

Respiratory Syncytial Virus Protects Against the Subsequent Development of Japanese Cedar Pollen-Induced Allergic Responses

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Respiratory syncytial virus (RSV) infection has been hypothesized to be a risk factor for the development of allergy and asthma, but epidemiologic studies in humans still remain inconclusive. The association between RSV infection and allergic diseases may be dependent on atopic background and previous history of RSV infection. In this study, the influence of the timing of RSV infection on the development of Japanese cedar pollen (JCP)-induced allergic responses was examined. BALB/c mice were intranasally infected with RSV before or after sensitization to JCP. Production of cytokines in the culture fluid of lung parenchyma cells and the level of antigen-specific antibodies in the serum were determined. It became clear that JCP was a strong inducer for the elicitation of Th2-type responses, characterized by production of interleukin (IL)-4 and IL-5 in the lung and JCP-specific IgE antibody in the serum. RSV infection, however, suppressed JCP-induced allergic responses by decreasing the production of Th2-like cytokines and Th2-type antibodies. This phenomenon was observed more clearly in the groups that were infected with RSV, 2 weeks or 2 days before sensitization to JCP. The inhibitory mechanism of RSV infection seems to be due to RSV-induced Th1 type dominant environment, which down-regulated the Th2-type responses subsequently induced by allergen sensitization. On the other hand, JCP-inoculation altered RSV-induced immune responses to shift from Th1- to Th2-type dominance, by inhibiting RSV-induced Th1-like cytokine production. These data provide evidence that under a certain condition, RSV infection may play a protective role in JCP-induced allergic responses. **J. Med. Virol. 79:1600–1605, 2007.** © 2007 Wiley-Liss, Inc.

KEY WORDS: respiratory syncytial virus (RSV); Japanese cedar pollen

(JCP); Th1/Th2-like cytokine; specific antibody

INTRODUCTION

Respiratory syncytial virus (RSV), a member of the family *Paramyxoviridae*, genus *Pneumovirinae*, is the most common cause of respiratory-tract infections in infants and young children [Everard and Milner, 1992]. Because severe RSV infection induces asthma-like symptoms (wheezing and airway hyperreactivity among others), epidemiological studies have been carried out to investigate the role of RSV infection in respiratory allergic diseases. Previous studies suggested that severe RSV bronchiolitis during infancy increased the prevalence of both asthma and allergic sensitization in children [Welliver and Duffy, 1993; Roman et al., 1997]. The evidence that RSV infection induced Th2-like cytokines and specific IgE antibody response led to the paradigm that allergen sensitization and RSV infection synergistically augmented the risk for the development of allergic disease. However, other studies demonstrated no association between RSV infection and an increased risk for developing allergic disease [Pullan and Hey, 1982; Carlsen et al., 1987]. It was reported recently that primary RSV infection in BALB/c mice induced a type 1 dominant cytokine profile, characterized by production of IL-12 and IFN- γ which down-regulated a Th2-like immune response [Graham et al., 1993; van Schaik et al., 2000; Peebles et al., 2001a].

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Thus, by reducing the capacity to mount a type 2 response, viral infection is expected to decrease the development of allergic sensitivity and asthma. The precise role of RSV infection in the development of allergic disease has remained a paradox.

Allergic asthma is a Th2-associated airway inflammation, characterized by the infiltration of eosinophils and the production of interleukin (IL)-4, IL-5, and IL-13 by T lymphocytes in response to certain antigens [Savelkoul and Neijens, 2000; Busse and Lemanske, 2001]. Several recent studies suggest that the time between viral [Tsitoura et al., 2000; Yamamoto et al., 2001] or bacterial [Chu et al., 2003] infection and exposure to allergens may be critical in the development of respiratory allergy in mice. To gain more insight on the association between RSV infection and allergen-induced Th2 responses, BALB/c mice were intranasally inoculated with live RSV before or after sensitization to an allergen of Japanese cedar (*Cryptomeria japonica*) pollen (JCP). JCP induces allergic rhinitis among more than 10% of the population in Japan in every spring.

