

Chronic aspiration shifts the immune response from Th1 to Th2 in a murine model of asthma

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ABSTRACT

Background Chronic aspiration associated with gastro oesophageal reflux disease (GERD) is thought to play a substantial role in the development of asthma, the incidence of which is dramatically increasing in industrially developed countries. The majority of data examining the association between aspiration and asthma has been obtained from epidemiological studies, which show that between 50 and 90% of individuals with asthma experience some element of GERD. This study describes the effect of chronic aspiration on a model of experimentally induced airway hypersensitivity in Balb/c mice.

Materials and methods Four experimental groups were utilized: Aspiration/Asthma, Sham/Asthma, Aspiration/Sham and Sham/Sham. Mice were sensitized with aerosolized 1% ovalbumin on days 1 to 10 (sensitization phase), followed by repeated exposure on days 31 to 40 (challenge phase). Aspiration events occurred on days 1, 8, 15, 22, 29, 36, 43 and 50. Animals were sacrificed on days 56 and 57.

Results Chronic aspiration of 10 μ L of murine gastric fluid per week for eight weeks produced an injury pattern distinct from that of acute aspiration, with lung injury characterized by hyperplasia, neutrophil infiltration of the bronchioles and relative parenchymal sparing. Aspiration during induction of ovalbumin-induced airway hypersensitivity was associated with a trend toward decreased production of antiovalbumin IgG, antiovalbumin IgE, and total IgE. Further, aspiration induced a substantial and significant increase in antiovalbumin IgG1/IgG2a ratios, consistent with a shift toward a predominantly Th2 response.

Conclusion These findings indicate that chronic aspiration has a profound effect on the nature of the immune response to aerosolized allergens in a model of experimentally induced airway hypersensitivity.

Keywords airway hypersensitivity, asthma, chronic aspiration, gastro oesophageal reflux, mice.

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Introduction

Numerous clinical and epidemiological studies indicate that chronic aspiration of gastric fluid exacerbates and can even lead to asthma in many cases [1–3]. Such aspiration likely occurs in modern societies as a result of lifestyle factors, including unhealthy diets and unrequited stress [4].

The majority of the data concerning gastro oesophageal reflux disease (GERD) and its pathological effects come from epidemiological studies. GERD is one of the most frequently encountered medical problems; up to 40% of adults in Western countries are reported to have the cardinal symptom, including chronic cough, heartburn and regurgitation [5]. One study indicates that 28% of patients with GERD will suffer from aspiration, and almost 60% of those will have significant respiratory symptoms or diseases [6]. GERD is said to be the most common cause of chronic intermittent aspiration [6].

GERD-associated aspiration is characterized by silent or occult events in which small amounts of gastrointestinal fluid are aspirated into the tracheobronchial tree [7]. When these seemingly occult events occur repetitively, the local cellular and humoral microenvironment may be altered, leading to a variety of immune-mediated disorders such as asthma [8].

Asthma is the leading cause of chronic childhood illness in the United States, affecting approximately 54 of 1000 children. Moreover, GERD in this population is increasingly being postulated as a potential causative agent [3,9,10]. Furthermore, the incidence of asthma in the adult population, estimated to be 4–6 per 1000 in women and 3–6 per 1000 in men, is on the rise, and it appears to be higher with advanced age [11]. Therefore, asthma is a significant health problem that extends across all age groups. One of the potential etiological factors associated with asthma that has

recently gained attention is GERD associated with the aspiration of gastric contents.

First implicated in 1976 [12], the association between GERD and asthma is being described in roughly 50 articles per year currently. To date, there are over 500 articles in the literature linking GERD to asthma. Since this association is especially strong when reflux is present, the implication is that frequent symptomatic or occult aspiration of gastric contents, as evidenced by oesophagitis, contributes to the development of various hyper-reactive airway diseases, particularly asthma.

The prevalence of GERD in asthmatic patients ranges from 30% to 80% in different studies [13,14]. A longitudinal study from the United Kingdom, consisting of 5653 patients with a first diagnosis of GERD and 9712 patients with a first diagnosis of asthma, revealed a strong link between GERD and asthma [15]. A more recent study also demonstrated that 87% of children with asthma suffer from pathological GERD, as evidenced by 24 h oesophageal pH-monitoring [16].

