Eosinophilia is induced in the colon of Th2-sensitized mice upon exposure to locally expressed antigen

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Eosinophilia is induced in the colon of Th2-sensitized mice upon exposure to locally expressed antigen. Am J Physiol Gastrointest Liver Physiol 293: G383–G390, 2007. First published April 12, 2007; doi:10.1152/ajpgi.00341.2006.—Eosinophil accumulation is a hallmark of allergic disease orchestrated by Th2-type cytokines (16). In addition to participating in pulmonary allergic diseases (e.g., asthma), eosinophils as resident and recruitable cells in the gastrointestinal (GI) mucosa play a critical role in responses to parasitic infection as well as in the regulation of gastrointestinal allergy (9, 45). Eosinophil accumulation is a profound cellular inflammation, often seen as a component of eosinophil-associated GI disorders (EGIDs). EGIDs involve most parts of the GI tract, orchestrated by Th2-type cytokines (16). Eosinophil accumulation is a hallmark of allergic disease (Crohn’s disease and ulcerative colitis) (5, 43, 46).

Although the underlying mechanisms involved in the development of GI eosinophilia still remains elusive, studies have shown that eosinophils are mainly recruited from the bone marrow through the blood circulation, with recruitment pivotally regulated by IL-5, a factor that is crucial in eosinophil generation, differentiation, and activation (6, 30, 46). Infiltration of these cells into local tissues is, however, regulated primarily by the chemokine eotaxin (12, 14, 35, 37). Eosinophil presence is believed to contribute directly to GI disease pathogenesis, leading to tissue destruction and clinical symptoms such as diarrhea, vomiting, and mucosal bleeding (40, 49). Some studies have demonstrated that eosinophil degranulation correlates with the severity of gastroenteritis (6, 52) and eosinophil infiltration into the mucosal lamina propria during active colitis is thought to contribute to the pathogenesis of the disease (10, 53).

It has been previously shown that when a host acquires a Th2-type immune response to a specific antigen, airway inflammation with marked Th2-like eosinophilia can be induced after aerosol challenge with the sensitizing antigen (41). In a limited number of murine models, it has been demonstrated that significant eosinophilic inflammation can occur in the upper GI tract (esophagus and stomach) and small intestines (23, 33, 34). Here we develop a mouse model of colonic eosinophilia to address whether similar eosinophilic inflammation can be induced in the large intestine associated with the Th2 response. The model reveals that mice sensitized with intraperitoneal (IP) injection of ovalbumin antigen (OVA) adsorbed to aluminum hydroxide mount Th2 type immune responses against the antigen both systemically and locally in the colon. After OVA challenge through adenovirus vector transient gene transfer (3–5 days of expression) of OVA to the rectal mucosal epithelium, sensitized but not naive mice developed notable inflammation in the colon, which peaked at day 5, and large lymphocytic follicles within the subepithelial regions of the colon significantly increased in number and size. Moreover, substantial eosinophilic inflammation was identified in the colon lamina propria. Eosinophilia was transient in this model and did not cause significant epithelial destruction. There was a decrease in the tissue inflammatory response over time and a return to normal structure and function of the colon by 15 days after antigen challenge. This study provides a new

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Innovative Methodology

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MATERIALS AND METHODS

Animals and cell lines. Female BALB/c (H-2d) mice aged 6–8 wk were purchased from Charles River Laboratories (Troy, NY) and housed in pathogen-free conditions at Central Animal Facility at McMaster University. All animal experiments were approved by the Animal Ethics Research Board of McMaster University and conducted according to regulations of the Canadian Council on Animal Care.

Adenovirus vectors. AdLuc is a replication-deficient adenovirus vector that expresses firefly luciferase (1). AdOVA is an adenovirus vector that expresses chicken OVA (Zhu Q, Thomson CW, Rosenthal K, McDermott MR, Collins SM, and Gauldie J, unpublished observations). Adenovirus vector were propagated in 293 cells and purified by cesium chloride gradient centrifugation (22). All virus stocks were aliquoted and stored at −70°C before use.

