Time course of pulmonary pathology, cytokine influx and their correlation on augmentation of antigen challenge by influenza A virus infection

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A murine model of influenza A virus exacerbation of allergen induced airway inflammation, pulmonary histopathological changes, bronchoalveolar lavage fluid (BALF) analysis, cytokine influx and the time course of these events have been studied. The present study was undertaken to determine the relative contributions of Th1/Th2 cytokines to the histopathological changes in the lungs observed at 9, 12, 24 and 48 hr following antigen challenge in mice previously immunized with influenza A virus. BALF analysis of acute phase group revealed statistically significant increase in neutrophils at 9 hr, macrophages at 12 hr, lymphocytes and eosinophils at 24 hr, as compared to OVA-sensitized control mice. These changes were associated with an alteration in the levels of IL-4, IL-5 and IFN-γ. A peak of IL-4 at 24 hr significantly enhanced bronchiolar and perivascular histopathology, whereas increased IL-5 level peaking at 24 hr was correlated with the enhanced infiltration of eosinophils in both BALF and lung tissue. There was simultaneous depletion of IL-10 an anti-inflammatory cytokine leading to persistence of pulmonary inflammation in case of acute phase group. Histopathology at 24 and 48 hr showed severe denudation of bronchiolar lining epithelium surrounded by dense chronic inflammatory infiltrate. Chronic interstitial infiltrate with focal loss of architecture, marked oedema, extravasation of RBCs from congested blood vessels and laying down of reticulin fibres was observed in acute phase. Thus, infection with influenza A virus on pre-existing asthmatic immunopathology elicits a cascade of Th2 cytokines with influx of inflammatory cells in BALF, mucosal and interstitial inflammation leading to asthma exacerbations

Keywords: Asthma, Cytokine, Immunopathology, Influenza A virus

Asthma is characterized by episodic symptoms and variable airflow obstruction that occur either spontaneously or in response to environmental exposures. Despite the advances made in treatments for asthma based on an understanding of allergen induced airway responses, acute exacerbations remain a major cause of morbidity and mortality. These exacerbations are frequently triggered by viral respiratory infections, which are a major cause of wheezing in infants and adult patients with asthma. Asthma exacerbations have different immunopathologies and emphasize the need to identify the pathway involved in order to improve their treatment.

Detailed immuno-pathological mechanisms underlying the interaction of respiratory virus infection and chronic asthmatic airway inflammation are currently unclear. The disease syndrome following infection by virus is a consequence both of direct harmful effects of virus itself and of immunopathology resulting from the host immune response. In an asthmatic individual exacerbation may occur because of functional interaction between viral pathology and asthmatic pathology through different mechanisms with the same end effect on function, or by sharing the same pathological mechanism in a synergistic fashion. In fact, it is likely that virus induced asthma exacerbations occur because of combination of these types of interaction.

Epidemiologic evidence linking viruses particularly, influenza A virus exacerbations getting even stronger have been seen in recent years. Influenza virus infection in some way amplifies subclinical allergic lower airway inflammation already present prior to infection. Influenza virus causes an acute infection with extensive lower airway involvement that is generally limited to the respiratory epithelium. Traditionally, antiviral responses have been characterized by Th1 phenotypic response with raised level of IFN-γ and recruitment of CD8+ cells. This is in contrast to Th2 response thought to be dominant in asthma, with increased levels of IL-4, IL-
5 and IL-13⁸. However, studies suggest that viral infection and respiratory allergies together appear to be synergistic as risk factors in producing symptoms of asthma exacerbations⁹. Pathogenicity of these diseases individually have been extensively studied and reported as well, but little is known regarding the immunopathology in asthma exacerbations induced by influenza virus.

The present study was designed to elucidate the correlation of pathological mechanisms with cytokine influx in influenza A virus induced infection in murine model of allergic asthma.

Materials and Methods

Animals—Specific pathogen free, 6-11 weeks of age male BALB/c mice (purchased from NIN Hyderabad, India) were used in the experiment. The animals were fed OVA free diet and kept under special pathogen free conditions in experimental facility of Vallabhbhai Patel Chest Institute. All experimental animals used in this study were maintained under the approved guidelines of the institutional animal ethical committee.

