

Purification of the antigenic components of pigeon dropping extract, the responsible agent for cellular immunity in pigeon breeder's disease

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Background: Pigeon breeder's disease (PBD) is a lung disease caused by inhalation of antigens derived from pigeons.

Objective: This study was undertaken to characterize the responsible component of pigeon dropping extract (PDE) for PBD.

Methods: First, crude PDE was applied to SDS-PAGE followed by immunoblotting by using antibodies in bronchoalveolar lavage (BAL) fluid. Second, 9 bands of PDE were separated by SDS-PAGE and used for antigen-induced PBMCs. Finally, amino-terminal sequencing was conducted on an isolated 21-kd protein by 2-dimensional electrophoresis.

Results: Immunoblots with BAL fluid from patients with PBD identified 9 bands. Similar patterns were observed by using BAL fluid from 10 control patients (9 with summer-type hypersensitivity pneumonitis or idiopathic pulmonary fibrosis and 1 asymptomatic breeder), except for the 21-kd protein, which was detected in 10 patients with PBD and 1 asymptomatic breeder. The stimulation indices of PBMCs determined by using proteins electroeluted from the 9 bands were higher in patients with PBD than in the 10 control patients. The 21-kd protein was separated into 5 spots by 2-dimensional electrophoresis; these spots were all reactive with BAL fluid from patients with PBD as determined by immunoblotting. The sequence of the 21-kd protein had 57% identity to a *Saccharomyces cerevisiae* chromosome X reading frame. A synthetic peptide, derived from the amino acid sequence of the N-terminal of the native protein, induced significant proliferation of PBMCs obtained from 5 patients with PBD, but not with PBMCs obtained from control patients.

Conclusion: The 21-kd protein is the only protein that identified individuals exposed to pigeons by immunoblotting. Only PBMCs from patients with PBD showed significant proliferation to the 21-kd protein and to the synthetic peptide on the basis of the N-terminal sequence of the native peptide. The 21-

kd protein will be an important antigen for studies on the epidemiology, diagnosis, and pathogenesis of PBD. (J Allergy Clin Immunol 1999;103:1158-65.)

Key words: Pigeon breeder's disease, antigen purification, sodium dodecylsulfate-polyacrylamide gel electrophoresis, 2-dimensional electrophoresis, immunoblotting, cellular immune response, N-terminal sequence of peptide, synthetic peptides

Pigeon breeder's disease (PBD) is an immunologically induced lung disease caused by repeated inhalation of materials derived from pigeons. Mononuclear cell infiltration of the lung interstitium is a common finding in PBD and sometimes develops into granulomas.^{1,2} The mechanisms involved in the pathogenesis of PBD are similar to those of the other types of hypersensitivity pneumonitis (HP) induced by other antigens.³⁻⁵ The immunologic mechanisms involved in the pathogenesis of HP are probably the result of an initial immune complex-mediated lung injury followed by cell-mediated hypersensitivity-induced tissue damage.^{6,7}

The presence of high titers of antibody to bird-related antigen is useful evidence in confirming the diagnosis of HP when the characteristic, clinical, radiologic, and functional features are present.⁸⁻¹⁰ However, the usefulness of antibodies in the diagnosis of HP is questionable because many exposed persons with no apparent lung disease also have antibodies to the causative antigen.^{11,12} On the other hand, cell-mediated hypersensitivity to the presumptive relevant antigens has been shown by the presence of specific sensitized T lymphocytes and is more important in distinguishing patients with PBD from their asymptomatic counterparts and in predicting the persistence of active disease.^{13,14}

The bird-related antigens include immunoglobulins (especially IgA or degraded IgA); intestinal mucin, which is present in bird droppings; or blooms, a waxy substance that coats the feathers of birds.^{15,16} A major problem in studies on the pathogenesis of HP is that complex antigenic mixtures have usually been used.

This study was undertaken to purify the component of pigeon dropping extract (PDE) for both humoral and cell-mediated hypersensitivity that is responsible for the elicitation of disease. Furthermore, the development of simple, cost-effective, and noninvasive methods would be more reliable for the diagnosis of PBD when synthesized peptides become available. Such purified compo-

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Abbreviations used

BAL:	Bronchoalveolar lavage
DW:	Distilled water
HP:	Hypersensitivity pneumonitis
IPF:	Idiopathic pulmonary fibrosis
PBD:	Pigeon breeder's disease
PDE:	Pigeon dropping extract
pI:	Isoelectric point
SHP:	Summer-type hypersensitivity pneumonitis

nents would also be more safe in skin testing and aerosol challenge of patients.