MATERIALS AND METHODS

Animals

Specific pathogen-free male BALB/c mice (6–7 weeks of age) were purchased from SLC (Hamamastu, Japan). Mice had fresh water and autoclaved food and were kept at 23°C under bio-clean conditions throughout all experiments. To avoid laboratory contamination, all virus- and allergen-inoculated mice were housed in negatively pressurized isolators equipped with a ventilation system through a high-efficiency particulate air filter (AH model; Nihon-Ika, Osaka, Japan). This work was approved by the Institutional Animal Care and Use Committee of Fukui University School of Medicine, Japan.

Virus

Human respiratory syncytial virus type A2 (RSV A2) was kindly supplied by Dr. K. Hashimoto, Fukushima Medical University School of Medicine, Fukushima, Japan. The virus was propagated in monolayers of HEp-2 cells (ATCC) grown in Eagle's minimum essential medium (Nissui Pharmacia, Tokyo, Japan) supplemented with 2% heat-inactivated fetal calf serum (FCS, Greiner, Frickenhausen, Germany). At the time point of the maximum cytopathic effect, cells were harvested and disrupted by sonication in the same culture medium. The suspension was clarified by centrifugation at 2,000g for 20 min at 4°C and the resulting supernatant was layered on top of a sucrose gradient (30% sucrose in 50 mM Tris buffered-normal saline solution containing 1 mM ethylenediamine-tetraacetate (pH 7.5)) and further centrifuged at 100,000g for 2 hr at 10°C. The pellet was resuspended in 10 mM phosphate-buffered saline (PBS) and stored in aliquots at –80°C. Virus titer was expressed as a 50% tissue culture infectious dose calculated using the method of Reed and Muench [1938].

To inactivate the virus, an aliquot of the viral suspension was irradiated with ultraviolet (UV) light for 15 min on ice [Reuman et al., 1990]. After irradiation, no infectivity of RSV could be detected.

Allergens

Japanese cedar (*Cryptomeria japonica*) pollen crude extract (JCP) was purchased from Cosmo Bio Co. (Tokyo, Japan).

Experimental Design

Six groups of mice were studied in this report (Fig. 1). Mice, under light anesthesia (pentobarbital sodium, 0.025 mg/g body weight, intraperitoneal administration), were infected intranasally with RSV in 20 µl of suspension containing 2×10^6 TCID₅₀ per mouse, or inoculated with 20 µl of PBS containing 20 µg of JCP per mouse at the different time points, according to the schedule shown in Figure 1. Samples were then collected at the described time points.

Preparation of Single-Cell Suspensions From the Lung

Mice were anesthetized and the lung was flushed in situ with 20 ml of PBS via cannulation of the heart to remove the intravascular blood pool. Minced lung tissues were incubated at 37°C for 1 hr on a rocker with 200 µg/ml collagenase D and 40 µg/ml DNase I (both from Roche Molecular Biochemicals, Mannheim, Germany) in 10 ml of RPMI 1640 medium supplemented with 10% FCS, 2×10^{-5} M 2-mercaptoethanol and antibiotics. Subsequently, the enzyme-digested lung tissues were passed through a stainless steel mesh. Single-cell suspensions from the digested lung were collected through density gradient centrifugation with lymphocyte-separation solution (Antibody Institute, Gumma, Japan) and resuspended in RPMI1640 medium.

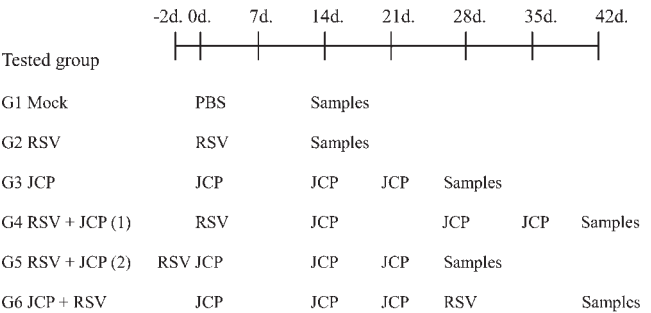


Fig. 1. Time line of experimental protocol. G1 mock group, mice were inoculated with PBS; G2 RSV group, mice were infected with live RSV at an inoculum dose of 2×10^6 TCID₅₀ per mouse; G3 JCP group, mice were inoculated with JCP at a dose of 20 µg per mouse; G4 RSV + JCP (1) group, mice were infected with RSV 2 weeks before JCP sensitization; G5 RSV + JCP (2) group, mice were infected with RSV 2 days before JCP sensitization; G6 JCP + RSV group, mice were infected with RSV after the final JCP sensitization. Five mice were used in each group.

