of colitis, rats receiving AdSIL-4 on day 1 and 3 post-TNB had increased circulating IL-4, decreased circulating IFN-γ, markedly fewer peritoneal adhesions, significantly less macroscopic damage, and reduced myeloperoxidase (MPO) activity in the distal colon. AdSIL-4 gene transfer into TBN-inflamed rats also inhibited the induction of NOS II in the colon, as shown by reverse transcription polymerase chain reaction and calcium-independent nitric oxide synthase activity. In contrast, rats treated with the AdS5 control, AdSLacZ, did not experience any reduction in the severity of colonic inflammation or in NOS II expression and nitric oxide production in the colon. Taken together, these data strongly support an immunomodulatory and antiinflammatory role for IL-4 in experimental colitis and may provide a novel approach for the delivery of immunoregulatory cytokines in IBD.

**Methods**

**Animals.** Specific pathogen-free, male, Sprague-Dawley rats (180–200 grams; Charles River Laboratories, Montreal, Canada) were caged in groups of two in a Level B clean room. All rats had ad libitum access to food and water before and during the study. The protocols used were in direct accordance with guidelines drafted by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

**Materials.** Human Ad5 lacking the E1 region of its genome which regulates replication were transfected with murine IL-4 cDNA (AdSIL-4) or with Escherichia coli Lac Z (AdSLacZ) according to detailed procedures published elsewhere (6, 8). β-galactosidase activity was determined using an assay system and authentic β-galactosidase purchased from Promega Corp. (Madison, WI). TNB was purchased from Eastman Kodak Co. (Rochester, NY). The following monoclonal antibodies used in the cytokine ELISAs were purchased from PharMingen (San Diego, CA): rat anti–mouse IL-4 (capture antibody; BVDV4-1D11), biotinylated rat anti–mouse IL-4 (detection antibody; BVDV6-24G2), purified rat anti–mouse IFN-γ (capture antibody; R4-6A2) and biotinylated rat anti–mouse IFN-γ (detection antibody; XM1G1.2). Nunc-immuno ELISA plates (MaxiSorp™) and recombinant murine IL-4 (rmIL-4) was obtained from Intermedico (Markham, Canada). NOSdetect™ assay kits were purchased from PDI BioScience Inc. (Aurora, Canada). Unless otherwise stated, all other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

**Induction of colitis.** While a mouse model of TNB colitis has recently been described (37), the rat model remains the better described and most frequently used experimental IBD model (38). We also used the rat model of TNB-colitis (25) because the role of nitric oxide in this model has been well established in our laboratory (32) and in others (35). Briefly, each rat (n = 54) was lightly anesthetized with Fluothane™ (halothane b.p.; Wyeth-Ayerst Canada, Inc., Montreal, Canada). 30 mg of TNB dissolved in 0.25 ml of 50% ethanol was instilled into the distal colon of each animal using a PE-50 cannula (Becton Dickinson Labware, Lincoln Park, NJ) attached to a 1-ml syringe. After instillation, each animal was returned to its respective cage for recovery from anesthesia. Using this procedure, > 95% of the rats retained the TNB-ethanol enema. However, if an animal quickly (i.e., in < 5 min) excluded this solution, it was omitted from the remainder of the study. Control or uninflamed rats (n = 24) received normal saline (0.9%; wt/vol) intracolonically in a similar manner.
TNB-treated and control rats were kept in separate cages over the course of the study.

