

A follow-up study of pulmonary function tests, bronchoalveolar lavage cells, and humoral and cellular immunity in bird fancier's lung

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Background and Objective: *The long-term outcome of bird fancier's lung appears to be variable. The objective of this study is to clarify the sequelae of disease process in bird fancier's lung, with special reference to the humoral and cellular immune responses after avoidance of direct antigen exposure.*

Methods: *Five patients with bird fancier's lung were studied for various parameters including pulmonary function tests, cellular profiles of bronchoalveolar lavage (BAL) fluids, determinations of antibodies in BAL fluids and sera, and antigen-induced proliferation of peripheral and bronchoalveolar lymphocytes during the 5 years of follow-up.*

Results: *Four of five patients showed improvement in pulmonary function, and one showed marked deterioration. This patient's room was close to the pigeon coop where her son was breeding pigeons, resulting in low-grade antigenic stimulation. Three patients demonstrated an increase in CD8⁺ cells in BAL fluid, but the remaining two showed an increase in CD4⁺ cells. The levels of IgA antibodies remained unchanged, whereas IgG levels started declining after the first 3 years of follow-up. Antigen-induced proliferation of BAL lymphocytes from all five patients and blood lymphocytes from four of five patients became weaker and gradually approached normal levels. One patient had pulmonary fibrosis and showed significant reduction in pulmonary functions but elevated reactivity of BAL lymphocytes to pigeon antigens.*

Conclusion: *This follow-up study demonstrates persistence of sensitized lymphocytes and antibody production in the respiratory tract and warrants careful evaluation of patients with bird fancier's lung, even after antigen avoidance. (J ALLERGY CLIN IMMUNOL 1995;96:122-9.)*

Key words: *Pulmonary function tests, bronchoalveolar lavage, humoral immunity, cellular immunity, bird fancier's lung*

Bird fancier's lung (BFL) is an immunologically induced lung disease caused by inhalation of organic materials derived from birds. The bird-related antigens include immunoglobulins, especially

IgA or degraded IgA and intestinal mucin, present in bird droppings and the bloom, a waxy substance that coats the feathers of birds.^{1,2}

The mechanisms involved in the pathogenesis of disease are identical to those of hypersensitivity pneumonitis (HP) induced by other antigens.³ The long-term outcome of patients who have HP appears to be variable. Although several patients have BFL that progresses to pulmonary fibrosis, only very few patients have summer-type HP that progresses to pulmonary fibrosis. The outcome of the disease depends on various factors: duration of

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Abbreviations used

BAL:	Bronchoalveolar lavage
BDE:	Budgerigar dropping extract
BFL:	Bird fancier's lung
DL _{co} :	Carbon monoxide diffusion in the lung
FEV ₁ :	Forced expiratory volume in 1 second
HP:	Hypersensitivity pneumonitis
OD:	Optical density
PBST:	Phosphate-buffered saline containing 0.05% Tween-20
PDE:	Pigeon dropping extract
SI:	Stimulation index
VC:	Vital capacity

exposure, concentration of inhaled antigens, chemical nature of the antigen, and age and genetic predisposition of the patient.⁴

The immunologic mechanisms involved in the pathogenesis of HP have been postulated to be due to an initial immune complex-mediated lung injury, followed by cell-mediated tissue damage.³ Evidence for an immune complex-mediated lung injury in patients is supported by the presence of high titers of antibody to the causative antigen and the presence of activated complement fragments in the bronchoalveolar lavage (BAL) fluids. Other evidence is the deposition of immune complexes detected in the damaged lungs of experimental animals.^{5,6} In contrast, the presence of cellular immune response is demonstrated by the granulomatous inflammatory process in the lung histology compatible with delayed-type hypersensitivity and by the presence of sensitized lymphocytes in both circulation and BAL fluids.⁷ In addition, cellular immunity has been suggested to be more important in distinguishing patients with BFL from their counterparts who are free of symptoms and in predicting the persistence of active disease.^{8,9}

However, a follow-up study of cell-mediated immune responses of the peripheral and bronchoalveolar lymphocytes is not currently available. Furthermore, the relationships between the disease process and immunologic findings, including humoral and cellular immunity, have not been investigated except in a few isolated studies, which demonstrated the persistence of antibodies in the circulation and BAL fluids after avoidance of exposure.^{10,11}

We have examined the clinical and immunologic findings, including cellular immunity, after avoidance of exposure. The results show the persistence of sensitized lymphocytes and antibody in the

respiratory tract and suggest that patients with BFL be carefully supervised even after avoidance of antigen by discontinuing their hobbies.

