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Use of co-immunoelectrodiffusion to detect presumed disease-associated precipitating antibodies, and time-course value of specific isotypes in bird-breeder's disease

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Abstract

Background and objective: The practical value of immunological diagnosis of bird-breeder's disease (BBD) is controversial, because of difficulties in distinguishing active disease patients from simple contact subjects. The aim of this study was to determine the diagnostic and prognostic value of (a) presumed disease-associated antibodies precipitating pigeon antigens (immunoglobulin A (IgAp) and P2 component), (b) characterization of specific isotypes (IgG, IgM, and IgA), and (c) antibody kinetics after antigen eradication. *Methods*: 405 subjects (775 sera) in contact with birds were studied [by means of co-immunoelectrodiffusion (Co-IED) and enzyme-linked immunofiltration (ELIFA)] with soluble extracts of pigeon droppings and squab crop milk. These patients were divided into two groups based on the final clinical evaluation of the patients' physicians, which was taken as the gold standard (positive in 90 and negative in 315 cases). *Results*: On the basis of this gold standard, the detection of presumed disease-associated precipitating antibodies by Co-IED had a specificity of 95.5%, a sensitivity of 98.7%, an accuracy of 98%, and positive and negative predictive values of 95.5% and 98.7%, respectively. Most of the patients with a final positive diagnosis of BBD had specific IgG, IgM, and IgA antibodies by ELIFA. After antigen eradication, anti IgAp and/or P2 antibodies disappeared more rapidly than other precipitating systems. *Conclusion*: Identification by Co-IED of precipitating immune complexes IgAp and/or P2 significantly reinforces the intrinsic credibility of immunological diagnosis of BBD. Compared to these presumed disease-associated precipitating antibodies, detection and time course of specific IgM, IgA antibodies, provided no additional diagnostic value or prognostic arguments to judge disease activity after antigen eradication.

Keywords: Immunological diagnosis; Precipitating antibodies; Specific isotypes; Bird-breeder's disease

Abbreviations: EAA, extrinsic allergic alveolitis; BBD, bird-breeder's disease; Ig, immunoglobulin; Co-IED, co-immunoelectrodiffusion; ELIFA, enzyme-linked-immuno-filtration-assay; Hg, hemagglutination; SEPE, soluble extract of pigeon excreta; SCM, squab crop milk.

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1. Introduction

The term extrinsic allergic alveolitis (EAA) covers a group of pulmonary diseases resulting from sensitization to inhaled organic particles. The pathogenesis is complex and involves host susceptibility factors (Boyd, 1990; Salvaggio, 1990, 1997; Allen and Spiteri, 1996; Calvert et al., 1999).

Bird-breeder's disease (BBD), the most frequent type of EAA, can be acute, subacute or chronic. Dyspnea is the main symptom, and is usually accompanied by dry cough. General signs (fever, fatigue, and weight loss) and pulmonary signs (diffuse crepitant rales) can also be present. BBD is potentially life-threatening (Gari et al., 1983; Tasaka et al., 1997). The diagnosis is based on clinical grounds, including an ensemble of nonspecific manifestations, such as symptoms, environmental factors, imaging, pulmonary function tests, bronchoalveolar lavage (BAL), and outcome after eradication of the presumed culprit antigen (often identified serologically) (Sandoval et al., 1990; Bourke and Boyd, 1997). The diagnostic use of serological tests is controversial, mainly because they cannot easily distinguish between active disease patients and simple contact subjects (Reynaud et al., 1990; Ward and White, 1997). In addition, published results of serological tests differ according to the author and method. The latter includes immunodiffusion (Andersen et al., 1982; Simpson et al., 1992), simple (Reynaud et al., 1990; Siested and Hansen, 1990) or two-dimensional immunoelectrophoresis (Longbottom, 1989; Siested and Hansen, 1990), counter-immunoelectrophoresis (Sandoval et al., 1990; Siested and Hansen, 1990) or co-immunoelectrodiffusion (Co-IED) (Pinon et al., 1982), hemagglutination (Hg) (Smets et al., 1981; Aubert et al., 1995), immunofluorescence (Eade et al., 1978; Smets et al., 1981, 1983), ELISA (Andersen et al., 1982; McSharry et al., 1984; Grammer et al., 1990; Sandoval et al., 1990; Simpson et al., 1992; Rodriguez de Castro et al., 1993; Yoshizawa et al., 1995a; Rodrigo et al., 2000), radio-immunoassay (Patterson et al., 1979; McSharry et al., 1984), enzyme-linked immunofiltration assay (ELIFA) (Pinon et al., 1985, 1990), Western blot (de Beer et al., 1990; Sandoval et al., 1990; Baldwin et al., 1999), and specific isotype characterization (Reynolds et al., 1991; Todd et al., 1993; Yoshizawa et al., 1994, 1995b; Mendoza et al., 1996; Baldwin et al., 1998a). The results also depend on the antigens used (Todd et al., 1991, 1993; Baldwin et al., 1998a,b, 1999) and the bird species incriminated [pigeons (Pg), turtledoves (Td), parrots (P), doves (Do), bengalis (B), canaries (Ca), mandarins (M), serins (S), chickens (Ck), ducks (Du), turkeys (Tk), etc.] (Tauer-Reich et al., 1994).

