

# Detection of specific antibodies to pigeon serum and bloom antigens by enzyme linked immunosorbent assay in pigeon breeder's disease

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## Abstract

**Background**—Pigeon breeder's disease is an extrinsic allergic alveolitis in the lungs of sensitised people, caused by hypersensitivity reactions to inhaled pigeon antigens. Antigens from different sources of the animal are used for diagnostic purposes, with serum being the most widely used. Bloom is rarely used; very little is known of its antigenicity and diagnostic performance, particularly when used with the enzyme linked immunosorbent assay (ELISA) method, which is the most popular test as it permits measurement of the antibody response.

**Methods**—To (a) standardise an ELISA for the measurement of specific IgG against pigeon serum and pigeon bloom extract; (b) to establish reference values for specific IgG in 73 non-exposed controls, (c) to show the presence of specific IgG against pigeon serum and bloom in serum samples of 17 patients with bird fancier's lung and 11 asymptomatic fanciers, and (d) to study the similarity of the two antigen sources by cross reactivity experiments.

**Results**—Reference values of specific IgG were defined with the 97.5 percentile (367.9 U/ml for pigeon serum and 953.7 U/ml for pigeon bloom extract). Of symptomatic patients 100% had values higher than the cut off for both antigens. In asymptomatic fanciers values were higher than the cut off for pigeon serum in 45% and bloom extract in 54%. Cross reactivity experiments showed that the two antigens differed in antigenic content although some components may be common to both.

**Conclusion**—The ELISA methods used proved to be useful tools for evaluating specific IgG antibody responses against both antigens. The diagnostic performance of both ELISA methods performed with these antigen sources was similar, showing very high sensitivity but moderate specificity. Although some antigenic similarity was found between pigeon serum and bloom extract, cross reactivity studies showed that various antigens seemed to be specific to the bloom extract. However, the antigens responsible for pigeon breeder's disease seem to be present in both antigenic sources.

(*Occup Environ Med* 2000;57:159–164)

Keywords: antibodies; pigeons; ELISA

Pigeon breeder's disease, first described by Reed *et al*<sup>1</sup> in 1965, is an extrinsic allergic alveolitis caused by hypersensitivity reactions to inhaled pigeon antigens in the lungs of sensitised people. The presence of specific antibodies in symptomatic people and delay between exposure and onset of symptoms suggest a pathogenetic type III hypersensitivity. Also, the finding that granuloma had formed in lung biopsies and the activation of T lymphocytes in these patients may also indicate cell mediated type IV hypersensitivity.<sup>2–3</sup> Diagnosis of these diseases is based on a characteristic clinical picture accompanied by: (a) presence of specific antibodies to the causal antigens, (b) positive skin prick test,<sup>6,7</sup> (c) resolution of symptoms after avoidance of contact with the pigeons,<sup>8</sup> and if required, (d) positive specific inhalation challenge. On occasions, a positive transbronchial, or rarely, open lung biopsy is necessary to confirm the diagnosis.

Owing to its simplicity and ease of performance, the identification of precipitating antibodies by countercurrent immunoelectrophoresis remains the most widely used technique in diagnosis (serodiagnosis) of this disease. However, this method lacks analytical sensitivity and provides no quantitative information on antibody concentrations. Consequently, current clinical trends are to substitute the precipitin technique by enzyme immunoassay methods which have shown high sensitivity and reliability, are measurable, and furthermore permit differentiation of the responsible immunoglobulin isotype.<sup>9–10</sup>

Antibody activity against a range of pigeon antigens has been described.<sup>11–13</sup> These antigens have been shown in pigeon serum, intestinal mucin,<sup>14,15</sup> and, particularly, in the bloom from pigeon feathers.<sup>2</sup> Pigeon serum is the antigen source most often used to measure the specific antibodies in this type of extrinsic allergic alveolitis.