METHODS

Preparation of PDE

Fresh pigeon droppings were collected and stirred with 20 volumes of PBS (0.15 mol/L $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4/0.15$ mol/L NaCl, pH 7.4) for 24 hours. The extract was centrifuged 3 times at 12,000 rpm for 30 minutes. The supernatant was collected and dialyzed against distilled water (DW) for 7 days and freeze-dried. This crude PDE was stored at -20°C until used.

Collection of patients' bronchoalveolar lavage fluids and PBMCs

The diagnosis of PBD was confirmed by history, clinical symptoms, white blood cell count, C-reactive protein, arterial blood gas analysis, anti-IgG and IgA antibodies to PDE in sera and bronchoalveolar lavage (BAL) fluid, chest x-ray findings, chest computed tomography, and lung function analysis. Patients with summer-type hypersensitivity pneumonitis (SHP) were individuals in whom HP to a species of *Trichosporon* developed, which was diagnosed as defined by Shimazu et al.³ We obtained informed consent from all personnel and approval of this study from institutional review boards for human study. BAL fluid was studied from 8 patients with acute PBD, 2 patients with chronic PBD, 1 asymptomatic pigeon breeder, 5 patients with SHP without a history of avian contact, and 5 patients with idiopathic pulmonary fibrosis (IPF) without a history of avian contact. PBMCs were isolated on a Ficoll-Hypaque density gradient from 5 patients with PBD (4 acute and 1 chronic), 5 patients with IPF, 5 normal volunteers, and 5 asymptomatic pigeon breeders (Fig 1).

SDS-PAGE

For SDS-PAGE, 2-dimensional electrophoresis, and Western blotting, 80 mg/mL crude PDE was dissolved in PBS and filtered through MINISART (Sartorius AG, Gottingen, Germany). Crude PDE was separated by SDS-PAGE with 10 mm high stacking gels composed of 4.95% acrylamide and bis acrylamide (T), with a cross-linking of 2.67% (C) (C, percentage ratio of bis-acrylamide to T), and 80 mm high running gels (T = 30% and C = 2.67 %) in the buffer system according to the Laemmli method.¹⁷ PDE was mixed with sample buffer in a ratio of 1:2. Samples were incubated at 95°C for 5 minutes before application, and 1 μL of the samples was loaded onto an 8-cm wide gel. Electrophoresis was performed at 100 V for approximately 45 minutes.

Two-dimensional electrophoresis

This procedure was performed according to the method described by O'Farrell.¹⁸

First dimension. An isoelectric focusing polyacrylamide gel (T = 30% and C = 5.4%) with an immobilized pH gradient from 3 to 10

was prepared in capillary tubes. The pH gradient was prepared with Bio-Lyte (Bio-Rad Laboratories, Hercules, Calif). Phosphoric acid (10 mmol/L) was used for the acidic solution, and 20 mmol/L NaOH was used for the basic solution. PDE was mixed with sample buffer in a ratio of 1:2 and incubated at 95°C for 5 minutes before application. Prefocusing was performed at 300 V for 10 minutes, 450 V for 10 minutes, and 600 V for 10 minutes. Prepared PDE was mixed with an equal amount of glycerol; 20 μL of this mixture was loaded onto tube gels, and 10 μL of sample buffer was overlaid on the sample. First-dimensional electrophoresis was performed at 750 V for 4 hours.

Second dimension. Fifteen percent polyacrylamide running gel was prepared as described previously. Electrophoresed tube gels were added to the wells, and electrophoresis was performed at 100 V for approximately 45 minutes.

Electroelution of SDS-polyacrylamide gel

Nine bands obtained with preparative stacking gels were cut into gel strips and destained with Copper destain solution (Bio-Rad). The gel strips were minced and placed into an electroeluting apparatus (Bio-Rad). Electroelution was performed at 60 mA for 4 hours, with the same running buffer as that used for SDS-PAGE. Each sample was collected, dialyzed against DW by using a Slide-A-Lyser Cassette (PIERCE, Rockford, Ill), and concentrated by use of Ultra-free (Millipore, Bedford, Mass). These samples were stored at -70°C until used.

