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Three-year follow-up study of allergy in workers in a mushroom factory

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Abstract Exposure to mushroom spores may cause many respiratory allergic diseases, however, there has been no serial study in a mushroom factory to address this problem. The aim of this study was to investigate the serial changes in respiratory allergy and the incidence of hypersensitivity pneumonitis (HP) in mushroom workers. A 3-year follow-up study, beginning in June 1996, was conducted in a newly operating mushroom factory in which one kind of mushroom is produced: *Hypsizigus marmoreus* (Bunashimeji). Allergic symptoms, chest roentgenogram, serum precipitins to the spores and soluble adhesion molecules in sera were evaluated once a year in 60 workers and 20 controls. Three out of the 60 subjects were diagnosed as having HP caused by inhalation of the mushroom spore and they were therefore excluded from this study, and the 57 non-HP subjects were evaluated. In this study 24 workers quit because of intolerable cough, runny nose, wheezing, sputum, fever elevation and/or shortness of breath at their place of work. During each year of this study as many as 70–80% of employees suffered some of the above symptoms, cough being the most frequent, and positive rate of serum precipitins to the spore revealed 30% in 1996, 93% in 1997 and 94% in 1998. From the June 1996 examination until the following May, serum soluble intercellular adhesion molecule-1 levels of the 15 workers who quit during that period were significantly higher than those in the 42 workers still employed in 1997 ($P < 0{\cdot}05$). Workers in Bunashimeji mushroom factories might be at critical risk of developing respiratory allergy. In our 3-year study, over 90% workers were sensitized to the spore, 40% quit because of the symptoms and 5% developed HP. It was suggested that workers should be counselled about the risk of mushroom allergy and precautionary measures should be taken to prevent its occurrence.

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INTRODUCTION

Mushroom spores can cause respiratory allergic diseases such as asthma and hypersensitivity pneumonitis (HP), and these disorders have been increasing in recent years (1–7). *Hypsizigus marmoreus*, called 'Bunashimeji' in Japanese, is one of the common edible mushrooms. As cultivation takes place indoors, when they are fully grown, the cultivating, harvesting and packing rooms are filled with the mushroom spores, because Bunashimeji is 'open' very early and releasing spores well before harvest. The size of the Bunashimeji spores is 4–6 μm and they can reach the alveoli of the lungs. We previously reported two cases with HP caused by the spore (8). Only one cross-sectional survey of mushroom workers has been published (9), and it stated that all mushroom farm workers should be counselled about the risk of developing HP and advised on the symptoms and warning signs of HP. However, there has been no follow-up study of the health problems of workers in this industry.

Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) are members of the immunoglobulin supergene family, and the principal ligands for leukocyte functional antigen-1 and vascular leukocyte antigen-4 (VLA-4), respectively. The soluble forms of these adhesion molecules might be useful markers for monitoring the activity of inflammatory and immune diseases (10–12).

To investigate the serial changes in respiratory allergy and the incidence of HP in mushroom workers, we con-

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ducted a 3-year follow-up study in a mushroom factory producing Bunashimeji. We also measured serum precipitins to the mushroom and serum soluble forms of adhesion molecules as evidence to support the existence of mushroom allergy.

MATERIALS AND METHODS

Study population

This factory, which produces only the Bunashimeji mushroom, had a total of 72 employees in June 1996; two were office workers, and the other 70 people were working in cultivation, harvesting and packing of the mushrooms. All their jobs involved rotating freely between the cultivation room, the harvesting and packing area, and the maintenance place every day. This factory produced mushrooms 6 days a week in all seasons. Out of the 70 workers exposed to mushroom spores, 60, aged between 34–66 years (49 ± 6 years), all of whom were women, were chosen to participate in this study after giving informed consent. A research ethics committee in Tomakomai Prefecture Hospital approved this study. Out of the 60 workers, 45 were non-smokers and 15 were current or former smokers. As a control group, we chose 20 age-and sex-matched control women (48 ± 7 years) who had just began working at a new, neighbouring, mushroom factory, but had never been engaged in cultivating Bunashimeji prior to that. These 20 subjects had been housewives or office workers before this employment, and no subjects with allergic symptoms were included.