Owing to the difficulty in obtaining antigenic extracts from these materials, studies aimed at measuring specific antibodies to antigenic sources other than serum are scarce<sup>16</sup>; furthermore, results are not conclusive.<sup>16,17</sup>

Bloom is a fine dust consisting of flattened squamous keratinised epidermal cells of pigeons,<sup>18</sup> which is readily deposited on hands and clothing during handling of the birds. It has been suggested that bloom has strongly antigenic properties.<sup>19</sup>

The aim of this study was to standardise an enzyme linked immunosorbent assay (ELISA) for the measurement of specific IgG against

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Accepted 15 October 1999

Table 1 Background details of pigeon fanciers in study

	Symptomatic patients	Asymptomatic fanciers
Subjects (n)	17	11
Age (mean, y)	43	33
Sex	14f/3m	11m
Smoking history:		
Current smoker	0	5
Ex-smoker	2	0
Non-smoker	15	6
Exposure to pigeons (mean):		
Pigeons kept (n)	24	87
Contact/week (h)	67	12
Duration pigeons kept (months)	147	216
Serum immunoglobulins (mean):		
IgG (mg/dl)	1277	1102
IgA (mg/dl)	279	202
IgM (mg/dl)	130	138
IgE (IU/ml)	42	31

two pigeon antigen sources (pigeon serum and pigeon bloom extract) to replace the precipitating antibody as the usual diagnostic method. We wanted to find whether differences exist between patients with pigeon breeder's disease and asymptomatic fanciers in the antigenic response to pigeon bloom and pigeon serum proteins, and to find whether antibodies to pigeon bloom extract would have greater diagnostic potential than antibodies to pigeon serum proteins. Thus, similarities and differences in antigenic composition were also studied.

## Materials and methods

### STUDY POPULATION

Seventeen patients with pigeon breeder's disease were studied (13 women, four men; mean age 35 years; range 22–58). All the patients fulfilled diagnostic criteria for extrinsic allergic alveolitis: all have precipitated antibodies (by counter-current immunoelectrophoresis) and had a positive inhalation test or a lung biopsy with changes typical of extrinsic allergic alveolitis. Eleven asymptomatic fanciers (all men; mean age 43 years; range 25–65) were also studied; of these, only four (39%) have precipitated antibodies. Details of these two groups are given in table 1.

Seventy three asymptomatic non-exposed people (54 women, 19 men; mean age 39 years; range 24–67) were studied as a control group.

The study was approved by the hospital ethics committee.

### ANTIGENS

Pigeon serum and bloom extract were used as antigens. Blood was collected from a vein under the wing of three pigeons and centrifuged; the serum protein concentration was measured by bicinchoninic acid assay (Pierce Chemicals, Rockford, Ill). Protein concentration was 2.15 g/dl. Bloom was obtained by aspiration, collecting the feather dust of 20 pigeons from a filter fitted onto a mini-vacuum cleaner.

Antigenic extract from pigeon bloom was prepared by defatting the raw material with anhydrous ether at 37°C in a Soxhlet apparatus (Corning, Stone, Staffordshire, England) for 4 hours.<sup>20</sup> Material was then dried and extracted (1:20 w/v) in 0.2 mol/l ammonium bicarbo-

nate, pH 7.9, overnight at 4°C. The solution was centrifuged and the supernatant dialysed overnight at 4°C against deionised water in a 3.500 molecular weight cut off membrane (Spectra/Por; Spectrum Medical Industries, Los Angeles, CA, USA) until a clear extract was obtained. The material was lyophilised and the protein concentration measured by the bicinchoninic acid assay method. The bloom extract had a protein concentration of 0.22 g/dl.

### ASSAYS FOR TOTAL IMMUNOGLOBULINS

Non-specific IgG, IgA, and IgM concentrations were measured by kinetic nephelometry (Array Protein System, Beckman-Instruments, Bre, CA, USA). Reference values established in our laboratory were: IgG 8.5–16 g/l; IgA 0.75–3.5 g/l; IgM 0.58–2.5 g/l. Concentrations of IgE were measured by the UniCAP 100 system (Pharmacia AB, Uppsala, Sweden). Reference values were <100 IU/ml.

