Detection of Indoor and Outdoor Avian Antigen in Management of Bird-Related Hypersensitivity Pneumonitis

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ABSTRACT

Background: In the management of hypersensitivity pneumonitis (HP), antigen avoidance is crucial to prevent the progression of disease. Indirect and unrecognized exposure to the antigen may continue for a long time if persistence of the causative antigen is not recognized. To make a correct assessment of the patients' environment, we tried to establish the methods to detect indoor and outdoor avian antigens.

Methods: Sixteen patients with bird-related HP, 4 asymptomatic breeders, and 6 healthy controls were examined. We prepared anti-pigeon dropping extracts (PDE) polyclonal antibody from rabbits. Air samples and house dust samples were analyzed by an antigen-capture ELISA with signal amplification using catalyzed reporter deposition.

Results: In air samples, avian antigen could be detected in patients with HP (0.73 ± 0.53 ng/m³) and asymptomatic breeders (0.63 ± 0.23 ng/m³). In house dust samples, the amount of avian antigen was higher in patients with HP (2.4 ± 1.8 μg/g) and asymptomatic breeders (4.1 ± 2.3 μg/g) than in the controls (0.1 ± 0.2 μg/g).

Conclusions: Detection of indoor and outdoor avian antigen might contribute to the correct diagnosis and appropriate managements of bird-related HP.

KEY WORDS

antigen avoidance, avian antigen, ELISA, hypersensitivity pneumonitis, pigeon dropping extracts

INTRODUCTION

Bird fancier's lung (BFL) is a type of hypersensitivity pneumonitis (HP), which is induced by inhalation of avian antigens. Immunoglobulins (IgA or degraded IgA) and intestinal mucin in bird dropping and bloom (a keratinous particles derived from bird feathers) are assumed to be causative avian antigens in BFL. In management of BFL, early diagnosis and avoidance of further exposure to the causative antigens are crucial. The diagnosis of acute BFL is relatively straightforward since the clinical features are usually obvious. However, chronic BFL is sometimes difficult to diagnose because most patients have few acute episodes. Several reports suggested that even a low exposure to wild birds and unrecognized exposure to feather duvets and others might lead to HP. In addition, symptoms and signs of HP sometimes persist even after discontinuation of raising birds.

Only a few studies have been reported on the environmental measurement of avian antigen in dust and air. Craig et al. reported that avian antigen could be detected in dust samples using an inhibition enzyme-linked immunoassay (ELISA) for prolonged periods of time after bird removal and environmental cleanup. Curtis et al. also reported that pigeon antigen could be found in dust and air samples from a pigeon-infected school by a direct competitive ELISA. However, these methods are not sensitive enough to measure a small amount of avian antigen.

This study was conducted to establish a method for measurement of a small amount of indoor and outdoor avian antigen by a catalyzed signal amplification system and to evaluate the clinical significance of this...
method in the management of bird-related HP.

METHODS

SUBJECTS

The environments of 16 patients with bird-related HP (15 chronic HP and one acute HP) were assessed by a site-visit and measurement of avian antigen (Table 1). Patients with chronic HP presented with an insidious type except in a patient with a recurrent type (Case 13). The diagnostic criteria for chronic bird-related HP included (i) a history of avian contact, (ii) antibodies and/or lymphocyte proliferation to avian antigen, (iii) reproduction of symptoms related to HP by an environmental provocation or a laboratory-controlled inhalation test, (iv) either evidence of pulmonary fibrosis on histopathological analysis or honeycombing on computed tomographic (CT) scans, (v) either progressive deterioration of a restrictive impairment on pulmonary function over one year or respiratory symptoms related to HP of 6 months duration. Samples from 4 asymptomatic breeders and 6 healthy controls were also examined. The study conformed to the declaration of Helsinki and was approved by the internal review board at our institution. Informed written consent was obtained for each subject.