METHODS

Subjects

Five patients with BFL (three pigeon breeders and two budgerigar fanciers) were studied repeatedly for various parameters during the 5 years of follow-up. Patients with pulmonary fibrosis and history of avian contact but no episodes of acute symptoms were excluded from the study. The diagnosis of BFL was based on the clinical history, chest roentgenogram, computed chest tomography, antibodies in the sera and BAL fluids to pigeon dropping extract (PDE) or budgerigar dropping extract (BDE), histologic examinations of tissue obtained by transbronchial lung biopsy, and results of pulmonary function tests. Informed consent was obtained from all patients.

All patients were nonsmokers, and they ranged in age from 40 to 64 years. Pigeon breeders had been breeding more than 50 pigeons in coops outside their homes, and budgerigar fanciers were keeping one to three birds inside their houses. None of the patients were being treated with corticosteroids when the examinations were performed, and all of the patients discontinued their hobbies when the diagnosis was communicated to them. In spite of the fact that one patient stopped taking care of pigeons, she demonstrated symptoms of fever, cough, dyspnea, and positive inflammatory signs (C-reactive protein, leukocytosis) after an environmental provocation test. However, her room was close to the pigeon coop where her son was breeding more than 70 pigeons.

Pulmonary function tests

The pulmonary function studies carried out included: vital capacity (VC), forced expiratory volume in 1 second (FEV₁), carbon monoxide diffusion in the lung (DL_{co}) and PaO₂. Because the interval between the repetition of the studies could influence the results, the results of pulmonary function tests were expressed as percent of predicted value.

BAL

BAL was performed as described previously,¹² and the lavage was repeated again in a few patients with BFL at varying intervals after avoidance of exposure. The cells from the BAL fluids were separated by centrifugation at 1000 rpm before the cells were phenotyped by a fluorescence-activated cell sorter. The cells were studied for in vitro antigen-induced proliferation of lymphocytes. The lavage fluid was studied for specific antibodies to avian antigens. Serial samples of sera were also obtained from all patients with BFL during the period of observation and were examined for specific antibodies.