### SPECIFIC IgG TO PIGEON SERUM AND BLOOM

Specific IgG was measured by an ELISA based on the method described by Metzger *et al*<sup>21</sup> modified with pigeon serum or bloom extract as the antigen. Wells of high binding microtitre plates (Costar, Cambridge, MA, USA) were incubated with 2 µg protein/well in 0.1M Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer, pH 9.6, at 4°C overnight. The wells were then washed three times with washing buffer (0.1M phosphate buffered saline, pH 7.5/0.005% Tween 20) and blocked with phosphate buffered saline/1% bovine serum albumin for 1 hour at 37°C. Washing the plates four times between steps, the specific IgG assays were performed in duplicate by incubating the serum samples at an appropriate dilution and the standard curve for 2 hours at 37°C. A solution of horseradish peroxidase labelled antihuman IgG (clone MH16-1ME, 0.5 mg/ml) diluted at 1:1000 was added and the plates incubated for 2 hours at 37°C. The reaction was developed with 3,3',5,5'-tetramethyl-benzidine (Sigma Chemicals), 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes at room temperature in the dark and stopped with 2M H<sub>2</sub>SO<sub>4</sub>, and the optical density at 450 nm was measured with a microplate reader (Titertek Multiskan Plus MKII).

Results were expressed as arbitrary units of ELISA using a reference pool serum with an assigned value of 5×10<sup>4</sup> U/ml for the two antigen sources. The pool was obtained with serum samples from precipitin positive symptomatic patients. Within run coefficients of variation for pigeon serum and bloom extract were 5.4% and 7.8%, respectively. Day to day coefficients of variation for the two antigen sources were 8.3% and 9.5%, respectively.

To study the behaviour of serum samples compared with the reference pool, titrations were performed of four serum samples with optical density values at 450 nm between 1.3 and 0.5 for pigeon serum and 2.5 and 0.5 for bloom extract. The curves obtained from the serum samples and reference serum were plotted for linear regression analysis.<sup>22</sup>

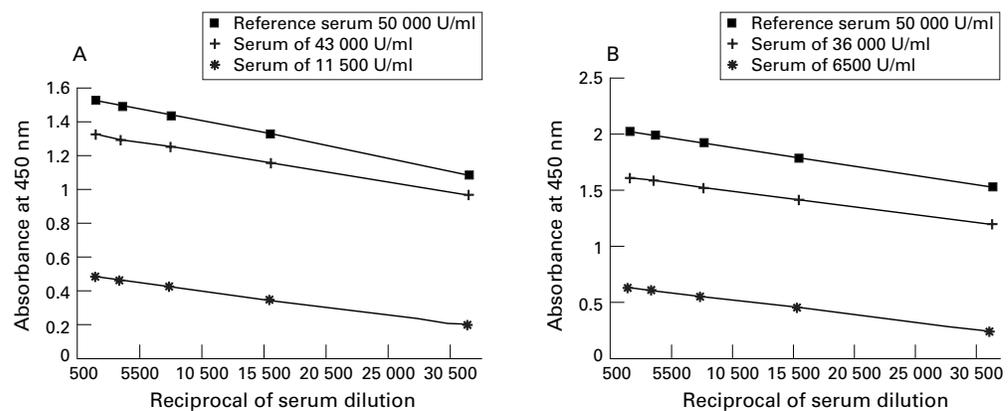


Figure 1 Regression lines for the reference serum and samples with different specific IgG antibody titres (A) to pigeon serum and (B) to pigeon bloom extract. No significant differences in the slopes of the lines were obtained by multiple regression analysis.

The minimum amount of specific IgG detectable with this assay was 0.4 U/ml for the two antigen sources. This value was obtained as the mean plus 3 SDs of the calibrator 0 performed in 30 determinations.