Study design

The follow-up investigations were performed in June 1996, June 1997 and June 1998. We assessed the subjects for six allergic symptoms by means of a questionnaire, chest roentgenogram, serum precipitin antibodies to the Bunashimeji's spores and serum sICAM-1. The self-administered questionnaire asked whether cough, sputum, wheezing, shortness of breath, fever or runny nose appeared after work in this mushroom factory. Chest films were interpreted by two thoracic radiologists.

Serum sVCAM-1 levels and the atopic status of the workers were evaluated only in the 1996 examination. Subjects were defined as non-atopic when they had no positive antigen specific serum IgE, and defined as atopic when they had at least one or more positive antigens to 12 common antigens tested by a commercial kit (CAP Phadiatop FEIA, Pharmacia and Upjohn, Uppsala, Sweden). In the 60 workers, 20 were atopic and the remaining 40 were non-atopic.

Mushroom exposure

Measurement of airborne spore for determination of β-D-glucan level was made by drawing air through a 110-mm diameter glass fiber of $0{\cdot}6\,\mu$m porosity (GB-100R, Toyo Roshi, Tokyo, Japan) at a flow rate of $500\,l\,min^{-1}$ for 30 min using a high volume air sampler. The air inlet of the sampler was attached at the subject's nose level. The filters were shaken for 10 min in 10 ml distilled water. Twenty-five microlitres of the extract and $100\,\mu$l glucan specific lysate (Fungitic G test; Seikagaku Kogyo, Japan) were added. The plate was incubated in a spectrometer, and the kinetics of the ensuing color reaction was read photometrically. Sampling consisting three measurements involved three filters in the cultivating room, three in the harvesting and packing area, and three in the office room. The limitation of detection was $20\,pg\,ml^{-1}$ for β-D-glucan. The mean concentrations of airborne β-D-glucan were $1{\cdot}0\,ng\,m^{-3}$ in the cultivating room, $3{\cdot}0\,ng\,m^{-3}$ in the harvesting and packing area and $0{\cdot}3\,ng\,m^{-3}$ in the office room.

Mushroom spore antigens preparation

The spores of Bunashimeji were collected from the mushroom in a sterilized method at this farm, and cultured in Sabouraud's glucose broth. The cultured spores and their proteins were extracted with 50% ammonium sulfate. The sample was centrifuged at 3000 rpm for 15 min and the pellet was dialysed with distilled water.

Serum precipitins to Bunashimeji

The double diffusion test for precipitating antibodies for HM was performed according to the method of Ouchterlony (13). Fifty microlitres of antigen and/or serum were added to opposite well of 1% agarose plates and monitored for 72 h at room temperature. The following antigens: *Micropolyspora faeni, Thermoactinomyces vulgaris* (prepared in our laboratory) and *Aspergillus fumigatus* (Hollister-Stier, Spokane, Washington, U.S.A.) were also tested in the same manner.

Measurement of serum sICAM-1 and sVCAM-1 levels

Double-determinant immunoassay (DDIA) using the FAST system (Becton Dickinson, Mountain View, CA, U.S.A.) was employed in order to measure the levels of sICAM-1 in serum. This DDIA for measurement of sICAM-1 has previously been described (14). Two monoclonal antibodies (CL207 and HA58) that recognize different epitopes of ICAM-1 were used in the DDIA. Briefly, the beads attached to the lip of a 96-well

microtitre plate, were first incubated with CL207 and then non-specific binding was blocked with PBS (pH=7·4) containing 3% bovine serum albumin at 37°C for 2 h. Aliquots of serum samples diluted 1 : 200 in PBS were then incubated with the bed for 2 h. After being washed with PBS containing 0·05% Tween 20, the beads were incubated with biotinylated HA58 for 2 h. Avidin-conjugated peroxidase (Vector, Burlingame, CA, U.S.A.) was diluted 1:1000 in 0.05 M PBS with 0.5 M NaCl pH 8.0, and incubated with the beds for 1 h. The degree of substrate reaction was determined with OPD at 495 nm in a Micro-ELISA Auto Reader MR 580 (Dynatech, Cambridge, MA, U.S.A.). Results were expressed as ng ml^{-1} calculated from the titration curve of the ICAM-1 antigen. Serum levels of sVCAM-1 were measured using a commercial ELISA kit (Human VCAM-1 ELISA kit, R&D Systems, MA, U.S.A.). ELISA was performed according to the manufacturers' instructions. Results were expressed in terms of ng ml^{-1} calculated from the titration curve of VCAM-1.