Assay of Cytokine Production

Single-cell suspensions obtained from the lung parenchyma of inoculated mice were re-stimulated *in vitro* by co-cultivating with UV-inactivated RSV or JCP for 48 hr. The supernatant were then harvested and assayed for IL-4, IL-5, and IFN- γ titers by using a mouse cytokine detection ELISA kit (BioSource International, Camarillo, CA) in accordance with the manufacturer's instructions. The minimum detectable concentrations of IL-4, IL-5, and IFN- γ were 5, 3, and 1 pg/ml, respectively.

Quantification of Antibody Level

JCP or RSV-specific antibody (IgE, IgG1, and IgG2a) and total IgE titer in the serum were measured using a mouse antibody quantitative kit (Bethyl Laboratories, Montgomery, TX). Test samples were incubated at 37°C for 1 hr on microplates coated with 10 μ g of JCP or 3 μ g of purified RSV proteins. Bound antibodies were reacted with horseradish peroxidase-conjugated goat anti-mouse IgE, IgG1, or IgG2a antiserum, respectively. After washing, the antigen-antibody complex was incubated with enzyme substrate of tetramethylbenzidine (Sigma, St. Louis, MO) and the reaction was stopped by 2 M H₂SO₄. The absorbance value at 450 nm was measured in an automated microplate reader.

Statistical Significance

The two-tailed Mann-Whitney *U*-test and Student's *t*-test were used to determine whether there was a significant difference ($P < 0.05$) between the experimental and control group. Statistical analyses were done by

using StatView J4.11 software (SAS Institute, Cary, NC).

RESULTS

Enhanced Th2 Cytokine Production in JCP-Sensitized Mice

To investigate the activity of JCP on the induction of allergic responses, the levels of IL-4, IL-5, and IFN- γ in the culture fluids of lung parenchyma cells were determined. As shown in G3 of Table I, JCP-sensitization induced a significant increase in the level of Th2-like cytokines (IL-4 and IL-5), particularly after *in vitro* boosting of the lung cells with the homologous allergen of JCP. Re-stimulation of the lung parenchyma cells with the heterogeneous antigen of UV-RSV did not influence JCP-induced Th2-like cytokine production, suggesting that there were no cross-reactive antigens between JCP and RSV protein. IFN- γ production was undetectable in JCP sensitized mice even after being re-stimulated with the same allergen. This result demonstrated that JCP was a strong inducer on the production of Th2-like, but not Th1-like, cytokines.

Suppression of JCP-Induced Th2-Like Cytokine Production by RSV Infection

To elucidate the role of RSV infection in the development of JCP-induced allergic responses, live RSV was intranasally inoculated into BALB/c mice before or after sensitization to JCP, in accordance with the experimental protocol (Fig. 1). The prior infection with RSV, either 2 weeks (expressed as RSV + JCP (1)) or 2 days (expressed as RSV + JCP (2)) before JCP sensitization,

TABLE I. Cytokine Secretion From the Lung Parenchyma Cells Re-Stimulated *In Vitro* With *Cryptomeria Japonica* Pollen (JCP) Crude Extract or UV-Inactivated Respiratory Syncytial Virus (UV-RSV)*