Influenza A infection—The mouse adapted strains of influenza A virus (A/udorn/31/72/H3N2) obtained from CDC, Atlanta was used and prepared as described earlier¹⁰. The diluted virus with the solution of 4.1×10³ PFU (H3N2) sublethal dose was inoculated intranasally¹¹,¹² with 50 µl of the virus solution under anesthesia with diethyl ether.

Antigen—OVA (Sigma Chemical Co.,) was dissolved in phosphate buffer saline (PBS; pH 7.4) to a concentration of 0.1% and mixed with an equivalent dose of alum adjuvant (10 mg/ml). The final concentration of OVA for sensitization was adjusted to 0.05%. For the OVA challenge, a solution of 2% OVA in PBS without alum was used ¹².

Experimental protocol—Mice were divided into four groups (in each group n = 5-7 animals were used). Group I (control): Mice were given injection of PBS (ip) on day 0, injected with PBS repeatedly on day 7 and 14, and were exposed to PBS aerosol on day 29-33. Group II (virus): Mice were given injection of PBS (ip) on day 0, injected with PBS repeatedly on day 7 and 14, instilled with influenza A virus on day 28 and were exposed to PBS aerosol on day 29-33. Group II: (aute phase): Mice were sensitized with 10 µg of OVA (ip) on day 0, resensitized with OVA on day 7 and 14, instilled with influenza A virus on day 28 and challenged with OVA on day 29-33.

Sensitization and challenge—Animals were sensitized by OVA 0.1 ml on day 0 and resensitized on day 7 and 14 through injections (ip). Sensitized animals were then challenged by inhalation of aerosolized OVA 2% in PBS by DeVilbiss 646 nebulizer for 30 min each day over five consecutive days¹¹,¹².

Extraction of serum and collection of BAL fluid—Mice were anaesthetized by diethyl ether, blood was collected from the heart in a vacuum tube, allowed to clot at 4°C and serum was obtained by centrifugation at 4°C for 15 min at 2,500 rpm. Following the blood collection, BALF was collected at 9, 12, 24, and 48 hr after final OVA challenge, trachea was cannulated with a stainless steel tube and through this tracheal cannula 2 ml of normal saline was injected into lungs 2-3 times and returning fluid was collected each time¹¹,¹².

BAL fluid cytology—For BAL fluid cytology, BALF was collected as mentioned above, and processed in cytocentrifuge. Supernatant was collected in a separate tube and cell pellet was washed twice and resuspended in 1 ml PBS (pH 7.4). The cells were then smeared onto a glass slide using a cytocentrifuge at 100 x g and stained with Diff-Quick (American Scientific Inc., USA). Total and differential leukocyte counts were performed according to standard morphological criteria with reference of at least 200 cells¹².

Histopathology of lungs—Lungs were removed at 9, 12, 24 and 48 hr after final OVA challenge and fixed by intratracheal instillation followed by immersion in 10% neutral buffered formalin solution. After fixation, 2 blocks from each lung were randomly sampled for histological evaluation. Sections (3 µm thick) were taken from each block and stained with hematoxylin and eosin¹²,¹³. Lung sections were also stained with PAS stain to observe goblet cell hyperplasia and Gomori’s reticulin staining was done to observe the laying of reticulin fibres¹⁴.

IgE assay in BAL fluid and serum—After collection of BALF and serum, levels of total IgE (BD OptEIA, CA, USA) after 24 hr of final challenge was measured using ELISA¹².

Kinetic assay of cytokines in BAL fluid—Kinetics of cytokines was also performed by collecting BALF at 9, 12, 24, 48 hr after the final challenge. Levels of
IL-4, IL-5, IL-10 and INF-γ cytokines (BD OptEIA, CA, USA) were measured using ELISAs according to the manufacturer’s instructions.

Statistical analysis—The data were analyzed with one and two way analysis of variance (ANOVA) followed by bonferroni’s multiple comparison tests. All the results are expressed as mean ± SE.

