

Induction of allergen-specific IL-2 responsiveness of lymphocytes after respiratory syncytial virus infection and prediction of onset of recurrent wheezing and bronchial asthma

Takeshi Noma, MD, Atuo Mori, MD, and Izumi Yoshizawa, PhD
Saitama, Japan

Background: In pediatric patients with bronchial asthma and/or atopic dermatitis, peripheral lymphocytes are activated if they are stimulated with the responsible antigen, resulting in induction of responsiveness to IL-2. Because some nursing infants experience recurrent wheezing after respiratory syncytial virus (RSV) infection, attention is being directed to progression of the disease to bronchial asthma.

Objective: The study was designed to elucidate the mechanism of the onset of allergic diseases after RSV infection.

Methods: We examined allergen-specific IL-2 responsiveness induced in lymphocytes in the peripheral blood of infants after infection by RSV. The relationship between the onset of recurrent wheezing and antigen-specific IL-2 responsiveness was analyzed in 25 pediatric patients who could be followed up for 3 years after RSV infection.

Results: Stimulation of lymphocytes with ovalbumin, α -casein, and mite (*Dermatophagoides farinae*) antigens induced significantly higher responsiveness to IL-2 in the RSV-infected infant group than in the healthy infant and disease control groups of the same age. There was no clear correlation between the IgE RAST scores for *D. farinae*, ovalbumin, and α -casein and IL-2 responsiveness. The families of RSV-infected infants had a high incidence of history of allergy (67%), but there was no significant difference in the incidence of patients with positive test results for IL-2 responsiveness between the groups with and without a familial history of allergy. The *D. farinae*-specific IL-2 responsiveness was significantly increased in the group with the symptom (16 patients) for a value of 1.64 ± 0.13 (mean $\pm$ SEM) compared with the value of 1.31 ± 0.21 in the asymptomatic group (9 patients). The incidence of patients with positive test results for IL-2 responsiveness was 68.8% in the symptomatic group and 44.4% in the asymptomatic group. Similarly, the ovalbumin-specific IL-2 responsiveness was significantly increased in the symptomatic group (1.63 ± 0.17) compared with the asymptomatic group (1.12 ± 0.26). The incidence of patients with positive test results was 62.5% and 22.2%, respectively. α -Casein-specific IL-2 responsiveness was also higher in the symptomatic group than in the asymptomatic group, but the difference was not statistically significant. In the patient groups without RSV infection, on the other hand, the *D. farinae*-, ovalbumin-, and α -casein-specific IL-2 responsiveness in the symptomatic group were all similar to that in the asymptomatic group; no significant increases were detected. **Conclusion:** The results indicated that after RSV infection, lymphocytes acquire specific susceptibility to *D. farinae*, a mite antigen, and food antigens, particularly ovalbumin. Hence, it is thought that positive IL-2 responsiveness specific for *D. farinae* and/or ovalbumin,

From Department of Pediatrics, Saitama Medical School, Saitama, Japan.

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Reprint requests: Takeshi Noma, MD, Kitasato University School of Medicine, 1-15-1, Kitasato, Sagamira, Kanagawa 228, Japan.

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detected several months after RSV infection, can be a prediction factor for the onset of allergic diseases, such as recurrent wheezing and bronchial asthma. (J Allergy Clin Immunol 1996;98:816-26.)

Key words: Recurrent wheezing, respiratory syncytial virus, ovalbumin, α -casein, *Dermatophagoides farinae*, propidium iodide staining, allergen-induced IL-2 responsiveness, bronchial asthma

Abbreviations used

Con A:	Concanavalin A
FA:	Immunofluorescent antibody
IFN- γ :	Interferon- γ
LSQ:	Lysing staining quenching
PI:	Propidium iodide
PPD:	Purified protein derivative
RSV:	Respiratory syncytial virus
SI:	Stimulation index

Respiratory syncytial virus (RSV) infection is an infectious disease of the respiratory tract seen in nursing infants.¹⁻⁴ Because some patients experience recurrent wheezing after RSV infection, attention is being directed to progression of the disease to bronchial asthma.⁵ On the other hand, in pediatric patients with bronchial asthma and/or atopic dermatitis, peripheral lymphocytes are activated if they are stimulated with the responsible antigen, resulting in induction of responsiveness to IL-2, a T-cell growth factor.⁶⁻⁹ The activated T cells act as helper cells in the IgE production system, the principal entity of the immediate-type allergic reaction, or the delayed-type hypersensitivity reaction system.¹⁰⁻¹³ Accordingly, determination of responsiveness of lymphocytes to IL-2 induced by stimulation with allergens is useful for screening the responsible antigens and assessment of the clinical condition,⁶⁻⁹ and it is thought that the responsiveness can be used for analyzing pathology and etiology of allergic diseases. With the objective of studying the mechanism of the onset of allergic diseases, peripheral lymphocytes of pediatric patients with RSV infection were stimulated with ovalbumin and α -casein, as antigens of food allergy, or a mite (*Dermatophagoides farinae*) antigen, as an inhalant antigen; and induction of responsiveness to IL-2 was studied. The relationship between the onset of recurrent wheezing or bronchial asthma after RSV infection and antigen-specific IL-2 responsiveness was also analyzed.

After RSV infection, the peripheral lymphocytes

acquired specific susceptibility to *D. farinae* and ovalbumin, and this finding indicates that *D. farinae*-specific or ovalbumin-specific IL-2 responsiveness can be a factor for predicting the onset of respective allergic diseases.