IMMUNOLOGICAL EXAMINATIONS

Pigeon dropping extracts (PDE) were obtained according to the method by Tebo et al. Fresh pigeon droppings were collected from a pigeon loft in Tokyo and stirred with 20 volumes of PBS for 24 hours. The fraction was extensively dialyzed against distilled water and lyophilized. Specific antibodies in sera and BAL fluids to PDE were measured by an ELISA as previously reported.

To examine lymphocyte proliferation to avian antigen, peripheral lymphocytes and bronchoalveolar lymphocytes (2 × 10⁵ cells) were cultured in quadruplicate with pigeon sera in 96-well plates for 5 days at 37°C. These cells were incubated with [³H]-thymidine for 20 hours and their radioactivity was counted by a scintillation counter. The results were expressed as a stimulation index, mean counts in stimulated cells divided by those in unstimulated cells. Each assay was considered positive when the stimulation index was more than 1.8.

The laboratory-controlled inhalation test was conducted as previously reported. Briefly, patients inhaled 2 ml of PDE (340 μg/ml) through a hand nebulizer. Tests were considered as positive when patients fulfilled three or more of the following criteria: (i) increased radiologic abnormalities, (ii) an increase in alveolar-arterial PO₂ difference (A-aDO₂) by more than 10 mmHg and/or a decrease of carbon monoxide diffusion capacity (DLco) by more than 20%, (iii) a decrease in vital capacity (VC) by more than 15%, (iv) an increase in peripheral leukocyte count by more than 30%, (v) an increase in C-reactive protein (CRP) by more than 1.0 mg/dl, (vi) an increase in body temperature more than 1.0°C and/or the development of systemic manifestations including chills and general fatigue, (vii) development of respiratory symptoms (cough and dyspnea).

COLLECTION OF AIRBONE DUST AND HOUSE DUST

Airborne dust was also collected by a high-volume air...
samplers (High Volume Air Sampler, Model HV-500F, Sibata Scientific Technology Ltd., Tokyo, Japan) with filters (Fiberfilm Filters T60A20, Pall Corporation, New York, NY, USA) at an air flow of 0.8 m$^3$/min. Sampling time ranged from one to 38 hours. The air sample was stored at -80°C until analysis.

House dust was collected by a domestic electronic vacuum cleaner. The dust was stored at -80°C until processed. Approximately one gram of the dust stirred with 40 ml of phosphate-buffered saline solution (PBS) for 24 hours at room temperature. After centrifugation at 10000 rpm, soluble antigen was filtered under sterile conditions at <0.45 μm (Millipore, Watford, UK).

PREPARATION OF POLYCLONAL ANTIBODY
Polyclonal antiserum to PDE was prepared according to the method by Coligan et al. Before immunization, 2 ml of serum was removed from a rabbit to use as a control preimmune fraction (pre-serum). On day 1, the rabbit was injected with a 1:1 mixture of PDE and Freund’s Complete Adjuvant (Sigma Chemicals, St. Louis, MO, USA). On day 29 and day 58, the rabbit was given additional injection of mixture of PDE and Freund’s incomplete adjuvant (Sigma Chemicals). The rabbit was then bled on day 68 and polyclonal antiserum (post-serum) was stored frozen at -80°C until used. The serum was purified by using an IgG purification kit (ImmunoPure [ProteinA] IgG Purification Kit, Pierce, Rockford, IL, USA) and conjugated with biotin (Biotin Labeling Kit-NH$_2$, Dojindo Laboratories, Kumamoto, Japan).