We report our experience with immunological diagnosis of BBD by means of Co-IED to detect serum antibodies (Pinon et al., 1982). We analyzed the diagnostic value of presumed disease-associated precipitating antibodies, on the basis of the final clinical diagnoses made by the patients' physicians. We also used ELIFA (Pinon et al., 1985, 1990) to characterize the specific immunoglobulin (Ig) isotypes (G, M, and A) contained in precipitating immune complexes, both at diagnosis and during follow-up.

2. Materials and methods

2.1. Patients

Seven hundred and seventy-five sera from 405 patients were sent to our laboratory for immunological diagnosis of BBD. The final diagnosis of the referring physicians (chest specialists, allergologists, and internists in public or private hospitals) was taken as the gold standard (Reynaud et al., 1990). It was based on the patient's habitat, the bird species incriminated, clinical manifestations, pulmonary imaging findings, blood gas values, spirometric data, carbon monoxide lung transfer capacity, bronchoalveolar lavage fluid analysis, and outcome after eradication of the presumed culprit antigen (Table 1). According to the final clinical diagnosis the patients were divided into two groups (G) (GI, positive, $n=90$; GII, negative, $n=315$). The groups were compared to the presence or absence of presumed disease-associated precipitating antibodies (arcs IgAp and/or P2, defined below) (Pinon et al., 1982). We also examined a control population composed of 100 healthy blood donors (100 sera) tested in the same conditions.

2.2. Antigens

Two antigen preparations were systematically used for all patients. The first was a soluble extract of

Table 1
Epidemiological, clinical and paraclinical data on patients ($n = 90$) with final clinically diagnosed BBD

Characteristic	Number of patients assessed		Number of cases	Percentage (relative to number assessed) (%)
Sex	90	Female	57	63
		Male	33	37
Habitat	90	Rural	51	57
		Urban	39	43
Species incriminated[a]	90	Pigeons (Pg)	44	49
		Turtledoves (Td)	33	38
		Parrots (P)	12	14
		Doves (Do)	9	10
		Canaries (Ca)	4	5
		Serins (S)	7	8
		Blackbird (BB)	1	1
		Chickens-Ducks-Geese-Turkeys (Ck-Du-G-Tk)	10	11
		Mandarins (M)	2	2
		Bengalis (B)	2	2
Symptoms	90	Cough	76	85
		Dyspnea	87	97
		Fever	37	41
		Altered general health	56	62
		Weight loss	33	38
Pulmonary imaging	82	Interstitial syndrome	64	78
		Normal	11	14
		Other	7	9
Blood gases	71	Hypoxia, normo- or hypocapnia	65	92
		Hypoxia hypercapnia	2	3
		Normal	4	6
Spirometry	61	Restrictive ventilatory syndrome	46	75
		Mixed ventilatory syndrome	11	18
		Obstructive ventilatory syndrome	1	2
		Normal	3	5
Carbon monoxide lung transfer capacity	27	Decreased	23	85
		Normal	4	15
BAL fluid analysis	44	Lymphocytes predominant	26	59
		Macrophages predominant	14	32
		Neutrophils predominant	4	9