Western blotting

After SDS-PAGE or 2-dimensional electrophoresis was performed, the proteins were blotted onto polyvinylidene difluoride membranes.^{19,20} These membranes were blocked with Block-Ace (Yukijirushi Nyugyou Corp, Sapporo, Japan) for 60 minutes and mixed with BAL fluids (either undiluted or 1:4 dilution) at 4°C overnight. These samples were washed and reacted with 1:5000 diluted biotin-goat anti-human IgG (Vector Laboratories, Inc, Burlingame, Calif) at room temperature (or 25°C) for 60 minutes followed by incubation with 1:27,000 diluted Streptavidin-alkaline phosphatase (Bio-Rad) at 25°C for 45 minutes. Alkaline phosphatase color development reagent (Bio-Rad) was added for 10 minutes and washed with DW.

PBMC proliferation test

PBMCs were resuspended in RPMI 1640 at $2 \times 10^6/\text{mL}$. One hundred microliters (2×10^5 cells) of the cell suspension was inoculated into 96-well, flat-bottom plates (Sumitomo Bakelite Co, Tokyo, Japan). One half microgram of fractionated PDE (Nos. 1 to 9) was added into each well and incubated for 120 hours. On day 6, PBMCs were pulsed with 1 μCi of ^3H -thymidine (Du Pont, Wilmington, Del) for 16 hours and harvested. The uptake of ^3H -thymidine was determined with a scintillation counter.

Amino acid sequence of PDE

PDE was separated by 2-dimensional electrophoresis followed by blotting onto polyvinylidene difluoride membranes as described previously. Blotted membranes were stained with Sulforhodamine B stain. Two spots (isoelectric points [pIs], 5.0 and 6.1) at molecular weights of about 21 kd were cut out for amino acid sequence procedure. A G1005A protein sequencer (Hewlett Packard Inc) was used for amino acid sequence analysis.

Preparation of synthetic peptide

A peptide with an amino acid sequence corresponding to one of the major avian antigens and a pI of 5.0 (Table I) was synthesized by the Fmoc (N-[9-fluorenyl]methoxycarbonyl) method on a Bio-Synthes BIOSYN 2000 peptide synthesizer. This peptide was puri-

		Fig.3 (A)	Fig.3 (B)	Fig.4	Fig.5	Fig.6
acute PBD	No.1	lane 1		(A)	○	○
	No.2	lane 2			○	○
	No.3	lane 3				
	No.4	lane 4				
	No.5	lane 5				
	No.6	lane 6				
	No.7	lane 7			○	○
	No.8	lane 8			○	○
chronic PBD	No.1	lane 9				
	No.2	lane 10			○	○
asymptomatic breeder	No.1	lane 11		(B)	○	○
	No.2				○	○
	No.3				○	○
	No.4				○	○
	No.5				○	○
SHP	No.1		lane 1			
	No.2		lane 2	(C)		
	No.3		lane 3			
	No.4		lane 4			
	No.5		lane 5			
IPF	No.1		lane 6		○	
	No.2		lane 7		○	
	No.3		lane 8		○	
	No.4		lane 9		○	
	No.5		lane 10		○	
normal control No.1~5					○	

FIG 1. Samples (BAL fluid and PBMCs) used for the experiments.

TABLE I. The N-terminal peptide sequences of the pI 5.0 and pI 6.1 PDE protein

pI 5.0 protein: ICNDP AQHPV THELA DPHXY
pI 6.1 protein: ICNDP ASHPV

The seventh amino acid residues (underlined) were different from each other.

fied by high-pressure liquid chromatography and had a single peak when examined by mass spectrometry.

In vitro proliferation of PBMCs with synthetic peptides

Synthetic peptides were dissolved at 3 different concentrations (10, 50, and 100 μ g/mL) with PBS. These synthetic peptides were added to 100 μ L (2×10^5 cells) of each cell suspension and incubated for 120 hours as described previously. Six days later, PBMCs were pulsed with 1 μ Ci of ^{3} H-thymidine (Du Pont) for 16 hours and

harvested. The uptake of ^{3} H-thymidine was determined with a scintillation counter.