**Study protocol.** Ad5LacZ or Ad5SIL-4, both at a concentration of 3.0 x 10⁶ plaque forming units (PFU)/ml of HBSS (pH 7.4), were injected into the peritoneal cavity of uninflamed (n = 6/group) or TNB-inflamed (n = 16/group) rats 1 h after the enema. 3 d later, a second injection of Ad5LacZ or Ad5SIL-4 (both at 3.0 x 10⁶ PFU/ml HBSS) was given to half of the control and TNB-inflamed rats. This route and time course of Ad5 delivery to these animals was based on previous studies. Braich et al. (6) have shown that an injection of Ad5 expressing luciferase via the peritoneal route resulted in Ad5 infection predominately in the liver (53% of total) and also in the spleen and peritoneum. Further, it has also been shown that cytokine production after Ad5-cytokine gene injection into the peritoneal cavity only persists for 4 d (6), we included groups that received Ad5LacZ or Ad5SIL-4 on day 1 and on day 3 of this study. While we have previously shown that Ad5LacZ and Ad5SIL-4 can be delivered selectively to the external surface of the intestinal tract (7), we did not use this technique in the present study because of its direct proinflammatory effect on the intestine. On day 6 of the study, all rats were anesthetized with Fluothane™. 1 ml of blood was removed by cardiac puncture and transferred to a 1.5-ml centrifuge tube on ice. Immediately after blood removal, rats were killed by cervical dislocation, and the entire length of the colon was excised through a longitudinal peritoneal opening. The colon was immediately placed in phosphate buffered saline containing 1% bovine serum albumin (Gibco BRL) and rinsed with phosphate buffered saline before homogenization.

**Assessment of colonic myeloperoxidase activity.** Tissues (200–400 mg) for determination of MPO activity were removed from the area of gross injury (i.e., 4–6 cm proximal to the anus) and snap frozen in liquid nitrogen. MPO is an enzyme located in neutrophils, eosinophils, and other cells of myeloid origin, and measures of MPO activity are commonly used as a marker of intestinal inflammation (38). MPO activity in samples of control and TNB-treated distal colon from Ad5-treated rats was determined using a previously published method (32). Briefly, samples were thawed, weighed, and homogenized in hexade cycltrimethylammonium bromide buffer. These homogenates were centrifuged and MPO activity was measured in the supernatants. MPO was expressed as units/milligram of tissue where 1 U corresponds to the activity of enzyme required to degrade 1 μmol of hydrogen peroxide in a minute at 24°C. Detection of inducible nitric oxide synthase by reverse transcriptase PCR. On day 6 of the study, samples of colon for mRNA isolation were removed 6 cm proximal to the anus in control rats or from the upper margin of the grossly damaged distal colon in the TNB-inflamed rats. Total cellular RNA was isolated using a previously described guanidium isothiocyanate method (40). The concentration of RNA was determined by absorbance at 260 nm and its purity was confirmed using the ratio of absorbency at 260 nm to that at 280 nm. RNA was stored at −70°C until used for reverse transcription-polymerase chain reaction (RT-PCR). mRNA was next reverse transcribed as previously described (40) to yield cDNA. 2-μl aliquots of cDNA (3 μg) were then mixed with 20 pmol each of sense (5′- TTC CCA AGT TTC TCG CAG C-3′) and antisense (5′-ATA GGA AAA GAC TGC ACC GAA GAT-3′) primers for NOS II (Promega Corp.), 0.4 μl of 10X Promega Buffer, 1.25 μl of dNTP (10 mM), 0.125 μl of 5X BRL AmpliTaq DNA Polymerase (Gibco BRL), 1 μl of cDNA template, and 1 μl of water. To ensure that equal amounts of RNA were loaded onto a 2% agarose gel with a 10X loading buffer, and then visualized under ultraviolet light after ethidium bromide staining. The 500-bp product corresponds to NOS II and the 302-bp product corresponds to β-glucuronidase.

Assessment of nitric oxide synthase activity. Nitric oxide synthase activity in control and TNB-inflamed colonic tissue was directly determined in Ad5-infected groups (n = 3/group) using an L-citrulline assay (32). L-citrulline is a coproduct of nitric oxide synthesis, and it is formed from l-arginine in a 1:1 stoichiometric reaction. On day 6 full thickness tissues from the distal one third of the colon (~4–6 cm of tissue) were homogenized, and supernatants of the homogenates were incubated with tritiated [3H]-l-arginine (Amersham, Arlington Heights, IL) and cofactors NADPH, FAD, FMN and BH₄ for 60 min at 37°C in the absence of CaCl₂. To ensure that l-citrulline formation was a consequence of nitric oxide generation, identical colonic homogenates with cofactors were incubated in the presence of Nω-nitro-l-arginine methyl ester HCl (L-NAME; 1 mM). [3H]-l-citrulline was eluted from columns containing an equilibrated resin. Using this procedure, ~90–95% of the [3H]-l-citrulline and <1% of the...
Table I. β-galactosidase Activity in Control and TNB-inflamed Rats on Day 6 after One or Two Intraperitoneal Injections of Ad5LacZ