METHODS

Subjects

Peripheral blood lymphocytes were collected from 30 pediatric patients (35 cases including duplication) arbitrarily selected from among patients younger than 24 months who were hospitalized, subjected to various tests, and diagnosed as having RSV infection during the period between October 1988 and February 1990 (Table I). The patients had primary RSV infection. None of them had atopic symptoms, and their IgE RAST scores were 0 except for a few scores obtained approximately 1 month after RSV infection (Table I). Lymphocyte specimens were also collected 1 to 8 months after RSV infection. As the control group, 14 pediatric patients with respiratory infections other than RSV infection were included in the study (Table I). Characteristics of the subjects who participated in this study are shown in Table I. Informed consent was obtained from each patient's parents. Clinical protocols were approved by the institutional review boards of participating institutions.

Diagnosis of RSV infection

For detection of RSV antigen, nasopharyngeal secretions were collected from both nostrils of the patients by using a tracheal aspiration kit (Sumitomo Bakelite Co., Tokyo, Japan). Each specimen was mixed well and centrifuged at 3000 rpm for 15 minutes. An ELISA was carried out with the supernatant, whereas the precipitate was subjected to the immunofluorescent antibody (FA) technique. In the ELISA an ortho-RSV test was used, the absorbance measured by an ELISA reader was compared with the cutoff value obtained from the absorbance of the control, and then judgment was made. In the FA, an ortho-RSV (FA) direct test was used, and a sample was judged to be positive for RSV antigen when specific apple-green fluorescent bodies were observed under a fluorescence microscope. In the test for detection of the serum RSV antibody, serum samples collected during the acute and recovery stages of RSV infection were allowed to undergo complement-fixing

TABLE I. List of patients with RSV infection and non-RSV infection

Patient No.	Age	Sex	Diagnosis	RSV antigen	Term after infection (mo)	Family history	IgE RAST score		
							Mite	EW	CM
1	20 d	M	Bronchiolitis	+	1	—	0	0	0
2	20 mo	F	Bronchiolitis	+	7	+	0	0	0
3	6 mo	F	Pneumonia	+	4	+	ND	ND	ND
4	1 yr	M	Bronchiolitis	+	7	—	0	0	0
5	2 mo	M	Bronchiolitis	+	1	+	0	0	0
					5				
					8				
6	1 mo	M	Pneumonia	+	2	+	0	2	0
					6				
7	6 mo	M	Pneumonia	+	1	—	0	0	0
8	2 yr	M	Bronchiolitis	+	1	—	2	0	0
					5				
9	1 yr	M	Pneumonia	+	3	+	0	0	0
10	1 yr	M	Pneumonia	+	6	+	0	0	0
11	6 mo	F	Pneumonia	+	5	+	0	0	0
12	1 yr	M	Pneumonia	+	4	—	ND	ND	ND
13	1 yr	M	Bronchiolitis	+	1	+	0	0	2
14	2 yr	F	Pneumonia	+	6	—	0	0	0
					7				
15	2 yr	F	Pneumonia		6	+	0	0	0
16	5 mo	F	Bronchiolitis	+	1	+	2	3	0
17	9 mo	M	Bronchiolitis	+	1	+	0	0	0
18	3 mo	M	Bronchiolitis	+	1	+	0	2	0
19	1 yr	F	Pneumonia	+	1	+	ND	ND	ND
20	1 yr	M	Pneumonia	+	1	+	0	0	0
21	3 mo	M	Bronchiolitis	+	2	—	0	0	0
			Hepatitis						
22	6 mo	M	Bronchiolitis	+	1	+	0	0	0
23	8 mo	F	Bronchiolitis	+	1	—	ND	ND	ND
24	7 mo	M	Bronchiolitis	+	1	+	ND	ND	ND
25	6 mo	M	Bronchiolitis	+	1	+	0	0	0
26	1 yr	F	Pneumonia	+	1	+	2	0	0
27	2 mo	M	Bronchiolitis	+	1	+	0	0	0
28	8 mo	M	Bronchiolitis	+	1	+	0	0	0
29	9 mo	M	Bronchiolitis	+	1	—	0	0	0
30	6 mo	M	Bronchiolitis	+	1	—	0	2	2
31	11 mo	M	URI	—	1	—	ND	ND	ND
32	2 yr	F	URI	—	1	—	ND	ND	ND
33	1 yr	M	Gastroenteritis	—	1	—	ND	ND	ND
34	1 yr	M	URI	—	1	—	ND	ND	ND
35	9 mo	F	URI	—	2	+	0	0	0
36	7 yr	M	Gastroenteritis	—	1	—	ND	ND	ND
37	10 mo	F	URI	—	1	+	0	0	0
38	2 yr	F	Measles	—	1	—	ND	ND	ND
39	8 mo	F	URI	—	1	—	ND	ND	ND
40	9 mo	M	Measles	—	1	—	0	0	0
41	11 mo	F	Measles	—	1	—	0	0	0
			Chicken pox						
42	1 mo	M	URI	—	1	—	ND	ND	ND
43	1 yr	F	Croup	—	7	—	0	0	0
44	2 yr	F	Croup	—	1	—	0	0	0

Patients 1 to 30 had RSV infection, and patients 31 to 44 had non-RSV infection.

EW, Egg white; CM, cow's milk; ND, not done; URI, upper respiratory tract infection.

