The Journal of Immunology

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J Immunol 2000; 165:3484-3491; ;
doi: 10.4049/jimmunol.165.6.3484
http://www.jimmunol.org/content/165/6/3484

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Respiratory Infection with Influenza A Virus Interferes with the Induction of Tolerance to Aeroallergens[1]

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Viral respiratory infections have been implicated in influencing allergen sensitization and the development of asthma, but their exact role remains controversial. Because respiratory exposure to Ag normally engenders T cell tolerance and prevents the development of airway hyperreactivity (AHR) and inflammation, we examined the effects of influenza A virus infection on tolerance induced by exposure to intranasal (i.n.) OVA and the subsequent development of AHR. We found that concurrent infection with influenza A abrogated tolerance induced by exposure to i.n. OVA, and instead led to the development of AHR accompanied by the production of OVA-specific IgE, IL-4, IL-5, IL-13, and IFN-γ. When both IL-4 and IL-5 were neutralized in this system, AHR was still induced, suggesting that influenza-induced cytokines such as IL-13, or mechanisms unrelated to cytokines, might be responsible for the development of AHR. The length of time between influenza A infection and i.n. exposure to OVA was crucial, because mice exposed to i.n. OVA 15–30 days after viral inoculation developed neither AHR nor OVA-specific tolerance. These mice instead acquired Th1-biased OVA-specific immune responses associated with vigorous OVA-induced T cell proliferation, and reduced production of OVA-specific IgE. The protective effect of influenza A on AHR was dependent on IFN-γ, because protection was abrogated with a neutralizing anti-IFN-γ mAb. These results suggest that viral respiratory infection interferes with the development of respiratory allergen-induced tolerance, and that the time interval between viral infection and allergen exposure is critical in determining whether viral infection will enhance, or protect against, the development of respiratory allergen sensitization and AHR. *The Journal of Immunology,* **2000, 165: 3484–3491.**

Asthma is a chronic inflammatory lung disease associated with intermittent airflow obstruction, airway hyperreactivity (AHR)[4] and infiltration of the respiratory mucosa with inflammatory cells, mainly eosinophils. Multiple factors are involved in the pathogenesis of asthma, but a critical element is the initiation of aberrant immune responsiveness against inhaled environmental respiratory allergens, characterized by the development of allergen-specific IgE, and the presence of allergen-specific $CD4^+$ T cells producing IL-4, IL-5, and IL-13, but not IFN-γ (1, 2). Both allergic and nonallergic individuals are exposed to environmental aeroallergens, but only allergic individuals develop allergen-specific Th2-biased immune responses to these allergens. Nonallergic individuals remain tolerant to allergen exposure because they lack allergen-specific IgE, lack functional immune reactivity to these allergens, or develop protective allergen-specific Th1-biased responses.

The development of asthma is linked not only to respiratory allergen sensitization, but also to viral respiratory tract infections in early childhood. It is well known that asthma symptoms such as wheezing worsen after respiratory viral infection (3, 4). Furthermore, viral infection, for example with respiratory syncytial virus, during the first years of life greatly enhances the risk of developing asthma in children (5, 6), and experimental data in murine systems support this idea (7). Moreover, viral infection and respiratory allergies together appear to be synergistic as risk factors in producing symptoms of wheezing (8). These studies suggest that specific interactions between respiratory allergies and viral infections exist, where viral illnesses in early childhood may enhance the development of allergen sensitization. However, this relationship remains controversial, because many other epidemiological studies suggest that viral and bacterial infections, in fact, protect against the development of asthma and allergy (9–12).

The purpose of this study was to determine the effects of infection with influenza A virus on the development of respiratory allergen sensitization and AHR in a murine model of asthma. We and others have demonstrated that respiratory exposure to a prototypic allergen, OVA (3), in naive mice leads to OVA-specific $CD4^+$ T cell unresponsiveness and lack of IgE responsiveness, due primarily to deletion and functional inactivation of Ag-specific $CD4^+$ T cells (13–15). Moreover, we recently demonstrated that such respiratory Ag-induced tolerance prevented the development of AHR and inflammation (16). Using this model system, we now report that concurrent influenza A virus infection and intranasal (i.n.) exposure to OVA abrogated the induction of OVA-induced tolerance, promoted the expansion of functionally active allergen-specific Th cells, enhanced the production of allergen-specific IgE, IL-4, IL-5, IL-13, and IFN-γ, and prompted the development of AHR. When both IL-4 and IL-5 were neutralized in this system, AHR was still induced, suggesting that influenza-induced cytokines such as IL-13 were responsible for the development of AHR.

The length of time between influenza A virus infection and i.n. exposure to OVA was crucial, because respiratory exposure to

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Received for publication January 28, 2000. Accepted for publication July 5, 2000.

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[1] This work was supported by National Institutes of Health Grant RO1 AI44699, and an American Lung Association Research Award to D.C.T.

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[4] Abbreviations used in this paper: AHR, airway hyperreactivity; a.f., allantoic fluid: BAL, bronchoalveolar lavage; i.n., intranasal, intranasally.

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allergen 15–30 days after viral infection, during recovery phase from the infection, abrogated tolerance induction but promoted a Th1-biased response. Moreover, this response was associated with reduced allergen-specific IgE production and normal airway reactivity. The protective effect of influenza A on AHR was dependent on IFN-γ, because it was abrogated with a neutralizing anti-IFN-γ mAb. Thus influenza A virus infection abolishes respiratory allergen-induced tolerance, but may enhance or protect against allergen-induced AHR depending on the timing of respiratory allergen exposure. These results strongly imply that respiratory viral infections in humans interrupt the normal tolerance-inducing mechanisms that protect against the development of allergic asthma, but may enhance subsequent protective Th1-biased responses. Therefore, these results may explain the conflicting results of clinical studies in humans demonstrating that respiratory viral infections either enhance the risk of, or prevent the development of, asthma.

Materials and Methods

Animals

Five- to 6-wk-old BALB/c or BALB/c IL-4$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in pathogen-free conditions at the laboratory animal facilities of Stanford University (Stanford, CA), in accordance with the guidelines of National Institute of Health. Mice used for experiments were sex and age matched.