<table>
<thead>
<tr>
<th>Tissue site</th>
<th>Control groups</th>
<th>TNB-colitis groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One dose Ad5</td>
<td>Two doses Ad5</td>
</tr>
<tr>
<td>Injection site</td>
<td>0.228±0.036</td>
<td>0.181±0.009</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0.436±0.010</td>
<td>0.552±0.019</td>
</tr>
<tr>
<td>Liver</td>
<td>4.989±0.217</td>
<td>4.160±0.171</td>
</tr>
<tr>
<td>Colon*</td>
<td>0</td>
<td>0.166±0.025</td>
</tr>
</tbody>
</table>

*β-galactosidase values were corrected for endogenous β-galactosidase activity from bacteria located in the lumen of the colon. β-galactosidase activity (milliunits) was measured in tissue homogenates of the intraperitoneal injection site, liver, diaphragm, and colon as described in the Methods section. Data represent mean±SEM of three rats per injection group.

[3H]-l-arginine was recovered. Therefore, background counts from the trace amounts of [3H]-l-arginine were subtracted from the counts of each sample before calculation of the total nitric oxide synthase activity in the homogenates. After the determination of protein in the tissue homogenates according to Lowry et al. (43), nitric oxide synthase enzyme activity was calculated using the following formula: adjusted counts (dpm/gram of protein) × 63.1 Ci mmol⁻¹ (sp act)/2.22 × 10⁶ min⁻¹ (conversion rate) × 60 min.

Data analysis. All data are expressed as mean±SEM; n refers to the quantity of animals. Statistical analysis was calculated using one-way ANOVA, and multiple comparisons were performed using the Newman-Keuls multiple comparison test. An associated probability of ≤ 5% was indicated as significant.

Results

β-galactosidase activity in Ad5LacZ-injected rats. To confirm that Ad5 infection was successful in control and TNB-inflamed rats and determine the relative tissue distribution of virus, Ad5 containing LacZ were injected intraperitoneally into control and colitic rats. The peritoneal lining, diaphragm, liver, and colon from Ad5LacZ-infected control and colitic rats were examined for β-galactosidase activity, and as shown in Table I, β-galactosidase activity was present in all tissues examined. No background β-galactosidase activity was measured in the peritoneal lining, diaphragm, or liver of noninfected rats from either inflammation group, but constitutive expression of this enzyme was present in colon samples taken from the same rats (not shown). The greatest expression of β-galactosidase activity after Ad5LacZ injection was observed in liver biopsies from control and colitic animals (Table I). Consistent increases in β-galactosidase activity between the groups that received one vs two injections of Ad5LacZ were not observed in the injection site, diaphragm, or liver. However, when corrected for endogenous bacterial β-galactosidase activity, clear increases in β-galactosidase activities were observed in colonic tissue taken from rats injected twice with Ad5LacZ when compared with β-galactosidase activity in colons removed from rats injected once with Ad5LacZ. These data suggested that TNB-colitis did not impair the ability of Ad5 to infect various tissues in the rat, and also showed that two injections of virus were associated with detectable infection of distal colon.