#### CROSS REACTIVITY STUDY BETWEEN BOTH ANTIGENS

The method was the same as that used for specific IgG measurement, with the following modifications: wells of high binding microtitre plates (Costar, Cambridge, MA, USA) were coated with pigeon serum or pigeon bloom extract. After washes (0.1 M phosphate buffer/0.1% Tween 20), the plates were incubated with 50 µl/well of an undiluted pool of serum from the allergic patients with a high concentration of specific IgG to both antigenic sources, and increasing concentrations (0–100 µg) of pigeon serum or pigeon bloom as inhibitor antigens, for 2 hours at 37°C. After three washes, horseradish peroxidase labelled anti-human IgG (clone MH16–1ME, 0.5 mg/ml) diluted at 1:1000 was incubated for 2 hours at 37°C. After washing, the reaction was developed with 3,3',5,5'-tetramethyl-benzidine (Sigma Chemicals), 3% H<sub>2</sub>O<sub>2</sub> for 20 minutes at room temperature in the dark. The reaction was stopped with 2M H<sub>2</sub>SO<sub>4</sub> and the optical density at 450 nm measured with a microplate reader (Titertek Multiskan Plus MKII).

The concentration of free antigen was plotted against percentage inhibition, and from this the concentration required to give 50% inhibition was calculated. Also, the inhibition lines had a measurable slope and comparison of the slopes of the extract was a measure of antigenic relatedness.

#### STATISTICAL ANALYSIS

The Kolmogorov-Smirnov test was used to check the fit of the data. Significant differences

between raw data and normal distribution were found. Therefore, non-parametric methods were used. Differences in mean values for each group were tested by the Mann-Whitney *U* test, and correlation coefficients between specific IgG against pigeon serum and bloom extract values were performed by the Spearman rank test. Multiple regression analysis with the SPSS statistic program was used to compare the similarity of the slopes of the regression lines obtained (linearity and cross reactivity studies). A *p* value <0.05 was considered to be significant.

## Results

#### LINEARITY OF THE ELISA METHOD

Every single serum sample was tested at seven different dilutions to create a curve. The regression lines were created with five points. Figure 1 shows the regression lines generated for pigeon serum (A) and pigeon bloom extract (B). No significant differences in the slopes of the lines were obtained by multiple regression analysis (*p*=0.52 and *p*=0.44, respectively). Thus it may be assumed that the behaviour of serum samples and the reference serum do not differ.

#### VALUES OF SPECIFIC IgG AGAINST PIGEON SERUM AND BLOOM EXTRACT IN THE CONTROL GROUP, IN PATIENTS WITH BIRD FANCIER'S LUNG, AND IN ASYMPTOMATIC FANCIERS

Concentrations of specific IgG against pigeon serum and bloom in the non-exposed control group are shown in table 2. The reference values were defined as the 97.5% percentile.

Table 3 shows concentrations of specific IgG against pigeon serum and bloom in patients with bird fancier's lung and in asymptomatic fanciers. Results were considered positive if they exceeded the reference value. Values of specific IgG to pigeon serum and bloom in the asymptomatic fanciers were higher than the cut off in 45% and 54%, respectively. All symptomatic patients (100%) had values higher than the cut off.

The highest correlation was found between specific IgG to pigeon serum and bloom extract in the asymptomatic group (*r*=0.84, *p*<0.01). Other significant correlations were

Table 2 Specific IgG against pigeon serum and pigeon bloom extract in the non-exposed control group

Extracts	Mean value	95% CI	Median	Range	Cut off†
Pigeon serum	116	114 to 160	108	33–645	367*
Bloom extract	473	460 to 549	475	215–1098	953

\**p*<0.005 *v* pigeon bloom extract.

†97.5th percentile.