Influenza A infection

Mice were anesthetized with methoxuflurane and inoculated i.n. with influenza A virus (attenuated strain HK-31 (H3N2)) in 40 μl PBS (diluted 1:5 v/v), generously provided by Dr. Peter Doherty (University of Tennessee, TN). The dose of virus used (240 hemagglutination U) causes severe, but nonlethal, pneumonia with complete viral clearance by day 10 after inoculation (17, 18). Control mice were treated with i.n. allantoic fluid (a.f.; also provided by Dr. Peter Doherty) diluted 1:5 in PBS.

Experimental protocols

Three, 15, or 30 days after infection, mice were lightly anesthetized and received 100 μg grade V OVA in 30 μl of PBS (Sigma, St. Louis, MO) i.n. on 3 consecutive days. Control mice received an equivalent volume of PBS i.n. Ten days after the last administration of i.n. OVA, the mice were immunized with 50 μg OVA in 2 mg aluminum hydroxide (alum) i.p. The mice were rechallenged 10 days later with 50 μg i.n. OVA three times (Fig. 1). In some experiments BALB/c mice received 2 mg anti-IFN-γ mAb i.p. (XMG1.2; generous gift of Dr. R. Coffman, DNAX Research Institute, Palo Alto, CA) 24 h before, and at the time of viral inoculation, as well as before the administration of i.n. OVA or PBS. In other experiments, BALB/c IL-4$^{-/-}$ mice were used and were treated with anti-IL-5 mAb (TRFK-5, 2 mg/dose i.p.; kind gift of Dr. R. Coffman, DNAX Research Institute) 24 h before administration of i.n. OVA or PBS. Control IL-4$^{-/-}$ mice received an isotype control Ab (4G10, 2 mg/dose; kindly provided by Dr. S. Levy, Stanford University).

Measurement of airway responsiveness

Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice placed in a whole body plethysmograph (model PLY 3211; Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by Penh using the following formula: Penh = (Te/RT-1) $\times$ (PEF/PIF), where Penh = enhanced pause (dimensionless), Te = expiratory time, RT = relaxation time, PEF = peak expiratory flow (ml/s), and PIF = peak inspiratory flow (ml/s), measured with a transducer (model TRD5100) and analyzed by Buxco system XA software (model SFT 1810). Measurements of methacholine responsiveness were obtained by monitoring Penh immediately after exposing mice for 2 min to nebulized 0.9% NaCl and of incremental doses of nebulized methacholine. Results were expressed for each methacholine concentration as the percentage of baseline Penh values after 0.9% NaCl exposure (19).

Analysis of airway inflammation

Following the sacrifice of mice, the trachea was cannulated, and the lungs were lavaged with 0.3 ml PBS (0.1% BSA, 0.5 mM EDTA) four times. The fluid was pooled, total cell numbers in the bronchoalveolar lavage (BAL) fluid were counted, and cytospin slides were prepared. The slides were stained with Diff-Quik (Dade Behring, Newark, DE) and cell differentials of at least 300 cells/slide were determined by light microscopy based on conventional morphologic criteria. In some animals, the lungs were removed, fixed in 10% formalin, routinely processed, and embedded in paraffin wax. Five-micrometer sections were prepared and stained with hematoxylin and eosin.

In vitro proliferation and cytokine assays

Lymph node cells were harvested, passed through a nylon mesh, and cultured (5×10^5 cells/well) with or without OVA in 0.2 ml DMEM (Sigma) supplemented with 10% FCS (Gemini Bioproducts, Calabasas, CA), 2 mM L-glutamine, 20 μg/ml gentamicin, and 5×10^{-5} M 2-ME. After 72 h, the cultures were pulsed with 1 μCi [^{3}H]thymidine for 12–16 h and the incorporated radioactivity was measured in a Betaplate scintillation counter (MicroBeta Trilux; Wallac, Gaithersburg, MD). To determine the secretion of cytokines in the cultures, supernatants were collected at day 4, and analyzed for levels of IL-4, IL-5, IL-13, and IFN-γ by ELISA.

Measurement of OVA-specific Igs

Mice were bled at the time of sacrifice and OVA-specific Abs were measured using OVA-specific ELISA. For the measurement of OVA-specific IgG1 and IgG2a, plates were coated overnight with 5 μg/ml OVA. After washing and blocking, serial dilutions of sera were added for 24 h. Subsequently the plates were incubated with HRP-conjugated goat anti-IgG subclass-specific Abs (Southern Biotechnology Associates, Birmingham, AL), washed, and developed by adding *o*-phenylenediamine substrate. The OD was determined at 492 nm. Anti-OVA IgG1 and IgG2a mAbs 6C1 and 3A11, respectively, were used as standards for quantification of each IgG subclass (20), and measurements were performed within the linear range of the standard curves. For the determination of OVA-specific IgE, 5 μg/ml rat anti-mouse IgE mAb EM95 (generously provided by Dr. Robert Coffman, DNAX Research Institute, and Dr. Z. Eshhar, Weizman Institute, Rhovot, Israel) was used to coat the plates. After the samples were applied for 24 h, 10 μg/ml biotinylated OVA was added for 2 h followed by a 1-h

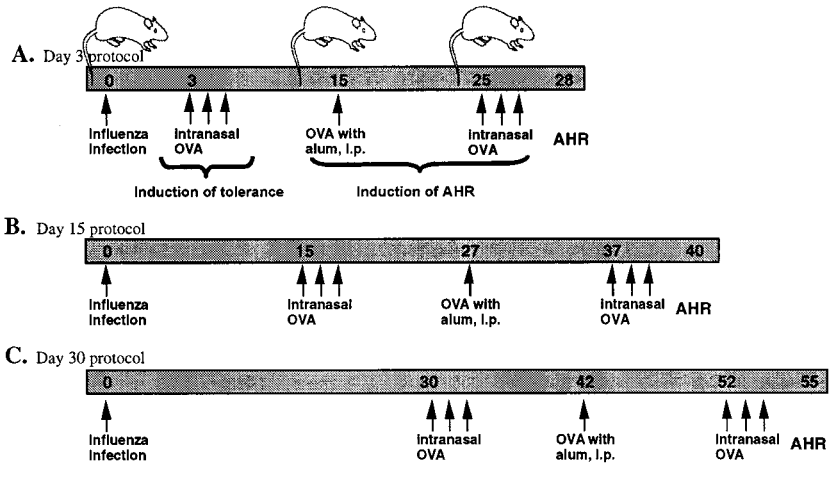

FIGURE 1. Protocols used for influenza A infection, induction of tolerance, and AHR. On day 0, BALB/c mice were inoculated with influenza A virus i.n. or sham inoculated with a.f. Three (*A*, Day 3 protocol), 15 (*B*, Day 15 protocol), or 30 days (*C*, Day 30 protocol) later, the mice were exposed three times to 100 μg i.n OVA or saline, and subsequently immunized with 50 μg OVA in alum. i.p. All mice were challenged 10 days later three times with 50 μg i.n. OVA before evaluation of AHR.