Despite the strong association between GERD and asthma, controversy exists as to whether the treatment of GERD would alleviate the symptoms and signs of asthma [13]. It is possible that the pathophysiology of GERD associated asthma may be different between paediatric and adult populations. For example, two ways by which GERD might induce or aggravate asthma have been suggested [17], including (i) acid in the oesophagus that causes an increase in bronchial hyper-responsiveness via the vagal reflex and (ii) microaspiration of gastric contents that damages the bronchial mucosa, resulting in inflammation of the mucosa and bronchial hyper-responsiveness. The first may be more important in paediatric patients and the latter in the adult population. A reason why the controversy still exists as to whether treatment of GERD would benefit asthma patients can be attributed to the lack of understanding of the cellular and immune mechanisms involved in GERD associated asthma.

Materials and methods

Asthma model

All procedures involving animals were approved by the Duke University Institutional Animal Care and Use Committee. We used a well established, laboratory model of airway hypersensitivity which involves the induction of an immune response against aerosolized ovalbumin [18]. The immune response includes production of ovalbumin-specific immunoglobulin and increased cytokine production. Although overt pathology indicative of asthma is not observed in this model, the model was selected because of its clinical relevance in terms of the immunological mechanism involved in the development of hypersensitivity [8,18,19]. The model entails a 10 day sensitization phase, followed 30 days later by a 10 day challenge phase. In summary,

10–12 week old female Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice were sensitized with aerosolized 1% ovalbumin in phosphate-buffered saline (PBS) for 10 min every day for days 1 to 10 (sensitization phase), followed by repeated exposure to the aerosolized allergen for 10 min each day on days 31 to 40 (challenge phase). Aspiration events occurred on days 1, 8, 15, 22, 29, 36, 43 and 50. In both the sensitization and challenge phases, mice were placed in an airtight inhalation chamber and exposed to an aerosolized solution of 1% ovalbumin (experimental groups) or aerosolized PBS (sham groups) for 10 min a day on 10 consecutive days. Following exposure, the chambers were vented and mice returned to their cages. Animals were sacrificed on days 56 and 57.

Preparation of gastric fluid

Whole gastric fluid was obtained from female Balb/c mice using the technique described previously in a rat model [20]. The gastric fluid used in these experiments was a pooled sample from multiple mice and was first strained through a 70 micron mesh to eliminate large particles and then stored at -80°C until needed.

Endotracheal intubation

Mice were sedated with a weight-based dose of pentobarbital administered by intraperitoneal injection. Animals were placed on a 45° slant board and their vocal cords visualized by insertion of a Welch-Allyn (Welch Allyn, Skaneateles Falls, NY, USA) otoscope into the oral cavity. A guide wire was passed through the vocal cords followed by a directly visualized intubation with an endotracheal tube. Correct intubation was verified by condensation forming on a mirror held over the proximal end of the endotracheal tube, indicating the passage of the mouse's exhaled air through the tube.

Aspiration events

10 μL of gastric fluid (experimental groups) or PBS (sham groups) were instilled down the endotracheal tube of intubated mice. Experiments in which blue dye was instilled down the endotracheal tube in identical fashion indicated that fluid is distributed to both lungs with this technique. Following the delivery of the fluid bolus, 200 μL of air was used to flush the endotracheal tube. Mice were then extubated and placed in the supine position to recover from sedation.

Sacrifice procedure

Mice were euthanized by intraperitoneal injection of high-dose pentobarbital. Whole blood was withdrawn from the inferior vena cava, and the serum separated and frozen at -80°C . Heart and lungs were removed *en bloc*, bronchoalveolar lavage (BAL) was then performed using 300 μL of 1% bovine serum albumin solution. Lung tissue was then sectioned and placed in formalin for histological slide preparation.