Results

Kinetic analysis of inflammatory cells in BALF—To study the kinetics of cellular response in the lung, bronchoalveolar lavages were collected at 9, 12, 24 and 48 hr after final challenge and were analyzed. The total cell number in BALF of mice challenged during the acute phase (group IV) of influenza A virus infection was significantly elevated as compared to other groups at all the time points except at 9 hr with the highest cell count peak at 24 hr (Fig.1a). In a differential cell count, the absolute number of neutrophils in group IV (acute phase), were found to peak at 9 hr, after which neutrophil counts started to decline, almost returned to control levels at 48 hr (Fig.1b), but neither lymphocytes nor eosinophils were recruited at this time interval into the airway lumen. Macrophages being the normal resident cells persist at all the time intervals with main peak at 12 hr (Fig.1c). The absolute number of lymphocytes started infiltrating early, showed a constant significant increase at 12 hr, reached a peak at 24 hr and then decreased (Fig.1d). This was followed by eosinophil influx with the main peak observed at 24 hr after final OVA challenge (Fig.1e). This increase in eosinophils was statistically significant.

Mice with OVA sensitizations (group III) and those instilled with influenza A virus (group II) also showed a significant increase in total and differential cell counts when compared with the normal controls (group I). A maximum increase in absolute number of macrophages was observed at 24 hr in OVA group and at 12 hr in virus group (Fig.1b), whereas, neutrophils, and lymphocytes followed the similar trend showing the peaks at 9 and 24 hr respectively in both groups. Virus group showed a significant increase in all inflammatory cells except eosinophils (Fig.1e), though it was less than acute phase (group IV). No eosinophils were observed in BALF of group I, and II.

Histopathological analysis of lungs—Histopathological analysis of the lungs performed at various time intervals after the final OVA challenge revealed changes in bronchial wall, parenchyma, interstitium and vasculature, in different groups. Major changes were observed at 24 hr and persisted till 48 hr after the final challenge. When compared to Control group, the lungs of group II (virus group) showed focal denudation of bronchiolar lining epithelium, plugging of airways with acute neutrophilic infiltrate and peribronchial moderately dense chronic inflammatory cell infiltrate. Interstitium showed chronic inflammation with congested vessels and interstitial widening (Fig. 2a and b). In group III, (OVA group) peribronchiolar and multifocal interstitial chronic inflammatory infiltrate comprising of lymphocytes, eosinophils, plasma cells and macrophages was seen. PAS stain revealed goblet cell hyperplasia (Fig. 2c and d). In group IV (acute phase), higher grade changes similar to that of group II and III were observed. Sections showed severe denudation of airway lining epithelium surrounded by intense chronic inflammatory infiltrate comprising predominantly of lymphocytes and macrophages. Interstitium showed acute on chronic inflammation comprising of neutrophils, lymphocytes, and eosinophils with focal loss of architecture, marked edema and extravasation of RBC’s at 48 hr. This chronic inflammation was also associated with haphazard laying down of reticulin fibers on reticulin stain (Fig. 2e and f).

Serum IgE level in BALF and serum—IgE level was assayed 24 hr after the final OVA challenge in all the groups. The level of IgE was detected in the group IV (acute phase) and group III (OVA group) and was significantly higher in group IV by two-fold increase. Level of IgE in group II (virus group) was not different from the respective control values. The data presented in Fig.3 shows the trend observed for IgE level i.e., acute > OVA > virus = control, reflecting allergic behaviour in Group IV (acute phase) and Group III (OVA).