Circulating IL-4 levels are increased after intraperitoneal Ad5IL-4 gene transfer. Further confirmation of Ad5 infection and transduction in control and TNB-inflamed rats was determined through the use of a murine IL-4 sandwich ELISA for the measurement of serum IL-4. The murine mAbs used to detect serum mIL-4 did not cross-react with rat recombinant IL-4 (not shown). On day 6 of the study, no IL-4 was detected in either group of control or TNB-inflamed rats injected with Ad5LacZ (Fig. 1, A and B). However, as summarized in Fig. 1, A and B, control and TNB-inflamed rats that received Ad5IL-4 had measurable amounts of immunoreactive serum IL-4. Control rats injected once with Ad5IL-4 had 1.2±0.47 ng mIL-4/ml of serum and two doses of Ad5IL-4 augmented levels of IL-4 to 2.3±1.24 ng/ml. In rats with TNB-colitis, serum levels of IL-4 were about threefold higher in animals that received one dose of Ad5IL-4 compared with rats that received two doses of Ad5IL-4 (9.3±2.7 vs 3.0±2.3 ng mIL-4/ml serum).

Numerous studies of therapeutic interventions in experimental and clinical IBD have shown the importance of mea-
suring changes in cytokine levels in intestinal tissue. These studies have highlighted that systemic changes in cytokine levels do not necessarily mirror alterations in these mediators at the site of intestinal inflammation. Although one injection of Ad5IL-4 in TNB-colitis rats gave the higher levels of serum IL-4, only the dual intraperitoneal injection of Ad5IL-4 was associated with detectable quantities of immunoreactive mIL-4 (3.5 \pm 0.7 \text{ng/ml}) in the distal colon (Fig. 2).

Advantages of using Ad5IL-4 reduce circulating and colon IFN-γ levels in TNB-treated rats. Elevations in IFN-γ have recently been described in a murine TNB-colitis model (37) and in the CD45RB<sup>hi</sup> T cell reconstitution colitis model (18). Further, resolution of colitis in both models is associated with a pronounced reduction in IFN-γ synthesis (27, 37). In the present study, IFN-γ was detected in a standard sandwich ELISA using murine anti–IFN-γ antibodies (i.e., capture and detection) that cross-reacted with rat IFN-γ. The cross-reactivity of these mAbs was confirmed using recombinant rat IFN-γ as a standard in each ELISA assay. Levels of circulating IFN-γ were typically present at the lower limit of detection in this ELISA (i.e., \sim \text{10 pg/ml}). Administration of Ad5LacZ did not affect the amount of immunoreactive IFN-γ present in the control groups (Fig. 3 A). However, control rats that were injected with one dose of Ad5IL-4 had levels of IFN-γ exceeding 5 ng/ml (Fig. 3 A) while control rats that were injected twice with the same Ad5 expressed 3\pm 1 ng IFN-γ/ml of serum. Marked elevations in IFN-γ were observed in TNB-inflamed rats (Fig. 3 B). Neither of the Ad5LacZ treatment groups (35\pm 10 and 24\pm 12 ng IFN-γ/ml of serum, respectively) nor the Ad5IL-4 group that received a single injection (28\pm 8 ng IFN-γ/ml of se-
rum) had circulating levels of IFN-γ that differed significantly from uninfected TNB rats (49±15 ng IFN-γ/ml of serum; not shown). However, in rats that received two doses of Ad5IL-4, circulating IFN-γ levels were reduced to 9±5 ng/ml.

Immunoreactive levels of IFN-γ in colonic homogenates from TNB-colitic rats are shown in Fig. 4. In colitic rats that received one or two injections of Ad5LacZ, IFN-γ levels were ≤ 1.0 ng/ml. However, in colitic rats that received one dose of Ad5IL-4, IFN-γ levels approached 2.0 ng/ml (Fig. 4). Colonic levels of IFN-γ were significantly decreased in colitic rats that received two injections of Ad5IL-4 (Fig. 4), consistent with the decreases in circulating IFN-γ in these rats.

