

Chronic aspiration shifts the immune response from adaptive immunity to innate immunity in a murine model of asthma

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Received: 19 January 2012 / Accepted: 10 April 2012
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Abstract

Objective and design The hypothesis that aspiration of gastric fluid drives the anti-ovalbumin response toward a Th2 reaction even in animals not prone to Th2 responses was evaluated.

Subjects Forty-eight male C57BL/6 mice were used.

Methods Mice were sensitized and challenged with ovalbumin starting 5 weeks prior to the initiation of weekly

aspirations of either gastric fluid or normal saline as a control. Weekly aspiration continued during the course of exposure to ovalbumin.

Treatment Aspiration consisted of 50 µl of gastric fluid with 50 µl of 0.9 % normal saline used as a control. Antigen exposure consisted of sensitization to ovalbumin via intraperitoneal injection on days 0 and 14 and challenge on day 21 with aerosolized antigen for 30 min.

Results No evidence of a shift toward a Th2 response as a result of gastric fluid aspiration was seen in the Th1-prone strain utilized, although a profound down-regulation of a broad array of T cell-associated cytokines and chemokines and up-regulation of macrophage-associated markers was observed as a result of aspiration.

Conclusions These data provide support for the hypothesis that the clinical association between asthma and gastroesophageal reflux disease (GERD) does not involve an exacerbation of asthma by GERD-associated aspiration of gastric fluid, but may cause immune reactions unrelated to the asthma pathology.

Responsible Editor: Michael Parnham.

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Keywords Gastroesophageal reflux · Aspiration ·
Asthma · Innate immunity · Adaptive immunity

Introduction

Gastroesophageal reflux disease (GERD) is a commonly diagnosed medical condition in developed countries. GERD affects nearly one-third of the adult population in those countries to some degree, and has become one of the most frequently encountered medical problems [1, 2]. The high incidence of this disease is potentially associated with a variety of culture-associated factors, including the timing, amount, and type of food consumption; the use of alcohol

and potentially some other drugs, stress, and obesity. These factors contribute to the weakening of the lower esophageal sphincter as a barrier to gastric fluid backing up from the stomach into the esophagus. Not only does GERD cause discomfort and in some cases disease in the esophagus, but GERD-associated aspiration may also cause, trigger, or exacerbate many pulmonary diseases such as chronic cough, bronchitis, bronchial asthma, pneumonitis, and interstitial fibrosis [3–5]. A substantial number of clinical and epidemiological studies have described the association between GERD and asthma, with several studies confirming the association between GERD and asthma using 24-h ambulatory pH monitoring [6–9]. GERD has been found in 30–80 % of patients with asthma, depending on the study [9–11]. A recent, systemic review of 28 epidemiological studies showed that the sample size-weighted average prevalence of GERD in patients with asthma was 59.2 %, whereas in controls it was 38.1 % [12]. However, despite the established association between GERD and asthma, a paucity of data on the direction of causality exists [12]. Studies evaluating the effects of proton pump inhibitors, which increase the pH of the gastric fluid, upon asthma have shown little effect, casting doubt on the hypothetical view that GERD might be involved in the exacerbation of asthma [13, 14]. On the other hand, the idea that GERD might induce asthma has not been evaluated, and experimental models aimed at evaluating the connection between chronic aspiration and lung function have only recently been developed [15–17]. Importantly, studies in those animal models indicate that it is the particulate matter in gastric fluid rather than the acid which mediates lung pathology [18], suggesting that the lack of any effect of proton pump inhibitors on asthma cannot be interpreted as an indicator that GERD does not affect asthma.

Previous studies using murine models of asthma in conjunction with chronic aspiration of gastric fluid have indicated that gastric fluid aspiration can alter the airway hypersensitivity response in mice [15–17]. Nemzek and Kim [17], for example, found that a single aspiration event with acid increased airway hyperresponsiveness for at least 8 h post-aspiration in mice with cockroach allergen-induced asthma. In another study tailored more to examine the effects of chronic rather than acute aspiration, our laboratory determined the effect of eight aspirations of gastric fluid administered once per week to mice with concomitant sensitization and challenge with ovalbumin [16]. For that study, sensitization was induced with 10 consecutive days of exposure of Balb/c mice to aerosolized ovalbumin. In that model, aspiration of gastric fluid was associated with a trend toward decreased production of anti-ovalbumin IgG and IgE [16]. Further, chronic aspiration of gastric fluid induced a significant increase in the ratio of anti-ovalbumin IgG1/IgG2a compared with controls not receiving gastric fluid

aspiration. Although the shift in IgG1/IgG2a ratios was consistent with a shift toward a predominantly Th2 response in the Balb/c mice, no increase in absolute levels of Ig production or increase in IgE, typical of Th2 responses, was observed [16]. In a follow-up study, we sensitized Balb/c mice to ovalbumin using intraperitoneal injection of antigen rather than exposure to aerosolized antigen, and evaluated the effects of chronic gastric fluid aspiration on those mice. We found that weekly aspiration of gastric fluid for 8 weeks was associated with increased production in the bronchoalveolar lavage (BAL) fluid of the macrophage/monocyte-associated cytokines TNF-alpha and IL-12. Strikingly, aspiration of gastric fluid led to a profound down-regulation of a broad array of T cell-associated cytokines, including interleukins 2, 4, 5, 6, 10, 13, and 23, and interferon gamma. [15]. These results suggest an interesting postulate that may impact human disease/asthma, whereby chronic aspiration of gastric fluid has the potential to drive the immune response in the airways from adaptive in nature to a more innate posture.