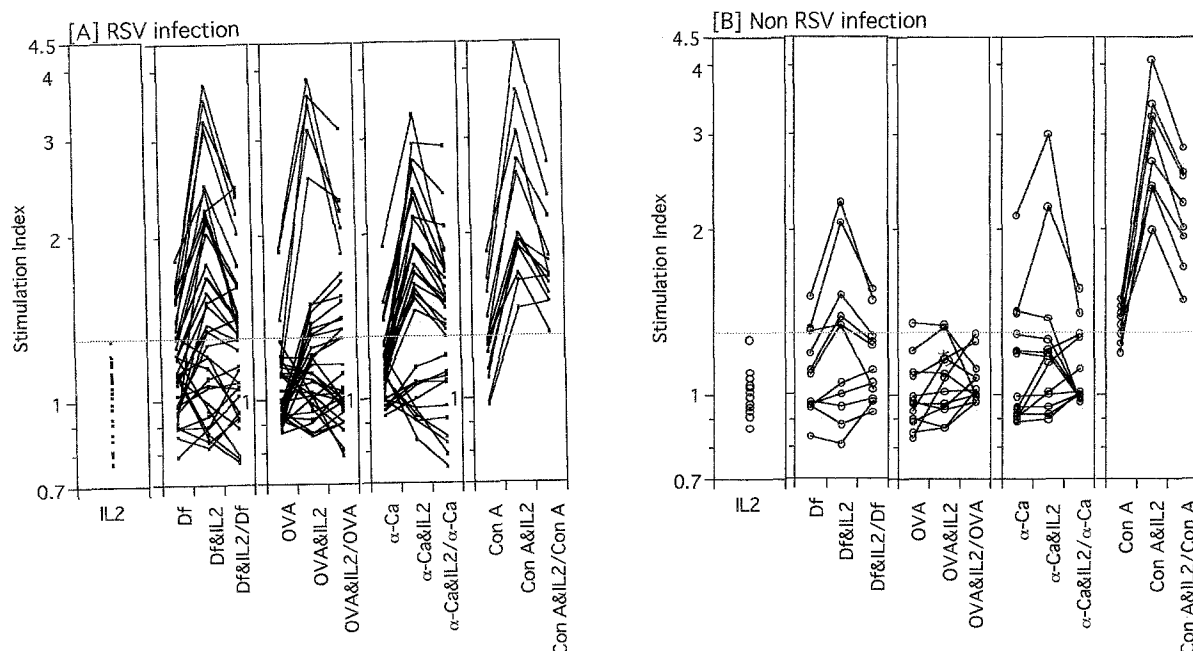


FIG. 1. Induced IL-2 responsiveness of lymphocytes from patients after RSV infection on stimulation with *D. farinae* (Df), ovalbumin (OVA), or α -casein (α -Ca) antigen alone or combined with IL-2. Data represent SIs as compared with cell growth of unstimulated cells. Df&IL2/Df, OVA&IL2/OVA, α -Ca&IL2/ α -Ca, and Con A&IL2/Con A represent induced IL-2 responsiveness as defined in the text. Filled squares, RSV infection; open circles, non-RSV infection.

reaction, and the patient was judged to be positive for RSV when increase in the antibody titer of at least a fourfold was observed. When only a single serum sample was available, the patient was judged to be positive for RSV when the antibody titer was at least 64 dilutions. In this study RSV infection was diagnosed when the patient had positive results for at least one of the tests (ELISA, FA, or complement-fixing).

Staining-quenching solution

One and a half milliliters of propidium iodide (PI) (Sigma Chemical Co., St. Louis, Mo.) stock solution (0.5 mg/ml in 4.9% ethylenediaminetetraacetic acid) was diluted with 7.5 ml of ethylenediaminetetraacetic acid solution (4.9%, pH 7.0; Wako Pure Chemical Industries Inc., Tokyo, Japan) and added to 1.5 ml of Triton X-100 (8%, vol/vol; Wako Pure Chemical Industries Inc.) for lysing lymphocytes. Drawing ink (0.1 ml of a 2^{10} -fold dilution) (Leica Inc., Wetzlar, Germany) was included to quench background fluorescence. This solution was kept at -20°C and referred to as lysing staining quenching (LSQ) medium.⁹

Assay for IL-2 responsiveness

Mononuclear cells (1×10^6), separated from heparinized peripheral blood by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation,¹⁴ were cultured in the presence of 100 $\mu\text{g}/\text{ml}$ ovalbumin (Sigma Chemical Co.), α -casein (Sigma), 10 $\mu\text{g}/\text{ml}$ *Dermatophagoides farinae* antigen (Torii & Co., Ltd., Tokyo, Japan), or

5 $\mu\text{g}/\text{ml}$ concanavalin A (Con A) (Sigma) and suspended in 1 ml of RPMI-1640 medium (Gibco, Grand Island, N.Y.) containing 40 mg/ml gentamicin and 10% heat-inactivated pooled human serum in a culture tube (Falcon Plastic 2054; Falcon, Oxnard, Calif.). After 5 days of culture (37°C , 10% CO_2), the media were replaced with fresh medium, and the cells were divided into 6 aliquots in 96-well flat-bottom microplates (Nunc, Roskilde, Denmark). These were cultured for an additional 3 days in the presence of 0.5 U/ml recombinant (r)IL-2 (Takeda Co. Ltd., Tokyo, Japan).²⁰ At the end point of the culture, 50 ml of the LSQ medium was added to the cell suspension, and then the mixture was kept for 2 hours at 37°C . Addition of PI to the lysed lymphocytes resulted in staining of double-stranded DNA but not of single-stranded DNA. Quenching of the medium's fluorescence was accomplished by the addition of drawing ink in the LSQ medium. The fluorescence intensity (long-pass filter, 580 nm) was determined for each well by using Leitz PATIMED equipment (Leica Inc., Rockleigh, N.J.).⁹ IL-2 responsiveness was evaluated as follows (Fig. 1)⁶⁻⁹:

$$\text{IL-2 responsiveness (SI)} = \frac{(\text{fluorescence intensity with IL-2} - \text{background})}{(\text{fluorescence intensity without IL-2} - \text{background})}$$

where SI is stimulation index, and background represents fluorescence intensity with LSQ medium alone. Coefficient value in the PI-staining method was relatively small ($<2.3\%$).