Table 3 Specific IgG against pigeon serum and pigeon bloom extract in patients with bird fancier's lung and asymptomatic fanciers

Extracts	Patients	Median	Range	Positive (%)
Pigeon serum	Asymptomatic	252	59–188145	45
	Symptomatic	19996	4134–440970	100
Bloom extract	Asymptomatic	979	505–253443	54
	Symptomatic	14718	4478–63480	100

found in the control group and patients ( $r=0.33$ ,  $p<0.01$  and  $r=0.57$ ,  $p=0.02$ , respectively).

#### COMPARISON OF SPECIFIC IgG AGAINST PIGEON SERUM AND BLOOM EXTRACT BETWEEN THE CONTROL GROUP, PATIENTS WITH BIRD FANCIER'S LUNG, AND ASYMPTOMATIC FANCIERS

The specific IgG concentration in the three groups studied is shown in figure 2. Significant differences were found between the non-exposed control group and the asymptomatic fanciers against pigeon serum and also against bloom extract ( $p<0.01$ ), and also between the non-exposed control group and symptomatic patients ( $p<0.01$ ). No significant differences were found between asymptomatic and symptomatic patients to both antigenic sources.

#### CROSS REACTIVITY EXPERIMENTS

Results of cross reactivity experiments between pigeon serum and bloom are shown in figure 3. Slopes of the regression lines obtained in the

inhibition experiments showed no significant differences by multiple regression analysis when the coated antigen was pigeon serum ( $p=0.06$ ). Significant differences were found when the coated antigen was pigeon bloom ( $p=0.02$ ). This did not seem to be due to cross reacting antibodies as the pigeon bloom concentration required to produce 50% inhibition when the coated antigen was pigeon serum was lower (135  $\mu\text{g}$ ) than the pigeon serum concentration needed to produce 50% inhibition when the coated antigen was bloom (1549  $\mu\text{g}$ ), suggesting that there is only minor cross reactivity between the two antigen sources.

Also, these results suggest that the relative potency of pigeon bloom was stronger than pigeon serum when the quantity of each extract required to produce 50% inhibition was compared.

#### Discussion

In this study the IgG response to both pigeon serum and pigeon bloom was quantitatively assessed by ELISA to measure whether responses differ between symptomatic and asymptomatic people. The results show that the ELISA method described in this study is a useful tool for evaluating the specific IgG antibodies, and also that although various antigens seemed to be specific for bloom extract, an

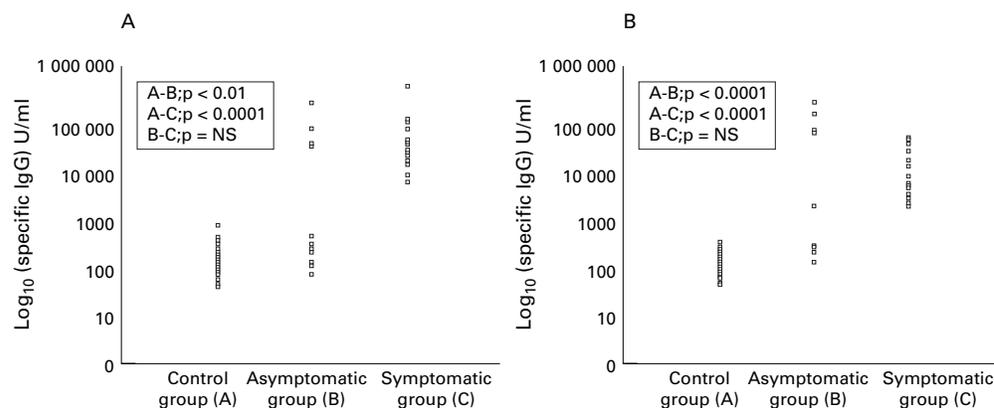


Figure 2 Specific IgG concentration to pigeon serum (1) and pigeon bloom extract (2) (A) in the control group, (B) asymptomatic fanciers, and (C) symptomatic patients. No significant differences were found between asymptomatic and symptomatic patients to both antigenic sources.