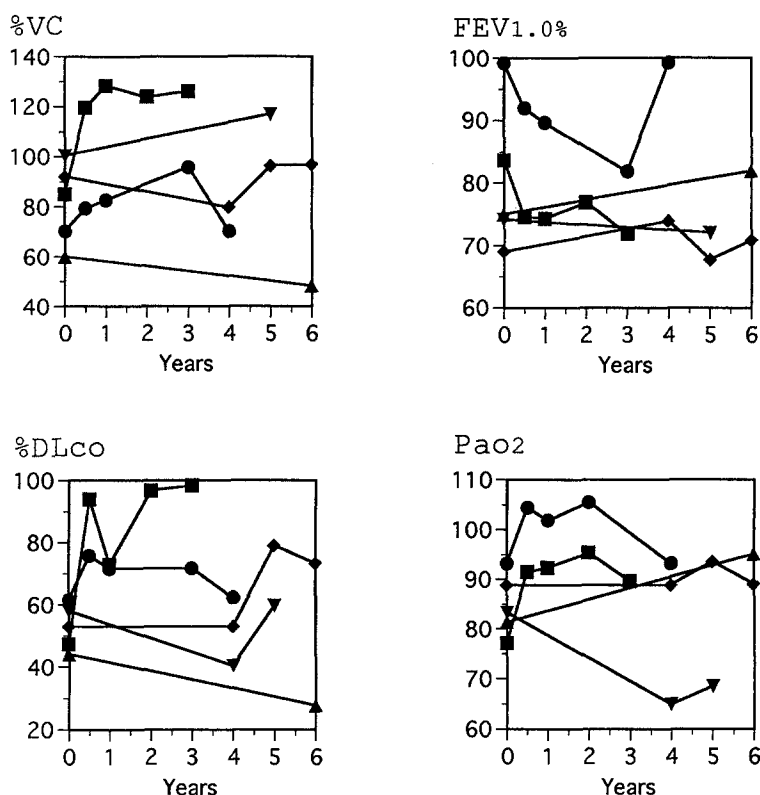


FIG. 1. Sequential changes in results of pulmonary function tests. Four patients showed improvement in percent VC, and one patient showed a deterioration in percent VC and percent DL_{co}. One patient showed deterioration from 44% to 27% in DL_{co} in 6 years, and another patient remained at the reduced levels of 58.0% and 59.7% in 5 years. DL_{co} in the remaining three patients recovered slowly during a various period depending on the individuals. Two patients who underwent repeat testing 6 months later showed improvement in Pao₂.

Antigen preparation

PDE and BDE were obtained for the detection of antibodies according to the methods described by Tebo et al.¹³ The extract was extensively dialyzed against distilled water and then lyophilized. Preliminary experiments showed cross-antigenicity between PDE and BDE as evidenced by the cross-absorption of antibodies to the antigen-coupled Sepharose 4B beads (Pharmacia, Uppsala, Sweden).

Determination of antibodies

IgG and IgA antibodies against the avian antigens were studied by ELISA. Briefly, each well of polystyrene plates (Immulon 2; Dynatech Laboratories Inc., Alexandria, Va.) was coated with 100 μ l of a 1 mg/ml concentration of the PDE in carbonate buffer (pH 9.6) overnight at 4° C. After the wells were washed three times with phosphate-buffered saline containing 0.05% Tween-20 (PBST), each well was treated with 0.5% bovine serum albumin-PBST to block the free binding surface of the wells. Because preliminary experiments showed that the optimal dilution with which to evaluate antibodies was 1:400 for sera and 1:8 for BAL fluids, test samples were diluted accordingly. One

hundred-microliter quantities of the samples were added to each well and incubated at 37° C for 1 hour. After the wells were washed with PBST, 100 μ l of a 1:1000 dilution of goat anti-human IgG or anti-human IgA coupled to horseradish peroxidase (Cappel, Malvern, Pa.) was added and incubated at room temperature for 1 hour. The wells were again washed, and the substrate (o-phenylenediamine and hydrogen peroxide [0.15% vol/vol]) was added, and the color developed was measured in an ELISA reader with 490 and 620 nm filters.

In vitro proliferation of peripheral and bronchoalveolar lymphocytes to avian antigen

The results of the preliminary studies showed that the optimal ratio of lymphocytes to adherent cells for antigen-induced lymphocyte proliferation was 10 to 1. Cultures in triplicate were made in 96-well flat-bottom plates in 200 μ l of complete RPMI 1640 medium (Gibco, Grand Island, N.Y.), containing fetal calf serum, streptomycin, and penicillin. Bronchoalveolar lymphocytes and peripheral lymphocytes (1×10^5)

were cultured with adherent cells in the presence of various amounts of avian antigens (pigeon sera or budgerigar sera from 1:10 dilution to 1:200 dilution) in a humidified incubator at 37° C with 5% CO₂ in air for 6 days. The cultures were then incubated with tritiated thymidine for 20 hours. The cells were harvested, and the incorporation of tritiated thymidine was estimated by counting the radioactivity in a beta-counter.