ANTIGEN CAPTURE ELISA WITH CATALYZED SIGNAL AMPLIFICATION SYSTEM
Antigen capture ELISA for avian antigen was constructed. One hundred μl of sodium carbonate buffer solution at pH9.6 containing 100 ng of polyclonal antiserum (pre- and post-serum) were coated on ELISA plates (Immulon 2HB Flat Bottom Microtiter Plates, Thermo Electron Inc., Milford, CT, USA) overnight at 4°C. The plates were washed three times with PBS with 0.05% Tween (PBST) and blocked for 1 hours with 1% bovine serum albumin and then washed three times with PBST. Then standard pigeon dropping extracts (concentration: 10$^{-7}$ to 10$^{-2}$ mg/ml) and samples were added to the plates and incubated at 37°C for 3 hours. The plates were washed six times and then 100 ng of biotin-labeled post-antiserum were added and incubated at 37°C for 30 minutes. The plates were washed six times and then VECTASTAIN Elite ABC Kits (Vector Laboratories, Burlingame, CA, USA) were used according to the manufacturer’s instructions. To amplify the signal generated by the enzyme horseradish peroxidase (HRP), the kit of ELAST ELISA Amplification System Tyramide (PerkinElmer Life And Analytical Science, Inc., Wellesley, MA, USA) was used according to the manufacturer’s instructions. After the amplification step, the plates were washed again six times with PBST and 100 μl of O-Phenylenediimine (OPD) solution was added. After 30 minutes the reaction was terminated by adding 50 μl of 4N H$_2$SO$_4$ and the color developed was measured in an ELISA reader with 490 and 655 nm filters.

STATISTICS
Statistical analysis was performed with post-hoc test using Scheffe’s F test. All expressed values are mean ± SEM unless stated otherwise. Statistical analyses were performed using a software package of Statcel 2 (OMS Publishing Inc., Saitama, Japan).

RESULTS
In the environment of an asymptomatic breeder who was breeding one pigeon in the veranda, avian antigen could be detected inside the room 2 meters away from the veranda. The amount of the antigen gradually increased depending upon the sampling time (Fig. 1).

We applied this method to a patient who was breeding about 200 racing pigeons in the loft (Case 1), which was located about 27 meters away from his house (Fig. 2). We collected air samples in the front of the pigeon loft and his house, and also obtained house dust samples inside his house. The amount of avian antigen in the air samples was 1.0 ng/m$^3$ in front of the pigeon loft and 0.3 ng/m$^3$ in front of his house. In the house dust sample, the amount of avian antigen was 3.0 μg/g (Fig. 2). These results supported the existence of avian antigen around his environment. After he quit breeding pigeons, his disease became stable without progression.

We obtained air samples from 5 patients with HP (Table 2), 4 asymptomatic breeders, and 6 healthy controls without obvious avian contacts. Avian antigen was detected in patients with HP (0.73 ± 0.53 ng/m$^3$) and asymptomatic breeders (0.63 ± 0.23 ng/m$^3$). However, no avian antigen could be detected in controls (Fig. 3).

We obtained house dust samples from 15 patients with HP (Table 2), 4 asymptomatic breeders, and 6 healthy controls without obvious avian contacts. The amount of avian antigen was significantly higher in patients with HP (2.4 ± 1.8 μg/g) and asymptomatic breeders (4.1 ± 2.3 μg/g) than in the controls (0.1 ± 0.2 μg/g) (Fig. 4). This system using PDE seemed to be applicable to detect various types of avian antigen, because the presumed causative antigen originated from birds other than pigeons in 8 of 15 patients (Table 2).

Three patients with bird-related HP (Case 1, 9, 15) had moved to other places which seemed to be free of birds to avoid of avian antigen. The amount of avian antigen in house dust samples was decreased in all cases, though a minute amount of the antigen was
Table 2  Measurement of avian antigen in patients with bird-related HP