Statistical analysis

Scheffé's F test for multiple-group comparison was used for the analysis of the proliferation of PBMCs with fractionated PDE. Results are expressed as means $\pm$ SEM. The Mann-Whitney U test for a 2-group comparison was used for the analysis of the proliferation of PBMCs with synthetic peptides. Results are expressed as means $\pm$ SEM. Probability (P) values of less than .05 were considered significant in both analyses.

RESULTS

Identification of major allergens in PDE

SDS-PAGE analysis of crude PDE revealed multiple protein bands (Fig 2). The spectrum of protein-staining

bands ranged from about 21 to 199 kD. To determine which proteins in PDE were responsible for specific IgG binding, Western blotting was performed with crude PDE and BAL fluid from patients with PBD and control patients. Analysis of the IgG immunoblots with BAL fluid from patients with PBD and 1 asymptomatic breeder identified IgG-binding proteins with molecular weights of about 199, 140, 110, 87, 60, 55, 44, 40, and 21 kD (Fig 3, A). Similar IgG immunoblots with BAL fluid from 5 patients with SHP and no history of avian contact and 5 patients with IPF revealed nonspecific IgG-binding proteins (Fig 3, B). The 21-kD protein was detected in BAL fluid from all 10 patients with PBD and 1 asymptomatic pigeon breeder, but not in BAL fluid from any of the patients with SHP and IPF.

Isolation and characterization of the 21-kD PDE protein

The 21-kD PDE protein was separated into 5 different pI spots by 2-dimensional electrophoresis. All 5 spots were positive as determined by the IgG immunoblots with BAL fluids from all patients with PBD (one is shown in Fig 4, A) and 1 asymptomatic pigeon breeder (Fig 4, B), but not with BAL fluid from 1 patient with SHP (Fig 4, C). Two of 5 spots with pIs of 5.0 and 6.1 were subjected to amino acid sequencing. The N-terminal peptide sequence (residues 1 to 20) of the 21-kD PDE protein (pI, 5.0) was compared with protein databases and had 57% sequence identity to *Saccharomyces cerevisiae* chromosome X reading frame (Table I). The N-terminal peptide sequence (residues 1 to 10) of the 21-kD PDE protein, with a pI of 6.1, was the same as the pI-5.0 protein, except for that the seventh amino acid residue was S instead of Q for pI 5.0. The pI-6.1 protein was compared with protein databases and demonstrated to have the same result as the pI-5.0 protein (Table I).

Cell-mediated hypersensitivity response to fractionated PDE antigens by PBMCs

The stimulation indices of PBMCs using all of the fractionated PDE were significantly higher in patients with PBD compared with patients with IPF, asymptomatic bird breeders, and normal volunteers (Fig 5). When using the 21-kD protein, there were no differences between the stimulation indices induced by the other 8 fractionated proteins.

In vitro proliferation of PBMCs with synthetic peptides

The synthetic peptides induced a higher stimulation index of PBMCs in patients with PBD compared with asymptomatic pigeon breeders at the concentrations of 50 and 100 µg/mL (Fig 6). These responses to the synthetic peptide were similar to or exceeded the stimulation indices to the native 21-kD peptide (Figs 5 and 6).

DISCUSSION

HP is an immunologic lung disease that results from repeated, intense exposure to a wide variety of inhaled

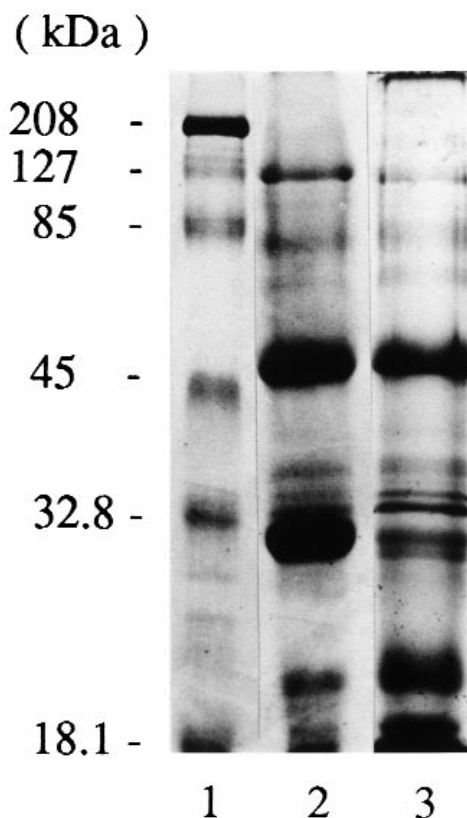


FIG 2. SDS-PAGE (15% polyacrylamide gel) patterns of PDE. Lane 1, Molecular weight standard marker; lane 2, CBB-R350 stain; lane 3, silver stain.