incubation with HRP-conjugated streptavidin (Southern Biotechnology Associates). Plates were developed with *o*-phenylenediamine substrate and the OD was determined at 492 nm. Sera from mice hyperimmunized with OVA in alum was used as standard for the OVA-specific IgE ELISA, and was first standardized for IgE levels against an anti-OVA IgE mAb generously provided by E. Gelfand (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO; Ref. 21).

Results

Influenza A infection interferes with the induction of CD4$^+$ T cell tolerance

We previously showed that i.n. administration of OVA induced peripheral CD4$^+$ T cell tolerance, characterized by inability of the specific CD4$^+$ T cells to expand and produce cytokines after immunogenic restimulation (14). The induction of i.n. tolerance effectively prevents the development of AHR and inflammation upon further contact with the allergen (16). To examine the effects of influenza A viral infection on these processes, we infected BALB/c mice with influenza A virus and administered OVA i.n. 3 days later (Fig. 1*A*). Ten days after exposure to i.n. OVA, the mice were treated with a protocol to induce AHR by immunization with OVA with alum i.p. and subsequent rechallenge with i.n. OVA. As expected, cells taken from bronchial lymph nodes of control mice previously exposed to a.f. followed by i.n. OVA (a.f./OVA) before i.p immunization with OVA, failed to proliferate when stimulated in vitro with OVA (Fig. 2). However, cells from mice infected with influenza before i.n. OVA exposure (Flu/OVA), proliferated vigorously, indicating that infection with the virus abrogated the induction of OVA-specific T cell unresponsiveness.

The cytokine profiles of the OVA-specific CD4$^+$ cells stimulated in vitro was examined. T cells from mice tolerized with i.n. OVA (a.f./OVA) failed to produce significant quantities of IL-4, IL-5, IL-13, or IFN-γ (Fig. 3), and did not secrete TGF-β or IL-10 (data not shown). In contrast, T cells from control mice treated only with our standard sensitization protocol to induce AHR (a.f./saline) produced high levels of IL-4, IL-5, and IL-13. Infection with influenza A before i.n. OVA exposure (Flu/OVA) markedly increased secretion of IL-4, IL-5, and IL-13 and particularly enhanced production of IFN-γ (Fig. 3). The levels of IL-10 and TNF-α measured in the culture supernatants were very low, while TGF-β production was not detected in any of the cultures performed (data not shown).

Infection with influenza A virus also affected the production of OVA-specific Ig. Mice tolerized by exposure to i.n. OVA (a.f./OVA) failed to produce OVA-specific IgE (Fig. 3; Ref. 13–15). However, influenza virus infection with concurrent exposure to i.n. OVA (Flu/OVA) resulted in secretion of high levels of OVA-specific IgE, equivalent to IgE levels observed in the fully sensitized control group (a.f./saline). These mice (Flu/OVA) also developed an increase in the levels of the OVA-specific IgG2a (Fig. 3) and IgG1 (data not shown), consistent with the induction by influenza A of an unrestricted T cell cytokine profile. These observations indicated that infection with influenza A virus abrogated the induction of i.n. tolerance, and allowed the development of allergic sensitization with the development of effector T cells with a mixed cytokine profile.

Concomitant exposure to i.n OVA and influenza A infection results in the development of AHR

Infection with influenza A virus not only interfered with i.n. tolerance induction, but also abrogated the protective effects of tolerance on the development of AHR. Thus, Fig. 4*A* shows that

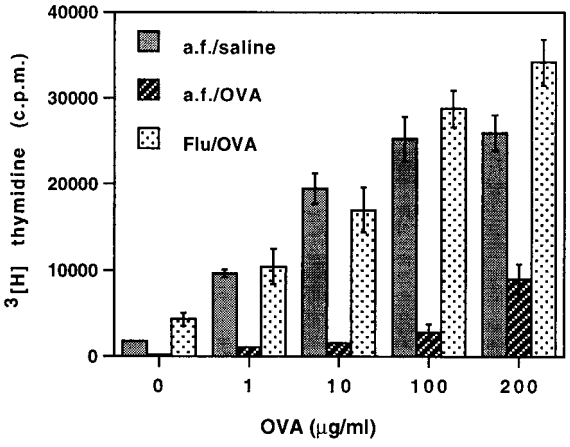

FIGURE 2. Influenza A infection abrogates the induction of CD4$^+$ T cell unresponsiveness. BALB/c mice were infected with influenza A virus or sham infected with a.f. Three days later, mice were exposed three times to 100 μg i.n. OVA or saline. On day 15, mice were immunized with 50 μg OVA in alum i.p. and rechallenged 10 days later three times with 50 μg i.n. OVA. *Flu/OVA*, Mice were infected with influenza A, then received i.n. OVA before treatment with the protocol to induce AHR. *a.f./OVA,* Mice were sham infected with a.f., then received i.n. OVA before treatment with protocol to induce AHR. *a.f./saline,* Mice were sham infected with a.f., then received i.n. saline before treatment with protocol to induce AHR. Forty-eight hours after the last dose of i.n. OVA, bronchial lymph node cells were harvested and cultured in vitro with increasing concentrations of OVA. The proliferation was determined by measuring the incorporation of [^{3}H]thymidine over the last 18 h of culture. Representative results using six to eight mice/group are shown. The results are expressed as mean cpm ± SD of triplicate cultures.

