Effect of Microbial Heat Shock Proteins on Airway Inflammation and Hyperresponsiveness

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Microbial heat shock proteins (hsp) have been associated with the generation and induction of Th1-type immune responses. We tested the effects of treatment with five different microbial hsp (Mycobacterium leprae, Streptococcus pneumoniae, Helicobacter pylori, bacillus Calmette-Guérin, and Mycobacterium tuberculosis) in a murine model of allergic airway inflammation and airway hyperresponsiveness (AHR). Mice were sensitized to OVA by i.p. injection and then challenged by OVA inhalation. Hsp were administered to each group by i.p. injection before sensitization and challenge. Sensitized and challenged mice developed increased serum levels of OVA-specific IgE with significant airway eosinophilia and heightened responsiveness to methacholine when compared with nonsensitized animals. Administration of M. leprae hsp prevented both development of AHR as well as bronchoalveolar lavage fluid eosinophilia in a dose-dependent manner. Treatment with M. leprae hsp also resulted in suppression of IL-4 and IL-5 production in bronchoalveolar lavage fluid, while IL-10 and IFN-γ production were increased. Furthermore, M. leprae hsp treatment significantly suppressed OVA-specific IgE production and goblet cell hyperplasia/mucin hyperproduction. In contrast, treatment with the other hsp failed to prevent changes in airway responsiveness, lung eosinophilia, or cytokine production. Depletion of γδ T lymphocytes before sensitization and challenge abolished the effect of M. leprae hsp treatment on AHR. These results indicate selective and distinctive properties among the hsp, and that M. leprae hsp may have a potential therapeutic role in the treatment of allergic airway inflammation and altered airway function. The Journal of Immunology, 2002, 169: 5300–5307.

Microbial heat shock proteins (hsp) are a highly conserved group of proteins produced by prokaryotic and eukaryotic cells, in response to a variety of environmental stressors (1–5). Although hsp are ubiquitous and highly homologous among different species, hsp constitute important target Ags of the immune response. Microbial hsp have been shown to be important immunogens stimulating both T and B cells (6). In recent years, it has become clear that members of the hsp families, mainly hsp60 and hsp70, are among the major Ags of pathogens that trigger an immune response (7). The hsp, immunodominant Ags of various microorganisms including mycobacteria, are a common target of T cell recognition (8) and increase reactivity of T cells (9–11). Especially microbial hsp have been associated with the generation and induction of Th1-type immune responses (12), and recent studies suggested that mycobacterial hsp65 induces immunostimulatory functions by increasing the production of monokines (13–15).

Allergic asthma is a common chronic disease with an increasing prevalence, and is characterized by airway hyperresponsiveness (AHR), airway inflammation, and high serum IgE levels. In asthmatic subjects, activation and accumulation of eosinophils, mast cells, and T cells are evident in bronchial biopsies and in bronchial lining fluid (16–19) and are considered to underlie the expression of the clinical disease. Because CD4+ T cells that produce IL-4, IL-5, and IL-13 (Th2 cells) have been found in the airways of asthmatics and because Th2 cytokines are required for the development of airway eosinophilia and IgE synthesis, it has been proposed that Th2 cells stimulate an inflammatory response that results in asthma (20).

Because hsp administration has been associated with generation of Th1-type immune responses, we hypothesized that hsp could serve as an immunomodulator and affect the outcome of allergic sensitization. In this study, we tested the efficacy of five different microbial hsp as immunomodulators in a murine model of airway inflammation and AHR. We previously characterized a mouse model of allergic AHR and found that systemic sensitization and repeated airway challenge with OVA result in a Th2-type inflammatory response in which elevated serum levels of IgE and IgG1, AHR, and eosinophilic airway inflammation were associated with increases in IL-4 and IL-5 in the bronchoalveolar lavage fluid (BALF). We used this model to investigate the effects of hsp on airway responsiveness, pulmonary inflammation, and local cytokine production in the airways.

Materials and Methods

Animals

Female BALB/c mice, 8–12 wk of age and free of pathogens, were obtained from The Jackson Laboratory (Bar Harbor, ME). The mice were...