RESULTS

Sequential follow-up of pulmonary function tests

All five patients with BFL underwent repeat pulmonary function tests on several occasions, and the results were expressed as percent VC, percent FEV₁, and percent DL_{CO} including the values of Pao₂. The results of the pulmonary function tests are shown in Fig. 1. One patient showed a deterioration in percent VC and percent DL_{CO}, whereas Pao₂ remained in the normal range of 81.4 to 95 mm Hg. All of the remaining four patients showed improvement in percent VC, although three of the four patients had values within the normal limits at the initial determination. Similarly, the percent FEV₁ values, although within the normal limits, showed a decrease in two patients from 83.6% to 71.9% and from 99.2% to 81.8%, respectively. Percent FEV₁ values of the other three patients were unchanged during the 6-year observation period. One patient showed a deterioration from 44% to 27.6% in percent DL_{CO} in 6 years, and another patient's values were unchanged and remained at the reduced levels of 58.0% and 59.7% in 5 years. The remaining two patients showed improved values within 2 years, but in the third patient values recovered slowly from 53% to 79% in 5 years. Four patients showed improvement in Pao₂, although the initial determinations of Pao₂ were within normal ranges. One patient has become more hypoxemic (from 83.3 to 68.5 mm Hg) 5 years later.

Lymphocyte phenotype distribution in BAL fluids

The total number of lymphocytes, including CD4 and CD8⁺ lymphocytes, in BAL fluid studied at different times during the 5-year follow-up period is shown in Fig. 2.

Total cells in BAL fluids during the acute stage of the disease were increased to $22.5 \pm 11.1 \times 10^6$ cells, 4 times greater than those of healthy volunteers with no history of direct avian contact. Lymphocytosis, predominantly an increase of T cells, was the most remarkable finding, showing $22.5 \times$

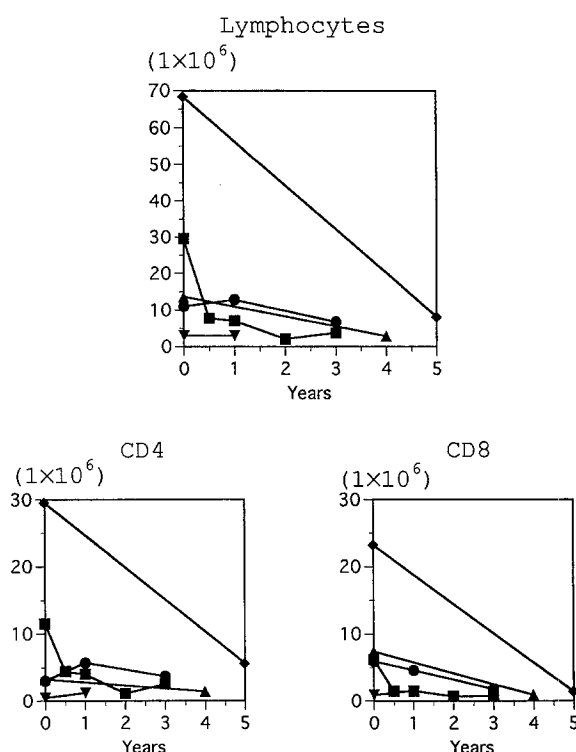


FIG. 2. Serial measurements of bronchoalveolar lymphocytes. Lymphocytosis, predominantly an increase in T cells, was the most remarkable finding, showing 22.5×10^5 cells. The BAL cells from patients with BFL are diverse, and the increase can be either CD4⁺ cells or CD8⁺ cells. Gradual decline in T-cell number, including CD4⁺ and CD8⁺ cells, was observed during the 5 years of observation in all patients.

10^5 cells, an increase 20 times greater than in healthy volunteers. Two patients showed a drastic increase in CD8⁺ cells, 62.6% of T cells with a CD4 to CD8 ratio of 0.51 and 61.8% of T cells with a CD4 to CD8 ratio of 0.51, respectively. The third patient showed slightly more CD8⁺ cells (39.2% of T cells in comparison with 22.2% in CD4⁺ cells), whereas the remaining two patients showed an increase of CD4⁺ cells (CD4 to CD8 ratio: 1.86 and 1.27, respectively). All patients showed a decline in the number of lymphocytes, particularly T cells. The CD4 to CD8 ratio approached normal values or showed a slight increase during the first 6 months of avoidance of exposure and eventually returned to almost normal values in 5 years.