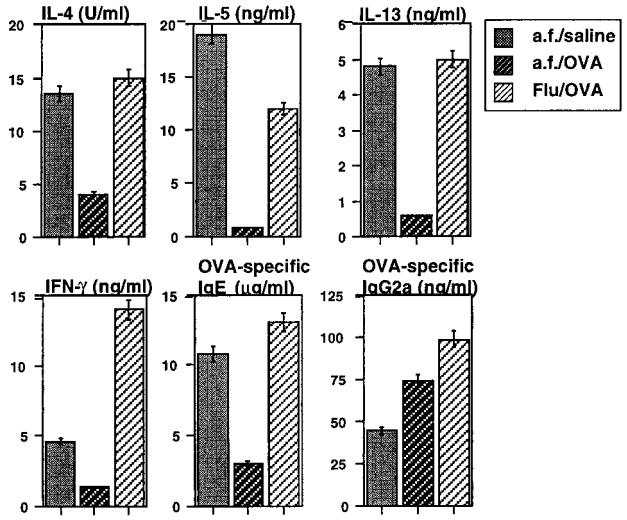

FIGURE 3. Concurrent exposure to influenza A virus and i.n. OVA generates OVA-specific T cells producing an unrestricted cytokine profile. BALB/c mice were treated as described in Fig. 2. *Flu/OVA,* Mice were infected with influenza A, then received i.n. OVA before treatment with the protocol to induce AHR. *a.f./OVA,* Mice were sham infected, then received i.n. OVA before treatment with protocol to induce AHR. *a.f./saline,* Mice were sham infected, then received i.n. saline before treatment with protocol to induce AHR. Forty-eight hours after the last exposure to i.n. OVA, bronchial lymph node cells were harvested and stimulated in vitro with increasing concentrations of OVA. In vitro cytokine production in response to 100 μg/ml OVA is shown. Cells cultured in vitro without OVA failed to produce cytokines. Results are expressed as mean ± SD for duplicate or triplicate cultures. Before sacrifice, serum was collected and quantitated for OVA-specific IgE and IgG2a by isotype-specific ELISA. Results are expressed as mean ± SD of the values for six to eight mice/group.

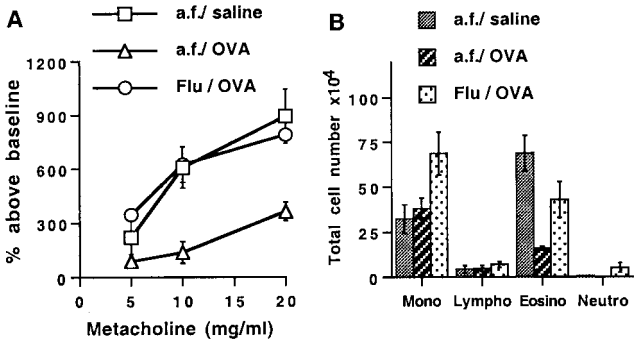

FIGURE 4. Concurrent exposure to influenza A virus and i.n. OVA accentuates the development of AHR and inflammation. BALB/c mice were treated as described in Fig. 2, and evaluated for AHR 24 h after the last i.n. dose of OVA. *A*, AHR in response to inhaled methacholine was measured in a whole-body plethysmograph. The results are expressed as mean ± SD of the percentage above baseline Penh values for six to eight mice/group. *B*, BAL was performed and the fluid was examined for content of macrophages/monocytes, lymphocytes, eosinophils, and neutrophils. The data are expressed as mean ± SD total number of each cell type/mouse. BAL fluid from normal untreated mice had $10–30 \times 10^4$ macrophages and $<0.5 \times 10^4$ eosinophils.

influenza A infection 3 days before exposure to i.n. OVA (Flu/OVA) resulted in a significant degree of AHR, similar to that observed in the fully sensitized control mice treated to induce AHR (a.f./saline). On the contrary, mice tolerized by the administration of i.n. OVA (a.f./OVA) before sensitization and challenge with OVA developed negligible AHR, as we have previously shown.

The degree of AHR in these mice correlated with the quantity of eosinophils found in the airway, as assessed in BAL fluid. Tolerized mice (a.f./OVA) had few inflammatory cells in BAL fluid, while mice infected with influenza before exposure to i.n. OVA (Flu/OVA) had a substantial increase in the levels of eosinophils and monocytes (Fig. 4*B*). As expected, control mice infected with influenza A, not exposed to the initial dose of i.n. OVA, but treated with the protocol to induce AHR developed a high degree of AHR, with significant airway eosinophilia (data not shown).

The degree of AHR and eosinophilia in the lungs of these mice also reflected the histology in the lungs of mice taken 1 day after the last administration of i.n. OVA. In control sensitized mice (a.f./saline), the bronchiolar airways were filled with mucus and were surrounded by an intense inflammatory infiltrate (Fig. 5*A*). In contrast, the tolerized mice, exposed to i.n. OVA (a.f./OVA) before immunization to induce AHR, had remarkably little evidence of inflammatory changes (Fig. 5*B*) and were protected from AHR. However, abrogation of i.n. tolerance and loss of protection against AHR due to influenza virus infection correlated with significant inflammatory histopathology in the lungs. In these mice (Flu/OVA), the high level of AHR correlated with dense peribronchiolar, perivascular, and interstitial inflammation, consisting mainly of lymphocytes, mononuclear cells, and eosinophils (Fig. 5*C*). Furthermore, many of the bronchiolar airways were filled with mucus