Various parameters associated with asthmatic responses, including airway remodeling, the cellular immune response and the humeral immune response, are dependent upon the strain of mouse utilized [19–21]. Several studies have illustrated that many factors, including genetic variability, interacting genetic loci, the activation of CD4⁺ T cells, and the rate of apoptosis of lung-derived eosinophils, influence the response to allergens in the airways [19, 22–24]. In particular, the propensity for Th1 versus Th2 responses is expected to strongly influence the nature of the asthmatic response, which is primarily Th2 in nature. With this in mind, Gueders et al. [25] compared the effects of ovalbumin sensitization and challenge in C57BL/6 and Balb/c mice. Both strains of mice have intact Th1 and Th2 responses, although C57BL/6 mice tend to favor Th1 responses, whereas Balb/c mice tend to favor Th2 responses. Gueders et al. observed that a wide range of T cell-associated cytokines were produced in the BAL fluid of Th1-prone (C57BL/6) mice to a substantially lesser degree than in the Th2-prone (Balb/c) mice. On the other hand, both eosinophils and neutrophils were observed in the BAL fluid of the Th1-prone mice to a much greater extent than in the BAL fluid of the Th2-prone mice. These data point toward fundamental differences in the airway hypersensitivity responses in Th1 versus Th2 prone mice, and suggest that studies involving the effects of GERD on Th1-prone mouse models would be of substantial interest. Thus, Th1-prone mice may be utilized to ask (1) whether chronic aspiration can drive a Th2 response even in a Th1-prone environment, and (2) whether chronic aspiration can drive the immune response from a more adaptive posture to a cytokine and cellular milieu characteristic of a heightened innate immune response in a Th1-prone environment.

In this study, we utilized Th1-prone C57BL/6J mice in a model of airway hypersensitivity, in conjunction with chronic gastric fluid aspiration to examine the hypotheses that chronic aspiration drives the immune response from adaptive to innate, and drives the remaining adaptive response toward a Th2 response even in a pro-Th1 environment. The effects of gastric fluid aspiration on the pathology associated with airway hypersensitivity were evaluated, and the nature of the immune response was characterized. The data strongly suggest that gastric fluid affects Th1-prone and Th2 prone environments differently, although some effects of gastric fluid aspiration on airway hyperresponsiveness are independent of the T-cell propensity for Th1 versus Th2 interactions.

Materials and methods

Laboratory mice

All procedures involving animals were approved by the Duke University Institutional Animal Care and Use Committee. Eight-week old male C57BL/6 mice ($n = 48$) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The experimental protocol was started at the 8th to 10th weeks of age.

Experimental design

In order to provide control groups which would allow for investigation of the effects of gastric fluid aspirations alone, asthma induction alone, and the combination of gastric fluid aspiration and asthma induction, mice received a combination of either normal saline or gastric fluid aspirations with either normal saline or antigen sensitization and challenge to make a total of four groups. The four groups, with $n = 12$ per group, were designated as follows: mice that received normal saline aspirations and alum/saline sensitizations and challenge (control group, NS/sham), mice that received gastric fluid aspirations and alum/saline sensitizations and challenge (GF/sham), mice that received normal saline aspirations and alum/ovalbumin sensitizations and challenge (NS/Asthma), and mice that received gastric fluid aspirations and alum/ovalbumin sensitizations and challenge (GF/Asthma).

Aspirations were conducted weekly for 8 weeks (days 1, 8, 15, 22, 29, 36, 43, and 50). In order to assess the influence of aspirations on the development of asthma, the asthma model utilized was initiated after 5 weeks of aspirations (i.e., the first sensitization to ovalbumin occurred after 5 weeks of gastric fluid aspiration). Sensitizations occurred on days 33 and 47, challenge occurred on day 54, and animals were sacrificed on day 56.

Collection and preparation of gastric fluid

Gastric fluid was obtained from male C57BL/6 mice using the technique we have previously described in a mouse model [15]. Briefly, a small midline incision was made over the upper abdomen and the peritoneal cavity entered. The proximal duodenum was ligated with a 2-0 silk suture. The abdominal cavity was then closed with suture, and the mice were returned to their cages for 4–10 h. Mice were then sacrificed, and the gastric fluid was collected in a 15-ml sterile conical tube. Gastric fluid was filtered through a 70- μ m strainer (BD Biosciences) to remove relatively large particulate matter, and then stored at -80°C until needed. Once all the gastric fluid was obtained, all samples were thawed and the pH checked before pooling. After pooling, pH was again checked (pH 3.6) and gastric fluid was aliquotted, frozen in liquid nitrogen, and stored at -80°C until needed for aspiration.

Aspiration events

Mice were sedated by exposure to aerosolized 4 % isoflurane for approximately 5 min. Animals were then placed supine on a board slanted at 45° with their two front teeth anchored on a loop of suture attached to the board. The tongue was then extended using forceps and 50 μ l of gastric fluid (experimental groups GF/sham and GF/Asthma) or 0.9 % normal saline (control groups NS/sham and NS/Asthma) were then instilled into the oropharynx. The nose was pinched following instillation of the fluid until the next breath, when it could be heard that the fluid was inhaled into the lungs. Once animals woke, they were returned to their cages.