antigens. The list of inhaled antigens for HP continues to enlarge.²¹ The responsible antigenic components have been identified as mannose and mannaoproteins of *Trichosporon asahii* and *Trichosporon mucoides* for SHP and as the carbohydrate moieties (glucose, mannose, and galactose units) of the glycoproteins from *Saccharopolyspora rectivirgula* (formerly *Micropolyspora faeni*) for farmer's lung.^{3-5,21-23}

We used PDE instead of pigeon serum for purification of the disease-associated antigen because (1) generally inhaled dusts are derived from bird droppings or fragmented feathers of birds (bloom); (2) bloom, a waxy substance that coats the feathers of birds, is included in droppings; and (3) IgA (contained in sera), fractionated IgA, and intestinal mucin are also included in droppings. In addition, we used patients' BAL fluid instead of sera as the source of antibodies because BAL fluid is superior to serum in terms of monitoring local pulmonary inflammation.²⁴⁻²⁶

The responsible antigenic components for PBD have been characterized by several investigators, and the results are variable and inconclusive, depending on the materials and the methods used for purification. De Beer et al²⁷ and Mendoza et al²⁸ used pigeon serum for purification of the disease-associated antigens and concluded that 29- to 32-kD substance and 220-kD antigens reacted specifically with patient's sera. McCormick et al²⁹ used