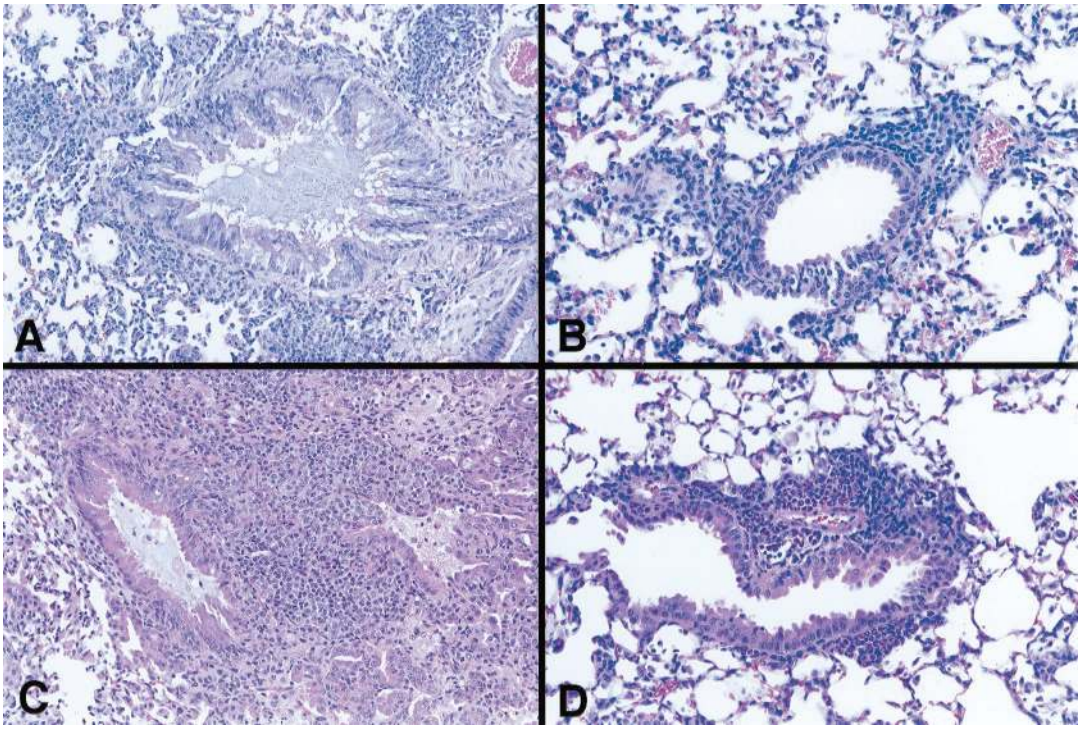

FIGURE 5. Lung histology of mice infected with influenza A and exposed to i.n. OVA. After the indicated treatment, all mice were immunized with OVA i.p. in alum and rechallenged with i.n. OVA three times. Twenty-four hours after the last dose of i.n. OVA, the lungs were removed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (×200). *A,* Before immunization, mice were given control a.f. and saline. A mononuclear cell infiltrate is present in peribronchial and perivascular areas, consisting primarily of eosinophils and lymphocytes. Abundant mucin is present within the bronchial lumen and within the cytoplasm of the bronchial epithelial cells. *B*, Before immunization, mice were exposed to i.n. OVA. Minimal peribronchiolar inflammation is present, composed primarily of mononuclear cells. The bronchial epithelial cells are normal, and the bronchial lumen is patent and free of mucus. *C*, Before immunization, mice were concurrently exposed to influenza A virus and i.n. OVA. Marked inflammation and airway mucus is present, with dense peribronchiolar and perivascular infiltrates, consisting of lymphocytes, eosinophils, and some neutrophils. The bronchial lumen is filled with mucus. *D*, Mice infected with influenza virus were exposed 15 days later to i.n. OVA. Moderate airway changes are seen in the lungs, with peribronchiolar infiltrates consisting of mononuclear cells and eosinophils. The histology of the lungs of mice that received saline in alum i.p., followed by i.n. OVA was essentially normal (16).

and contained disrupted epithelium. Thus, influenza A virus infection abrogated the induction of i.n. tolerance, which resulted in exacerbation of allergen-induced respiratory inflammation.

Exacerbation of AHR by influenza virus is independent of IL-4 and IL-5

Because influenza A virus infection induced the production of a broad spectrum of cytokines, including IL-4 and IL-5, which are important in the induction of AHR (22–24), we asked whether the exacerbation of AHR and inflammation by influenza A in our system was dependent on these two cytokines. For this purpose, IL-4$^{-/-}$ BALB/c mice were infected with influenza A virus and exposed to i.n. OVA before immunization with our protocol to induce AHR. To eliminate the influence of IL-5 on the development of the inflammatory response, the mice were also treated with a neutralizing anti-IL-5 mAb during the initial i.n. treatment, as well as during the later i.n. challenges. Control IL-4$^{-/-}$ and anti-IL-5 mAb-treated mice developed significant AHR when immunized with our protocol to induce AHR (Fig. 6*A*). This suggested that the presence of IL-4 and IL-5 was not an absolute requirement for the induction of AHR (25). Additionally, exposure of such mice lacking active IL-4 and IL-5 to i.n. OVA in the absence of infection (a.f./OVA), led to the inhibition of AHR (Fig. 6*A*). In this group, the reduction in AHR was associated with a reduced T cell proliferative response (16), indicating that the induction of i.n. tolerance was independent of the presence of IL-4 or IL-5. In contrast, the IL-4$^{-/-}$, anti-IL-5 mAb-treated mice (as well as wild-type mice), when exposed concurrently with i.n. OVA and influenza A virus (Flu/OVA), developed significant AHR (Fig. 6*A*). This implied that the induction of AHR by influenza virus was also independent of IL-4 and IL-5.

Although the development of AHR was not influenced by the absence of active IL-4 and IL-5, the character of the inflammatory infiltrate in the lung was greatly affected. In particular, the number of eosinophils in BAL fluid from mice lacking IL-4 and IL-5 was markedly reduced (Fig. 6*B*). Similarly, examination of their lung histology revealed inflammatory infiltrates with almost no eosinophils and no mucus production, while the pulmonary infiltrates present in wild-type controls contained numerous eosinophils and significant mucus production (data not shown). These observations indicated that IL-4 and IL-5 regulated the recruitment of eosinophils and mucus production in the lung, but suggested that cytokines other than IL-4 and IL-5 (e.g., IL-13) produced in response to infection with influenza A, or mechanisms unrelated to cytokines, controlled the development AHR.