Asthma model

We used a slightly altered version of an established laboratory model of airway hypersensitivity which involves the induction of an immune response against aerosolized ovalbumin [20]. The model consists of sensitization to allergen via intraperitoneal (IP) injection on days 0 and 14 and challenge on day 21 with aerosolized antigen for 1 h. The procedure we utilized entailed a challenge with aerosolized ovalbumin for 30 min rather than 1 h as previously described, in an effort to potentially increase the sensitivity of the procedure to any potential effect of chronic aspiration. Because the sensitization to ovalbumin was initiated after 5 weeks of aspirations, day 0 of the ovalbumin sensitization procedure corresponded to day 33 of our experimental protocol.

Mice in the experimental groups (groups NS/Asthma and GF/Asthma) were sensitized on days 33 and 47 by IP injection of 10.0 μ g ovalbumin (albumin from chicken egg

white, grade V; Sigma, St. Louis, MO, USA) emulsified in 2.0 mg of aluminum hydroxide and 2.0 mg of magnesium hydroxide (Imject Alum; Pierce Biotechnology, Rockford, IL, USA) in a total volume of 100 μ l. Control mice (groups NS/sham and GF/sham) received IP injection of 2.0 mg of aluminum hydroxide and 2.0 mg of magnesium hydroxide in a total volume of 100 μ l 0.9 % normal saline (Duke University Hospital, Durham, NC, USA) on days 33 and 47. On day 54, mice were placed in an airtight inhalation chamber and challenged with either an aerosolized solution of 1 % ovalbumin in 0.9 % normal saline (experimental groups NS/Asthma and GF/Asthma) or 0.9 % normal saline (control groups NS/sham and GF/sham) for 30 min. Following exposure, the chambers were vented and mice returned to their cages.

Sacrifice procedure

Mice were sacrificed by a procedure described previously [15]. Briefly, mice were anesthetized by exposure to aerosolized 5 % isoflurane. Whole blood was withdrawn from the inferior vena cava, and the serum was separated and frozen at -80°C .

Heart and lungs were then removed en bloc and BAL [16] was then performed using 1.0 ml of a solution of 0.9 % normal saline with protease inhibitor (2 protease inhibitor cocktail tablets dissolved in 15 ml 0.9 % normal saline; Roche Diagnostics, Indianapolis, IN, USA). BAL samples were centrifuged for 5 min at 2,040g and 4°C , flash frozen in liquid nitrogen and stored at -80°C until needed. Lung tissue was placed in formalin for histologic slide preparation.

Histology

Tissue specimens were fixed in 10 % neutral-buffered formalin for 24 h and in 70 % ethanol for at least 24 h, then processed into paraffin blocks for microscopic examination. Tissues were sectioned at 5- μ m thickness and mounted onto positively charged microscopy slides (Erie Scientific, Portsmouth, NH, USA). Sections for histological analysis were then stained with haematoxylin and eosin (H&E) to detect inflammatory infiltrates and with periodic acid Schiff staining (PAS) for mucus production. Images were obtained using the Leica light microscope with a Sony Cyber-shot digital camera. The ratio of the area of the airway associated epithelium (EA) over the length of the airway basement membrane (BM) [13] was evaluated as a measure of bronchiolar epithelial damage [26, 27]. The EA and the BM were measured using an image recorder connected to a computerized image system (Quantimet 500). The number of goblet cells containing mucus was quantified as the percentage of PAS-positive cells/total cells

counted under the microscope. Perivascular inflammation was assessed by a semi-quantitative scoring system.

Enzyme-linked immunosorbent assay (ELISA)

The relative concentrations of mouse antiovalbumin IgE, IgG1, IgG2a, IgG2b, and IgG3 in serum were evaluated by ELISA as follows: 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were incubated with 50 μ l/well of PBS containing 7.5 μ g/ml ovalbumin. Plates were incubated overnight at 4°C and then washed six times with 200 μ l/well of PBS. Plates were then blocked with 200 μ l/well of PBS containing 0.1 % bovine serum albumin (BSA). Next, appropriately diluted mouse serum (50 μ l/well) was added and incubated for 3 h at room temperature ($22\text{--}24^{\circ}\text{C}$). Each concentration was run in duplicate and sera were sufficiently diluted such that the readout from the ELISA was linearly proportional to the serum concentration. After incubation for 3 h, wells were washed six times with 200 μ l/well of PBS, and an alkaline phosphatase-conjugated goat antibody specific for the appropriate immunoglobulin (for IgG1, IgG2a or IgE determination) was added to each well and incubated for 1 h at room temperature. For evaluation of antiovalbumin IgG2b and IgG3 subclass levels, subclass specific, unconjugated goat antibodies (Sigma-Aldrich, St. Louis, MO, USA) were used, followed by washing and incubation with an alkaline phosphatase conjugated rabbit antibody specific for goat IgG (Sigma-Aldrich). Wells were then washed six times with PBS and the plates were developed using *p*-Nitrophenyl phosphate as the substrate in a diethanolamine buffer. The absorbance at 405 nm was measured and the relative antibody concentration was taken to be the change in absorbance as a function of serum concentration. Results from different groups were compared using a two-tailed *t* test.