Histology

Tissue specimens were fixed in 10% neutral-buffered formalin for at least 24 h, and then processed into paraffin blocks for microscopic examination. Tissues were then sectioned at 5 microns thickness and mounted onto positively charged microscopy slides (Erie Scientific Company, Portsmouth, NH, USA). Sections for histological analysis were then stained with either a haematoxylin and eosin stain or Mason's trichrome stain.

Enzyme-linked immunosorbent assay (ELISA)

The relative concentrations of mouse antiovalbumin IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, and IgE in serum were evaluated by ELISA as follows: 96-well Maxisorp plates (Nunc, Roskilde, Denmark) were incubated with 50 μL well⁻¹ of PBS containing 7.5 $\mu\text{g mL}^{-1}$ ovalbumin. Plates were incubated overnight at 4 °C and then washed three times with 200 μL well⁻¹ of PBS. Plates were then blocked with 200 μL well⁻¹ of PBS containing 0.1% bovine serum albumin (BSA). Next, appropriately diluted mouse serum (50 μL well⁻¹) was added and incubated for 3 hours. 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 at room temperature, wells were washed three times with 200 μL well⁻¹ of PBS and an alkaline phosphatase conjugated goat antibody specific for the appropriate immunoglobulin (for IgG or IgE determination) was added to each well and incubated for 1 h at room temperature. For evaluation of antiovalbumin IgG 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, St. Louis, MO, USA). Wells were then washed four 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.

The relative concentrations of mouse IgG and IgE (not antigen specific) were also evaluated by ELISA. The procedure for evaluation of total IgG levels and total IgE levels was identical to that described above for determination of antiovalbumin IgG and IgE, except the plates were initially coated with goat antimouse IgG or goat antimouse IgE rather than ovalbumin.

Cytokine analysis

Cytokines were assayed using the Procarta Cytokine Assay Kit (Panomics, Inc., 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) -1B, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, Tumour Necrosis Factor (TNF)- α , Interferon (INF)- γ , and MIP (Macrophage Inflammatory Protein)

-1A. The standard curve for sample cytokine concentration determination was used. Twenty-five micro litres of BAL fluid was used per assay. Assays were run in duplicate (two wells per sample).

Experimental design

Four experimental groups were utilized. Mice receiving aspiration of gastric fluid and exposure to aerosolized antigen (Aspiration/Asthma); mice receiving aspiration of saline and exposure to aerosolized antigen (Sham/Asthma); mice receiving aspiration of gastric fluid and exposure to aerosolized saline (Aspiration/Sham); and mice receiving aspiration of saline and exposure to aerosolized saline (Sham/Sham).

Results

Histological analysis

As shown in Fig. 1, our murine model of chronic aspiration resulted in a pneumonitis characterized by an inflammatory cellular infiltrate that included the presence of neutrophils. The pneumonitis was observed in mice with airway hypersensitivity

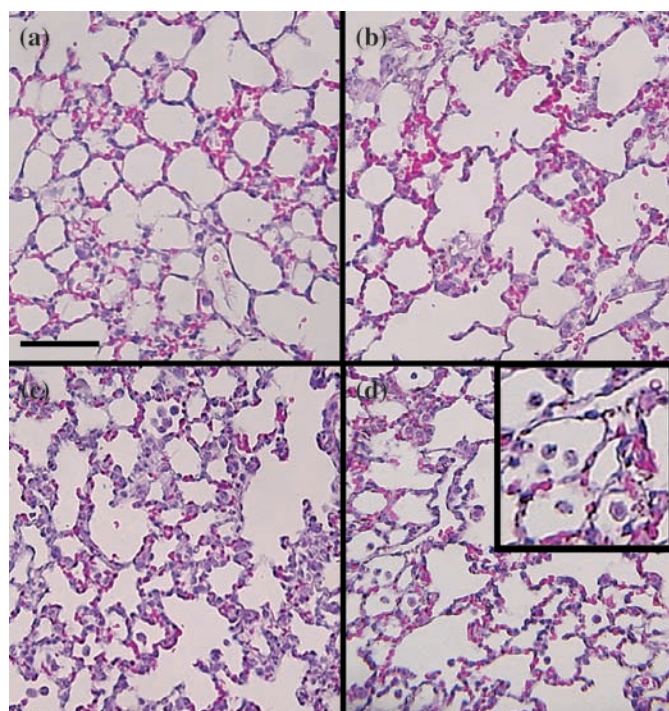


Figure 1 Histological analysis of mouse lungs treated with chronic gastric fluid aspiration (panels c and d) and/or with sensitization and challenge with aerosolized ovalbumin (panels b and d). The bar in panel a represents 50 microns. The inset in panel d shows a close-up (twofold magnification relative to the rest of the photo) of the infiltrating cells.