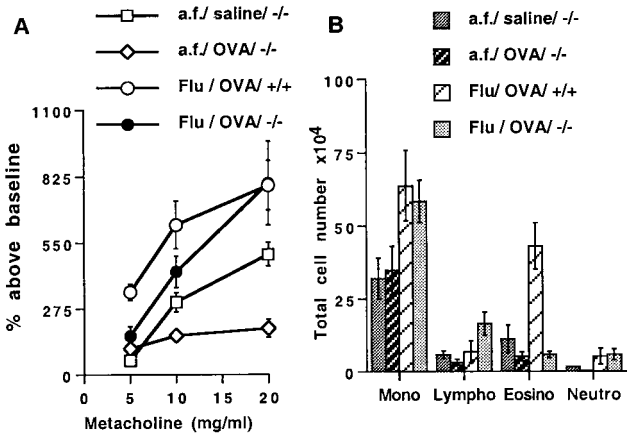

FIGURE 6. Induction of AHR and inflammation by influenza virus is independent of IL-4 and IL-5. IL-4$^{-/-}$ mice received anti-IL-5 mAb prior and during all of the i.n. challenges, and the mice were treated as described in Fig. 2. *A*, Twenty-four hours after the last i.n. dose of OVA, AHR in response to methacholine was assessed. The results are expressed as mean ± SD of the percentage above baseline Penh values for six to eight mice/group. *B*, BAL was performed, and the fluid was examined for content of monocytes, lymphocytes, eosinophils, and neutrophils. The data are expressed as mean ± SD of the total number of each cell type/mouse.

Exposure to i.n. OVA late after influenza A infection does not induce T cell tolerance, but protects against the development of AHR

We next determined whether exposure to i.n. OVA late after influenza A virus infection affected the induction of tolerance. For this purpose, mice received i.n. OVA 15 or 30 days after inoculation with virus before treatment with our standard protocol to induce AHR (Fig. 1, *B* and *C*). As expected, T cells from control mice exposed to a.f. followed by i.n. OVA (a.f./OVA) before i.p immunization with OVA failed to proliferate when restimulated in vitro (Fig. 7). However, T cells from mice infected with influenza A then given i.n. OVA 15 days (Fig. 7) as well as 30 days (data not shown) later (Flu/OVA) proliferated as vigorously in vitro in response to OVA as did T cells from control sensitized mice (a.f./saline), indicating that the induction of i.n. tolerance was still abrogated by infection with influenza A.

The cytokine profile of the T cells from these mice responding to OVA in vitro was examined. The production of IL-4, IL-5, and IL-13 by T cells from mice exposed to i.n. OVA late after inoculation with virus (Flu/OVA) was reduced, with a more profound decrease observed in mice that received i.n. OVA 30 days after infection (Fig. 8). In addition, the down-regulation in Th2 cytokine secretion was associated with enhanced IFN-γ production. Moreover, OVA-specific IgE in serum from these mice was significantly reduced compared with control sensitized mice, with a more significant decrease observed in mice that received OVA 30 days

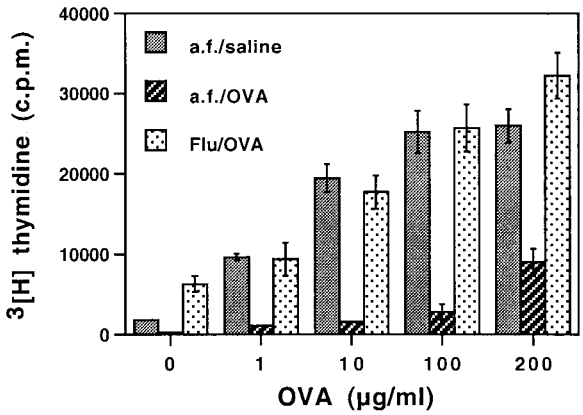

FIGURE 7. Exposure to i.n. OVA late after influenza A infection does not induce T cell tolerance. BALB/c mice were exposed to i.n. OVA or saline 15 days after infection with influenza A virus. The mice were subsequently immunized i.p. with OVA in alum and rechallenged i.n. with OVA 10 days later. Forty-eight hours after the last administration of i.n. OVA, bronchial lymph node cells were harvested and cultured in vitro with increasing concentrations of OVA. *Flu/OVA,* Mice were infected with influenza A, then received i.n. OVA before treatment with the protocol to induce AHR. *a.f./OVA,* Mice were sham infected, then received i.n. OVA before treatment with protocol to induce AHR. *a.f./saline,* Mice were sham infected, then received i.n. saline before treatment with protocol to induce AHR. Proliferation was determined by measuring the incorporation of [^{3}H]thymidine over the last 18 h of culture. Representative results using six to eight mice/group are shown. The results are expressed as mean cpm ± SD of triplicate cultures.

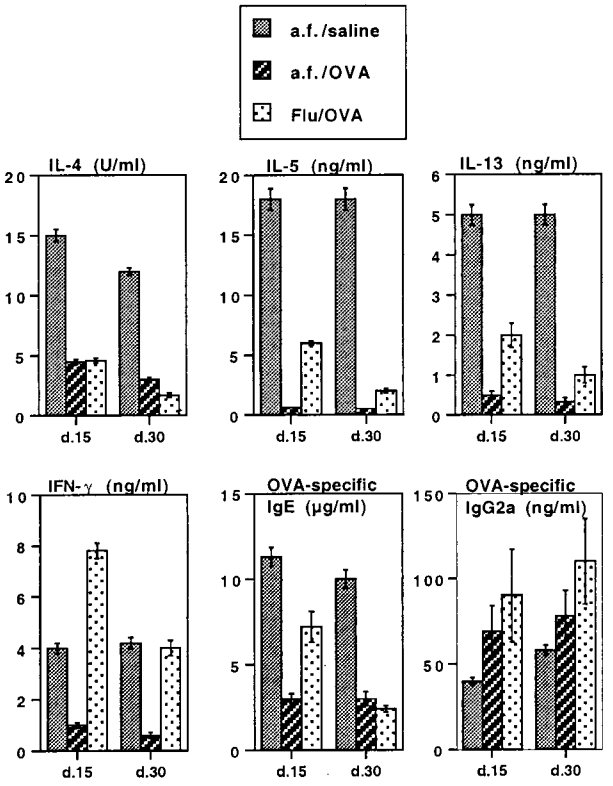

FIGURE 8. Exposure to i.n. OVA late after influenza A infection induces Th1-prominent immunity. BALB/c mice were treated as described in Fig. 7. Forty-eight hours after the last exposure to i.n. OVA bronchial lymph node cells were harvested and stimulated in vitro with increasing concentrations of OVA. In vitro cytokine production in response to 100 μg/ml OVA is shown. Cells cultured in vitro without OVA failed to produce cytokines. Results are expressed as mean ± SD for duplicate or triplicate cultures. Before sacrifice, serum was collected and quantitated for OVA-specific IgE and IgG2a by isotype-specific ELISA. Results are expressed as mean ± SD of the values for six to eight mice/group.

after viral infection (Fig. 8). Additionally, serum OVA-specific IgG2a levels in the infected mice (Flu/OVA) exceeded those of the control sensitized (a.f./saline) and tolerized groups (a.f./OVA; Fig. 8).