Cytokine analysis

Cytokines were assayed using the Procarta Cytokine Assay Kit (Panomics, Fremont, CA, USA) according to the manufacturer's instructions. The assay was run using a Luminex 100 instrument (Bio-Rad, Hercules, CA, USA). The cytokines examined were as follows: Interleukin (IL)-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IL-23, interferon (IFN)— α , IFN- β , IFN- γ , eotaxin, macrophage inflammatory protein (MIP)-1 α , RANTES, tumor necrosis factor (TNF)- α , transcription growth factor (TGF)- β , and granulocyte macrophage-colony stimulating factor (GM-CSF). The standard curve for sample cytokine concentration determination was used, with a detection limit of 1 pg/ml/cytokine or less. An amount of 50 μ l of BAL fluid was

used per assay. Assays were run in duplicate (two wells per sample), and serum from at least eight animals in each group was evaluated. (Serum of all animals could not be evaluated because of a paucity of serum.) Although absolute values of cytokines are reported, these values have the caveat that the actual “epithelial lining fluid”, the fluid present in the lung prior to lavage, is diluted in the BAL fluid by at least 90 % and probably by 99 % or more [28]. Thus, values of cytokines in the actual lung are expected to be higher than those found in the assay. Results from different groups were compared using a 2-tailed *t* test.

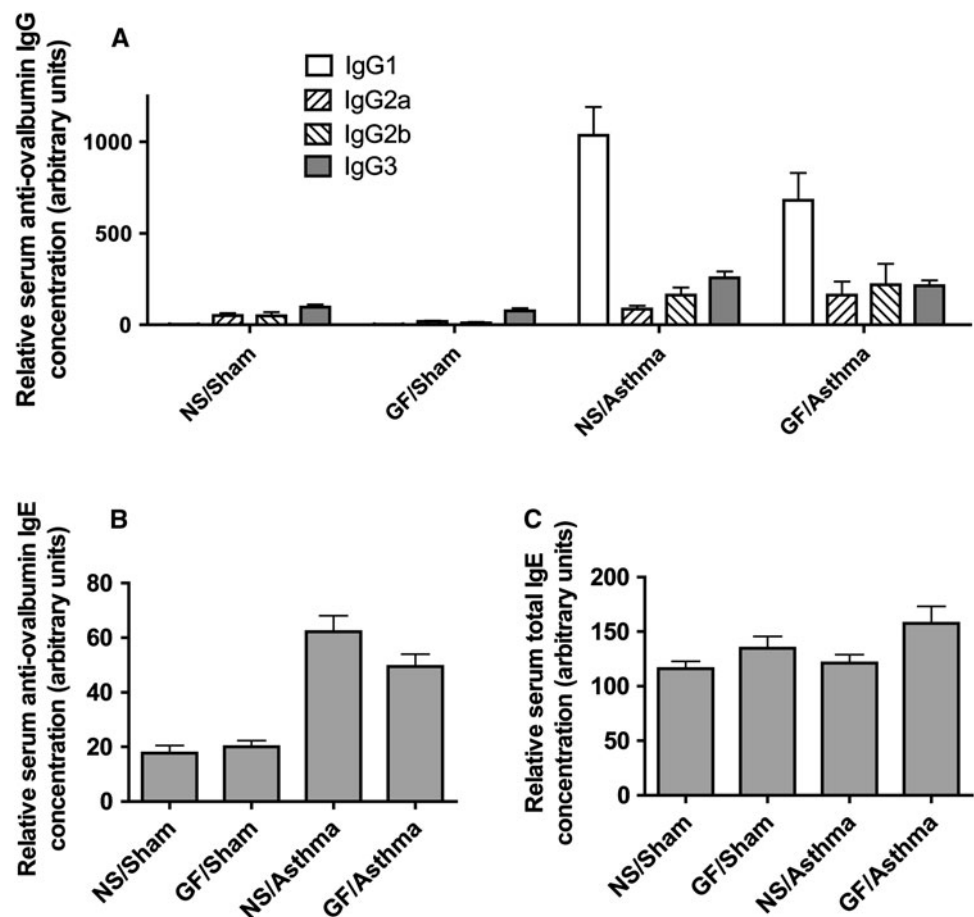
Results

Effects of chronic aspiration on the humoral response of C57BL/6 mice following ovalbumin sensitization and challenge

As expected, exposure to ovalbumin elicited potent anti-ovalbumin IgG (Fig. 1a) and IgE (Fig. 1b) responses in all C57BL/6 mice, regardless of the aspiration of gastric fluid. The amount of anti-ovalbumin IgE and anti-ovalbumin

IgG1 production in animals receiving chronic gastric fluid aspiration was not greater than that in controls not receiving gastric fluid aspiration. Interestingly, there was a slight (about 20 %) but not statistically significant decrease in the average amount of anti-ovalbumin IgE and anti-ovalbumin IgG1 production in animals receiving chronic gastric fluid aspiration compared to animals not receiving gastric fluid aspiration. Thus, chronic gastric fluid aspiration might, to a degree, attenuate the humoral response induced by exposure to ovalbumin, although additional studies with more animals will be required to test this possibility. This finding is supported by our two previous reports using Balb/c mice, one using aerosolized antigen for sensitization, and the other using IP injection for sensitization [15, 16]. The reports, by Barbas et al. and by Thomas et al., found decreases of about 40 and 30 %, respectively, of anti-ovalbumin IgE production in animals receiving chronic gastric fluid aspiration compared to animals not receiving gastric fluid aspiration. However, in these reports, like the present report, statistical significance was not reached, suggesting that the variation in the population is large relative to the change induced by aspiration.