[a] Possible contact with several bird species.

pigeon excreta (Ag-SEPE). Lipids were removed by grinding and ether extraction, and the excreta were then macerated for 8 days in COCA liquid (4 g/l NaCl; 0.7 g/l sodium bicarbonate; 4 g/l phenol). Finally, the preparation was filtered, dialyzed for 12 h against distilled water, and freeze-dried. The second preparation was rich in pigeon IgA (IgAp) (Goudswaard et al., 1977, 1978), which is abundant in squab crop milk (Ag-SCM). The milk was diluted (1:3, v/v) in normal saline, homogenized, and centrifuged at 3000 *g*. The supernatant was delipidated by adding 0.02 volumes of 10% dextran sulphate and 0.1 volume of 1 M CaCl2. After centrifugation at $3000 \times g$ for 5 min, the supernatant was dialyzed against 0.01 M Tris–HCl, pH 8, containing 2% EDTA and 1% NaCl. The dialysate, concentrated fivefold, was assayed for protein content then aliquoted and stored at -80 °C (Pinon et al., 1982).

When the patient data accompanying the serum sample mentioned exposure to chickens or ducks, the appropriate antigens, prepared in the same way as the pigeon excreta antigen, were also tested.

2.3. Co-immunoelectrodiffusion (Co-IED)

Co-IED was performed on cellulose acetate membranes (2.5 × 14 cm) (Sartorius, France) (Pinon et al., 1982). The antigen (15 μl) was deposited as a line on the cathodic side, perpendicular to the direction of migration. A drop of three sera (15 μl) was on the anodic side of the membrane, with a reference serum between two test sera (Fig. 1). Co-migration of the three sera was run for 2 h 15 min at 140 V in 0.05 M Tris–glycine buffer, pH 9 (generator and cuvettes: Sebia, Issy les Moulineaux, France). The membranes were then washed for 20 min and stained with Coomassie blue. Simple and fully developed in 3 h, this co-migration technique is analytical. Each test serum is examined for the generation of precipitating arcs called "IgAp" (formed by pigeon IgA present in Ag-SCM) and "P2" (Ag-SEPE). The arcs generated by the test sera are identified as IgAp (Fig. 1) or P2 (Fig. 2) by their continuity with pre-identified arcs generated by the reference positive serum (this continuity indicates the presence of a same antigenic component in the immune complex formed by antibodies with the same specificity) (Pinon et al., 1982) (Figs. 1, 2). To characterize the arc P2 for glycoprotein or mucin component, we stained the membranes with Schiff's reagent or used a Mab anti-mucin (Seraclone-Biotest—Germany) as positive control in Co-IED. Semi-quantitative information can be obtained for follow-up studies, by co-migration of serial serum samples from the same patient (Fig. 2).

2.4. Enzyme-linked immunofiltration assay (ELIFA)

When Co-IED is positive, the isotypes of precipitating antibodies (IgG, IgM, or IgA) can be determined by means of ELIFA, a two-phase method (Pinon et al., 1985). The first phase is a Co-IED assay identical to that described above but without the Coomassie-blue staining step. The precipitating arcs are revealed by immunofiltration (through the membrane) of alkaline phosphatase-labeled anti-human (IgG, μ or α chain) antibodies. For that, the part of the cellulose acetate membrane corresponding to the region of antigen–antibody migration is cut out and placed in an ELIFA cell controlled by an automat