The reduction in Th2 cytokine production in mice that received i.n. OVA 15 or 30 days after viral infection (Flu/OVA) was associated with protection against the development of AHR. Thus, airway reactivity in these mice was negligible and similar to that observed in the i.n. tolerized mice (a.f./OVA; day 15, Fig. 9*A*; day 30, data not shown). Additionally, the number of eosinophils observed in BAL fluid in these mice was reduced, particularly in mice that received i.n. OVA 30 days after viral infection (Fig. 9*B*). Similarly, a reduction in the inflammatory infiltrate was observed in the lungs of these mice (Fig. 5*D*). These results taken together indicate that influenza A alters the pulmonary environment, disrupting its capacity to induce T cell unresponsiveness, and later shifting immune responsiveness toward a Th1 pattern, which protects against the development of AHR.

Protection from AHR in mice exposed to i.n. OVA late after influenza infection is dependent on IFN-γ

We examined the mechanism by which exposure to i.n. OVA late after influenza A infection protected the mice from the development of AHR. To this end, mice were treated with a neutralizing anti-IFN-γ mAb during viral infection, as well as during all exposures to i.n. OVA. Fig. 10*A* shows that absence of active IFN-γ in influenza A-infected mice exposed to i.n. OVA 15 or 30 days later (Flu/OVA/anti-IFN-γ) reversed the protective effects of the virus on AHR. The increase in airways reactivity in these mice was associated with a notable increase in airway eosinophilia (Fig. 10*B*). Thus, protection from AHR in this system was very much dependent on the presence of IFN-γ, produced as a consequence of influenza A virus infection.

Discussion

Viral infections occurring during early childhood have been implicated in influencing the development of respiratory allergy, but their specific effects upon allergen sensitization, either protective or deleterious, have been controversial. In the present study we examined this issue using influenza A virus infection in a murine model of asthma. Our results indicated that viral infection around the time of respiratory exposure to aeroallergens disrupted the establishment of allergen-specific immunological tolerance. Thus, respiratory exposure to OVA 3 days after influenza A viral infection resulted in allergen sensitization, expansion of allergen-specific $CD4^+$ T cells, and was associated with the development of AHR and the production of OVA-specific IgE, IL-4, IL-5, IL-13, and IFN-γ. However, respiratory exposure to allergen late after influenza A infection, during the recovery period from infection, disrupted tolerance induction, but promoted an IFN-γ-predominant response which was associated with protection from the development of AHR. Thus, the respiratory effects of influenza A infection depended on the phase of infection during which the primary exposure to OVA took place.

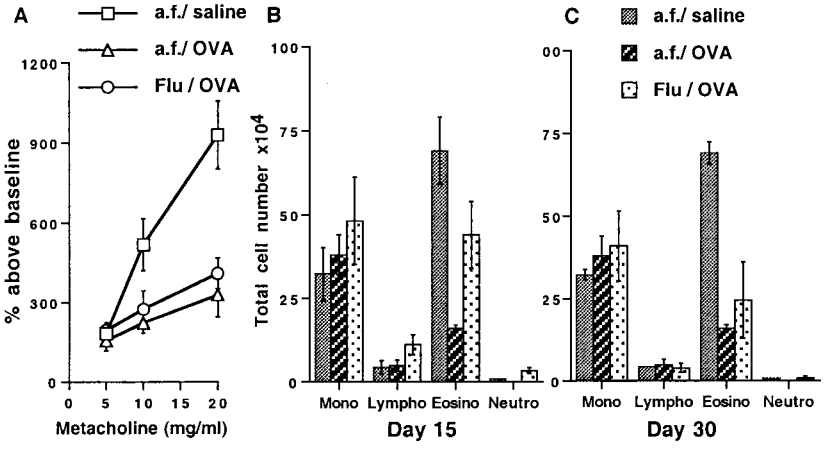

FIGURE 9. Exposure to i.n. OVA late after influenza A infection protects against the development of AHR. BALB/c mice were treated as described in Fig. 7, and evaluated for AHR 24 h after the last dose of i.n. OVA. *A*, AHR in response to inhaled methacholine was assessed in mice completing the day 15 protocol. The results are expressed as mean ± SD of the percentage above baseline Penh values for six to eight mice/group. *B*, BAL was performed in mice completing the day 15 protocol, and the fluid was examined for content of monocytes, lymphocytes, eosinophils, and neutrophils. The data are expressed as mean ± SD of the total number of each cell type/mouse. *C*, BAL fluid analysis from mice completing the day 30 protocol.

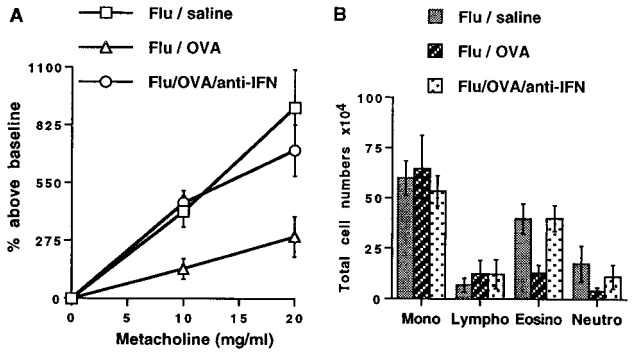

FIGURE 10. Protection from AHR in mice exposed to i.n. OVA late after influenza A infection is dependent on IFN-γ. BALB/c mice were treated with anti-IFN-γ mAb before infection with influenza A virus and during all challenges with i.n. Ag. Mice were immunized as described in Fig. 7, and were examined for AHR. *A*, AHR was assessed and the results are presented as mean ± SD of the percentage above baseline Penh values for six to eight mice/group. *B*, BAL was performed, and the fluid was examined for content of monocytes, lymphocytes, eosinophils, and neutrophils. The data are expressed as mean ± SD of the total number of each cell type/mouse.