Fig. 1 Relative concentrations of various subclasses of anti-ovalbumin IgG (a) and of IgE (b) in the serum of animals from each experimental group. c Relative concentrations of total IgE in the serum of animals from each experimental group. Concentrations were determined by ELISA as described in the materials and methods. The increase in levels of all anti-ovalbumin immunoglobulins in response to sensitization (a, b) was statistically significant ($p < 0.05$), whereas aspiration did not result in a statistically significant difference, regardless of sensitization. The standard errors are shown



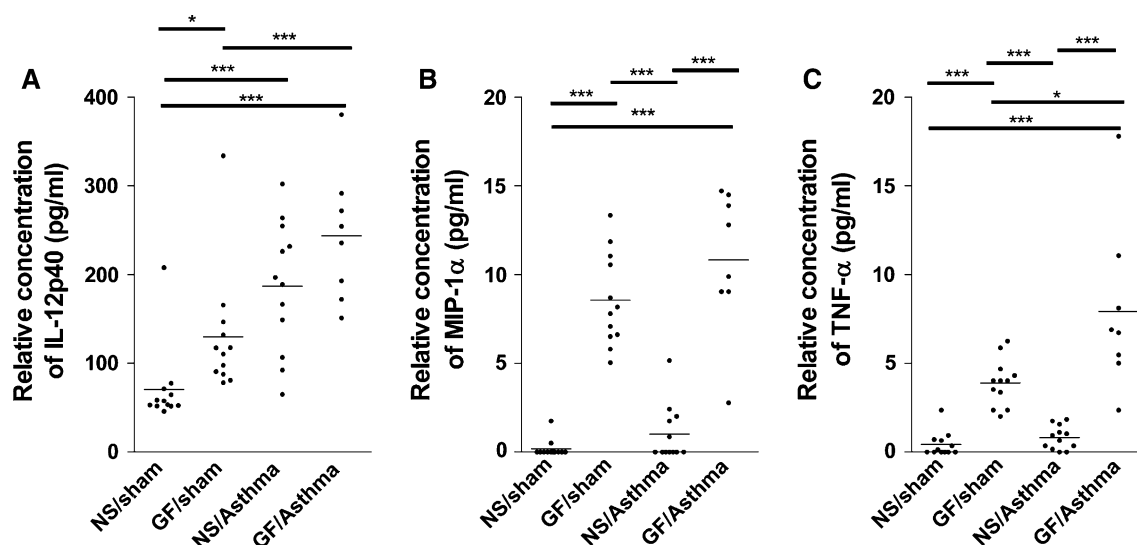


Fig. 2 GF-associated increase in the concentration of TNF- α , IL-12p40 and MIP-1 α in the BAL fluid. A significant ($p < 0.5$) increase in the mean production of cytokines was observed in the face of

chronic aspiration in mice both with and without the induction of airway hypersensitivity, with the exception of IL-12p40 in mice with induced airway hypersensitivity. * $p < 0.05$, *** $p < 0.005$

Effect of chronic aspiration on the secretion of cytokines in the BAL fluid

As shown in Fig. 2, chronic aspiration caused a profound increase in levels of TNF- α , IL-12p40, and MIP-1 α in the BAL fluid of mice both with and without sensitization and challenge with ovalbumin. The aspiration-induced increase of these cytokines, which are all associated with macrophage activity, is a likely result of the giant cell formation that is prominent feature of the pathology associated with chronic aspiration in this model (see below for assessment of histology).

As expected, following sensitization and challenge with ovalbumin, the level of several Th2-associated cytokines increased significantly in the BAL fluid, including IL-4, IL-5, IL-12p40, IL-13, and GM-CSF. (Fig. 3; $p < 0.5$, NS/asthma vs. NS/sham group) In addition, sensitization and challenge with ovalbumin also induced a significant increase in the levels of IL-2 and IL-6 in the BAL fluid (Fig. 3b, e). These cytokines are key mediators of airway inflammation and allergic reactions through their ability to activate and probably regulate T cells [29–31], with both cytokines being increased in the serum of patients during acute asthma attacks.

Chronic aspiration caused a significant decrease in the concentration of three cytokines in the BAL fluid of animals challenged with ovalbumin (Fig. 3). Down-regulated cytokines were the pro-inflammatory mediators IL-1 α and IL-2 and the allergic mediator IL-13. The down-regulation of the potent T cell-stimulating cytokine IL-2 was particularly noticeable, with an approximately fivefold reduction in mean cytokine production. There was also a non-

significant trend of reduction in production of another allergic mediator, IL-5. This result is corroborated by a previous study using Balb/c mice which showed a significant decrease in IL-5 production in the face of aspiration [15]. Similar trends were observed for the presence of IL-4, IL-6, and GM-CSF in the BAL fluid, although the degree of abrogation by gastric fluid aspiration was not statistically significant. Thus, with the exception of IL-12p40 (Fig. 2a), chronic aspiration may suppress the production of all cytokines that were found to increase in the face of sensitization and challenge with ovalbumin. These data might suggest that, in this model, the chronic aspiration of gastric fluid may suppress a considerable portion of the allergen-specific response to ovalbumin. This idea is consistent with the apparent aspiration-induced decrease in ovalbumin-specific Ig production that has been observed in several of our studies, as described above. However, additional studies in C57BL/6J mice using more animals will be necessary to confirm this observation in this strain.