Diagnosis of HP by Bunashimeji spores

HP was diagnosed by the following criteria: (1) episodes of cough, dyspnoea and fever occurring some hours after work; (2) auscultatory evidence of crepitations; (3) diffuse reticulonodular opacities on chest radiograph or computer tomography; (4) pulmonary function test revealing a reduction in vital capacity, carbon monoxide diffusing capacity, and arterial PaO_2; (5) presence of serum precipitins to Bunashimeji and no precipitins to the antigens of *Micropolyspora faeni* and *Thermoactinomyces vulgaris;* (6) positive result for *in vitro* blastogenesis of bronchoalveolar lavage fluid (BALF) lymphocytes to the mushroom antigens; (7) an increased number of T-lymphocyte in BALF; (8) the presence on a transbronchial biopsy of bronchiolitis and interstitial pnuemonitis, frequently with granuloma formation; (9) resolution of the episodic respiratory symptoms after cessation of exposure the spore of Bunashimeji.

Statistical analysis

Data on serum soluble adhesion molecules are presented as mean $\pm$ SD. The chi-square test was used to test the comparison of the positive rates of the precipitins between the workers and controls. Comparisons of serum sICAM-1 and sVACM-1 levels in 1996 among the three groups shown in Fig. 1 and serial changes in serum sICAM-1 levels revealed in Table 1 were assessed by analysis of variance (ANOVA) and Scheffe's test. Serum adhesion molecules in the workers and quitters were compared with the Mann–Whitney *U*-test. Statistical significance was assumed for *P*-values lower than 0·05.

RESULTS

During this study three of the 60 subjects were diagnosed with HP induced by the spore of Bunashimeji, and two of these cases had already been documented (8). In this survey, abnormal shadow could be detected on chest roentgenogram in only these three HP patients.

Consequently, these three patients were excluded from the evaluation and the 57 mushroom workers without HP were assessed. None of the control subjects had the symptoms at the work place or serum precipitins to the Bunashimeji mushroom. Serial changes in the number of symptomatic workers, the frequency of each symptom, serum sICAM-1 levels, and positive rate of serum precipitins are shown in Table 1. Fifteen workers quit from this factory during 1 year from June 1996 to May 1997 and nine more subjects during the following year. A total of 24 employees (40% of the original 60 subjects) stopped working because they could no longer tolerate the symptoms during this study. The incidence of allergic symptoms at the workplace was 80% of the subjects in 1996, 76% of those still employed in 1997 and 73% in 1998. Thirty per cent of the workers were sensitized to the mushroom spore in the 1996 examination, and in 1997 and 1998 this figure had become 93% and 94% respectively of the workers still in employment. No precipitins in sera to the antigens of *Micropolyspora faeni, Thermoactinomyces vulgaris* or *Aspergillus fumigatus* were found. Serial changes in serum sICAM-1 levels were not significantly different among the three measurements (Table 1).

The serum sVCAM-1 levels of 17 workers who precipitin positive were significantly higher than those of both 40 negative subjects and controls (534 ± 74 ng ml^{-1} *vs*. 465 ± 94 and 463 ± 68; $P=0{\cdot}033$, $P=0{\cdot}013$, respectively) (Fig. 1). The levels of sICAM-1 in precipitin positive subjects were significantly higher than those of controls (149 ± 50 ng ml^{-1} *vs.* 116 ± 27; $P=0{\cdot}012$) (Fig. 1). The positive rate of precipitins to the Bunashimeji spore in 1996 in smokers was higher than that in non-smokers, but not significantly (36% *vs*. 16%, NS).

Serum sICAM-1 levels of the 15 quitters, but not sVCAM-1 levels, were higher than those of the other 42 subjects (156 ± 13 ng ml^{-1} *vs*. 130 ± 6, $P=0{\cdot}044$) (Fig. 2). No significant differences were observed in serum sICAM-1 or sVCAM-1 levels between 13 smokers and 44 non-smokers, or between 19 atopic workers and 38 non-atopic subjects.