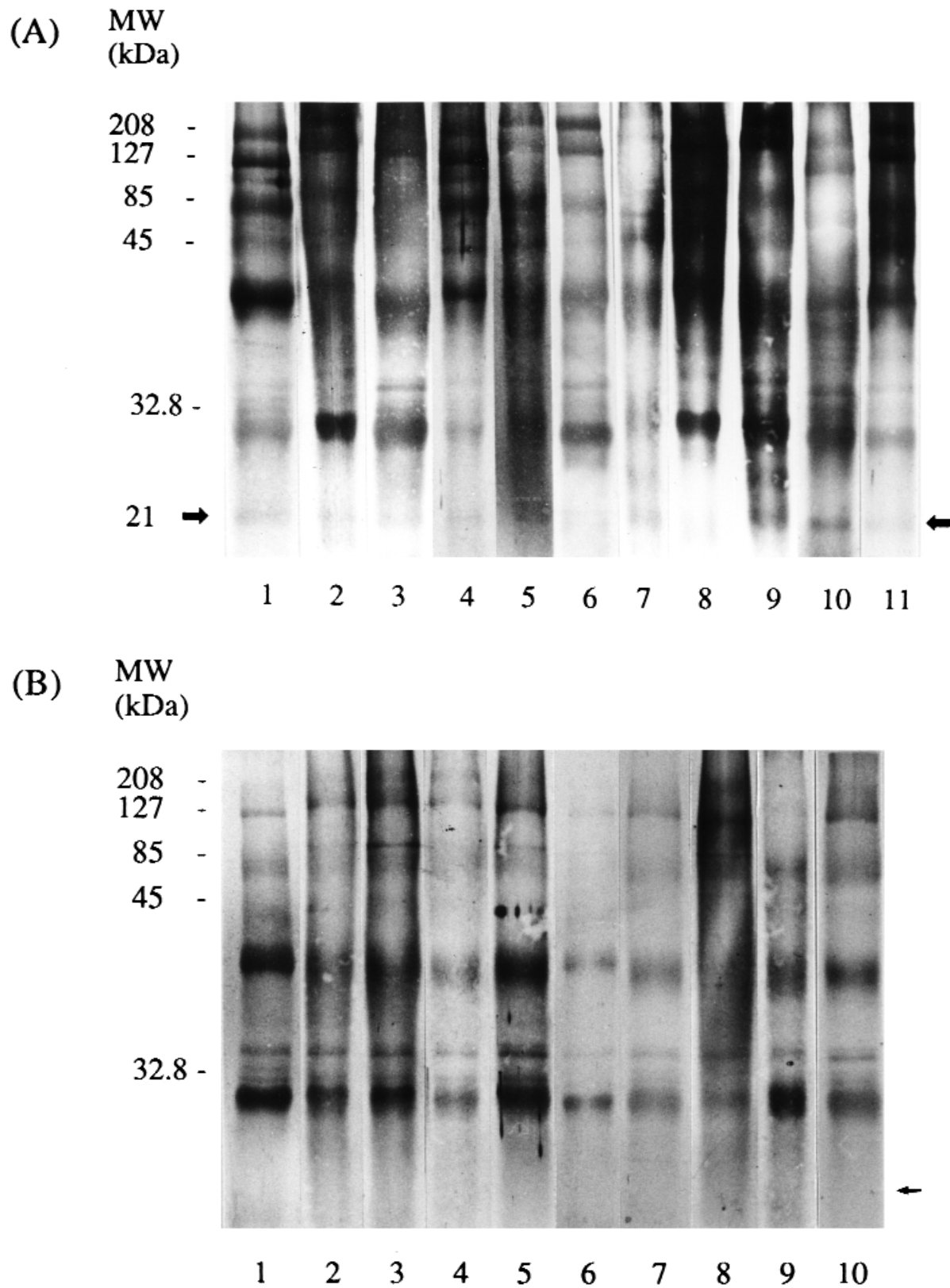


FIG 3. IgG immunoblotting patterns with BAL fluids and PDE. **A**, Lanes 1 to 8, Patients with acute PBD; lanes 9 and 10, patients with chronic PBD; lane 11, one asymptomatic pigeon breeder. **B**, Lanes 1 to 5, Patients with SHP; lanes 6 to 10, patients with IPF. The 21-kd protein (arrows) was detected in all of the patients with PBD and 1 asymptomatic breeder, but not in the control patients.

PDE as a source of antigen and demonstrated a qualitative difference in precipitation patterns with sera of symptomatic patients by using crossed immunoelectrophoresis. Calvanico³⁰ reported that the disease-specific component of PDE was a single polypeptide with a molecular weight of about 50 kD and a pI between 3.5 and 5.1. All of the above results were obtained by using either PDE or pigeon sera as a source of antigen and patients' sera as a source of antibody; BAL fluids were not used. This difference is critical because the local pulmonary immune response is remarkably different from the systemic immune response.^{24,26,31} Furthermore, the above studies were conducted by assessing the antibody response, except for the study by Mendoza et al²⁸ who extracted antigens from pigeon sera and evaluated both humoral and cellular hypersensitivity.

The development of antibodies to pigeon antigens does not distinguish patients with PBD from asymptomatic breeders.^{11,12} In this study BAL fluid from one asymptomatic breeder, but not unexposed control patients, showed antibody reactivity to the 21-kD protein, suggesting that an antibody response to this protein can only identify individuals exposed to pigeons.

The chronic granulomatous pulmonary inflammatory response observed in patients with HP suggests a role for cell-mediated hypersensitivity in this disease. The persistence of cell-mediated hypersensitivity as assessed by the antigen-induced proliferation of sensitized lymphocytes to the causative antigen is required for sustained disease.³² In addition, the *in vitro* proliferation of peripheral and BAL lymphocytes to avian antigens is taken as a reliable method to demonstrate differences between patients with PBD from their asymptomatic counterparts.^{13,14,26} Collectively, these studies suggest that cell-mediated hypersensitivity plays a major role in the pathogenesis of HP.

PBMCs from patients with PBD were reactive to all 9 fractions of PDE, and there was no difference in stimulation indices between each fraction. PBMCs from 4 of 5 asymptomatic breeders had no reactivity to all 9 fractions, including the 21-kD fraction, although one asymptomatic counterpart showed a very weak response, ranging from 1.5 through 1.8 stimulation index. Thus although the 21-kD protein is useful for distinguishing symptomatic from asymptomatic breeders, it is not a specific antigen when compared with the other PDE fractions. Mendez-Samperio et al³³ reported that multiple peptides were recognized as T-cell epitopes in patients with leprosy. In addition, Mendoza et al²⁸ reported that several antigenic components of different molecular weights contribute to the overall polyclonal T-cell response to pigeon serum antigens. Therefore our results may be explicable on the basis of the multiple epitopes recognized by T cells and consistent with the previous observations.