Analysis of history of familial allergy

The parent or guardian of each patient was interviewed, and patient's familial history of allergy was judged to be positive when a major allergic disease (bronchial asthma, allergic rhinitis, atopic dermatitis, or recurrent urticaria) was present in a second-degree relative of the patient.

Statistical analysis

The data obtained were tested for the difference with the control data by the two-tailed Student's *t* test. The IL-2 responsiveness (in terms of stimulation index [SI]) was defined as positive when there was a significant difference between the SIs of the stimulated and unstimulated samples. Disease-specific (i.e., antigen-specific) IL-2 responsiveness (in terms of SI) was defined as positive when there was a significant difference compared with the healthy group. Namely, the IL-2 responsiveness (SI) of the same age group was 1.22 ± 0.10 (mean $\pm$ SEM) ($n = 10$) for ovalbumin, 1.19 ± 0.06 ($n = 12$) for *D. farinae*, and 1.15 ± 0.26 ($n = 4$) for α -casein. In this study an SI of 1.30 or higher was defined as positive.

RESULTS

Induction of IL-2 responsiveness by allergen stimulation of peripheral lymphocytes of pediatric patients with RSV infection

D. farinae, ovalbumin, or α -casein antigen was added to each lymphocyte specimen collected more than 1 month after RSV infection; the mixture was incubated for 5 days; rIL-2 was added to the mixture, which was incubated for another 3 days; and then IL-2 responsiveness was analyzed. As a result, IL-2 responsiveness was not induced in the absence of an antigen, whereas it was induced in 19 of 34 cases (56%) by *D. farinae*, 16 of 34 cases (47%) by ovalbumin, and 20 of 34 cases (63%) by α -casein (Fig. 1, *A*). When lymphocytes collected from patients with respiratory infections other than RSV infection were tested, addition of *D. farinae*, ovalbumin, or α -casein antigen induced IL-2 responsiveness in 2 of 14 patients (14%), 0 of 13 patients (0%), and 2 of 13 patients (15%), respectively (Fig. 1, *B*). Thus compared with the patient group with RSV infection, no significant increase in the IL-2 responsiveness was observed. In all cases stimulation with Con A induced IL-2 responsiveness (Fig. 1, *A* and *B*). In the healthy group of the same age, stimulation with Con A similarly induced IL-2 responsiveness, but neither *D. farinae*, nor ovalbumin, nor α -casein stimulation induced IL-2 responsiveness.

To evaluate IL-2 dose-dependency of cell growth of antigen-stimulated lymphocytes from patients, cells from a random selection of RSV and

control patient samples (4 cases for each group), which were stimulated with each antigen, were cultured for 5 days, then serial dilutions of rIL-2 (0.031, 0.063, 0.125, 0.250, 0.50, 1.00, 2.00 U/ml) were assayed. The fluorescence intensity increased corresponding to the IL-2 concentration added and showed almost maximum values at or above a dose of 0.5 U/ml (data not shown). This IL-2 response is not different between patient group with RSV infection and control patient samples (data not shown). In contrast to IL-2, serial dilutions of rIL-4 (3.1, 6.3, 12.5, 25, 50, 100, 500 U/ml) (Genzyme, Boston, Mass.) were added to the cell cultures and assayed for cell growth. The fluorescence intensity did not increase corresponding to IL-4 at any concentration (data not shown). The results indicate that antigen-induced IL-2 responsiveness is specific for the IL-2 cytokine.

We further studied in vitro kinetics of IL-2 responsiveness in the different patient groups. Results showed no differences in intensity of responses when we cultured the cells for 4, 5, 6, and 7 days in first cultures (data not shown). There were also no differences in the activity when we cultured the cells for 3 and 4 days in second cultures (data not shown).

In the subsequent study, said induction was analyzed with respect to the relationship with time after RSV infection. When specimens collected 1 month after infection were analyzed, many patients showed a high responsiveness (data not shown). Although the induced IL-2 responsiveness tended to decline gradually thereafter, IL-2 responsiveness was inducible even 6 to 7 months after infection in some patients (data not shown).

Induction of IL-2 responsiveness by purified protein derivative stimulation of peripheral lymphocytes of pediatric patients with RSV infection

To verify the relationship between allergen-induced IL-2 responsiveness and subsequent clinical symptoms, the response to bacteria-derived antigen, including purified protein derivative (PPD), was evaluated in subjects arbitrarily selected from a patient group. PPD (0.1, 1.0, 5.0 μ g/ml) was capable of inducing IL-2 responsiveness in a dose-dependent manner in both normal subjects and the patient group showing allergen-induced IL-2 responsiveness when the subjects demonstrated a positive skin reaction to PPD (mean diameter, ≥ 10 mm). However, the response was not noticed in any subject not sensitized to PPD (Fig. 2). The results indicated that the re-