DISCUSSION

Mushrooms are usually cultivated indoors in Japan and workers are exposed to a high concentration of spores such as *Lentinus edodes* (Shiitake) or *Pholiota nameko* (Nameko). Our results revealed that in the first year 80% of the workers noticed symptoms associated with

Table I. Serial changes in symptoms, serum soluble ICAM-1 levels and precipitin antibodies in 57 mushroom workers hypersensitivity pneumonitis

	June 1996	June 1997	June 1998
Workers still employed (*n*)	57	42	33
Quitters (*n*)	–	15	24
Symptoms in workers still employed	46 (80%)	32 (76%)	24 (73%)
Cough	42	29	20
Sputum	10	7	6
Wheezing	12	8	6
Fever	9	6	4
Shortness of breath	9	6	5
Runny nose	23	15	10
Serum sICAM-1 (mean ± SD)	142 ± 47 ng ml^{-1}	122 ± 32 ng ml^{-1}	109 ± 32 ng ml^{-1}
Positive serum precipitins	17 (30%)	39 (93%)	31 (94%)

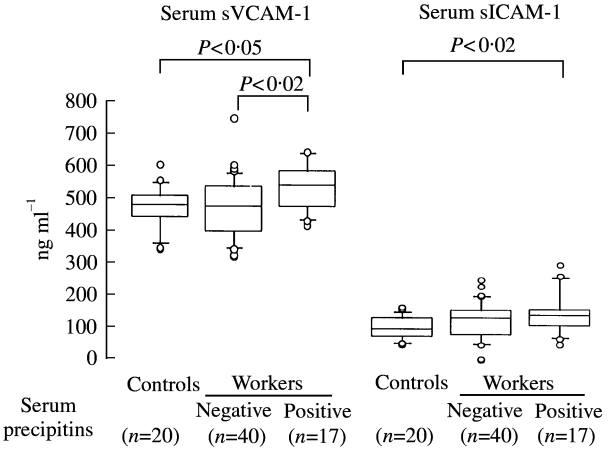

Fig. 1. Comparisons of serum sVCAM-1 (left) and sICAM-1 (right) levels in June 1996 examination among three groups using the ANOVA; controls, workers with negative serum precipitins to the mushroom, and workers with positive precipitins. Box plot represent the median and 25th–75th percentiles; error bars span 10th–90th percentiles.

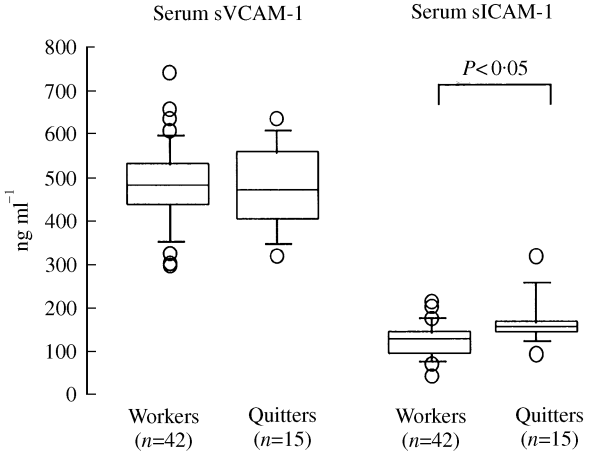

Fig. 2. Comparisons of serum sVCAM-1 (left) and sICAM-1 (right) values in the 1996 examination between workers still employed in 1997 and quitters after the 1996 examination. Box plot represent the median and 25th–75th percentiles; error bars span 10th–90th percentiles.