Th2 precondition and antigen challenge. As described in previous studies (41), mice were sensitized to OVA by 2 IP injections of 8 or 100 μg OVA (Grade V, Sigma-Aldrich, Oakville, ON, Canada) absorbed to 4 μg aluminium hydroxide [Al(OH)3; Sigma-Aldrich] in PBS given 5 days apart. Seven days after the second sensitization, mice were challenged with AdOVA either intranasally (IN) or intrarectally (IR). Tissues were collected at days 5, 10, 15, and 20 post-challenge for histological analysis.

ELISA. ELISA was performed as previously described (4, 13). Briefly, sera, lung, or colorectal homogenates were serially diluted and incubated in OVA protein (Grade V, Sigma-Aldrich, St. Louis, MO) precoated plates for 2 h. The plates were then incubated with alkaline phosphatase-conjugated anti-mouse IgG1 or IgG2a detection antibodies (Southern Biotechnology Associates, Birmingham, AL) for 2 h and color was developed by incubating p-nitrophenyl phosphate for 30 min in darkness. The optical density was read at 405 nm on TECAN (Research Triangle Park, NC). Antibody titers were derived from the inverse dilution at which the sample yielded an optical density twice that of the background of control specimens from nonimmunized mice.

Luciferase assay. Luciferase activity was measured by using a luciferase assay system kit (Promega, Madison, WI) according to manufacturer’s instructions. Briefly, colons were homogenized with a homogenizer (POLYTRON, Kinematica, Cincinnati, OH) and placed in cell culture lysis reagent. After centrifugation, 20 μl of supernatant were plated on a LumiNunc MicroWell plate (Nalge Nunc, Rochester, NY) and assayed on a Tropix TR-717 microplate luminometer (Applied Biosystems, Bedford, MA) following the addition of beetler luciferin (beetle luciferin) in PBS given 5 days after instillation (Fig. 1A). A to verify that antigen expression from the colonic epithelial cells through gene transfer by adenovirus vectors can induce inflammatory responses in the lungs similar to those induced by aerosolization of OVA protein as described previously (41), 5 × 10⁸ PFU of adenovirus vector expressing OVA (AdOVA) were given by IN route once, 7 days after sensitization. We found that IN AdOVA challenge produced profound pulmonary inflammation 5 days after instillation (Fig. 1B). Compared with histological lung sections taken from sensitized mice challenged with AdBH1 (a control adenovirus vector without heterologous gene), which were identical to those from naive mice (Fig. 1B, left), AdOVA-transfected lungs of sensitized mice showed a marked immune response, characterized by lymphocytic infiltration surrounding the pulmonary blood vessels and bronchi (Fig. 1B, right). Eosinophils were rarely seen in control lungs (Fig. 1B, left) but were readily visualized by Congo red stain in AdOVA challenged lungs (Fig. 1B, right). Lymphocytosis and increased eosinophil counts were also observed in the BAL fluid taken from these AdOVA-challenged mice (data not shown). Thus IN antigen challenge of Th2-sensitized mice using adenovirus vectors specifically induced antigen-specific inflammatory responses in a similar manner to repeated antigen aerosolization to the upper airway as previously described (41).

Colonic Th2 precondition and adenovirus-mediated gene transfer to the colon. We next investigated whether mice sensitized with IP injections of OVA and Al(OH)₃ had a Th2-type response in the colon and, if so, whether Th2 sensitization would cause differences in quantity of gene transfer to the colon mucosa compared with naive controls. Similar to observations in the lung, levels of OVA-specific IgG1 antibodies in colonic tissue increased after sensitization, whereas...
IgG2a antibodies were undetectable (Fig. 1). This suggests that, similar to the lung, the colonic mucosal immune system can also be preconditioned to develop Th2-type responses specific for the antigen after sensitization by IP route.