We further purified the 21-kD PDE protein, which induced both humoral and cellular hypersensitivity, and separated it by isoelectric focusing electrophoresis. This protein was separated into 5 different pI spots, and 2 of 5

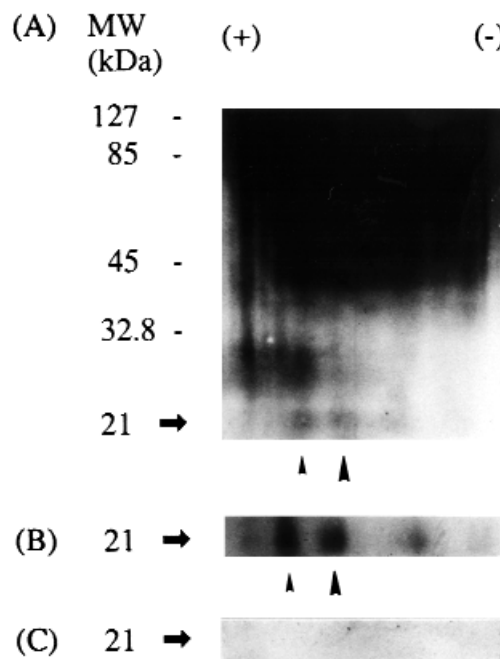


FIG 4. IgG immunoblots with BAL fluid from patients with acute PBD (**A**), 1 asymptomatic pigeon breeder (**B**), and patients with SHP and PDE separated by 2-dimensional electrophoresis (**C**). The 21-kD PDE protein was separated into 5 different pI spots. These spots were detected with the BAL fluid from 1 patient with PBD and 1 asymptomatic pigeon breeder. *Small arrowhead*; pI 6.1 protein; *large arrowhead*; pI 5.0 protein.

spots were examined for amino acid sequence. No completely matched sequence to these 2 proteins was found in the database. However, the N-terminal amino acid sequences of pI 5.0 and 6.1 protein identity revealed 57% homology with *Saccharomyces cerevisiae* chromosome X reading frame. When homology is greater than 80%, it suggests complete matching. However, the 57% identity suggests that either the protein we purified from PDE as an avian antigen was *Saccharomyces cerevisiae* chromosome X reading frame or this is a novel protein. Individuals with no history of avian contact could be sensitized to *Saccharomyces cerevisiae* because of exposure to various foods containing this protein. However, only patients with PBD showed reactivity to this protein, suggesting that this is a protein different from *Saccharomyces cerevisiae*. Further studies will be required, such as a complete amino acid sequence and comparison with antigens in pigeon serum and blooms.

Amino acid sequences of these 2 spots were identical except for one residue, suggesting that these 2 proteins are point mutations at the seventh residue. After amino acid sequencing, we synthesized the peptides and proved that this peptide induced a significant proliferation of PBMCs only in the patients with PBD. These observations suggest that the 21-kD protein we purified from PDE is an important T-cell epitope of avian antigen that is involved in the pathogenesis of PBD. This 21-kD PDE protein may be an important antigen component for the