In healthy, nonallergic individuals, mechanisms exist that limit immune responses to nonreplicating, environmental Ags encountered at the respiratory mucosal surface. The respiratory mucosa is continuously exposed to a wide variety of environmental Ags, and indiscriminate immunological responses to innocuous Ags could be detrimental, and cause tissue injury that interferes with respiratory gas exchange. We and others have demonstrated that exposure of the respiratory mucosa to nonreplicating Ags in the absence of inflammatory signals, results in peripheral immunological tolerance, characterized by a reduced T cell proliferative response upon subsequent encounter with the same Ag (13, 14, 26). Exposure to respiratory Ag transiently activates Ag-specific $CD4^+$ T cells, but this is followed by a degree of T cell deletion and by functional inactivation of any remaining Ag-specific $CD4^+$ T cells (14, 26). Moreover, the induction of allergen-specific tolerance by respiratory exposure to allergen prevents subsequent production of allergen-specific IgE and prevents the development of allergen-induced AHR (16), strongly suggesting that immunological tolerance induced by respiratory exposure to allergen protects against the development of allergic disease and asthma.

The functional outcome of primary contact with aeroallergen is of critical significance for the regulation of allergic reactivity, because the initial contact with allergen can generate either tolerance or immune responsiveness. Infection with influenza A generates an inflammatory process in the lung parenchyma and associated lymphoid tissues that is characterized early on by increased levels of both Th1 and Th2 cytokines (18, 27) and by the activation and maturation of dendritic cells (28). Exposure of the respiratory mucosa to OVA under these circumstances did not result in tolerance induction, but rather facilitated allergen sensitization, enhanced the production of OVA-specific IgG2a and IgE, and the expansion of OVA-specific CD4 T cells secreting high amounts of IL-4, IL-5, IL-13, and IFN-γ. Moreover, mice infected with influenza A and concurrently exposed to i.n. OVA developed significant AHR and eosinophilic inflammation. These results demonstrated that influenza virus infection disrupted allergen-induced tolerance and allowed OVA sensitization resulting in the development of AHR.

In our system, neutralization of IL-4 and IL-5 in mice exposed to i.n. OVA early after infection with influenza A virus (by treating IL-4 knockout mice with anti-IL-5 mAb) did not abrogate the induction of AHR. Nevertheless, the absence of IL-4 and IL-5 was associated with greatly reduced lung eosinophilia, confirming the important role of these two cytokines in the recruitment of eosinophils in the airways, but not in the development of AHR (25, 29). The observed AHR in these mice correlated with the presence of IL-13, suggesting that the induction of IL-13 by influenza A infection might be responsible for the development of AHR. IL-13 has been shown recently to play a primary role in the induction of AHR in murine models of asthma (30, 31), and was produced by bronchial lymph node T cells when our mice were exposed to OVA 3 days but not 30 days after influenza A infection. Alternatively, other cytokines, for example IL-9, or other nonimmunological mechanisms related to the cytopathic effect of the virus on respiratory epithelium and smooth muscle might be responsible for the elevated bronchial reactivity (6, 25, 29). In addition, virus-induced $CD8^+$ T cells producing Th2 cytokines might aggravate the development of AHR (32–34).

The AHR induced in infected mice exposed to i.n. OVA 3 days after viral inoculation could not be reversed by the Th1-like mediators, such as IFN-γ, which were also co-produced during the influenza A virus infection. These Th1-like factors, including cytolytic CD8 cells, are prominent in virus-infected mice, are required for eliminating influenza A virus (35), and might direct the differentiation of bystander responses toward a Th1 predominance. However, it appears that the presence of high levels of Th2 cytokines at the initial stage of infection dominated over the effects of the Th1 cytokines (36), resulting in an allergic inflammatory response and AHR. Alternatively, it is possible that when both Th1 and Th2 cytokines are present in large quantities, they are synergistic or codominant in producing inflammatory responses (19, 37, 38). This is consistent with observations demonstrating that Th1 cells participate concomitantly with Th2 cells in the airways of asthmatic individuals, and appear to enhance the development of allergic inflammation (19, 39, 40).

Although influenza virus is completely cleared from the lungs within 7–10 days after the initiation of infection (17), the inflammatory effects of influenza A virus in the lung persisted for several weeks after the initiation of infection. Although early exposure to i.n. OVA enhanced the development of AHR, exposure of mice to i.n. OVA late after influenza A infection protected against AHR. Thus, mice exposed to i.n. OVA 30 days after influenza A infection were not tolerized, but developed an IFN-γ-dominant OVA-specific immune response, accompanied by reduced airway inflammation and reactivity, and with production of little or no IL-4, IL-5, or IL-13. In these mice, a degree of airway inflammation was present, characterized by the accumulation of monocytes, lymphocytes, neutrophils, and some eosinophils. The protective effect of influenza A virus on AHR was dependent on IFN-γ, because it was reversed by the administration of a neutralizing anti-IFN-γ mAb. These results indicate that influenza virus infection can direct bystander responses toward the Th1 pathway, abrogating respiratory allergen-induced tolerance, and producing allergen-specific immune responses that protect against the development of AHR, but only at a time point late after infection. Although potent allergen-specific Th1 responses in the lung produce an inflammatory process that can result in parenchymal tissue damage (19), the effects of the IFN-γ-dominant profile observed in these mice may have been mitigated by the simultaneous local production of inhibitory or protective cytokines such as IL-10 (41, 42).

In summary, our results clearly indicate that respiratory infection with influenza A alters the pulmonary environment and disrupts its capacity to induce allergen-specific T cell unresponsiveness. The interruption of respiratory-induced tolerance resulted in the generation of functional allergen-specific Th cell immunity, the

features of which depended on the stage of infection when primary respiratory contact with the allergen occurred. Concurrent exposure to allergen produced a pathological Th2-dominant response, associated with the development of AHR, whereas late exposure to allergen resulted in Th1 predominance that protected against the development of AHR. These results demonstrate that respiratory viral infection may provide either protective or deleterious effects with regard to allergen-sensitization and the development of AHR.

Acknowledgments

We thank Dr. P. Doherty for the HK-31(H3N2) strain of influenza A virus and the a.f. controls. We also thank Drs. R. Coffman, DNAX Research Institute; S. Levy, Stanford University; and E. Gelfand, National Jewish Center for Immunology and Respiratory Medicine for invaluable reagents.

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