To investigate gene transfer to the colon, 10^9 PFU of adenovirus vector expressing luciferase (AdLuc) were given by the IR route, 7 days after Th2 sensitization. At days 2, 4, 6, and 8 after vector challenge, entire colons were removed and cut into distal, middle, and proximal sections of equal length for luciferase measurement. Significant levels of luciferase expression were detected and were shown to persist for ~6–7 days (Fig. 2). However, no differences in luciferase gene expression were found between sensitized and naive mice (Fig. 2), suggesting that Th2 sensitization does not lead to alterations in gene transfer ability or modulation of gene expression systems at the colonic mucosa.

**Induction of colonic inflammatory response after antigen challenge at the colonic mucosa.** Eosinophilia of the upper GI tract and small intestine can be experimentally induced in mice through oral feeding of allergen (23, 34). We here examined whether colonic eosinophilia can be induced in these Th2-sensitized mice upon exposure to the OVA antigen at the mucosa of the large intestine. Seven days after sensitization as described above, mice were challenged IR with 10^9 PFU of AdOVA or AdBHG (a control Ad without heterologous genes). Five days later, lungs were paraffin-embedded, sectioned, and stained and were examined with a light microscope. B: sensitized mice were challenged IN with AdBHG (left) or AdOVA (right). Lung sections were stained with hematoxylin and eosin (H&E; magnification of ×50 in diameter). C: sensitized mice were challenged IN with AdBHG (left) or AdOVA (right) and lungs were stained with Congo red/hematoxylin for eosinophils (a magnification of ×400 in diameter). AdOVA-infected lungs showed extensive peribronchial and perivascular inflammations (A, right) and a typical appearance of eosinophilia (B, right; eosinophils in brownish color). Results represent 1 of 5 independent experiments.
colonic lymphoid nodules using the Leica Q500IW image processing and analysis system and Leica Qwin Pro version 2.3 software. All lymphoid nodules were counted under light microscopy and the total cross section of full-length area was measured on one slide from each mouse under the same instrument settings. Numbers of lymphoid nodules and total areas were compared between naive/no challenge, sensitized/AdBHGH challenge, and sensitized/AdOVA challenge groups. It was found that AdOVA challenge resulted in a two- to threefold increase of both parameters measured, whereas challenge with the AdBHGH control vector did not have significant impact on histological changes compared with the naive control (Fig. 4).

Eosinophilic infiltration was examined by using Congo red stain on colon sections obtained from challenged mice. Challenge of sensitized mice with the control vector AdBHGH by the IR route did not induce a significant appearance of eosinophils in the colon (Fig. 5A). However, upon IR challenge with AdOVA, a large number of eosinophils was present in the colonic lamina propria at day 5 postinfection (Fig. 5B). This suggests that colonic eosinophilia can be induced in association with local antigen exposure as seen in the lung in Th2-sensitized animals.

The colonic inflammatory response was also examined in sensitized mice at days 10, 15, and 20 postchallenge with AdOVA. Analysis of these colonic tissues with respect to lymphoid follicles and eosinophils revealed that at these later time points inflammation was markedly decreased. There were no overt histological changes at day 15 after challenge compared with controls, suggesting that inflammation began to resolve by day 10–15.

Eosinophils are involved in the inflammatory response by discharging their granule-specific proteins at site of inflammation (45). We thus examined colon sections by transmission electron microscopy and asked whether infiltrated eosinophils were activated in the colonic mucosa after antigen challenge. Compared with naive (Fig. 6A) and sensitized animals with irrelevant antigen challenge (Fig. 6B), colonic eosinophils of OVA-challenged mice displayed a moderate degree of piece-meal degranulation, which is indicated by increased numbers of granules with reduced electron translucent matrix density or halos (Fig. 6C). Although these intracellular granules were significantly increased in number than those of controls (Fig. 6D), we did not observe extensive degranulation such as loss of electron dense core or morphologically altered granules.