Two intraperitoneal injections of Ad5IL-4 markedly attenuates macroscopic and microscopic injury in TNB-colitis. Examination of the peritoneal cavity in a rat with acute (i.e., on day 6) TNB colitis characteristically reveals multiple adhesions between the colon and other organs, and a markedly dilated and thickened distal colon (32). A view of the mucosal surface of the colon typically revealed frank ulceration, particularly in the area of the colon exposed initially to the TNB and ethanol enema. In the present study, we saw no deviation from this macroscopic picture in colitic rats treated with Ad5LacZ. Out of a total macroscopic damage score of 10, damage scores in these groups were 7±1 and 8±2, respectively, and these values were similar to those obtained previously in noninfected TNB-treated rats (32). Intraperitoneal injection of control rats with one or two doses of Ad5LacZ (not shown) or Ad5IL-4 had no effect on the macroscopic appearance of the colon. Rats that received one ip injection of Ad5IL-4 exhibited distal colonic injury similar to that seen in the Ad5LacZ-treated groups with a macroscopic damage score of 6±3. However, in rats injected twice with Ad5IL-4, there was a marked improvement in the macroscopic picture, and this was reflected in a significantly lower macroscopic injury score of 3±1. Also in contrast to the other treatment groups, no adhesions between the colon and other organs in the peritoneal cavity were evident. The therapeutic effect of two peritoneal injections of Ad5IL-4 was confirmed by a histologic examination of tissues from the distal colon of TNB-treated rats. While transverse sections of distal colon revealed marked transmural injury in tissues from the other three Ad5-injected groups, an intact colonic architecture and an absence of neutrophil infiltration were observed in the double-injected Ad5IL-4 group. Fig. 5, A–H illustrates the histologic appearance of the colon in each of the Ad5 treatment groups. As shown in the first four panels of Fig. 5, nothing abnormal was observed in colon samples taken from Ad5-injected control rats. After 6 d of colitis the microscopic picture of the colon was vastly different. Note the presence of deep penetrating ulcers and the marked infiltrate characterized by neutrophils, eosinophils and lymphocytes in the Ad5LacZ groups (Fig. 5, E and F) and the single injection Ad5IL-4 group (Fig. 5 G). Fig. 5 H illustrates the characteristic appearance of colonic tissues removed from colitic rats that received two injections of Ad5IL-4. A marked reduction in tissue injury and inflammatory cell infiltrates was readily apparent in all sections of colon examined from these rats.

Granulocyte accumulation in TNB-treated colon is inhibited by Ad5IL-4 treatment. Administration of Ad5LacZ or Ad5IL-4 to control rats did not affect MPO activity in the distal colon (Fig. 6 A). Similar to the histologic observations, two injections of Ad5IL-4 in TNB-colitis rats significantly reduced by approximately threefold the amount of MPO activity in the distal colon (Fig. 6 B). However, MPO activity in this Ad5-injected TNB group was still elevated above those observed in the controls.

Ad5IL-4 gene transfer inhibits NOS II gene expression and colonic nitric oxide synthesis. Fig. 7 illustrates the expression of NOS II and β-glucuronidase in the four Ad5-infected groups examined in this study. Although β-glucuronidase gene expression was present in all RNA samples subjected to RT-PCR, NOS II was absent in all control colonic samples from Ad5LacZ- and Ad5IL-4–injected rats. In contrast, NOS II gene expression was apparent in all colonic tissues removed from TNB-inflamed rats, but NOS II gene expression was diminished in colon samples removed from rats injected twice with Ad5IL-4 (Fig. 7). Colonic calcium-independent or NOS II nitric oxide synthesis, as determined by the measurement of [3H]-citrulline formation in colonic homogenates without exogenous CaCl₂, was < 10 nmol/min per gram of protein in control rats injected twice with Ad5LacZ (Fig. 8). Two injections of Ad5IL-4 in control rats reduced colonic nitric oxide synthesis approximately fivefold. On day 6 after TNB administration and after two injections of Ad5LacZ, NOS II nitric oxide synthesis was increased fivefold (Fig. 8). The increase in NOS II activity observed here was similar to those reported previously in the TNB colitis model (32, 35). In rats injected twice with Ad5IL-4, NOS II nitric oxide synthesis was significantly reduced by ~ 50%. The presence of L-NAME during the 60-min reaction reduced nitric oxide synthase activity to levels observed in Ad5LacZ-injected, control rats (Fig. 8).