induction and in mice without induction (sham induction). Consistent with previous reports [18], there was no apparent effect of the aerosolized ovalbumin exposure on the histology of animals without gastric fluid aspirations. Further, exposure to aerosolized ovalbumin caused no overt changes in pathology in lungs that received gastric fluid aspiration.

Effects of chronic aspiration on the humoral response during airway hypersensitivity induction

Induction of airway hypersensitivity by exposure to ovalbumin elicited potent antiovalbumin IgG (Fig. 2a) and IgE (Fig. 2b)

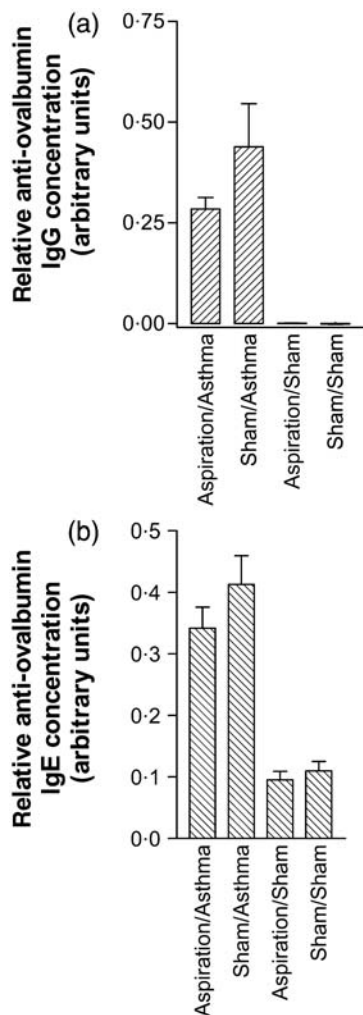


Figure 2 Relative concentrations of antiovalbumin IgG (a) and IgE (b) in the serum of animals from each experimental group. Concentrations were determined by ELISA as described in the Materials and Methods. The standard errors are shown. Aspiration/Asthma $N = 12$, Sham/Asthma $N = 11$, Aspiration/Sham $N = 7$, Sham/Sham $N = 9$.

responses in animals receiving chronic aspiration of gastric fluid and in animals receiving chronic aspiration of saline. Interestingly, there was a slight (about 30%) but not statistically significant decrease in the average amount of antiovalbumin IgG and IgE production in animals receiving chronic gastric fluid aspiration compared to animals not receiving gastric fluid aspiration. Further, there was a slight but not statistically significant decrease in levels of total (not ovalbumin specific) IgE in animals treated with chronic gastric fluid aspiration, regardless of the presence or absence of airway hypersensitivity induction (Fig. 3). These observations may suggest that chronic gastric fluid aspiration