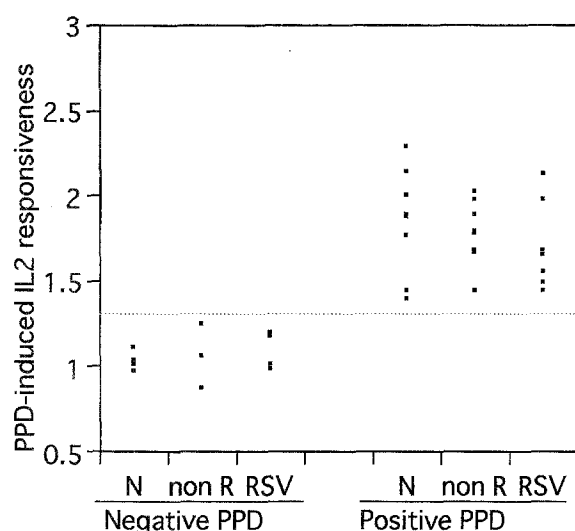


FIG. 2. Induced IL-2 responsiveness of lymphocytes on stimulation with PPD. Peripheral blood mononuclear cells from normal subjects and patients arbitrarily selected from the patient group were added to a 1.0 $\mu\text{g}/\text{ml}$ dose of PPD and were cultured for induction of IL-2 responsiveness for 5 days. *Positive PPD*, Positive skin reaction to PPD (mean diameter, ≥ 10 mm); *N*, normal subjects; *non R*, patients with non-RSV infection; *RSV*, patients with RSV infection showing allergen-induced IL-2 responsiveness.

sponses induced by allergens such as *D. farinae*, ovalbumin, and α -casein were specific for subjects with RSV infection.

Relationship between rate of induction of IL-2 responsiveness by stimulation with allergens, familial history of allergy, and serum IgE level

Each of the 116 pediatric patients was analyzed with respect to familial history of allergy and serum IgE level. According to the analysis, familial history of allergy was present in 64.7% (75 of 116) of patients infected with RSV. The incidence of patients with a high IgE level (0 to 11 months of age, ≥ 30 IU/ml; 12 to 23 months of age, ≥ 100 IU/ml) was small (20%, 21 of 105), and there was no definite tendency in terms of the correlation with IL-2 responsiveness. Similarly, there was no clear correlation between the IgE RAST scores for *D. farinae*, ovalbumin, and α -casein and IL-2 responsiveness; but induction of specific IL-2 responsiveness was observed even in patients with a score of 0 (Fig. 3).

The rate of induction of IL-2 responsiveness in the patient group with familial history of allergy tended to be higher for each antigen (*D. farinae*, ovalbumin, and α -casein) compared with that in the patient group with no familial history of al-

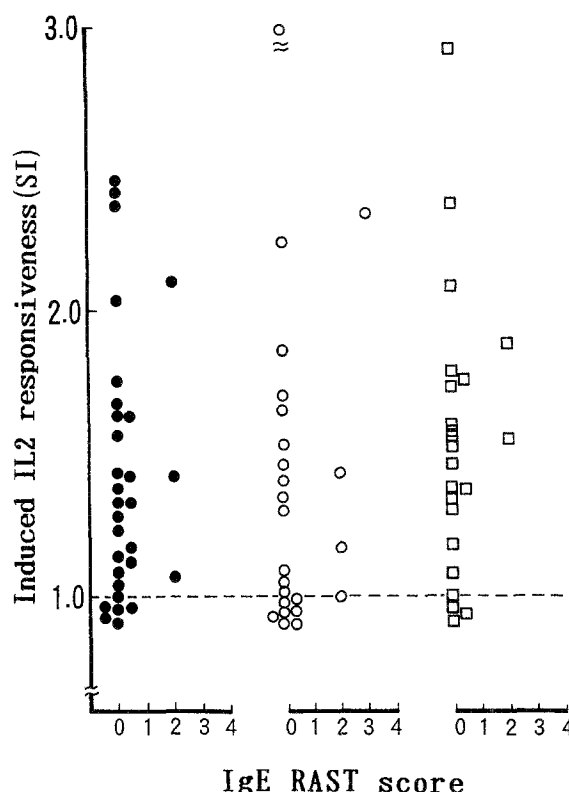


FIG. 3. Antigen-specific induction of IL-2 responsiveness is unrelated to IgE RAST score. *Filled circles*, *D. farinae*; *open circles*, ovalbumin; *open squares*, α -casein.

lergy, but the difference between the two groups was not statistically significant (Table II). We could not find any difference in the rate, even when we compared the in vitro kinetics of IL-2 responsiveness for the groups (data not shown). Thus it was concluded that the two groups do not differ with respect to the rate of induction of IL-2 responsiveness.

Relationship between onset of recurrent wheezing after RSV infection and antigen-specific IL-2 responsiveness

Twenty-five patients who were followed up for 3 years after RSV infection were classified into two groups: a group in which patients manifested some atopic symptoms and recurrent wheezing after leaving the hospital without known causes other than RSV infection (symptomatic group, 16 patients) and a group in which patients did not manifest such atopic symptoms (asymptomatic group, 9 patients). One to seven months after RSV infection, peripheral blood was collected from each group, and lymphocytes were analyzed with respect to specific IL-2 responsiveness for each antigen (Fig. 4). The *D. farinae*-specific IL-2 re-