Sequential measurements of antibodies to PDE or BDE

IgG antibodies to pigeon antigens showed a slight decrease from 1.75 ± 0.21 (optical density [OD]) to 1.23 ± 0.27 (OD) in four patients during

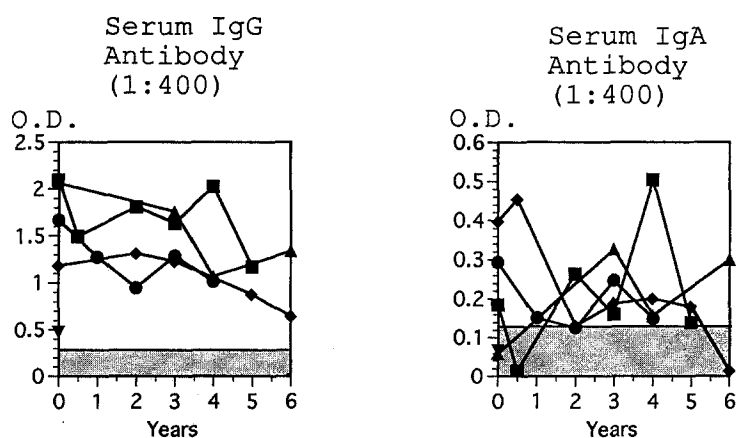


FIG. 3. Sequential measurements of antibodies to PDE in sera. IgG antibodies to avian antigen showed a slight decrease in four patients during the 4-year observation period. Sera of one patient showed weakly positive IgG antibody to BDE. IgA antibodies in sera were very low and varied considerably. *Shaded areas* indicate values (mean $\pm$ 2 SD) for healthy control subjects.

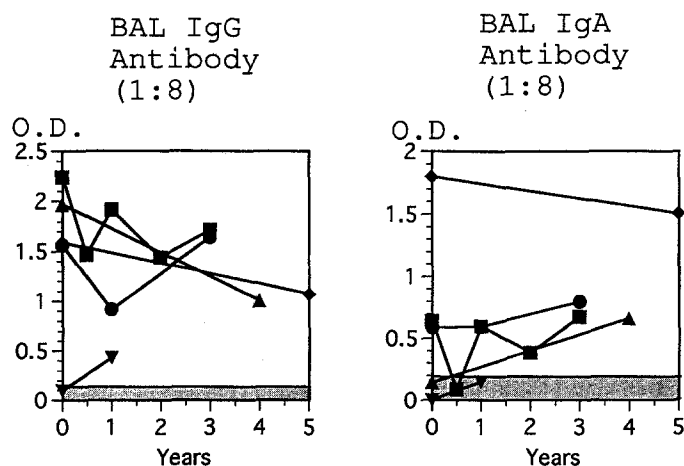


FIG. 4. Sequential measurements of antibodies to PDE in BAL fluid. IgG antibodies in BAL fluids showed a decline in four patients after the third year. IgA antibodies in BAL fluids of four patients remained at the same level during the 5-year observation period. *Shaded areas* indicate values (mean $\pm$ 2 SD) for healthy control subjects. BAL, Bronchoalveolar lavage.

the 4 years (Fig. 3). Beyond 4 years, the antibody showed a tendency to decline. Although, sera of one patient showed a low level of IgG antibody (OD, 0.416) to BDE, a provocation test with budgerigar sera reproduced acute symptoms including fever, cough, and dyspnea. IgA antibodies in sera to avian antigens were very low and showed considerable variability (Fig. 4).

Like serum IgG antibodies, IgG antibodies (OD, 1.84 ± 0.16) in BAL fluids also showed a decline in four patients after the third year, although until that time considerable antibody production was detected (Fig. 3). IgA antibodies (OD, 0.79 ± 0.35) in BAL fluid of four patients remained at the same level during the 5 years of observation (Fig. 4).

In vitro antigen-induced proliferation of peripheral and bronchoalveolar lymphocytes

The magnitude of proliferation was expressed as stimulation index (SI). The SI is calculated as follows: Uptake of tritiated thymidine in antigen-stimulated cultures $\div$ Uptake of tritiated thymidine in control cultures without antigen. The indices of bronchoalveolar lymphocytes stimulated with avian antigens were lower than the indices of peripheral lymphocytes (Fig. 5).