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3 Abbreviations used in this paper: hsp, heat shock protein; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BALF, BAL fluid; BCG, bacillus Calmette-Guérin; Cd, dynamic compliance; IPN, i.p. sensitization and neutilization; MBP, major basic protein; MCh, methacholine; Neb, nebulized; PAS, periodic acid Schiff; PBNL, peribronchial lymph node; Penb, enhanced pause; RL, airway resistance.

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maintained on OVA-free diets. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

**Sensitization, airway challenge, hsp treatment, and depletion of \( \gamma/\delta \) T lymphocytes**

Mice receiving the following treatment were studied: 1) airway challenge with OVA alone in nonsensitized animals (nebulized (Neb)); 2) sensitization with OVA and OVA airway challenge (i.p. sensitization and nebulization (IPN)). Mice were sensitized by i.p. injection of 20 µg OVA (grade V; Sigma-Aldrich, St. Louis, MO) suspended in 2.25 mg aluminum hydroxide (Alun Imject; Pierce, Rockford, IL) in a total volume of 100 µl on days 1 and 4. Mice were challenged via the airways using nebulized OVA (1% in PBS) for 20 min on days 24, 25, and 26 with an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA).

hsp were obtained from StressGen Biotechnologies (Victoria, Canada).Five microbial hsp from different microorganisms were used in this study (Mycobacterium leprae, Streptococcus pneumoniae, Helicobacter pylori, bacillus Calmette-Guérin (BCG), and Mycobacterium tuberculosis). The purified, hsp contained <50 ELISA units endotoxin/µg protein. On days 1, 14, and 24, 100 µg hsp, dissolved in 100 µl PBS, was administered to each group, both inspiratory and expiratory sides of two ventilators. Ventilation was maintained as a constant tidal volume of air (0.2 ml). Lung function was assessed as percent change of baseline (PBS) Penh. Baseline Penh values were determined as a change in airway function after aerosolized MCh challenge. As a positive control, the sections were stained with periodic acid Schiff (PAS) for fixed to microscope slides and deparaffinized. The sections were stained with paraffin acid Schiff (PAS) for fixation with OVA (grade 50 µl/ml in PBS) and incubated with medium alone or OVA (100 µg/ml) for 48 h in a humidified atmosphere of 5% CO\(_2\) at 37°C. Cell-free supernatants were harvested and stored at −20°C pending cytokine ELISA.

**Histological and immunohistochemistry studies**

After obtaining the BALF, lungs were inflated through the trachea with 2 ml of 10% Formalin and then fixed in the same solution by immersion. Tissue sections, 5 µm thick, were affixed to microscope slides and deparaffinized. The sections were stained with periodic acid Schiff (PAS) for identification of mucus-containing cells, and examined under light microscopy. Eosinophils were identified by immunohistochemistry using a rabbit anti-mouse eosinophilic major basic protein (MBP) Ab (provided by J. J. Lee, Mayo Clinic, Scottsdale, AZ), as previously described (26). The slides were examined in a blinded fashion with a Nikon microscope equipped with a fluorescent filter system (Nikon, Garden City, NY).

**Statistical analysis**

ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by Tukey-Kramer honest significant difference test; \( p \) values for significance were set to 0.05. Values for all measurements were expressed as the mean ± SEM.

**Bronchoalveolar lavage (BAL) and measurement of BALF cytokines**

After assessment of airway responsiveness, lungs were lavaged via the tracheal cannula with PBS (1 × 1 ml, 37°C). The number of BAL cells was measured by cell counter (Coulter Counter: Coulter, Hialeah, FL). Cytospin slides were stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA) and differentiated in a blinded fashion by counting 200 cells counted under immersion oil at 1000 × magnification. Cytokine levels in BALF supernatants were determined by ELISA, as previously described (24). Measurements of IL-10 and IFN-γ were performed by using OptEIA kits (BD PharMingen, San Diego, CA), following the manufacturer’s protocol. The limits of detection were 4 pg/ml for IL-4 and IL-5, and 10 pg/ml for IL-10 and IFN-γ.