Histological analysis

Assessment of sections stained with H&E (Fig. 4) revealed that chronic aspiration was associated with giant cell formation in the lungs, along with an inflammatory cellular infiltrate that included neutrophils. Similar results were observed regardless of sensitization and challenge with ovalbumin (Figs. 4, 5b). In contrast, mice without gastric fluid aspiration showed no sign of giant cell formation, regardless of sensitization and challenge with ovalbumin (Fig. 5b). Examination of mucin production by goblet cells, as assessed by a PAS stain, revealed that ovalbumin-

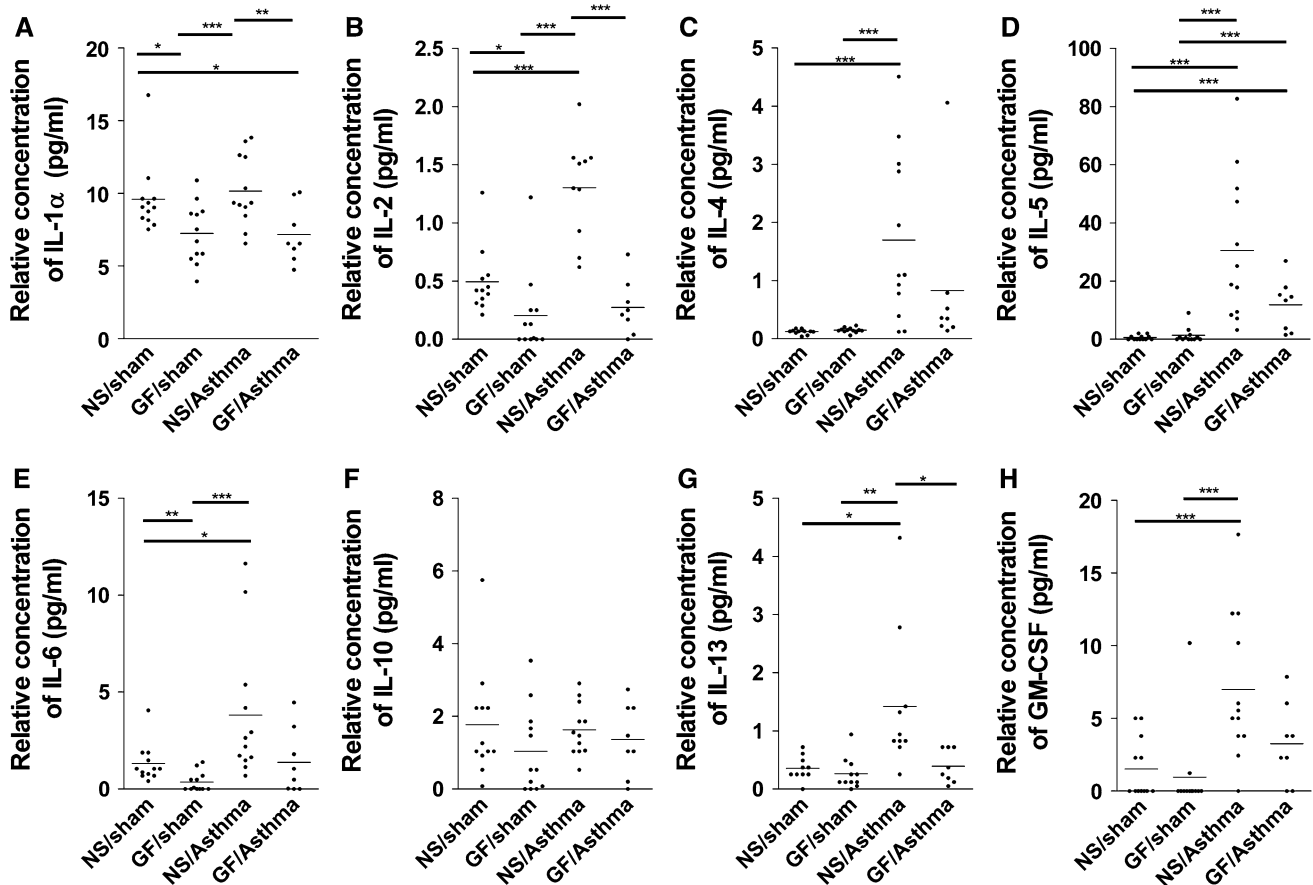


Fig. 3 Decreased concentrations of various T cell-associated cytokines in the BAL fluid as a result of chronic aspiration. A significant ($p < 0.5$) decrease in the mean production of cytokines was observed in the face of chronic aspiration in mice without airway hypersensitivity (a, b, e), and in mice with airway hypersensitivity (a, b, g). A

significant ($p < 0.5$) increase in the mean production of cytokines was also observed in the face of airway hypersensitivity induction (b, c, d, e, g, h) comparing to control mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

challenged mice had increased numbers of PAS-positive cells in their airways compared to numbers in the non-challenged mice (Figs. 4, 5). The aspiration of gastric fluid in these mice following sensitization and challenge with ovalbumin did not increase mucus cell hyperplasia. However, in the absence of ovalbumin sensitization, chronic aspiration of gastric fluid was associated with increased number of PAS-positive cells (Fig. 5a). On the other hand, peri-bronchial inflammatory cell infiltration was not increased by chronic aspiration of gastric fluid (Fig. 5c), and epithelial cell integrity was not affected by chronic gastric fluid aspiration (data not shown).