Stimulation index

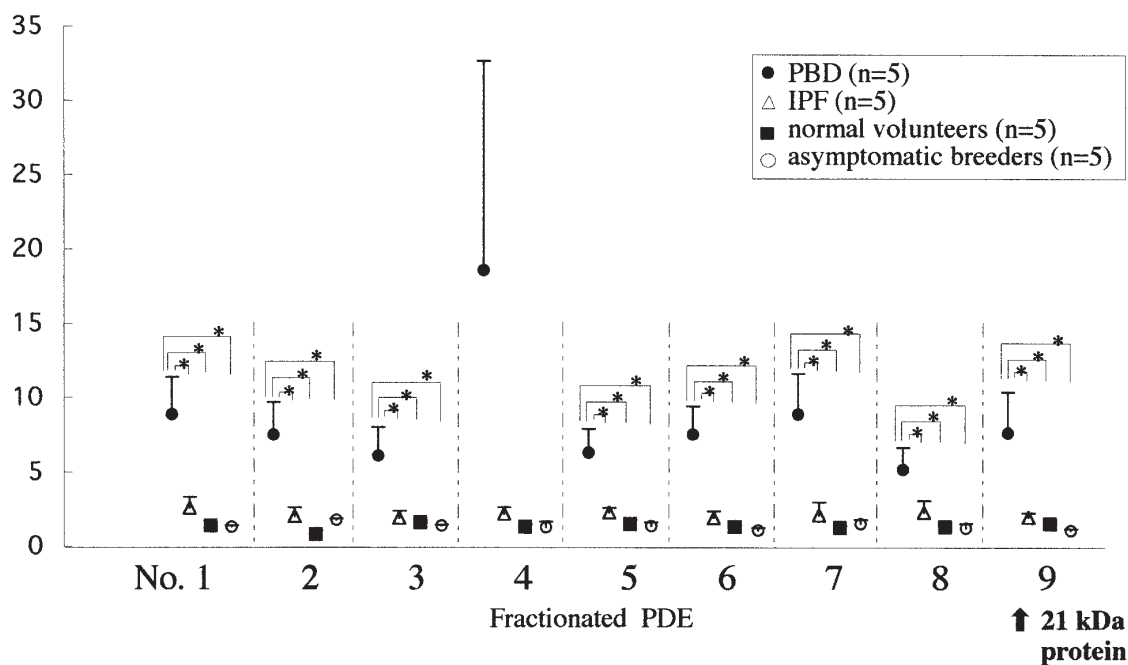
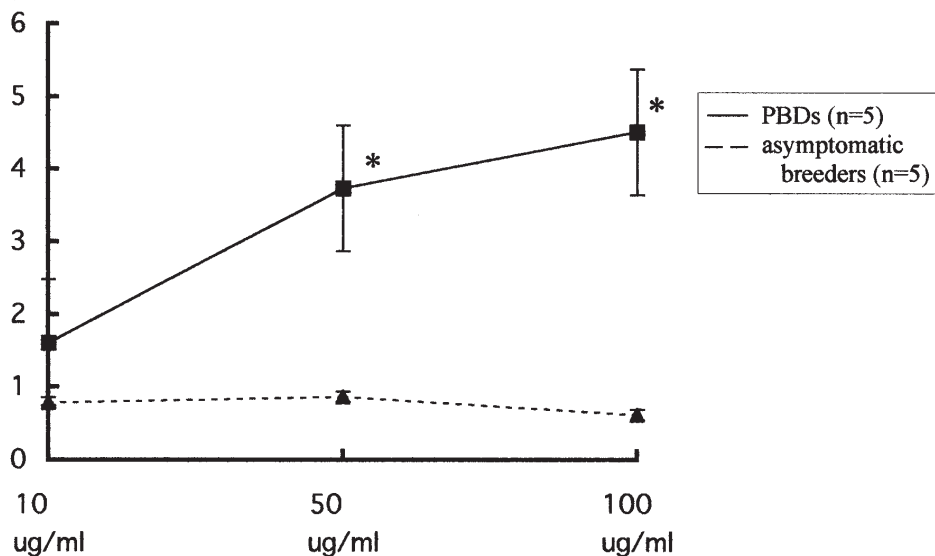


FIG 5. Blastogenic responses of PBMCs by fractionated PDE (Nos. 1 to 9). A significantly high stimulation index was observed only in patients with PBD, but not in patients with IPF, normal volunteers, or asymptomatic breeders. The ninth fraction of PDE indicates the 21-kd protein. *Filled circles*, Patients with PBD; *open triangles*, patients with IPF; *filled rectangles*, normal volunteers; *open circles*, asymptomatic breeders. Results are expressed as means $\pm$ SEM. * $P < .05$.

Stimulation index



Concentration of synthetic peptide

FIG 6. Blastogenic responses of PBMCs by synthetic peptide. Synthetic peptide induced a higher stimulation index of PBMCs in patients with PBD compared with asymptomatic bird breeders at concentrations of 50 and 100 μ g/mL. *Solid line*, Patients with PBD; *broken line*, asymptomatic pigeon breeders. Results are expressed as means $\pm$ SEM. * $P < .05$.

elicitation of PBD. It will also be useful for further studies on the epidemiology, diagnosis, and pathogenesis of PBD.

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