Kinetic analysis of cytokines in BALF—The kinetic study of cytokines in BALF was also performed at various time periods (9, 12, 24, and 48 hr). The level of IFN-γ (Fig.4a) showed a two-fold increase at 12 hr in virus (Groups II), whereas acute phase (group IV) also showed a similar trend, though it was lesser than group II. The level of IL-4 started increasing at 12 hr reaching a maximum at 24 hr then showed decrease from the peak level at 48 hr with the maximum rise in acute phase (Group IV) (Fig.4b). A two-fold increase in IL-5 level was observed at 24 hr in case of acute
Fig. 1—Effect on kinetics of cellular response in BALF of influenza A virus mediated allergic asthma. [I=control group; II=influenza virus infected group; III=OVA group; IV=acute phase group (a): shows BALF cell analysis at 9, 12, 24 and 48 hr after final OVA challenge. The absolute values of total cell number in BALF of mice in acute phase (group IV) was significantly higher at all time points with main peak at 24 hr as compared to other three groups ($P<0.001$) with 11 fold increase in comparison to control. Mice from group II (virus instilled) and group III (OVA challenged) also showed a significant increase in the total cell counts when compared with normal control group I, (a maximum of 6 fold increase at 12 hr in virus and 24 hr in OVA groups, $P<0.001$). (b): In a differential cell count, group IV (acute phase), the absolute number of neutrophils were found to peak at 9 hr ($P<0.001$) with 2 fold increase as compared to control values (group I), then returned to control values at 48 hr ($P>0.05$). Group II and group III also showed the peak at 9 hrs though values were lesser than group IV (group II, 25.67 to group IV, 33.00; $P<0.05$) and (group III, 20.67 to group IV, 33.00; $P<0.001$). (c): Macrophages in acute phase group persisted at all time intervals with the main peak at 12 hr and increased an average of 14 & 2 fold (from 5 to 73.00 & 37.33 to 73.33; $P<0.001$) as compared to control and OVA groups. Acute phase group also showed an increase in macrophage level in comparison to virus group but the values didn’t reach to level of significance (from 64.00 to 73.33; $P>0.05$). In group II (virus) a maximum increase in absolute number of macrophages was observed at 12 and 24 hr in group III (from 5 to 64.00; $P<0.001$ and 6.000 to 49.00; $P<0.001$) as compared to control values. (d): The absolute number of lymphocytes in acute phase (group IV) reached a peak at 24 hr and an average of 2 fold increase was observed (from 42.00 to 80.67; $P<0.001$) in comparison to group II and III and then began decreasing, group II and III also spiked at 24 hr as compared to control group I ($P<0.001$). (e): The eosinophil influx with the main peak observed at 24 hr after the final OVA challenge in group III and IV. This increase in eosinophils was statistically significant ($P<0.01$). The values were double in group IV as compared to group III (from 4.000 to 8.000; $P<0.001$). No eosinophils were observed in BALF of group I, and group II]
phase (group IV), a significant increase was also observed at 24 hr in case of OVA (group III), while no change was observed in virus (group II; Fig. 4c). IL-10 level (Fig. 4d) peaked at 12 hr in Group II (virus), while group III (OVA) and group IV (acute phase) showed slight increase at 12 hr.
Respiratory viruses are important triggers of asthma exacerbations. The mechanism involved in virus-induced asthma exacerbations are complex and incompletely understood. The pathology observed in airways depends on virus type and host immune response. Influenza A virus infection and replication in the respiratory tract directly injures the nasal and tracheobronchial epithelium, possibly as a result of virus-induced apoptosis. Influence of influenza virus infection on the ongoing immunopathogenetic mechanisms in asthmatic airway and possible causal relationship between them are the subjects of intense interest. The present study, therefore, attempts to systematically characterize the course of allergen-induced immunopathology, cytokine influx and correlate with pathologic features on BALF analysis and histopathology after instillation of influenza A virus in sensitized, resensitized and challenged Balb/c mice.

We report a model of influenza A virus mediated allergic asthma that reproduces many characteristics features of the disease. The mice of acute phase...
developed an inflammatory cell infiltration of the airways which was predominantly neutrophilic and monocytic in nature during the early hours after the final challenge, and thereafter became eosinophilic. The recruitment and infiltration of neutrophils peaked at 9 hr after the antigen challenge in acute phase group. This neutrophilia was more predominant and intense when compared to the mice challenged with OVA only (group III). These results are consistent with the studies carried out in a murine model by Christian Taube et al. who have suggested that neutrophil infiltration occurs early after the allergen challenge and is allergen-specific. The observed neutrophilia was also in concordance with the studies on viruses which have reported that neutrophil recruitment is the early innate immune response against the viral infection and also plays a causative role in bronchoconstriction by cytokine release. Thus, maximum increase in neutrophil count observed in acute phase could possibly be due to the synergistic effect of viral infection and allergen exposure.