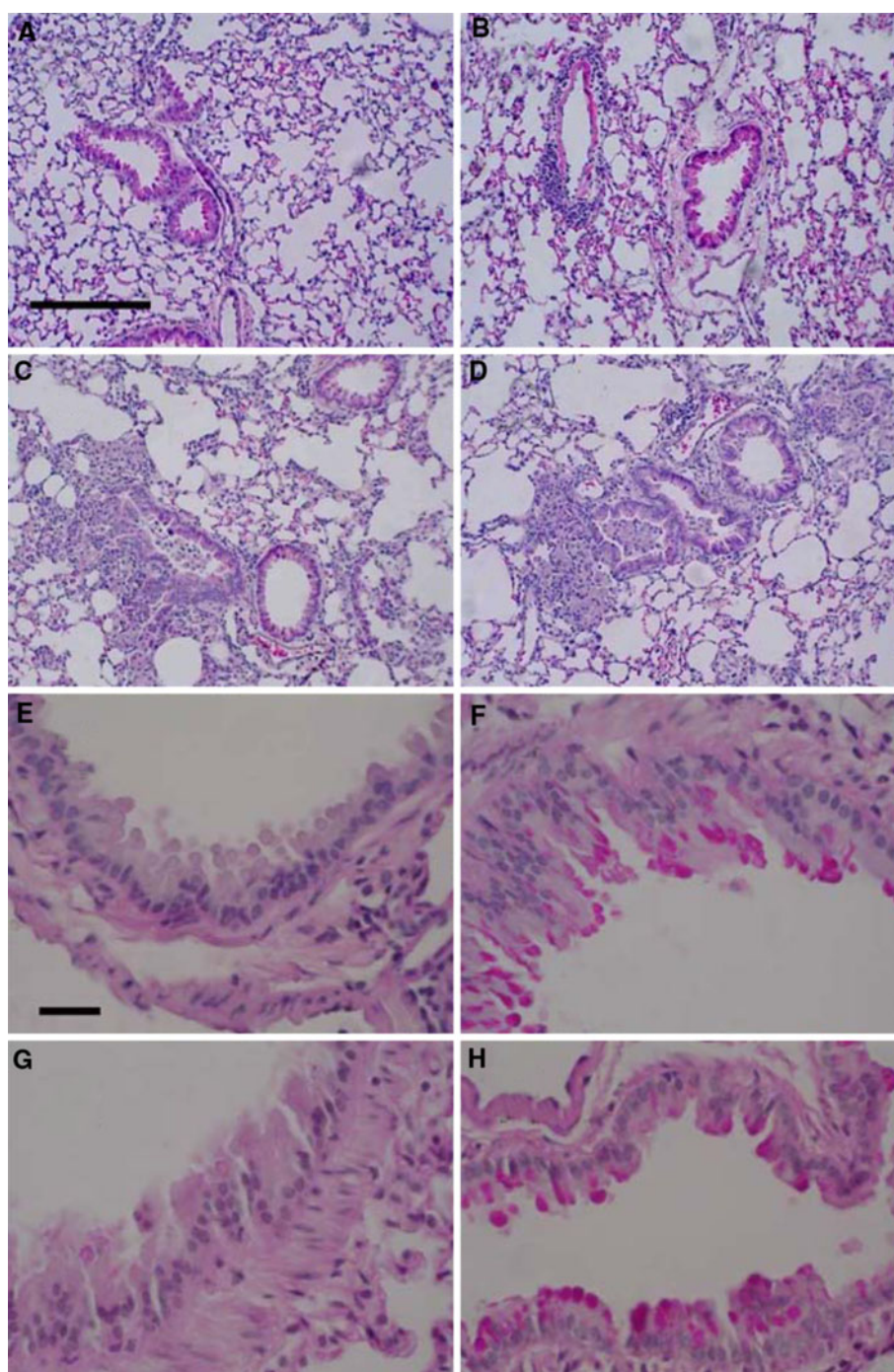
Discussion

Previous studies by our laboratory have shown a profound impact of chronic gastric fluid aspiration on airway hyperresponsiveness in murine models of asthma. These models utilized the Balb/c strain of mouse, which is a Th2

responder, and the results were consistent with a shift toward a Th2 response. In light of the potentially profound effects of the immunological environment on the association between asthma and GERD, we designed this study to probe the effects of chronic gastric fluid aspiration on airway hyperresponsiveness using a Th1-prone model. In particular, we hypothesized that chronic aspiration of gastric fluid may be less effective at driving a Th2 response in the C57BL/6 genetic background, which is Th1-prone. If this hypothesis is proven correct, it has substantial bearing on the pathogenesis of asthma in humans, in which the Th2 response is central.

Consistent with our prior studies in other rodent models [15, 16], chronic gastric fluid aspiration resulted in a prominent inflammatory infiltrate with giant cell formation. It seems highly likely that this change in the inflammatory milieu will have a profound effect on other process such as the hypersensitive response to allergens in the lung. Studies in a rat model have shown that the particulate matter in the gastric fluid is pivotal in the development of a macrophage-

Fig. 4 Histologic analysis of mouse lungs treated with (c, d, g, h) and without (a, b, e, f) chronic gastric fluid aspiration and with (right hand panels) and without (left hand panels) sensitization and challenge with ovalbumin. H/E staining (a–d) showed substantial giant cell formation in mice with chronic gastric fluid aspiration, and perivascular infiltration of inflammatory cells in mice with induced airway hypersensitivity. PAS staining (e–h) revealed increased levels of saccharide associated with epithelial cells in mice having induced airway hypersensitivity compared to those without induced airway hypersensitivity. The bar in (a) indicates 200 μ m and applies to (a–d). The bar in (e) indicates 25 μ m and applies to (e–h)



laden pathology [32]. Our previous in vitro study showed that Toll-like receptor (TLR)-4-dependent pathway and NF- κ B are both involved in mouse macrophage activation by gastric fluid, further supporting the role of gastric fluid-associated bacteria in the pathogenesis [33]. Another study showed that lipopolysaccharides (LPS) enhance the cellular response to allergen in patients with allergic asthma [34]. In contrast, the suppression of acid has little effect on asthmatic symptoms [35, 36] indicating that, to the extent that gastric fluid aspiration may affect asthma, the acidity

of the aspirate is not a critical factor. Consistent with this idea, previous work in our laboratory showed that aspiration-associated pathology in the lung was not dependent on the acidity of the aspirate [32]. With this in mind, further studies aimed at evaluating the effects of various components within gastric fluid (e.g., acid, bacteria, food, and enzymes) upon airway inflammation and hyperresponsiveness may have important implications for clinical treatments of asthma and may provide insight into the underlying mechanisms associated with the pathogenesis of