**Measurement of serum OVA-specific Ab and total IgE**

Total IgE levels and OVA-specific IgE, IgG1, and IgG2a Ab levels in the serum were measured by ELISA, as previously described (25). Briefly, Immonul-2 plates were coated with 5 µg/ml OVA. After addition of serum samples, a biotinylated anti-IgE Ab (02122D; BD Pharmingen) was used as the detecting Ab, and the reaction was amplified with avidin-HRP ( Sigma-Aldrich). To detect IgG1 and IgG2a, alkaline phosphatase-labeled Abs (02003 E and 02013 E; BD Pharmingen) were used. The OVA-specific Ab titers of samples were related to an internal pooled standard, which was arbitrarily assigned to 500 ELISA units. The total IgE level was calculated by comparison with a known mouse IgE standard (55 3481; BD Pharmingen). The limit of detection was 100 pg/ml for IgE.

**In vitro cytokine production assays**

Peribronchial lymph node (PBNL) cells were purified, as previously described (26). Cells were plated at 4 × 10⁶/ml in 96-well round-bottom tissue culture plates in triplicate and incubated with medium alone or OVA (100 µg/ml) for 48 h in a humidified atmosphere of 5% CO\(_2\) at 37°C. Cell-free supernatants were harvested and stored at −20°C pending cytokine ELISA.

**FIGURE 1.** *M. leprae* hsp prevents the development of AHR. Groups of mice were sensitized, challenged, and treated, as described in Materials and Methods. Control mice (Neb) received three aerosol challenges to OVA alone. Airway reactivity to increasing concentrations of inhaled MCh was measured 48 h after the last challenge in unrestrained, conscious mice, and Penh values were determined. Mean ± SEM of Penh values are the results obtained from eight mice (two independent experiments) and are expressed as the percentage of baseline Penh values observed after PBS exposure. * Significant difference (\( p < 0.05 \)) between *M. leprae*-treated group and sensitized/challenged group (IPN) and other hsp-treated groups. HP, H. pylori; ML, M. leprae; SP, S. pneumoniae; MT, M. tuberculosis.
Results

M. leprae hsp prevents the development of AHR

To assess the effects of hsp treatment on the development of AHR after systemic sensitization and airway challenge with OVA, we measured in vivo airway responsiveness to aerosolized MCh by whole body barometric plethysmography. Fig. 1 illustrates that mice that were sensitized and challenged with OVA and treated with PBS i.p. (control) demonstrated a significant dose-dependent increase in Penh in response to inhaled MCh when compared with control mice. Mice that were treated with M. leprae hsp showed significantly reduced Penh compared with mice treated with PBS ($p < 0.05$). In contrast, the other hsp failed to show significant inhibitory effects on the development of AHR, even at doses up to 200 μg. Increasing or decreasing the dose of M. leprae hsp (200, 50, or 10 μg) showed dose-dependent effects of the hsp on prevention of development of AHR (Fig. 2A). The development of AHR after sensitization and airway challenge was not affected when M. leprae hsp was administered only before sensitization or only before airway challenge (data not shown).

Treatment with M. leprae hsp prevents airway eosinophilia

Eosinophilic airway inflammation is an important outcome of allergen-induced airway sensitization and challenge. To determine the effects of microbial hsp on the inflammatory response following allergic sensitization and challenge, the cellular content of BALF was assessed. Mice sensitized and challenged with OVA developed significant airway inflammation with a predominance of eosinophils. Control mice that received 3 days of aerosol challenge to OVA alone had almost no eosinophils in the BALF. Administration of M. leprae hsp before sensitization and challenge prevented the increase in eosinophil numbers in the BALF (Fig. 3). The other microbial hsp failed to show a significant inhibitory effect on eosinophil accumulation in lungs. Altering the dose of M. leprae hsp (200, 50, or 10 μg) showed a dose-dependent effect on BALF eosinophil numbers (Fig. 2B). Mice treated with hsp, with the exception of BCG hsp-treated mice, showed significantly ($p < 0.05$) higher numbers of macrophages compared with the untreated sensitized and challenged mice. When M. leprae hsp was administered alone before sensitization or before airway challenge, no effect was seen on eosinophil numbers compared with PBS-treated, sensitized, and challenged mice (data not shown).