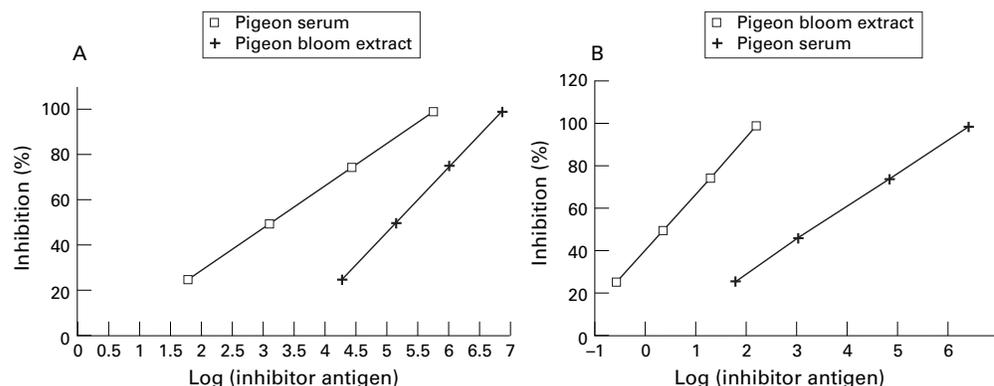


Figure 3 Cross reactivity experiments for specific IgG antibodies, using as solid phase: (A) pigeon serum and (B) pigeon bloom extract. The pigeon bloom concentration that produced 50% inhibition when the coated antigen was pigeon serum was lower (135  $\mu\text{g}$ ) than the pigeon serum concentration that produced 50% inhibition when the coated antigen was bloom extract (1549  $\mu\text{g}$ ).

antigenic similarity was found between these two antigenic sources.

Specific IgG measurement to avian antigens by ELISA method in the diagnosis of pigeon breeder's disease constitutes an advance compared with classic precipitating antibody measurement as it has better analytical sensitivity (10–1000 ng/ml) and specificity.<sup>9</sup> Also, the ELISA is more useful for the detection of antibodies against pigeon antigens in fluids with a low protein content—such as bronchoalveolar lavage—as reported by Sandoval *et al.*<sup>16</sup> These authors, in a comparative study for the detection of specific antibodies by counter-current immunoelectrophoresis and ELISA in the bronchoalveolar lavage of patients with extrinsic allergic alveolitis, found that 12% and 100% were positive, respectively. Simpson *et al.*,<sup>23</sup> in a study of specific antibodies against pigeon serum by double immunodiffusion and ELISA in serum and bronchoalveolar lavage of patients with pigeon breeder's disease, also showed the ELISA method to have higher sensitivity ( $10^3$  times) than the double immunodiffusion method.

In the absence of standards, which are currently unavailable, for measuring specific IgG against avian antigens, the results of our study were expressed in arbitrary U/ml referred to our own reference serum with an assigned value of  $5 \times 10^4$  U/ml for the two antigen sources. This reference pool was obtained with serum samples from symptomatic patients with positive antibody results by counter-current immunoelectrophoresis. The results of multiple regression analysis of the linearity study showed the validity of this standard, as the behaviour of serum samples in a large interval of dilutions and the reference serum do not differ (fig 1).

The ELISA method used in the present study showed for pigeon serum and pigeon bloom that 100% of symptomatic fanciers had values higher than the cut off established in this study, whereas in asymptomatic fanciers the percentages were 45% and 54%, respectively. These percentages are similar to those reported by Andersen *et al.*<sup>24</sup> with a counter-current immunoelectrophoresis method who found precipitating antibodies in 100% of symptomatic patients and 60% of asymptomatic fanciers. Beer *et al.*<sup>25</sup> with western blot, an expensive and time consuming procedure, found that all the symptomatic patients as well as 57% of the asymptomatic fanciers were positive to pigeon serum.