DISCUSSION

Previous studies imply that food allergy contributes to the development of GI eosinophilia (36, 48). Eosinophil infiltration can be triggered by hypersensitivity to cow’s milk, and the tissue eosinophilia decreases on withdrawal of milk from the diet (21, 28, 32, 48) and Th2, but not Th1, cytokine profiles were found in patients with milk hypersensitivity (3). Rothenburg and colleagues (23, 33, 34) developed a murine model of eosinophil-associated allergy, in which mice sensitized with OVA and aluminum hydroxide are fed with the same antigen by oral route. They identified eosinophils in the upper GI mucosa and related the findings to the presence of Th2-type cytokines and specific chemokines such as eotaxin (23, 24). Naturally, the presence of luminal bacteria probably drives immune responses toward either a Th1 or Th2 cytokine bias, and such cytokine imbalance might promote the disease (19, 20). The recognition of microbial components by intestinal epithelial cells through Toll-like receptors may play an important role in establishing the polarized cytokine profile (26), and CD4+ T cells may be essential in producing these cytokines (2, 25, 58). We here demonstrate that Th2-sensitized mice, but not naive mice, could develop colonic eosinophilic inflammation after antigen challenge by the rectal route. Therefore, preexisting biased Th2 immune response and expression of associated antigen appear responsible for the development of colonic eosinophilic inflammation and may also apply to a number of human GI conditions.

Studies (11, 55; Zhu Q, Thomson CW, Rosenthal K, McDermott MR, Collins SM, and Gauldie J, unpublished observation) have shown that adenovirus vector transfer of antigen genes to the colon results in efficient cell-associated gene expression in the epithelial layer with some expression extending to the lamina propria. In this regard, adenovirus vector proved to be a useful tool for antigen transfer and expression at the lower GI mucosa. In the present study, we challenged mice sensitized to OVA with adenovirus vector encoding the OVA gene by the intrarectal route and showed that antigen transfer and expression induced significant lymphocytic and eosinophilic infiltration, whereas an identical vector that lacks OVA gene (AdBHGH) had no appreciable histological effects. Thus antigen expression in the large intestine mucosa of Th2-sensitized mice induces colonic inflammation. We found that adenovirus vector-transferred gene product was expressed and detectable for up to 1 wk in the colon. Peak antigen expression was detected during the initial 1–3 days after IR administration, after which the levels reduced to baseline by 1 wk. The presence of eosinophilic inflammation is dependent on the presence of antigen as the inflammation subsided coincident with the decreased antigen presence in the colon. Antigen presence triggers colonic inflammation and, very likely, is responsible for the entire course of pathological alterations.
Eosinophils can be activated to release a series of cytotoxic granule proteins causing tissue damage, and accumulation of these eosinophils is thought to be detrimental (15, 54) and to be central to the pathogenesis of colitis (10, 39). We did not see the profound inflammation sustained for more than 1 wk nor the development of colonic ulceration, significant weight loss, or diarrhea, although granules with electron translucent matrix were significantly increased in the infiltrated eosinophils. However, no extensive eosinophil degranulation was found. These data suggest that induced eosinophils in the colon alone do not progress to tissue damage and require at least a “second” signal for activation/degranulation. Moreover, the pathological alterations (eosinophilia) are reversible and do not lead to prolonged tissue destruction or remodeling.

Several factors might be contributory to the results. First, exposure to only one antigen might be not sufficient to induce

![Fig. 3. Inflammatory responses in the colon of sensitized mice 5 days after antigen challenge with adenovirus vector by IR route. Seven days after OVA (8 μg) sensitization, mice were challenged IR with 10^9 PFU of AdOVA or AdBHG. At day 5 after IR challenge, animals were killed and colons were paraffin embedded, sectioned, and stained with H&E and were examined with a light microscope. A: gross examination (top) and colon section (bottom) from sensitized mice challenged with AdBHG. B: gross examination (top) and colon sections (bottom) from sensitized mice challenged with AdOVA. C: part of colon is shown for distribution of lymphoid follicles. In AdBHG-infected mice, there were only 1 or 2 visible lymphoid nodules, relatively small in size, and indistinguishable from those seen in naive animals. In AdOVA-infected mice, lymphocytes infiltrated the colon. The lymphoid follicles were enlarged and more than 2 follicles found. Results represent 1 of 5 independent experiments. Magnification of ×50 in diameter.](image)