Discussion

The present study demonstrates that increased in vivo IL-4 levels through Ad5 gene delivery had a marked therapeutic effect in an acute model of experimental colitis. Every parameter examined indicated that a dual treatment of rats with Ad5IL-4 resulted in significantly reduced amounts of circulating and tissue IFN-γ, and in a significant improvement in the histologic appearance of the distal colon. Further, the reduction in distal colonic injury was associated with a decrease in NOS II gene expression and calcium-independent nitric oxide synthesis in the colon. The delivery of Ad5IL-4 to rats allowed us to directly determine the in vivo therapeutic effect of IL-4 in a well-characterized model of distal colonic inflammation. While there is concern about an immune response mounted against a foreign murine protein (44) and the Ad5 vector itself (45), these studies were acute (i.e., 6 d) in duration permitting a rather limited immune response by the rats to these elements. Further, the rat model of TNB-colitis provides many advantages over the murine model recently described (37, 46). The intestinal injury in rat TNB-colitis is mediated almost exclusively by infiltrating neutrophils (47), the deleterious role of increased nitric oxide synthesis in this model is well characterized (32, 35), and rats with TNB colitis respond to many of the therapies currently used in the clinical treatment of IBD (26). Taken together, these findings illustrate that immunoregulatory cytokines may have utility in the treatment of inflammatory diseases of the bowel.

Why use a strategy of cytokine gene delivery by Ad5 rather than the direct delivery of recombinant cytokine? The use of an Ad5 vector offers many advantages over the bolus delivery of recombinant cytokine. For a study extending > 6 d in duration the amounts of recombinant cytokine required would be pro-

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ter the inflammatory (i.e., IFN-γ) levels exceeded 8 ng/ml, this increase in IL-4 was not sufficient to alter the cytokine response to cytokine signals in the intestinal tract has deleterious effects. Further, recombinant cytokines are rapidly cleared from the circulation resulting in a marked fluctuation in the amount of the biologically active cytokine over the course of a study. In contrast, the quantity of cytokine synthesized after Ad5 infection can be regulated by the titer of virus delivered and by the route of Ad5 administration (39). Using Ad5-based gene delivery techniques it has been shown that transitory gene replenishment, vaccination, modulation of cell proliferation, and cytokine delivery is possible (see reference 4 for review). Although previous studies using recombinant Ad5 expressing cytokines have shown the potential pathological role of cytokine over expression in the lung and intestinal tract (6–8), this technique has also demonstrated therapeutic potential in disease processes such as cancer (9). The present study further emphasized the importance of elucidating the therapeutic dose and timeframe of Ad5IL-4 administration. The therapeutic effects of a relatively small amount of adenovirus (i.e., \(3.0 \times 10^9\)) were only apparent when delivered in two separate injections 3 d apart, and these effects appeared to be related to the overexpression of IL-4 in the colon. Although one injection of Ad5IL-4 resulted in levels of circulating IL-4 that exceeded 8 ng/ml, this increase in IL-4 was not sufficient to alter the inflammatory (i.e., IFN-γ levels and MPO activity) and histologic parameters in this model of colitis. It is not readily apparent why circulating levels of IL-4 in colitic rats injected once with Ad5IL-4 were eightfold higher than levels of control rats that received a similar amount of adenovirus. However, these findings may be related, in part, to the effect of elevated IFN-γ on factors that regulate the transcription and/or translation of the IL-4 transgene. Since considerable evidence has accumulated showing that Th1-type (i.e., T cell responses characterized by IFN-γ synthesis) and Th2-type responses (i.e., T cell responses characterized by IL-4 synthesis) cross-regulate one another (47), further examination of the role of elevated IFN-γ during transgene manipulation in colitis is certainly warranted.