The responsiveness of peripheral blood lymphocytes (SI, 8.0 ± 2.6) to antigen stimulation progressively weakened in three of five patients. One patient showed enhanced response to pigeon sera 3 years after avoidance of exposure, whereas an-

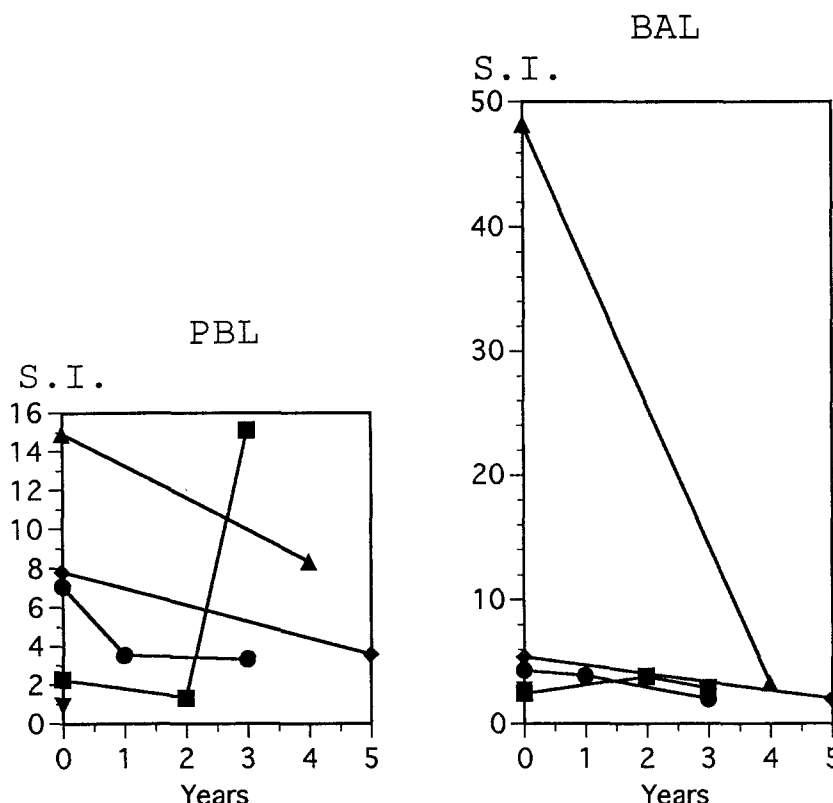


FIG. 5. In vitro proliferation of peripheral and bronchoalveolar lymphocytes to avian antigens. The responses of peripheral blood lymphocytes to antigen stimulation progressively weakened in three of five patients. One patient who had pulmonary fibrosis associated with a decrease of percent VC and percent DL_{CO} demonstrated significantly enhanced stimulation in response to pigeon serum antigen compared with the other four patients. The response of bronchoalveolar lymphocytes to avian antigen gradually diminished and returned to normal values during 5 years. One patient who had pulmonary fibrosis showed a markedly enhanced response to pigeon serum. *PBL*, Peripheral blood lymphocytes; *BAL*, bronchoalveolar lavage.

other patient, who had pulmonary fibrosis associated with a decrease of percent VC and percent DL_{CO} , demonstrated significantly enhanced stimulation (SI, 14.9) in response to pigeon serum antigen compared with the other four patients. In four patients the response of bronchoalveolar lymphocytes (SI, 15.1 ± 11.1) to avian serum gradually diminished and returned to normal values during 5 years of follow-up, but in one patient, who had pulmonary fibrosis with deterioration of pulmonary function, a marked enhancement in the response (SI, 48.2) to pigeon serum was detected.