Simpson *et al.*<sup>23</sup> with an ELISA method and a pigeon faecal extract as antigen, found a positive reaction in 100% of 1:100 diluted serum samples of patients with the disease, whereas a positive immunodiffusion reaction was detected with undiluted serum samples in only 80% of these patients. At present, the use of faecal extract as an antigen source has been ruled out owing to the possible positive precipitins when the patient's serum reacts to the teichoic acid of the *Staphylococcus* capsule, which is a frequent contaminant of bird droppings.<sup>26</sup>

To our knowledge, no studies have been published which focus on measuring specific

IgG antibodies to pigeon bloom extract by an ELISA method. Thus, we have been unable to compare our results, as authors who study this antigenic source use other techniques.<sup>24 25</sup>

The results of the present study show that both patients who develop the disease and asymptomatic exposed people synthesise high concentrations of class IgG antibodies against both pigeon serum and bloom extract. With the ELISA method standardised in this study, values of specific IgG to pigeon bloom extract are triple those to pigeon serum in the control group, and the percentage of asymptomatic breeders with antibodies was also higher for the pigeon bloom extract. These results are similar to those reported by Banham *et al.*,<sup>19</sup> which would suggest that pigeon bloom is a more potent antigen source than pigeon serum.

Furthermore, the cross reactivity studies conducted in our work with inhibition experiments between pigeon serum and bloom extract show that a greater concentration of pigeon serum is required to inhibit pigeon bloom than the other way round. This could indicate that bloom is a more potent antigenic source than pigeon serum and that the extracts differ in antigen content. Longbottom *et al.*,<sup>18</sup> in a study to investigate the antigenicity of a pigeon bloom extract by crossed immunoelectrophoresis, identified 29 antigenic components in the bloom and only 10 in pigeon serum. Although some antigenic similarity was found between pigeon bloom and serum, various antigens seemed to be specific. These results concur with those of our study and would corroborate our previous comments. More recently, Baldwin *et al.*<sup>5</sup> studied the response of IgG and specific IgG subclasses to pigeon serum and intestinal mucin in a group of symptomatic people and another group of asymptomatic exposed subjects and found higher specific IgG values against intestinal mucin than against pigeon serum in both groups. However, in patients with extrinsic allergic alveolitis, contrary to Baldwin *et al.*,<sup>3</sup> values of specific IgG to both antigenic sources are similar; this induces us to think that the antigen which causes the symptoms is present in both pigeon serum and bloom extract.

A notable point of the work of Baldwin *et al.* is that they found no significant differences in the composition of specific IgG subclasses against pigeon serum between symptomatic and asymptomatic people, but differences were found in the composition of subclasses against mucin, with significantly higher IgG1 titres in the patient group. These results concur with those found by our group in patients with extrinsic allergic alveolitis caused by inhalation of mollusc shell dust<sup>27</sup> where the specific IgG1 against mollusc shell dust extract was the more abundant subclass, suggesting that it could be the principal antibody involved in the pathogenesis of this disease. These results support the importance of the study of specific IgG subclasses against different avian antigen sources, as the results of specific IgG do not seem to show any difference in response between the group of patients with symptoms and asymptomatic subjects.

In conclusion, this study shows that: (a) specific IgG measurement by an ELISA method is more sensitive for detecting exposure to antigens than the precipitating antibodies study, (b) the ELISA described proved to be a useful tool for evaluating specific IgG responses against pigeon serum and pigeon bloom, and (c) although bloom has some antigens different from pigeon serum, in patients with extrinsic allergic alveolitis, the results obtained in this study show that the antigens responsible for the disease are found in both pigeon serum and bloom extract.

Further studies should be conducted with measurement of specific IgG subclasses against both antigen sources, as their profiles may provide greater understanding of the pathogenetic mechanisms and of how to explain why only some exposed people develop the disease. In this line, studies are currently underway in our laboratory in an attempt to find by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot the identification and purification of some antigenic proteins of these avian antigenic sources recognised by serum samples of affected people.

This research has been supported in part by a grant from Fondo de Investigaciones Sanitarias (FIS 93/0448). We thank Cristina O'Hara for help with the english translation of the manuscript.

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