Tested group	Stimulated with	Titer of cytokines (pg/ml)		
		IL-4	IL-5	IFN- γ
G1 Mock	JCP	<5.0	24.9 $\pm$ 12.5	<1.0
	UV-RSV	<5.0	<3.0	<1.0
	None	<5.0	<3.0	<1.0
G2 RSV	JCP	<5.0	340.3 $\pm$ 43.3	<1.0
	UV-RSV	<5.0	68.9 $\pm$ 9.3	5.2 $\pm$ 1.6
	None	<5.0	31.2 $\pm$ 0.8	<1.0
G3 JCP	JCP	195.7 $\pm$ 87.2	1584.6 $\pm$ 31.2	<1.0
	UV-RSV	<5.0	66.3 $\pm$ 59.7	<1.0
	None	<5.0	77.6 $\pm$ 54.1	<1.0
G4 RSV + JCP (1)	JCP	65.7 $\pm$ 19.4 ^a	1058.4 $\pm$ 608.1	<1.0
	UV-RSV	<5.0	<3.0	<1.0
	None	<5.0	<3.0 ^a	<1.0
G5 RSV + JCP (2)	JCP	69.6 $\pm$ 23.7 ^a	1037.5 $\pm$ 856.1	<1.0
	UV-RSV	<5.0	<3.0	<1.0
	None	<5.0	<3.0 ^a	<1.0
G6 JCP + RSV	JCP	156.9 $\pm$ 43.6	1624.2 $\pm$ 987.6	<1.0
	UV-RSV	<5.0	<3.0	<1.0
	None	<5.0	<3.0 ^a	<1.0

*Lung parenchyma cells of BALB/c mice in each tested group were obtained at the time point indicated in Figure 1. The lung cell suspension obtained from one mouse was divided into three equal parts. The first part was re-stimulated *in vitro* with JCP allergen (100 μ g/ml), the second part with UV-inactivated RSV (the original, 1×10^6 TCID₅₀/ml) and the last part with mock-treatment for 48 hr. Concentrations of IL-4, IL-5, and IFN- γ in the culture supernatants were determined by ELISA. Each value represents the mean $\pm$ SD of five culture supernatant samples obtained from five mice.

^aSignificant difference ($P < 0.01$, by Mann-Whitney *U*-test), compared with the G3 JCP group.

decreased JCP-induced Th2-like cytokine production, especially the production of IL-4 (Table I, G4 and G5). The amount of IL-4 in the culture fluids of the lung parenchyma cells stimulated in vitro with the homologous allergen of JCP, was reduced from 195.7 ± 87.2 in JCP-sensitized mice to 65.7 ± 19.4 in RSV + JCP (1) mice and to 69.6 ± 23.7 in RSV + JCP (2) mice ($P < 0.01$). The titer of IL-5 was decreased from 77.6 ± 54.1 pg/ml in JCP-sensitized mice to <3.0 pg/ml in RSV plus JCP-mice.

However, RSV infection after sensitization to JCP, has a weak inhibitory effect on JCP-induced Th2-like cytokine production (Table I, G6). The titer of IL-4 and IL-5 in the culture fluids of JCP-sensitized mice were reduced slightly by the subsequent RSV infection.

Decrease in the Level of JCP-Induced Th2-Type Antibodies by RSV Infection

Because the production of antigen-specific Th2-type antibodies is prerequisite for the immunologic response leading to allergic diseases, the antibody titer in the serum of tested mice was determined using the ELISA method. JCP-sensitized mice exhibited Th2-type dominant antibody responses, characterized by increased titers of total serum IgE, JCP-specific IgE as well as JCP-specific IgG1 (Table II, G3). By prior RSV infection the levels of these allergy-related antibodies significantly diminished. JCP-specific IgE and IgG1 antibody became undetectable and the total serum IgE was decreased to the normal level (Table II, G4 and G5). RSV infection after sensitization to JCP showed a slight inhibitory activity on JCP-induced Th2-type antibody response (Table II, G6), suggesting that the timing of RSV infection appeared to be more critical on the suppression of JCP-induced antibody response.

The level of JCP-specific IgG2a antibody, a kind of Th1-type antibody, was undetectable in the serum of all tested groups (Table II). This demonstrated that humoral immune responses induced by JCP-sensitization were essentially shifted to Th2-type dominance.

Influence of JCP Inoculation on RSV-Induced Immune Responses

RSV infection alone induced a small amount of IFN- γ and a middle level of IL-5 production in all five BALB/c

mice tested after in vitro boosting of the lung cells with the homologous antigen of UV-RSV (Table I, G2). However, JCP in vivo inoculation, before or after RSV infection, decreased RSV-induced Th1 and Th2 cytokine production. The titer of IFN- γ and IL-5 was undetectable in the culture fluids of the lung parenchyma cells of RSV-infected JCP-sensitized mice, even after re-stimulation in vitro with the homologous antigen of UV-RSV (Table I, G4, G5, and G6).