Careful attention has been directed at identifying strategies to limit the inflammatory response and immune dysregulation during IBD. Toward this goal, progress has been made in understanding the role of cell-mediated immune responses in the intestinal mucosa in the regulation of cytokine homeostasis, particularly related to those cytokines involved in T cell function. Gene knock-out murine models of spontaneous IBD (48) have given important clues as to regulation of mucosal immune system, and demonstrate that an imbalance in the Th cell response to cytokine signals in the intestinal tract has deleterious consequences. In gene-competent rodent models with experimentally induced IBD, recent findings support a therapeutic role for anticytokine antibodies or recombinant immunomodulatory cytokines. Neurath et al. (37) found that monoclonal antibodies directed against murine IL-12 abrogated established TNB-colitis in mice, and that this therapeutic effect was associated with a marked reduction in the synthesis of Th1 cytokines (i.e., IFN-γ and IL-2) in vitro. These observations coincide with those in the present study, in that resolution of colitis following two injections of Ad5IL-4 was associated with a marked reduction in circulating IFN-γ levels. Herfath et al. (49) injected recombinant murine IL-10 (rmIL-10) into rats with granulomatous colitis, and observed a beneficial effect of this treatment after 17 d. Rats receiving rmIL-10 had significantly decreased macroscopic damage scores in the colon, liver and joints, and decreased gene expression for IL-1, IL-6,
TNFα, and IFN-γ in mesenteric lymph node and liver. However, not all attempts to attenuate experimental IBD with exogenous cytokine administration have shown similar therapeutic effects. The twice daily injection of rhIL-10 into colitic rats failed to attenuate the mucosal injury and nitric oxide synthesis observed on day 5 after TNB (50). This study, unlike the others mentioned above, addressed the effects of exogenous rhIL-10 delivery on nitric oxide synthesis in the gut because it is established that nitric oxide exerts a deleterious effect on many tissues, including the gastrointestinal tract (55). Experimentally, we have previously shown that the oral administration of L-NAME attenuated most of the mucosal injury observed on day 6 after TNB (32). A major caveat of this and other studies (33, 35, 36, 51) that have used this therapeutic strategy is that L-NAME and other nitric oxide synthase inhibitors such as L-NMMA and aminoguanadine do not selectively inhibit NOS II activity. A selective approach to regulating NOS II activity is found in the ability of IL-4, IL-10, and TGF-β to transcriptionally regulate NOS II expression in many cells (57, 58). In the intestinal tract, Kolios et al. (59) demonstrated that IL-4, but not IL-10, concentration dependently inhibited the cytokine-induced expression of NOS II and nitric oxide synthesis by human colonic epithelial cells. In the present study, although we cannot comment specifically on the NOS II-expressing cells that were directly affected by the upregulation of IL-4, two injections of

\[\text{Nitric Oxide Synthesis Activity (mmol/min/g of protein)}\]

Figure 8. Calcium-independent NOS II nitric oxide synthesis in colons taken from control and colitic rats injected on day 1 and day 3 of the study with Ad5LacZ (open bar) or Ad5IL-4 (solid bar). Data bars represent mean ± SEM of three rats per dosing group. \(* P < 0.05\) compared with double-injected Ad5LacZ rats with TNB colitis.
Ad5IL-4 markedly reduced ROS II gene expression and ROS II synthetic capacity. Thus, direct inhibitory effects of IL-4 on the expression of ROS II in the inflamed intestine may provide additional beneficial effects.

In conclusion, these findings represent the first demonstration of therapeutic Ad5 gene transfer in experimental IBD, and emphasize the need for further studies on the therapeutic potential of cytokine gene transfer in clinical IBD. The transfer of IL-4 to IBD patients using gene therapy may compensate for the recently described mucosal deficit in IL-4 synthesis in these patients (27). However, the effectiveness of human Ad5 expressing human IL-4 in IBD may be hampered by recent observations that certain elements of the immune system in IBD patients have diminished responsiveness to the con-trainflammatory actions of IL-4 (20, 60). Because IL-13 has many similar immunoregulatory characteristics of IL-4 but rec-

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