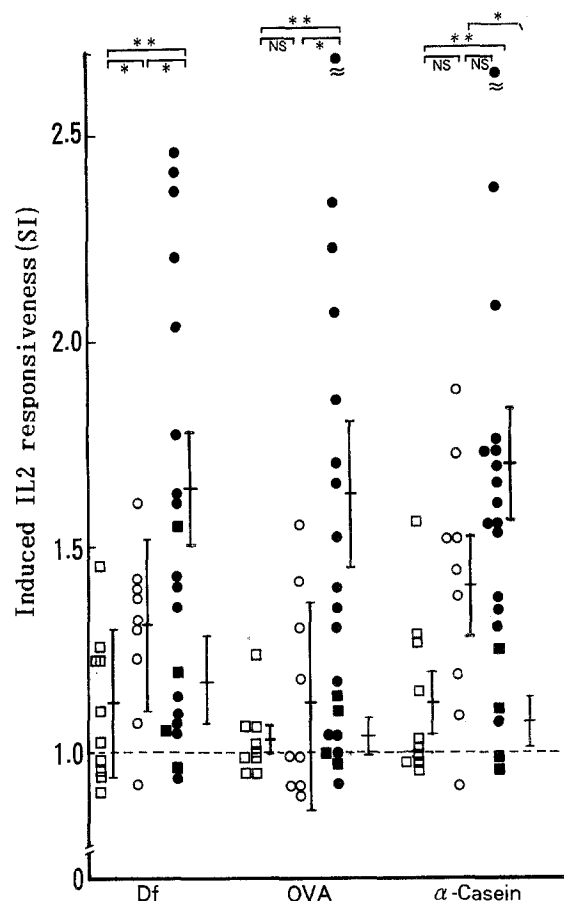


FIG. 4. Infants after RSV infection showing allergen-induced IL-2 responsiveness had recurrent wheezing or bronchial asthma within a few years. Filled circles, Wheezing group after RSV infection; open circles, healthy group after RSV infection; filled squares, wheezing group after non-RSV infection; open squares, healthy group after non-RSV infection. Data are expressed as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

sponsiveness was significantly ($p < 0.05$) increased in the symptomatic group (1.64 ± 0.13 , $n = 16$) compared with the asymptomatic group (1.31 ± 0.21 , $n = 9$). In the symptomatic group, the incidence of patients positive for *D. farinae*-specific IL-2 responsiveness was 68.8% (11 of 16). The corresponding incidence in the asymptomatic group was 44.4% (4 of 9), but their responsiveness was low. In the patient group with no RSV infection (14 patients), on the other hand, the *D. farinae*-specific IL-2 responsiveness was 1.20 ± 0.14 ($n = 4$) in the symptomatic group (4 patients), and only one patient had a positive result for this test. This value in the symptomatic group was nearly equal to 1.12 ± 0.18 ($n = 10$) in the asymptomatic group (10 patients); no significant increase was observed in the symptomatic group.

TABLE II. Frequency of induced IL-2 responsiveness in patients after RSV infection

Family history	Antigen used for stimulation		
	<i>D. farinae</i>	Ovalbumin	α -Casein
Negative	5/12 (42%)	5/12 (42%)	6/12 (50%)
Positive	14/23 (61%)	11/22 (50%)	15/20 (75%)

The ovalbumin-specific IL-2 responsiveness in the patient group with RSV infection was also significantly ($p < 0.05$) increased in the symptomatic group (1.63 ± 0.17 , $n = 16$), compared with that in the asymptomatic group (1.12 ± 0.26 ; $n = 9$). The ovalbumin-specific IL-2 responsiveness was positive in 62.6% (10 of 16 patients) of the symptomatic group, whereas it was 22.2% (2 of 9 patients) in the asymptomatic group; but the responsiveness was low, as seen with *D. farinae* antigen. In the patient groups without RSV infection (14 patients), the ovalbumin-specific IL-2 responsiveness was 1.05 ± 0.04 ($n = 4$) in the symptomatic group (4 patients), which was equal to 1.03 ± 0.03 ($n = 10$) in the asymptomatic group (10 patients); the symptomatic group showed no significant increase.

The α -casein-specific IL-2 responsiveness in the patient group infected with RSV was similarly increased in the symptomatic group (1.70 ± 0.12 , $n = 16$), compared with that in the asymptomatic group (1.40 ± 0.12 , $n = 9$), but the difference was not statistically significant. In the patient groups without RSV infection, the α -casein-specific IL-2 responsiveness was 1.08 ± 0.07 ($n = 4$) in the symptomatic group, which did not differ from 1.12 ± 0.07 ($n = 10$) in the asymptomatic group; no increase in the responsiveness was observed. The patterns of IL-2 responsiveness to *D. farinae*, ovalbumin, and α -casein were the same for each of the patient groups. This response pattern was true for individual patients.

On the basis of these results, it was inferred that lymphocytes are in a state likely to be activated by food or mite antigens after RSV infection. Therefore it is thought that a patient infected by RSV is likely to experience recurrent wheezing and/or bronchial asthma in the future if the patient has positive test results for the *D. farinae*-specific or ovalbumin-specific IL-2 responsiveness within several months after RSV infection (rate of onset in *D. farinae*-positive patients: 73.2%, 11 with onset among 15 patients with positive response; onset in ovalbumin-positive patients: 83.3%, 10 with onset among 12 patients with positive response). In

addition, recurrent wheezing may also appear in the asymptomatic group with RSV infection if they are followed up for more than 3 years, the follow-up period of this study.