M. leprae hsp treatment abolishes eosinophil accumulation in the peribronchial/perivascular tissue

In parallel to examination of BALF, lung tissue was examined. Evidence of eosinophil infiltration and the effects of microbial hsp were further investigated through histological examination of tissue stained with anti-MBP (Fig. 4). In mice challenged alone, few eosinophils were detected in the peribronchial and perivascular tissues (Fig. 4A). In contrast, sensitization and subsequent airway
challenge resulted in significant increases in numbers of peribronchial and perivascular eosinophils (Fig. 4B). Treatment with \textit{M. leprae} hsp abolished the eosinophil accumulation in the peribronchial and perivascular areas (Fig. 4C). The other hsp did not significantly alter eosinophil infiltration (data not shown).

\textit{Treatment with \textit{M. leprae} hsp alters cytokine production in BALF.}

Th2 cytokine production by T cells plays a key regulatory role in the induction of allergic airway inflammation and AHR. To evaluate the effects of microbial hsp in the prevention of induction of Th2 responses, we measured concentrations of IL-4, IL-5, IL-10, and IFN-\(\gamma\), 48 h after the last OVA challenge in BALF. As shown in Fig. 5, sensitization and challenge resulted in increases in IL-4 and IL-5 and significant decreases (\(p < 0.05\)) in IL-10 and IFN-\(\gamma\) levels. \textit{M. leprae} hsp treatment of sensitized/challenged mice significantly (\(p < 0.05\)) reduced the levels of IL-4 and IL-5 in the BALF compared with untreated mice (IPN group) (Fig. 5). In contrast, \textit{M. leprae} hsp treatment significantly increased both IFN-\(\gamma\) and IL-10 levels in BALF compared with IPN group. There were no significant differences between treated and untreated groups regarding the other hsp (data not shown).

\textit{M. leprae hsp treatment down-regulates production of IL-4 and IL-5 by PBLN cells in response to OVA in vitro.}

To monitor local cytokine production, mononuclear cells, isolated from PBLNs, were cultured and stimulated with OVA. Concentrations of IL-4 and IL-5 in culture supernatants were measured by ELISA. Sensitization and subsequent airway challenge with OVA resulted in increased production of IL-4 and IL-5 by PBLN cells in culture (Fig. 6). PBLN cells from mice treated with \textit{M. leprae} hsp
showed significantly lower IL-4 and IL-5 production in OVA-stimulated cultures compared with untreated mice.

M. leprae hsp inhibits production of OVA-specific IgE in serum

Serum levels of OVA-specific and total IgE were measured 48 h after the last airway challenge. Sensitization and challenge with OVA resulted in significantly increased serum levels of OVA-specific IgE and total IgE when compared with mice challenged alone (p < 0.05). Treatment of mice with 100 μg of M. leprae hsp reduced OVA-specific IgE serum (but not total IgE) levels when compared with untreated controls (Fig. 7). No such effects were observed after treatment with the other hsp. Increasing the M. lepra hsp dose (200 μg/injection) did not further reduce OVA-specific IgE levels (data not shown).

M. leprae hsp prevents goblet cell hyperplasia and mucin hyperproduction in airway epithelium

Lung sections were stained with PAS to identify mucus-containing cells in the airway epithelium. A large number of cells staining positive for mucus were found in sensitized and challenged mice compared with mice challenged alone (Fig. 8, A and B). Treatment with M. leprae hsp inhibited goblet cell hyperplasia and mucin hyperproduction (Fig. 8C), whereas such differences were not seen following treatment with the other hsp (data not shown).