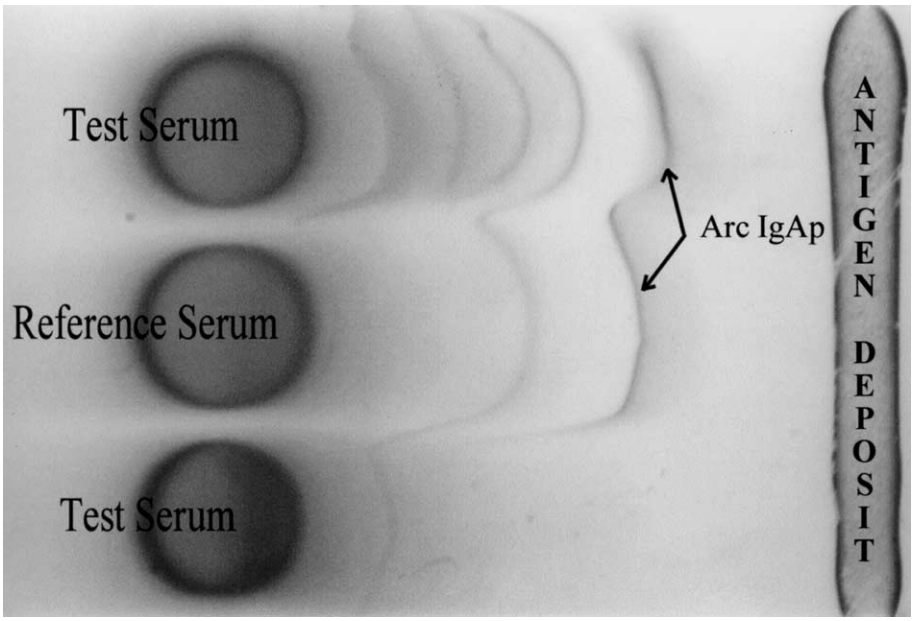

Fig. 1. Co-immunoelectrodiffusion on cellulose acetate membrane (Coomassie blue staining): detection of precipitating immune complexes (including arc IgAp) with the squab crop milk antigen (Ag-SCM). Note: (a) the highest test serum, IgAp positive (active disease patient); (b) the lowest test serum with only one arc different from IgAp (contact subject).

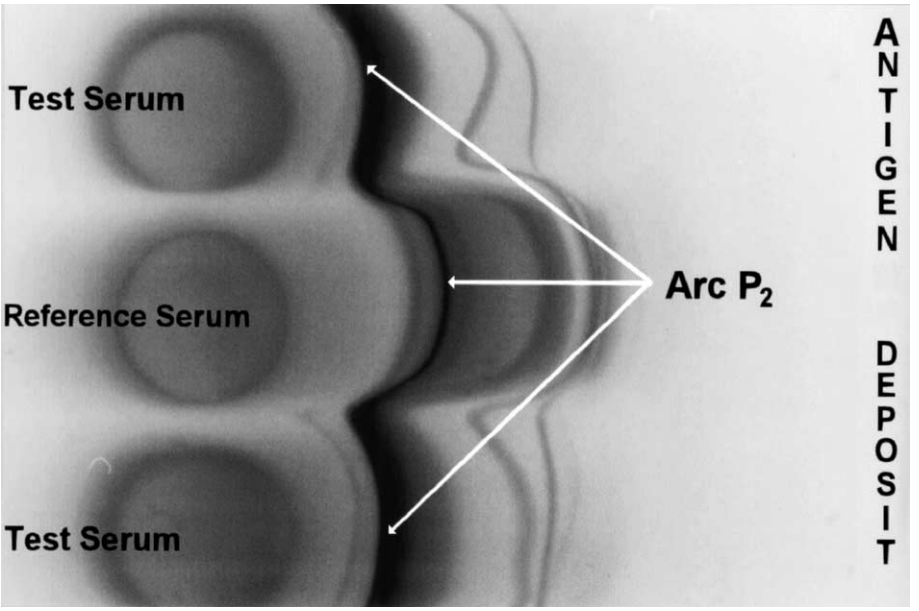

Fig. 2. Co-immunoelectrodiffusion on cellulose acetate membrane (Coomassie blue staining): detection of precipitating immune complexes (including arc P2) with the soluble extract of pigeon excreta (Ag-SEPE). Note that, for a given arc, the higher the concentration of specific antibodies (reference serum), the further the immune complex precipitates from the serum deposit.

which programs the washing, labeling, and impregnation steps in revelation buffer; this entire process takes only 30 min after the Co-IED phase (Pinon et al., 1990).