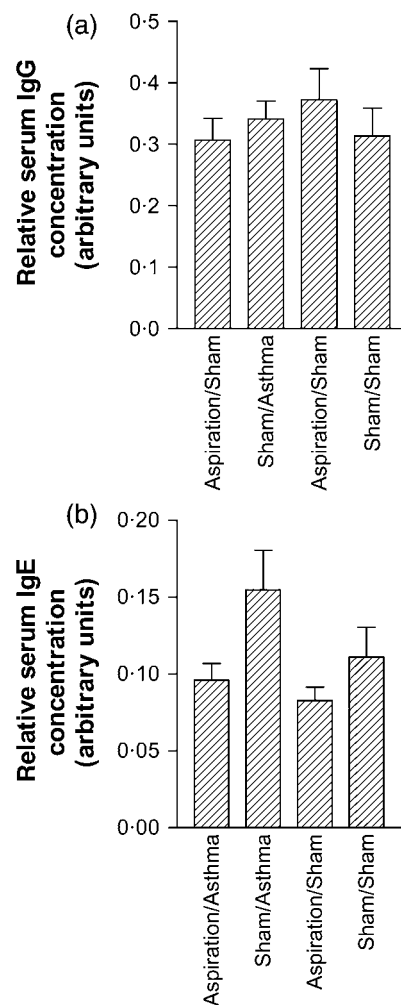


Figure 3 Relative concentrations of IgG (a) and IgE (b) in the serum of animals from each experimental group. Concentrations were determined by ELISA as described in the Materials and Methods. The standard errors are shown. Aspiration/Asthma $N = 12$, Sham/Asthma $N = 11$, Aspiration/Sham $N = 7$, Sham/Sham $N = 9$.

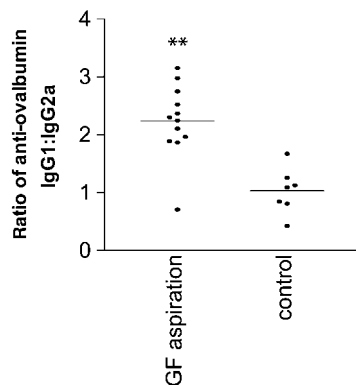


Figure 4 The ratio of antiovalbumin IgG1/IgG2a in animals receiving chronic aspiration of gastric fluid (GF) and in those receiving chronic aspiration of saline (control) during airway hypersensitivity induction using ovalbumin as the allergen. The bar indicates the mean in each group. Aspiration/Asthma $N = 12$, Sham/Asthma $N = 11$.

re-directs the immune response away from a humoral response. Additional studies will be needed to test this hypothesis.

To assess the type of humoral response in the mice following sensitization and challenge with ovalbumin, we evaluated the subtypes of antiovalbumin IgG produced by mice receiving chronic gastric fluid aspiration and in mice receiving chronic sham aspirations. As shown in Fig. 4, chronic aspiration induced a profound (> 2-fold) and significant ($P = 0.0003$) increase in the ratio of antiovalbumin IgG1 to antiovalbumin IgG2a in the serum of the mice. The IgG1/IgG2a ratio can be taken to be an indirect indication of the Th2/Th1 balance operating *in vivo*. With an IgG1/IgG2a ratio > 1 being indicative of a Th2 response, this change in ratio suggests that chronic aspiration induces a shift toward a Th2 response in this experimental model.

Effects of chronic aspiration on cytokine production during airway hypersensitivity induction

We examined cytokine levels in both the serum and bronchoalveolar lavage (BAL) fluid from the animals. In the serum, we detected low levels of IL-1B and IL-12. Additionally, IL-1B, IL-4, IL-5, TNF- α , and INF- γ were noted in the BAL fluid. However, only in the BAL fluid did we observe a statistically significant ($P = 0.04$) increase in IL-12 levels in all treated groups compared to controls receiving sham aspiration and sham airway hypersensitivity induction (Fig. 5). Although not statistically significant, we observed the same pattern in serum samples, with mean levels of IL-12 being higher in all experimental groups compared to the double-sham control group (data not shown). The increase in IL-12 was most notable in those animals exposed to both inhaled allergen and gastric fluid aspiration (Fig. 5).

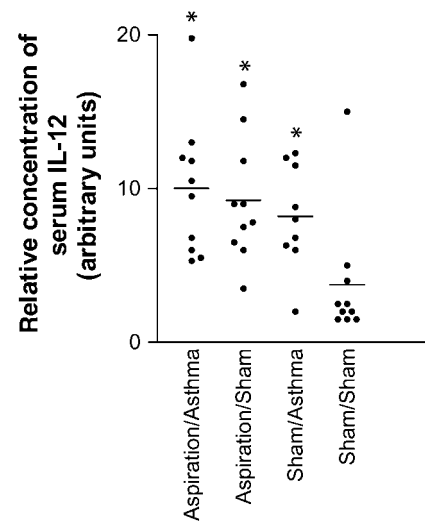


Figure 5 Relative concentrations of IL-12 in the serum of animals from each experimental group ($P = 0.004$). Concentrations were determined by quantitative bead array immunoassay as described in the Materials and Methods. The bar indicates the mean in each group. Aspiration/Asthma $N = 12$, Sham/Asthma $N = 11$, Aspiration/Sham $N = 7$, Sham/Sham $N = 9$.