![Fig. 4. Increased number and total area of lymphoid nodules after antigen challenge with adenovirus vector by IR in sensitized mice. OVA (8 μg)-sensitized mice were challenged with AdOVA or AdBHG 7 days after sensitization. Five days later, colons were removed and analyzed under a microscope. A: number of visible lymphoid nodules were counted on one histological section from each group. Increasing number of lymphoid nodules was observed after AdOVA IR challenge compared with controls (**P < 0.01, n = 5/group). B: cross-sectional area of all lymphoid nodules (total area) were analyzed morphometrically by using image analysis software. There was an increase in total area of lymphoid nodules after AdOVA IR challenge (**P < 0.01, n = 5/group).](image)
activation or degranulation. An early observation in human disease revealed that sequential dietary eliminations reduced clinical symptom each time (31), implying that multiple allergens are involved to cause disease. Second, the antigen is only present for up to 1 wk after challenge and this might be not long enough to promote disease development (ulceration). Third, the route of challenge might result in different pathological changes. It was shown that challenge of systemic sensitized mice by the intragastric route induced diarrhea, which was accompanied by a dramatic infiltration of eosinophils, mast cells, and CD4⁺ Th2 cells into the large but not the small intestine (29). It remains to be determined whether these colonic eosinophils can generate severe or irreversible pathological changes upon a more chronic antigen exposure. Because the efficacy of gene expression on repeated adenovirus gene transfer is dramatically reduced owing to the immunogenicity of the vector, different serotypes of adenovirus or other types of antigen-delivery vehicles may be used for chronic antigen challenge. Also, it warrants investigation whether these cells participate only in antigen presentation, which can potentially promote local inflammation and destroy mucosal cells as shown in the respiratory system (50).

In addition to colonic eosinophilia, we also identified lymphoid hyperplasia and the development of multiple lymphoid...
nODULES IN THE COLON ON ANTIGEN EXPRESSION. IN THE LARGE INTESTINE, M CELLS OVERLAY AGGREGATED LYMPHOCYTE FOLLICLES (42, 44) AND SMALLER ISOLATED LYMPHOCYTE FOLLICLES (17). THIS AGGREGATED LYMPHOID RESPONSE IS REMISSENT OF PREVIOUSLY FOUND LYMPHOID HYPERPLASIA IN COLITIS IN MICE (7, 8) AND HUMANS (18, 57). IT IS POSSIBLE THAT THESE MUCOSAL LYMPHOID STRUCTURES ARE INVOLVED IN THE DEVELOPMENT OF COLONIC INFLAMMATION. IT HAS BEEN RECENTLY SHOWN IN THE LUNG THAT MICE LACKING SECONDARY LYMPHOID ORGANS CAN GENERATE MUCOSAL LYMPHOID AGGREGATES, CALLED INDUCIBLE BRONCHUS-ASSOCIATED LYMPHOID TISSUE, BENEATH THE EPITHELIUM AT THE BRANCHES OF THE BRONCHI, ALLOWING THE DEVELOPMENT OF PROTECTIVE LOCAL IMMUNITY UNTIL INFECTION (38). ADAPTIVELY TRANSFERRED CD4+ T CELLS CAN UNDERGO CLONAL EXPANSION IN THE LAMINA PROPRIA AND THE EPITHELIAL LAYER OF BOTH SMALL AND LARGE INTESTINES (47). THEREBY, SCATTERED LYMPHOCYTES MIGHT ALSO BE ABLE TO EXPAND IN THE LAMINA PROPRIA AFTER ANTIGEN CHALLENGE, LEADING TO A SIGNIFICANT NUMBER OF LYMPHOCYTE FOLLICLES IN THE MUCOSA.

In conclusion, Th2-sensitized mice challenged with antigen via the rectal route can induce colonic inflammatory responses, featured by significant eosinophil infiltration and lymphoid hyperplasia in the lamina propria, which is similar to human EGID. This murine model might provide a new understanding concerning the influence of a preexisting antigen-specific Th2-type response on subsequent antigen exposure causing eosinophilic inflammation in the large intestine mucosa and also indicates the potential for reversal of inflammation (eosinophilia) without progression to overt tissue damage.

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GRANTS

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