2.5. Hemagglutination

Quantitative passive hemagglutination was based on the use of rhesus-negative group O red cells sensitized by Ag-SCM. Sera were diluted 1:2 in 1:2 in phosphate buffer in microtiter plates (initial dilution 1:50) (Smets et al., 1981). The results were read automatically after 2 h of incubation (Aubert et al., 1995). The final titer corresponds to the reciprocal of the last positive dilution.

3. Results

Ninety patients had a final positive clinical diagnosis of BBD (G I; #1–90), and 315 patients had a final negative clinical diagnosis of BBD (G II; #91–405). On the basis of presumed disease-associated precipitating antibody detection (arcs IgAp and/or P2), there were 86 true-positive immunological diagnoses (patients #1–86) and 4 false-negative immunological diagnoses (#87–90). Two of these false-negative immunological results concerned patients (#87 and 88) who had been in contact with pigeons, and the other two concerned patients (#89 and 90) with a history of chicken-duck exposure. Following receipt of the final clinical diagnosis, stored serum from the false-negative patient #90 was retested with a homologous antigen (Ag-Ck-Du) by Co-IED, revealing nine precipitating arcs. Antigen co-migration (semi-deposit of Ag-Ck-Du and semi-deposit of Ag-SEPE or Ag-SCM) showed no IgAp- or P2-like arcs among the arcs obtained with Ag-Ck-Du. Hemagglutination titers in patients with a final positive diagnosis ranged from 50 to 1 000 000. Among 315 patients with a final negative diagnosis of BBD, 311 had no arc or only arcs different from IgAp and/or P2 (true-negative immunological diagnosis, patients #95–405) while four had presumed disease-associated precipitating antibody arcs (false-positive immunological diagnosis, patients #91-94). Two of these subjects (#91 and 92) had been in contact with pigeons, and the other two (#93 and 94) with parrots.

Table 2
Immunological follow-up of 10 patients with a final clinical diagnosis of BBD: changes in Co-immunelectrodiffusion (Co-IED), hemagglutination (Hg) and ELIFA results [soluble extract of pigeon excreta antigen (Ag-SEPE) or squab crop milk extract antigen (Ag-SCM)]

Patient# age (years)	Follow-up (bird species)	Co-IED (Ag-SEPE); Hg (Ag-SCM)		Kinetics of the arc IgAp (Ag-SCM)	ELIFA (Ag-SEPE)	
		First serum, number of arcs (titer)	Last serum, number of arcs (titer)		Isotypes on arcs other than P2	Isotypes of arc P2
55 (32)	30 months (P)	4 arcs (12 800)	3 arcs (1600)	disappearance between 5° and 9° months	• IgG, M, A on 1st serum • IgG, A at 9 months • IgG at 24 months	• IgG, M, A on 1st serum • IgG, IgA at 1 month • IgG at 5 months • Disappearance at 24 months
58 (56)	18 months (C, P)	4 arcs (6400)	2 arcs (25 000)	disappearance between 2° and 6° months	• IgG, M, A on 1st serum • Persistence of IgG, M, A	• IgG, M, A on 1st serum • Persistence of IgG, M, A
61 (68)	13 months (Td, P, Ck-Du)	8 arcs (12 800)	7 arcs (ND)*	persistence at 13 months	• IgG, M, A on 1st serum • Persistence of IgG, A	• IgG, M, A on 1st serum • Persistence of IgG, M, A
62 (72)	32 months (Pg)	10 arcs (100 000)	5 arcs (ND)*	persistence at 32 months	• IgG, M, A on 1st serum • Persistence of IgG, M, A	• IgG, M, A on 1st serum • Decrease in last serum
65 (65)	52 months (Industrial chicken breeder)	7 arcs (25 000)	4 arcs (6400)	disappearance between 30° and 46° months	• IgG, M, A on 1st serum	• IgG, M, A on 1st serum