DISCUSSION

The response of lymphocytes to allergens is typically observed as blastogenesis reaction of lymphocytes of patients with delayed-type allergic diseases when the lymphocytes are cultured in the presence of the antigen. Allergen-specific lymphocytes proliferating in a patient with allergy, (i.e., T cells induced and activated with an allergen) react to IL-2, proliferate, and continue to exist. This phenomenon is thought to be important for elucidation of the pathology and etiology of allergic diseases.⁶ The authors reported earlier that: (1) lymphocytes of healthy persons retain nonresponsiveness to added IL-2 even when they are stimulated with an allergen, such as ovalbumin or mite antigen; (2) responsiveness to IL-2 is induced in lymphocytes of patients with atopic dermatitis or bronchial asthma who have been sensitized to ovalbumin, *D. farinae*, and/or other antigens; and (3) the IL-2 responsiveness induced with the allergen is specific to that antigen.⁶⁻⁹ In this study as well, induction of antigen-specific IL-2 responsiveness in the peripheral lymphocytes of infants with RSV infection was confirmed, and not only food antigens such as ovalbumin and α -casein but also such inhalant antigens as *D. farinae* are thought to be capable of inducing IL-2 responsiveness. In fact, the presence of lymphocytes sensitized against RSV can be demonstrated by blastogenesis reaction of lymphocytes collected from infants 1 month after they had been infected by RSV.¹⁵ The activation of functional CD4⁺ peripheral blood T-cell repertoires may be relevant to RSV infection; compartmentalization of T cells with different specificities may be induced. Fluorescence-activated cell sorter analysis of the cells showed that the IL-2-absorbing and IL-2-responding cells mainly consisted of CD3⁺2⁺4⁺8⁺45RA⁺ cells. β -Chains (P75) of IL-2 receptors were involved in *D. farinae*- or ovalbumin-induced IL-2 responsiveness of the patients' lymphocytes, whereas the α -chains (p55) were expressed on normal lymphocytes stimulated with the same antigens as well. The results of this study indicate that RSV-sensitized lymphocytes may acquire the ability to induce susceptibility to inhalant or food antigens, probably by secreting cytokines such as IL-4, which is able to induce T_{H2}-type helper T cells.¹⁶ The possible mechanisms for this may be that viruses

such as RSV, but not other respiratory viruses, can induce IL-12 production by phagocytic cells so that IL-4 production by allergen-exposed T cells might be augmented in early infancy. Although in this study the ability of RSV-infected cells to produce IL-12 could not be evaluated, this hypothesis is supported by a report that CD4⁺45RO⁻ cord blood, but not adult lymphocytes, stimulated with anti-CD3 antibody is capable of inducing IL-4 production in addition to interferon- γ (IFN- γ) production in the presence of IL-12.¹⁷ It is inferred that this increased susceptibility is deeply involved in the onset of atopic diseases.

Con A-stimulated cells acquired IL-2 responsiveness, and the cells divided approximately twofold or threefold in 8 days. Ovalbumin- or *D. farinae*-activated lymphocytes from patients with atopic dermatitis or bronchial asthma specifically induced the response, and the cell number increased in the same fashion. In contrast, unstimulated lymphocytes after 5 days' culture were never observed to proliferate as measured by increased fluorescence intensity, and the cell growth as measured by increased number was never observed, even in those cases in which rIL-2 was added to the cell suspension over the course of a second culture for 3 days. In contrast, unstimulated cells also showed increased tritiated thymidine uptake on stimulation with rIL-2 as compared with that without rIL-2, indicating that IL-2 responsiveness as measured by tritiated thymidine uptake of the cells was increased to an extent comparable to that of the antigen-activated cells. Therefore the tritiated thymidine uptake method for evaluation of antigen specificity of the induced IL-2 responsiveness of the activated cells could not substitute for the cell enumeration as determined by PI staining.⁹

In contrast, the presence of antigen-sensitive cells had been demonstrated by tritiated thymidine uptake of antigen-added cells so far. However, the in vitro responses have been sometimes evaluated unsuccessfully in the in vitro assay with freshly obtained lymphocytes, because the response has been sometimes markedly low as compared with unstimulated cells in human systems. In this study, therefore, we chose antigen-induced IL-2 responsiveness by lymphocytes for evaluation of antigen-induced lymphocyte activation.

As already reported,⁶⁻⁸ the study on the relationship between IgE RAST scores for *D. farinae*, ovalbumin, and α -casein and specific IL-2 responsiveness to each antigen failed to demonstrate a correlation. In addition, induction of specific IL-2 responsiveness was observed even in patients

whose score was 0. Although this phenomenon is in agreement with a finding that induction of antigen-specific IL-2 responsiveness in T cells is correlated with the activity of allergic diseases,⁶ it is also in agreement with reports that induction of specific IL-2 responsiveness does not necessarily reflect IgE-type allergic reactions.⁶⁻⁹

The study on the relationship between induced IL-2 responsiveness and the time elapsed after RSV infection revealed a tendency for IL-2 responsiveness to peak 1 month after infection and gradually decrease thereafter. In nursing infants, regardless of the presence or absence of RSV infection, IL-2 responsiveness induced such allergens as ovalbumin and *D. farinae* to be slightly increased in many cases. In infants with allergic diseases, however, this responsiveness is greater than that in healthy infants, and a decrease in the responsiveness is also delayed compared with healthy infants.⁶ Although distribution by age was not presented in this study, some patients showed a high IL-2 responsiveness at 1 month after RSV infection and some showed a delayed decrease in IL-2 responsiveness as evidenced by a positive level of response even 5 to 7 months after infection. These patients are thought to be in a state in which T cells are readily activated with allergens after RSV infection. Hence, it is thought that such patients may experience recurrent wheezing in the future.

In the analysis of the relationship between IL-2 responsiveness and familial history of allergy, there was no significant difference in the incidence of patients positive for IL-2 responsiveness between the groups with and without familial history of allergy. Of patients in whom IL-2 responsiveness was induced, those with a familial history of allergy (74% for *D. farinae*, 69% for ovalbumin, and 75% for α -casein) outnumbered those without such history, but this is thought to be natural because approximately 65% of the population had familial history of allergy. Although the presence of familial history of allergy does not seem to directly affect the inducibility of IL-2 responsiveness, it is a fact that IL-2 responsiveness was induced at a higher rate in patients with this history, and this suggests that the immune response to some allergens is potentially increased in patients with familial history of allergy and that RSV infection may trigger actualization of this potential. The authors failed to find a correlation between antigenic responsiveness among the patients and familial history of allergy. This may not be the case when a patient is from a family with more severe atopy and/or

objective evidence suggestive of atopy including clearly elevated serum IgE levels. The issues remain to be elucidated.