Discussion

We demonstrated that chronic aspiration of gastric fluid results in overt pathology evidenced by the infiltration of neutrophils and by the hypertrophy and hyperplasia of the bronchial basement membrane. The pathology is thus substantially different from acute aspiration injury, which is characterized by parenchymal injury, alveolar thickening, distortion and neutrophil infiltration. These results are important since this is the first report of a model of chronic aspiration in mice. Given the well characterized nature of the immune system of mice and the extensive availability of reagents to evaluate the immunological response in mice, we hope that this model of chronic aspiration will prove useful for a wide variety of studies involving asthma as well as other chronic aspiration associated conditions. For example, we anticipate that future studies will involve models of asthma that (a) generate overt pathology from allergen exposure and (b) provide a means of evaluation of the effects of aspiration on that process.

Of substantial interest is our demonstration that the aspiration of gastric fluid by itself results in a shift toward a Th2 response based on changes in the IgG1:IgG2 ratio. Th2 responses have been implicated in allergen atopy and autoimmune responses. Cytokine production during a Th2 response is thought to inhibit cell-mediated responses and to enhance the humoral response. Th2-oriented cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which provide help to B cells and induce class switch to IgE and IgG1,

as well as supporting eosinophils and mast cells. This is significant since it suggests that the underlying immune mechanism by which chronic aspiration results in injury is identical to that of asthma. While it would be premature to assert that GERD results in asthma, the fact that both appear to function via a Th2 response is intriguing.

Our cytokine analysis provided some interesting results. We observed increased levels of IL-12 in the BAL fluid from animals that underwent either chronic aspiration and allergen exposure or both chronic aspiration and allergen exposure compared to sham controls. Similar trends were observed in the serum of the animals. IL-12 is a proinflammatory cytokine that is crucial in the initiation and augmentation of the presentation of exogenous antigen to T cells by dendritic cells, and typically plays a role in the reorienting of a response to a Th1 pattern. In addition, the cellular infiltrate we observed is predominantly neutrophils, whereas an infiltration of predominantly eosinophils is often characteristic of a Th2 response. Thus, it seems likely that chronic aspiration in this model, which apparently drives a Th2 response, has profound effects on the immunology of asthma involving both Th1 and Th2 related pathways.

The work described herein has yielded rather unexpected results that have potentially interesting implications for our understanding of how GERD affects the pathogenesis of asthma. In addition, these results have potential implications for research involving the effects of GERD on other pulmonary pathologies. Studies of the effects of GERD on lung transplant allografts, for example, have been in progress for several years, and thus, our knowledge in this area was previously more advanced than our knowledge of the effects of GERD on asthma. However, studies of the effects of GERD on pulmonary allografts are limited by the difficulty of the procedures and the difficulty of assessing immunological parameters in the rat lung allotransplant model. Thus, our understanding of the immunological basis underlying the effects of GERD on asthma in an experimental model has already, in some regards, outdistanced our understanding of the effects of GERD on pulmonary allografts in experimental models. In light of this, we expect that our results will have a substantial impact on our understanding of not only the effects of GERD in asthma, but also of the effects of GERD in pulmonary allograft failure.

Prior to this work, there existed no experimental model investigating the relationship between *chronic* aspiration and asthma. It is our hope that this work will provide an avenue with which to further investigate the immune as well as non-immune mechanisms underlying the effects of gastro oesophageal reflux associated chronic aspiration on the development of asthma. Of particular interest will be studies aimed at evaluating the extent to which various gastric fluid components contribute to the exacerbation of asthma or the predisposition for asthma.

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