DISCUSSION

BFL is an immunologic lung disease associated with exposure to bird-related antigens.^{1,2} The long-term outcome of BFL is variable, because some patients may have pulmonary fibrosis and others may improve despite the reduced but continued antigen exposure. The study of the longitu-

dinal course of 18 cases of acute pigeon breeder's lung revealed that most subjects showed improvement in pulmonary function test results and chest roentgenograms in spite of the continued exposure to pigeons.¹⁴

In this study of patients with BFL, four of five improved or remained at similar levels of pulmonary function, and one patient with pigeon breeder's lung deteriorated in percent VC and percent DL_{CO} , although all patients discontinued their hobbies. Low-grade, protracted antigenic stimulation is likely to occur in budgerigar fanciers because a small number of birds kept in a house may result in an insidious disease, leading to irreversible pulmonary damage. However, this study implicated the worst outcome of residual or progressive abnormalities of pulmonary function tests even in pigeon breeders when they are unexpectedly exposed to the low dose of antigen. Although complete cessation of antigen exposure may lead to regression of

the disease process, frequent evaluation of the patient is warranted in spite of avoidance of direct antigen exposure. In support of our observation, a recent study by Perez-Padilla et al.¹⁵ has shown that in a considerable proportion of patients with chronic BFL advanced interstitial lung disease developed.¹⁵

The lymphocyte phenotype studies of BAL cells from patients with HP induced by other antigens show a marked increase of lymphocytes, predominantly CD8⁺ cells, whereas the BAL cells from patients with BFL are diverse, and the increase can be in either CD4⁺ cells or CD8⁺ cells, although T lymphocytosis in BAL cells is common. The phenotypes of T-cell subsets in BAL fluids from patients with BFL in this study showed an increase of CD4⁺ cells in two cases and a drastic or moderate increase of CD8⁺ cells in three cases. However, a decreased CD4⁺ to CD8⁺ ratio in patients with HP may even increase to more elevated values in some cases for a certain period.¹² Therefore phenotypes of T-cell subsets may merely reflect the stage of disease process. The predominance of CD4⁺ T-cell subsets in BAL fluids of patients with HP is more likely to be associated with the development of pulmonary fibrosis than CD8 predominance.¹⁶ However, no definitive conclusions can be made, because this study did not include patients who have pulmonary fibrosis associated with avian contact without a history of acute episodes. Gradual decline of T-cell numbers, including CD4 and CD8 cells, was observed during the 5 years of observation in all patients, although the clinical course was not uniform.

The persistence of antibody production was observed in this study. A follow-up study of antibody to PDE or BDE showed the persistence of high levels of IgG antibody in sera and BAL fluids and of IgA antibody in BAL fluids. In particular, the latter isotype in BAL fluids was unchanged during the 5 years of observation. Because all patients discontinued their hobbies, all direct exposure ceased. Because immune memory has been considered to be short-lived in the absence of antigen, bird antigen persisting in the home environment in small quantities after removal of the bird or continuous sensitization, to a lesser extent, occurring through environmental contamination by wild birds living in flocks or consumption of hen eggs, which may contain cross-reactive avian antigens, results in continued antibody production in patients with BFL.¹⁷⁻²⁰ Bice et al.²¹ have recently shown in dogs that the long-term antibody production in

the lungs and in vitro production of specific antibody by immune cells from the immunized lung lobes can be achieved without further antigen challenges. Therefore local immune reactions may persist for a long period in the absence of antigen.

The in vitro responses of peripheral and bronchoalveolar lymphocytes to pigeon or budgerigar sera have been investigated to distinguish patients with symptomatic BFL from pigeon breeders without symptoms.⁸ The proliferation of peripheral and BAL lymphocytes to avian antigens has been considered to be a specific method to demonstrate differences between pigeon breeders with symptoms and those without symptoms, although several investigators have indicated that the levels of antibody to the causative antigen may be useful to differentiate these two groups.^{22, 23} A follow-up study of the antigen-specific proliferation indicated that cell-mediated immunity to the specific antigen persists for a period of 5 years, declining gradually to unresponsiveness in BAL, but not in peripheral blood lymphocytes after avoidance of exposure to birds.

One patient had pulmonary fibrosis as a result of low-grade, protracted exposure to antigen derived from the pigeon but revealed the most significantly enhanced lymphocyte proliferation to pigeon sera compared with the other four patients at the initial evaluation. At present, it is not known whether this observation is pathognomonic for the disease process.

In conclusion, whether the presence of sensitized cells and antibodies to avian antigens in the lung or peripheral blood is sufficient for the development of active disease remains unknown.

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