On the other hand, RSV-infection alone induced a stronger Th1-type antibody (IgG2a) response and a middle level of Th2-type antibody (IgG1) production in the serum of mice (Table III, G2). Although JCP inoculation enhanced both of the RSV-specific IgG2a and IgG1 antibody production, the ratio of IgG2a: IgG1 was decreased from 23.1 in RSV-infected mice to 13.5 and 19.4 in RSV-infected, JCP-sensitized mice (Table III, G4 and G5), and to 14.5 in JCP-sensitized, RSV-infected mice ($P < 0.05$; Table III, G6). It seems likely that JCP-sensitization, before or after RSV infection, changed RSV-induced humoral immune responses to shift toward Th2-type dominance.

Furthermore, JCP-sensitization enhanced the total serum IgE from 66.3 pg/ml in RSV alone mice to 80.6 and 98.2 pg/ml in RSV plus JCP mice, and to 126.5 pg/ml in JCP plus RSV mice ($P < 0.05$; Table III). However, no RSV-specific IgE antibody response was detected in all groups tested.

DISCUSSION

The association between RSV infection in infancy and the development of respiratory allergy later in life is still inconclusive [Graham et al., 1993; Welliver and Duffy, 1993; Peebles et al., 2001a]. This article focused on the timing of RSV infection in the development of JCP-induced allergic reactions. Prior RSV infection, either 2 weeks or 2 days before sensitization to JCP, diminished the production of allergen-induced Th2-like cytokine, total serum IgE, and allergen-specific IgE and IgG1 antibodies. It is well known that Th1/Th2-like cytokine plays an important role in the regulation of a certain allergen-induced allergic reaction. Type 2 cytokine profile is a critical component of allergen-induced allergic disease. Increased level of IL-4 in BAL

TABLE II. Production of Total Serum IgE and JCP-Specific Serum Ig Isotypes by Intranasal Inoculation of BALB/c Mice With *Cryptomeria Japonica* Pollen (JCP) Crude Extract and Respiratory Syncytial Virus (RSV)*

Tested group	Antibody titer (ng/ml) in serum			
	Total IgE	JCP-specific IgE	JCP-specific IgG1	JCP-specific IgG2a
G1 Mock	77.1 ± 26.7	<3.9	<3.9	<3.9
G2 RSV	66.3 ± 23.1	<3.9	<3.9	<3.9
G3 JCP	142.8 ± 76.9	20.3 ± 8.8	16.5 ± 6.7	<3.9
G4 RSV + JCP (1)	80.6 ± 11.5^a	$<3.9^a$	$<3.9^a$	<3.9
G5 RSV + JCP (2)	98.2 ± 54.5^a	$<3.9^a$	$<3.9^a$	<3.9
G6 JCP + RSV	126.5 ± 53.1	13.3 ± 6.1	14.6 ± 8.2	<3.9

*Serum was collected at the time indicated in Figure 1. Total IgE and JCP-specific IgE, IgG1, and IgG2a were detected by ELISA kit. Each value represents the mean $\pm$ SD of five serum samples obtained from five mice.

^aSignificant difference ($P < 0.01$, by Mann-Whitney *U*-test), compared with the G3 JCP group.

TABLE III. Production of Total Serum IgE and RSV-Specific Serum Ig Isotypes in BALB/c Mice Intranasally Inoculated With *Cryptomeria Japonica* Pollen (JCP) Crude Extract and Respiratory Syncytial Virus (RSV)*