<table>
<thead>
<tr>
<th>Case</th>
<th>Sampling place</th>
<th>Presumed causative antigen</th>
<th>Amount of avian Ag in 1m³ of air (ng)</th>
<th>Amount of avian Ag in 1g of dust (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>House</td>
<td>200 racing pigeons 70 meters away from house</td>
<td>0.8</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>Workplace</td>
<td>A bird shop next door</td>
<td>1.6</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>House</td>
<td>Racing pigeons raised by a neighbor</td>
<td>N.D.</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>House</td>
<td>Breeding parakeets</td>
<td>N.D.</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>House</td>
<td>Pigeons raised by a neighbor</td>
<td>N.D.</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>Workplace</td>
<td>Birds in his workplace</td>
<td>N.D.</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>House</td>
<td>Many birds in neighbor’s veranda</td>
<td>N.D.</td>
<td>3.0</td>
</tr>
<tr>
<td>8</td>
<td>House</td>
<td>Pigeons raised by a neighbor</td>
<td>N.D.</td>
<td>5.2</td>
</tr>
<tr>
<td>9</td>
<td>House</td>
<td>Use of poultry manure</td>
<td>N.D.</td>
<td>6.3</td>
</tr>
<tr>
<td>10</td>
<td>House</td>
<td>Wild birds in the yard</td>
<td>N.D.</td>
<td>1.1</td>
</tr>
<tr>
<td>11</td>
<td>House</td>
<td>Wild birds around house</td>
<td>0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>12</td>
<td>House</td>
<td>Wild birds in the yard</td>
<td>N.D.</td>
<td>0.2</td>
</tr>
<tr>
<td>13</td>
<td>House</td>
<td>Pigeons in a railway station</td>
<td>N.D.</td>
<td>3.9</td>
</tr>
<tr>
<td>14</td>
<td>House</td>
<td>200 racing pigeons 27 meters away from house</td>
<td>0.3</td>
<td>3.0</td>
</tr>
<tr>
<td>15</td>
<td>House</td>
<td>Wild birds around house</td>
<td>N.D.</td>
<td>2.0</td>
</tr>
<tr>
<td>16</td>
<td>House</td>
<td>Breeding 10 pigeons</td>
<td>0.3</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Ag, antigen; N.D., not done.

still detectable in two cases (Fig. 5).

**DISCUSSION**

In the International Consensus Statements of ATS/ERS, chronic HP is one of the most important disease that must be differentiated from idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia (NSIP). We previously reported that 47% of patients with chronic BFL were initially misdiagnosed as having IPF or NSIP in our series. In management of patients with HP, antigen avoidance is crucial to prevent the progression of disease. There are many obstacles to complete antigen avoidance in bird-related HP. First, it is relatively difficult to persuade patients to quit breeding birds because they are reluctant to understand the relationship between the occurrence of disease and bird breeding. Second, they have no chance to recognize the causative antigen when exposure to the antigen is indirect. In recent years, indirect and/or unrecognized exposures to birds including pigeons raised by neighbors, a flock of pigeons and/or wild birds in parks, shrines and railway sta-
Environmental Avian Antigen

**Fig. 3** The amount of avian antigen in air samples. Air samples were collected in front of each patient’s house for 6 to 24 hours. C, control; HP, hypersensitivity pneumonitis; AB, asymptomatic breeder. Small bar: mean. *p < 0.05, **p < 0.01.

**Fig. 4** The amount of avian antigen in house dust samples. C, control; HP, hypersensitivity pneumonitis; AB, asymptomatic breeder. Small bar: mean. *p < 0.05, **p < 0.01.

...dust and applied an antigen capture ELISA with catalyzed signal amplification system. As a consequence, we could detect a small amount of avian antigen in dust and air samples from patients with bird-related HP and asymptomatic bird breeders. In the present study, we used anti-PDE polyclonal antibodies, because the exact epitope of the antigen responsible for BFL remains incompletely understood. In addition, antibodies against avian antigen are known to be cross reactive in various kinds of birds.

Avian antigen was reported to persist in the patient's house 6 months after removal of all birds. Even after the patients with BFL quit breeding birds, existence of a minute amount of avian antigen might contribute to the progression of disease. To accomplish complete avoidance of the causative antigen, it should be important to evaluate a small amount of antigen in each patient’s environment. In this study, a minute amount of avian antigen could be detected in the environments of 2 of 3 patients who moved to other places for antigen avoidance. These two patients seemed to slowly progress to an end-stage lung, whereas one patient without detectable avian antigen remained in stable condition. Relationships between the amount of avian antigen and the prognosis of bird-related HP should be further examined.

In conclusion, we were able to detect avian antigen in various environments. This method might be useful to prove existence of avian antigen in indoor and outdoor environments of patients with bird-related HP.

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