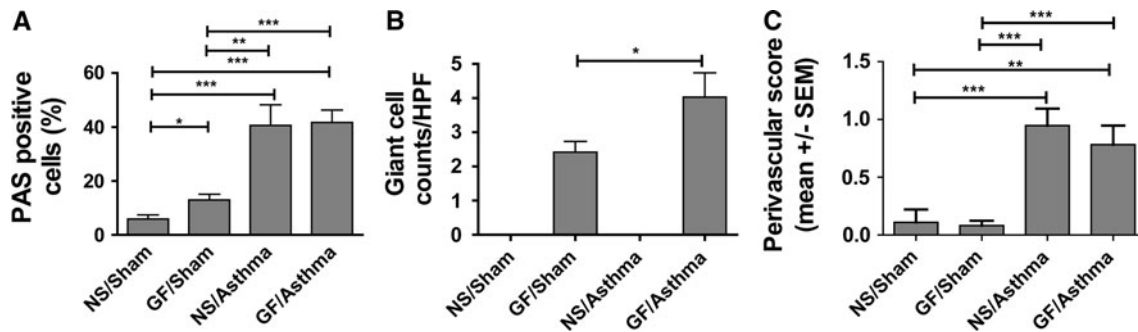


Fig. 5 Overt pathology associated with chronic gastric fluid aspiration and induced airway hypersensitivity (**a**). The number of goblet cells containing mucus was quantified as the percentage of PAS-positive cells/total cells counted under the microscope. **b** The number of giant cells infiltrating in lung parenchyma and peribronchial areas

was quantified as number per high-power field (HPF) of specimen. **c** Peribronchial inflammatory cell infiltration was assessed in a blinded fashion using a semiquantitative scale. All vessels in each section were evaluated, with an average of 11 vessels/section. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

asthma. Of particular interest is the aspiration of bile, which has specific effects on airway epithelium, including significantly increased TGF- β_1 production [37]. Our procedure for gastric fluid collection, involving ligation of the duodenum, prevents the inclusion of substantial quantities of bile, suggesting that further experiments focused explicitly on the role of bile aspiration in asthma are warranted.

Since the effects of acute and chronic aspiration are substantially different [32], it seems possible that the suppression of the T-cell response is dependent on chronic as opposed to acute aspiration. However, it remains unknown what effects a single, acute aspiration event would have in the model of airway hypersensitivity we utilized, and further study will be necessary to determine the effect of a single aspiration event on induced airway hypersensitivity. In addition, more frequent aspiration or aspiration of longer duration than that probed in this study may be worthwhile; aspiration in patients likely occurs more frequently than once per week, and for more than 8 weeks in duration. Thus, although studies using our model of aspiration under anesthesia preclude more frequent aspiration, it seems likely that such studies may be very worthwhile. One possible means by which this may be accomplished is the induction of aspiration using a surgical approach [38]. However, such models cannot distinguish between the effect of actual aspiration and the effects of vagally-mediated reflex bronchospasm, which does not require aspiration.

Potentially of great importance is the observation that chronic aspiration of gastric fluid suppressed levels of T cell-associated cytokines and chemokines, especially IL-2 in mice with induced airway hypersensitivity. This study, in combination with other studies using different mouse strains [15, 16], suggests that the production of other T cell-associated cytokines, including IL-4, IL-5, IL-6, IL-13,

and GM-CSF, might be downregulated to some extent by chronic aspiration. With this in mind, it seems likely that aspiration of gastric fluid does not exacerbate asthma per se. Thus, if GERD does exacerbate asthma, it may be through vagally-mediated reflex bronchospasm, as has been proposed [39].

Although the immune response to ovalbumin is profoundly affected by chronic aspiration based on the production of a range of cytokines, the effect on immunoglobulin is less pronounced. For example, although a trend in lowered production of anti-ovalbumin IgE production was observed as a result of chronic aspiration, the difference between experimental groups was not significant ($p = 0.10$). Two previous studies by our laboratory have shown the same tendency for chronic aspiration to reduce the humoral response to allergen, particularly in terms of serum levels of anti-ovalbumin IgE [15, 16]. In both previous studies, like the current study, considerable variability in the model was observed, and decreases in anti-ovalbumin IgE levels as a result of chronic aspiration never reached a level of statistical significance ($p < 0.05$), suggesting that an increased number of animals may be useful in verifying the observations. Thus, while aspiration profoundly decreases the T cell-associated immune response in some regards, in other regards the effect is less pronounced and more variable.

Acknowledgments This work was supported by the Society of American Gastrointestinal Endoscopic Surgeons Research Grant, and in part by the Parks Protocol Memorial Fund, the American Association for Thoracic Surgery Second Dwight Harken Research Scholarship, the American College of Surgeons Faculty Research Fellowship Award, the Duke Heart Center Career Development Award, and the Fannie E. Rippel Foundation. We thank Roxanne Wilson and Julie Fuller for their technical assistance.

Conflict of interest The authors have no competing interests to declare.

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