the mushrooms. In 1 year from June 1996 to June 1997, 15 of the 60 workers quit because their respiratory symptoms became intolerable. These results suggested that this mushroom factory is a significant risk factor for allergic diseases. Moreover, serum precipitins to the Bunashimeji mushroom were positive in 30% of the subjects in the first year, but in the second year this figure was, surprisingly, as high as 93%. A cross-sectional health study of mushroom farm workers in Florida (9), where *Agaricus bisporus* was the prevalent spore, demonstrated that only 15 out of 227 workers (7·5%) had positive serum precipitating antibody to *Agaricus bisporus*. Another study of several 'Shiitake' mushroom farms in Japan revealed that three out of 37 workers (8·1%) had positive precipitins to the antigens of 'Shiitake' (2). In contrast however, antibodies were found not only in almost all affected persons but also in 50% of asymptomatic persons who worked with the mushroom (15). In one study, in which subjects had not engaged in mushroom production, serum precipitating antibodies provided a useful marker of exposure to mushroom spores, but were not specific to an allergic respiratory disease or their predictors (16,17). In our study, no workers had previously engaged in mushroom production, therefore the high incidence of symptoms and high positive precipitin rate might have been due to the inhalation of a high concentration of the spore. These results suggested that contemporary mushroom factories are of significant risk for sensitization to mushroom antigens. Moreover, all mushroom antigens can cause respiratory allergic diseases.

Mushroom antigens are known to produce allergic reactions of all the Gell and Coombs types I–IV (18), but the overall prevalence and incidence of mushroom allergic diseases remains unknown. The complaints of common mushroom workers include the following symptoms: dyspnoea, fever, chill, coughing, malaise, chest pain and headache, appearing 5–10 h after antigen provocation (19). Our results revealed that 80% of 57 workers had one or more of these symptoms, whereas in the Florida mushroom report only 21% of 259 workers had them (9). In our study, the symptoms most frequently reported were cough (60–73%), followed by runny nose (30–40%), wheezing (18–24%) and in the Florida study cough was 17%, fatigue 16% and myalgia 15%. The difference in the prevalence may have been due to the subject selection procedures. We selected a subgroup that might have been inhaling a high concentration of mushroom spores, but this was not the case in the Florida study. Recent studies have indicated that occupational anaphylactic reaction, asthma and extrinsic allergic alveolitis by the inhalation of mushroom spores were frequent among atopic asthmatics (20,21). However there were no such trends in the present study. Two of the three HP patients were non-atopic, and there were no significant differences in circulating levels of sICAM-1 or sVCAM-1 between atopic and non-atopic subjects.

The serum sICAM-1 concentrations of quitters were higher than those of workers still employed in the factory and the levels in precipitin positive workers were higher than those in controls. These results supported the fact that allergic reactions existed in mushroom workers. Serial changes in the incidence of allergic symptoms and serum sICAM-1 levels in workers without HP had a tendency to decrease. This might have been caused by exclusion of the quitters, who had high levels of sICAM-1, meaning that the relative increase in the health of these mushroom workers was in fact a statistical illusion. On the other hand, circulating sVCAM-1 levels were significantly higher in precipitin positive workers in this study. VCAM-1 is enhanced by Th2 cytokines like IL-4 and IL-13 (22,23). IL-4 is also capable of up-regulating the VLA-4-VCAM-1 pathway, which may play a part in both eosinophil and T-cell migration (24). Nakajima *et al*. (25) reported that the expression of VCAM-1 was induced for the first time on the endothelium after inhaled antigen challenge in sensitized mouse; however ICAM-1 had already been expressed to some extent before the challenge. One possible explanation for the elevation of sVCAM-1, but not of sICAM-1 in precipitin positive workers could be that VCAM-1 expression might be enhanced for the first time by the spore-induced Th2-cytokine immunoreaction, which would up-regulate antibody production including precipitins to Bunashimeji.

The level of airborne spore for determination of β-D-glucan was about 10 times higher in the harvesting and packing area as compared with that in the office room. However, workers in this study may have been exposed to a similar level of mushroom spores because all their jobs involved rotating freely between the cultivation room and the harvesting and packing area every day. After this survey, the employees were required to wear protective masks (8), and a part of the harvesting and packing process became automated.

In conclusion, three subjects developed HP by inhalation of the mushroom spore in this 3-year follow-up study. About 70–80% of the workers had allergic symptoms, 93% of the employees were sensitized to the spore within 2 years and 40% of the original workers quit because of the symptoms. These clinical findings were supported by the elevation of soluble adhesion molecules in sera. Contemporary mushroom factories are places where there is substantial risk of the occurrence of occupational allergic diseases.

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