Depletion of γ/δ T lymphocytes abolishes the effect of M. leprae hsp treatment on AHR

Hsp can activate γ/δ T cells (27) and γ/δ T cells can be negative regulators of airway responsiveness (21). To assess the role of γ/δ T lymphocytes in mediating the effect of treatment with M. leprae hsp on AHR, we depleted γ/δ T lymphocytes by injection of TCR-δ mAb 1 day before initial sensitization. Airway responsiveness to MCh was measured in anesthetized, tracheotomized, and ventilated animals 48 h after the last airway challenge using whole body plethysmography. There were no significant differences in baseline Rrs and Cdyn values between the different groups. Mice injected with the Ab and then subsequently sensitized and challenged showed the same degree of airway responsiveness to inhaled MCh as the sensitized and challenged alone mice (Fig. 9). Treatment with 100 μg M. leprae hsp prevented the increases in Rrs and reductions in Cdyn throughout the MCh dose-response curve, results similar to the findings monitoring Penh (Figs. 1 and 2). However, M. leprae-treated mice that received the anti-δ Ab developed the same degree of AHR as the sensitized and challenged mice (which received control hamster IgG) (Fig. 9). Following anti-δ treatment, BALF eosinophil numbers and cytokine levels remained unchanged (data not shown).

Discussion

In this study, we show that M. leprae hsp was effective in preventing the development of AHR and airway inflammation in a murine model of allergic airway inflammation. Moreover, when compared with four other microbial hsp, the effects on airway function and inflammation and cytokine production were restricted to treatment with M. leprae hsp. M. leprae hsp administered to sensitized and challenged mice resulted in decreases in eosinophilic inflammation, Th2 cytokine production, Ag-specific IgE, goblet cell hyperplasia, and in normalization of airway function.

During specific types of inflammation, including arthritis and severe asthma (28), a stress response is induced, which results in the production of hsp (2). Cytokines can stimulate T lymphocytes to produce hsp70 (5), and arachidonate and PGA, activate heat shock transcription factors (29–31). Production of hsp presumably protects cells against the inimical effects of stress (1, 32). Of possible relevance to asthma, allergen-induced inflammation might result in stress induction in airway epithelium (33), and alveolar macrophage phagocytosis of eosinophils elicits heat shock protein synthesis (34). Recently, hsp70 up-regulation was demonstrated to be present in patients with bronchial asthma (35). In this study, we demonstrate that treatment of mice with M. leprae hsp before sensitization and airway allergen challenge significantly inhibited the development of AHR. Furthermore, eosinophilia in the lung tissue and airways was prevented after treatment with M. leprae hsp, but not with the other hsp. These findings imply that despite their enormous conservation in amino acid sequences (36), microbial hsp differ in their immunological properties.

T cells play a key role in allergic asthma because upon recognition of allergen they are capable of releasing large amounts of Th2-type cytokines, including IL-4 and IL-13, which are required for IgE production and goblet cell hyperplasia, and IL-5, which is essential to the development of tissue eosinophilia (37). T cells are activated (17, 19, 38, 39) and express mRNA encoding IL-4, IL-5 (40), and IL-4, and IL-5 are produced by a subtype of Th cells (41), which predominates in chronic asthma (42, 43). Because allergic asthma is thought to be regulated by Th2 cells, interfering with this arm of the immune response may be an attractive mode for intervention (44, 45). T cells capable of recognizing hsp are induced during the natural establishment of microflora on the skin or in the gut and are capable of recognizing conserved epitopes such as hsp. These responses to hsp provide an initial defense against infectious agents before the development of immunity to novel Ags has begun (46). We show that M. leprae hsp treatment altered the local cytokine profile following allergen challenge of sensitized mice, decreasing IL-5 production and increasing IL-10 and IFN-γ levels in the BALF. These results were confirmed in in vitro cultures of
involves Th1-like activity in mice. These findings parallel those reports in which microbial hsp targets immature APC to make them particularly effective in similar models of OVA-induced airway eosinophilia, local IL-5 production, and AHR, suggestively through what others have referred to as effective inhibitor in similar models of OVA-induced airway eosinophilia and AHR (50, 51). Other cell types, such as dendritic cells, might also play a role in the activity of M. leprae hsp. Recently, Todryk et al. (51) showed that induction of hsp expression induces an infiltrate of Th2 cytokine expression (IFN-γ, TNF-α, and IL-12), and enhances immunogenicity via a Th1 cell-mediated mechanism. They also demonstrated that hsp targets immature APC to make them significantly more capable of capturing Ags, and triggering a Th1 response.