Further the lymphocyte count was also found to increase after 24 hr of final allergen challenge in acute phase group. This increase coincided with BALF neutropenia (Fig.1b) that declined after the main peak at 9 hr. Since T-lymphocytes are believed to have a key role in pathogenesis of asthma, the effect of viruses on allergen sensitized airways with increased recruitment of lymphocytes at 24 hr, seen in our study is of particular importance. This pattern of cell recruitment in acute phase is consistent with a progressive increase in the influence of the Th2 lymphocyte subset, on Influenza A virus infection with characteristic production of cytokines such as IL-4 and IL-5.

Release of cytokines such as, IL-4 and IL-5, is appreciated to be the key event in orchestrating, perpetuating and amplifying the inflammatory response in asthma. In the present study, IL-5 level in BAL fluid of acute phase group increased between 9 and 24 hr after allergen challenge and peaked at 24 hr with associated increase in eosinophil counts. Though a similar change was observed in OVA group, the peak was much steeper than acute phase. The level of eosinophilia in BAL was seen to correlate with the disease severity on histopathology. IL-4 level was also observed to increase at 24 hr and thought to influence the airway eosinophilia indirectly by its ability to suppress IFN-γ production. Increased IL-4 level and suppression of IFN-γ production in acute phase has been significantly correlated with parenchymal damage on histopathology.

The normal CD4+ T-cell response to virus infection is thought to be of T-helper (Th)1 type and associated with increase in IFN-γ, a major Th1 cytokine, secreted from macrophages. In the present study, rise of macrophage levels at 12 hr was observed to be lower in acute phase as compared to virus group, possibly because of the down-regulation of Th1 by Th2 in acute phase group. Thus, demonstrating that on viral infection within an airway with a pre-existing Th2-type allergic asthmatic microenvironment, there is an inhibition of the normal effective Th1-type antiviral immune responses, leading to exacerbation of interstitial inflammation and cell death.

This was associated with a diminished production of IL-10 in acute phase, at all time intervals except for a minor peak at 12 hr which was also lower compared to virus group. IL-10 being an anti-inflammatory cytokine is known to oppose the inflammatory behaviour of asthma. Its decrease in acute phase group seen in the present study can be correlated with progression of interstitial inflammation to cell necrosis and loss of parenchymal architecture. This finding was similar to a human study where subjects with allergy showed an intrinsic inability to up-regulate IL-10 production in response to respiratory viral infection. Further IgE level was also elevated which depicts the allergic behaviour in acute phase group.

Degree of cytokine influx and BAL fluid cytology correlated with severity of histopathological changes at various time periods in acute phase group. Biopsy revealed highest changes in alveolar architecture at 24 hr which persisted till 48 hr after final challenge. Severe denudation of airway lining epithelium surrounded by intense chronic inflammatory infiltrate was seen. Interstitial showed chronic inflammatory infiltrate comprising lymphocytes, eosinophils and macrophages with loss of architecture, marked oedema, extravasation of RBC’s. Reticulin staining demonstrated haphazard laying down of reticulin fibres that may lead to prefibrotic changes during viral exacerbations of the sensitized airways. This could be associated with the severity of the exacerbation in acute phase group and needs to be evaluated by future studies.

Thus, the study defines the time course of events of pulmonary pathological changes due to potential
synergistic effects of respiratory viral infection on allergen sensitized airway at different time points after the antigen challenge. The exacerbations by influenza A viral infection are initiated by tracheobronchial epithelium damage with epithelial denudation and inflammatory infiltration through airway epithelium. The consequent cytokine response pattern is consistent with a progressive increase in the influence of Th2 lymphocyte subset, with characteristic production of cytokines such as IL-4 and IL-5. This leads to a typical cytokine mediated neutrophil response pattern and exaggerated airway response. Interstitial and parenchymal damage may also be mediated by a deficient IL-10 response in influenza A viral infection are initiated by allergen sensitized airway at different time points after the antigen challenge. The exacerbations by influenza A viral infection increases IgE production and airway responsiveness in aerosolized antigen-exposed mice, J Aller Clin Immunol, 102 (1998) 732.


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