Tested group	Antibody titer (ng/ml) in serum				Ratio of IgG2a: IgG1
	Total IgE	RSV-specific IgE	RSV-specific IgG1	RSV-specific IgG2a	
G1 Mock	77.1 ± 26.7	<3.9	<3.9	<3.9	
G2 RSV	66.3 ± 23.1	<3.9	191.6 ± 53.4	4416.8 ± 325.4	23.1
G3 JCP	142.8 ± 76.9	<3.9	<3.9	<3.9	
G4 RSV + JCP (1)	80.6 ± 11.5 ^a	<3.9	562.9 ± 213.7 ^a	7577.5 ± 562.2 ^a	13.5
G5 RSV + JCP (2)	98.2 ± 54.5 ^a	<3.9	476.5 ± 178.9 ^a	9255.5 ± 2598.0 ^a	19.4
G6 JCP + RSV	126.5 ± 53.1 ^a	<3.9	272.8 ± 43.6 ^a	3935.1 ± 216.8	14.5

*Serum was collected at the indicated time after the final inoculation (Fig. 1). RSV-specific IgE, IgG1 and IgG2a were detected by ELISA kit. Each value represents the mean ± SD of five serum samples obtained from five mice.

^aSignificant difference ($P < 0.05$, by Mann-Whitney U -test), compared with the G2 RSV group.

fluid [Walker et al., 1992] and increased expression of IL-4 mRNA in BAL cells [Robinson et al., 1992] and bronchial biopsies [Ying et al., 1995] have been demonstrated in patients with atopic asthma. Mice treated with anti-IL-4 antibodies or mice genetically deficient in IL-4 or IL-5 exhibited a decreased level of allergen-induced airway hyperreactivity (AHR) and eosinophilia [Brusselle et al., 1994; Kips et al., 1995; Corry et al., 1996]. In the present study, JCP-sensitization induced an increase in the production of allergen-induced Th2-like cytokine and allergen-specific Th2-type antibodies (Tables I and II). However, prior RSV infection decreased production of both the cytokines and antibodies (Tables I and II). It has been reported that primary live RSV infection induces a type 1 dominant cytokine profile [Graham et al., 1993; Anderson et al., 1994], characterized by increased IFN- γ production, which plays an important regulatory role in the differentiation of CD4⁺ T cells to Th1-like cells and down-regulates Th2-like responses and allergic reaction [Abbas et al., 1996]. In fact, as shown in G2 of Table I, RSV infection induced IFN- γ production in the culture fluids of the lung parenchyma cells, although the titer was not so high. Furthermore, Th1-type dominant humoral immune responses were observed in the serum of RSV infected mice (Table III). It seems likely that prior RSV infection establishes a type 1 dominant immune environment which down-regulates type 2 immune responses subsequently elicited by JCP sensitization. The present findings are consistent with Peebles's report that RSV infection before OVA-sensitization decreased allergen-induced AHR, production of IL-13 in lung tissue and lung eosinophilia, by an IFN- γ response [Peebles et al., 2001b]. However, the protective efficacy of RSV infection against allergic responses might not be maintained for a longer period, since the immunity induced by primary RSV infection usually vanishes shortly [Henderson et al., 1979; Hall et al., 1991].

The "hygiene hypothesis" explains in part the reason why allergic diseases have been increasing over the past three decades. The infections suffered during the very early stage of life induce an intense type 1 immune response that acts protective against the subsequent

development of atopy [Openshaw and Walzl, 1999; Folkerts et al., 2002; Busse and Lemanske, 2001]. Schoolchildren with a positive tuberculin response exhibit a lower rate of asthma development, lower serum IgE levels and cytokine profiles biased toward the Th1 type than children who show a faint response of delayed hypersensitivity to *Mycobacterium tuberculosis* [Shirakawa et al., 1997]. In epidemiology studies infections with measles virus [Shaheen et al., 1996] and hepatitis A virus [Matricardi et al., 1997] seem to be protective against the development of allergic disease.

Under the present experimental conditions, RSV infection after JCP sensitization appeared insufficient to modulate the allergic Th2 responses in the same way that prior RSV-infection did. The production of JCP-induced Th2-like cytokine and specific Th2-type antibodies was diminished only slightly by the subsequent RSV infection, suggesting that the tough Th2 environment once established by JCP-sensitization could not easily be modified by RSV infection. On the other hand, JCP-inoculation altered RSV-induced responses to shift to Th2-type dominance, by suppressing RSV-induced cytokine production of IFN- γ and by enhancing JCP-induced Th2-like cytokines.

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