Sensitization and challenge normally result in a decrease in BALF IL-10 levels, a cytokine associated with anti-inflammatory and anti-inflammatory activities. M. leprae hsp resulted in increased IL-10 levels in the sensitized and challenged mice. Such data suggest that the cross-reactivity between M. leprae hsp and host hsp may activate regulatory T cells to release important regulatory cytokines such as IL-10, and as a result, the increase in IL-10 may have played a role in the down-regulation of Th2 cytokine production (52). Intrapерitoneal administration of M. leprae hsp generated regulatory mechanisms that reduced the activity of cells present in the lymph nodes responsible for allergic airway inflammation induction (52). This notion is supported by the in vitro observations of cytokine production in cultures of PBLN cells from M. leprae-treated mice in which OVA-stimulated IL-4 and IL-5 production was inhibited.

It is unclear at the present time and somewhat surprising that the significant regulatory effects on airway responsiveness, inflammation, and cytokine production were so restricted to M. leprae hsp. Simple explanations such as differences in endotoxin content were eliminated, as endotoxin content did not differ among the various hsp (data not shown). Despite extensive homology, M. leprae hsp65-specific epitopes have been described, which are distinguishable from the analogous sequences, for example, from the 65-kDa M. tuberculosis protein (53). Whether or not these unique sequences serve as the T cell-stimulating sequences, conferring protection in the allergen-induced models, remains to be determined. Although BCG hsp was not generally effective in our model, infection with an attenuated form of M. bovis BCG was an effective inhibitor in similar models of OVA-induced airway eosinophilia, local IL-5 production, and AHR, suggestively through increased IFN-γ production (54, 55). These differences between BCG hsp and BCG infection/imunization could reflect the potency of mycobacterial DNA (CpG oligonucleotides or immunostimulatory sequences) in triggering a Th1 response and preventing lung inflammation and AHR (56, 57).

In summary, M. leprae hsp was very effective in the regulation of inflammation in this murine model of airway inflammation and AHR. Following treatment with M. leprae hsp, the mice failed to develop allergen-induced Th2 responses, lung eosinophilia, and especially mycobacterial hsp65 (48). Born et al. (49) showed that γδ T cells could specifically respond to a small synthetic peptide corresponding to the same region of M. leprae hsp in vitro and in vivo (50). γδ T cells have been shown to negatively regulate airway responsiveness (21). Thus, one possibility is that administration of M. leprae hsp could activate γδ T cells, resulting in the down-regulation or prevention of AHR. To investigate this hypothesis, we used an anti-δ mAb before the hsp administration and sensitization. This Ab has been shown to deplete γδ T cells (21) and was confirmed in this study. Indeed, injection of this Ab virtually eliminated the effects of M. leprae hsp on AHR, suggesting a very critical role of γδ T cells in mediating the effects of M. leprae in this model as previously described. As described previously (21), the effects of this Ab were not mediated through alteration in lung inflammation as eosinophil numbers and cytokine levels remained unchanged.

OVA-stimulated mononuclear cells from PBLN. Downstream of these cytokine effects were specific consequences including reduced eosinophilic numbers in the BALF and lung tissue, and reduced serum levels of Ag-specific IgE; staining of the epithelium confirmed the elimination of goblet cell hyperplasia and mucin hyperproduction that followed allergen challenge of sensitized mice. These findings parallel those reports in which microbial hsp have been associated with the generation and induction of Th1-type immune responses (12) and induced Th1-like activity in CD4+ T cells from leprosy patients and healthy individuals (47).

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AHR. The activity of *M. leprae* hsp especially on the reduction of AHR appeared, at least in part, to be mediated through γδ T cells. The results of this study suggest that hsp administration can modify allergen-induced airway inflammation and hypersensitivity following allergen sensitization and challenge.

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