To analyze the relationship between onset of recurrent wheezing after RSV infection and antigen-specific IL-2 responsiveness, 25 patients who could be followed up for 3 years after RSV infection were classified into two groups: a group in which patients manifested some atopic symptoms and recurrent wheezing after leaving the hospital without known causes other than RSV infection (symptomatic group, 16 patients) and a group in which patients did not manifest such atopic symptoms (asymptomatic group, 9 patients). Peripheral blood was collected from each group 1 month after RSV infection, and lymphocytes were analyzed with respect to specific IL-2 responsiveness for each antigen. The *D. farinae*-specific IL-2 responsiveness was significantly increased in the symptomatic group compared with the asymptomatic group. In the symptomatic group, the incidence of patients positive for the *D. farinae*-specific IL-2 responsiveness was about 70%. The corresponding incidence in the asymptomatic group was about 45%, but this responsiveness was lower than that in the symptomatic group. In the asymptomatic group as well, the incidence of patients positive for this responsiveness was relatively large, and thus it is thought that onset of recurrent wheezing might also be observed in this group if the observation period were to be extended. In fact, 73.2% (11 with onset and 15 with positive test results) of patients who had positive test results for IL-2 responsiveness several months after RSV infection experienced later onset of recurrent wheezing or bronchial asthma.

The ovalbumin-specific IL-2 responsiveness was also significantly increased in the symptomatic group with RSV infection compared with the asymptomatic group with RSV infection. About 60% of the patients in the symptomatic group with positive test results for the ovalbumin-specific IL-2 responsiveness. The corresponding incidence in the asymptomatic group was about 20%, but the responsiveness was lower than that in the symptomatic group, as was the case for the ovalbumin-specific IL-2 responsiveness. The incidence of patients who later experienced onset of recurrent wheezing or bronchial asthma was a high 83.3% (10 patients with onset/12 patients with positive test results). In the case of the patient group without RSV infection (14 patients), on the other hand, the *D. farinae*-specific and ovalbumin-specific IL-2 responsiveness in the symptomatic group

(4 patients) was equal to that in the asymptomatic group (10 patients), and no significant increase was detected. The α -casein-specific IL-2 responsiveness in the symptomatic group was also slightly increased compared with that in the asymptomatic group, but the difference was not statistically significant. In contrast, responses to the test allergens were observed to be higher in the healthy group after RSV infection than those in the non-RSV-infected wheezing group (Fig. 4). Actually, there was a statistical difference in the α -casein-induced IL-2 responsiveness ($p = 0.033$) but not in *D. farinae*- and ovalbumin-induced IL-2 responsiveness. The results indicated that lymphocytes of the group after RSV infection may be more sensitive to allergen, especially α -casein, than those after non-RSV infection, although the intensity of activated lymphocytes is incapable of inducing the disease. At approximately 1 month after RSV infection, lymphocytes are thought to be in a state likely to be activated against food or mite antigens. If a patient tests positive for IL-2 responsiveness specific for mite (*D. farinae*) antigen or food antigen, particularly ovalbumin antigen, it is thought that the patient is very likely to experience onset of allergic diseases, such as recurrent wheezing and bronchial asthma, in the future.

In animal models the serum IgG and IgE antibody response to antigen, including ragweed and ovalbumin antigen, after primary exposure by the respiratory mucosal surface demonstrated higher levels in mice and rats previously infected with RSV^{18, 19} or influenza virus.²⁰ In our human system frequency of the patients with symptoms and positive IgE RAST scores (≥ 2) against mite, egg white, or cow's milk (25.0%) did not increase as compared with that of patients with positive scores evaluated approximately 1 month after RSV infection (24.0%, 6 patients of 25, Table I). Antibody response including IgG and IgA to allergen remain to be elucidated in further studies.

Although the mechanism behind these phenomena has not yet been elucidated, it is thought that infection by RSV results in immunologic activation, rather than depression, of the vital body and potentiates immune response involved in allergic reactions.

Allergen-primed antigen-presenting cells were capable of inducing at least two kinds of helper T cells (T_{H1} , T_{H2}) in animal models.^{10, 11} Activated helper T_{H2} cells are involved in immediate hyperactivity, and T_{H1} cells mediate delayed-type hypersensitivity in allergic reactions. The T_{H2} -specific cytokine IL-4 enhances IgE production by induc-

ing class switch and promotes the expression of low-affinity Fc ϵ receptor II.^{13, 14}

T_{H1} -specific IFN- γ and T_{H2} -specific IL-4 act in a reciprocal manner. IFN- γ inhibits IL-4-induced IgE production, and IL-4 also inhibits IFN- γ production.¹⁴⁻¹⁶ In contrast, allergen-activated T_{H1} cells are capable of producing IL-2, and the activated helper T cells respond to IL-2 in an auto-crine or paracrine manner and then initiate their functions in lymphokine-mediated allergic responses in patients with atopic diseases.^{12, 13} As described above, activation of helper T cells (T_{H1} or T_{H2})^{10, 11} involved in allergic reactions and immune response of allergic reactions is induced in lymphocytes after RSV infection; and hence induction of an increase in the secretion of cytokines (e.g., IL-2, IL-3, granulocyte-macrophage colony-stimulating factor, IL-4, IL-5, IL-6, and IFN- γ) and their imbalance¹⁶ are predicted. These problems, including involvement of these cytokines, should be analyzed in the future.

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