					• IgG, A at 30 months • IgG at 46 months	• IgG, A at 4 months • IgG at 25 months
67 (56)	13 months (Pg)	3 arcs (1600)	2 arcs (1600)	disappearance before 4° months	• IgG, M, A on 1st serum • IgG at 6 months	• IgG, M, A on 1st serum • IgG, A at 6 months • IgG at 13 months
71 (4)	48 months (C)	5 arcs (100)	0 arc (50)	disappearance between 24° and 48° months	• IgG, M, A on 1st serum • IgG and M at 3 months • IgG at 6 months • Negative at 48 months	• IgG, M, A on 1st serum • IgG at 3 months • Negative at 24 months
75 (55)	24 months (Pg)	3 arcs (12 800)	1 weak arc (1600)	disappearance between 15° and 18° months	• IgG and IgA on 1st serum • IgG at 18 months • Negative at 24 months	• IgG, A on 1st serum • IgG at 15 months • Negative at 24 months
76 (85)	10 months (C)	4 arcs (400)	2 arcs (400)	disappearance between 1° and 9° months	• IgG and M on 1st serum • IgG at 1 month	• No P2 arc
84 (19)	32 months (Td, S)	7 arcs (12 800)	3 arcs (3200)	disappearance between 10° and 24° months	• IgG, M, A on 1st serum • IgG, A at 4 months • IgG at 10 months	• IgG, M, A on 1st serum • Negative at 4 months

ND, not done.

Hemagglutinin titers were between negative and 1600 in the patients with final negative diagnoses. No IgAp or P2 arcs were found in the 100 healthy control subjects.

The diagnostic value of presumed disease-associated precipitating antibodies was estimated from the numbers of true-positive ($n=86$), true-negative ($n=311$), false-positive ($n=4$), and false-negative ($n=4$) results in a total of 405 subjects. The sensitivity (Se) was 95.5%, the specificity (Sp) 98.7%, the accuracy 98%, the positive predictive value 95.5%, and the negative predictive value 98.7%. Note that if seropositivity had been defined on the basis of three arcs, five patients (#1, 18, 27, 31, 52) in group I would have been wrongly considered negative and 24 patients in group II would have been wrongly considered positive.

Specific isotypes (IgG, IgM, IgA) were determined by ELIFA in the first positive serum samples from 30 subjects with a final positive diagnosis (#55–80 and 83–86). The analysis of arcs formed between Ag-SEPE and IgG, IgM, and/or IgA antibodies shows that several isotypes can be detected in a given arc. Ten of these 30 patients were monitored for between 10 and 52 months after diagnosis, allowing us to analyze: (a) changes in the hemagglutination titer and differences in the arcs generated by the first and last serum samples, (b) the kinetics expression of the arcs IgAp and P2, and (c) the persistence of specific isotypes detected by ELIFA. At the end of follow-up, arc IgAp had totally disappeared in 8 of 10 cases, while arc P2 had disappeared in four of nine cases. The disappearance of IgM and IgA antibodies was variable from subject to another (Table 2).

4. Discussion

Exposure to avian antigens can generate circulating precipitins. These precipitins can be present in active disease patients and also in simple contact subjects, sometimes at high levels (Yoshizawa et al., 1995b; Allen and Spiteri, 1996). The definitive diagnosis of bird-breeder's disease was based on an ensemble of arguments (Reynaud et al., 1990), including a history of exposure to birds, symptoms and signs, BAL findings, and spirometry (Sandoval et al., 1990).

The characteristics of our patients with BBD are in keeping with the results of previous epidemiological studies (female predominance, variable contact period, respective frequency of clinical manifestations, age range, etc.). On the basis of the final clinical diagnosis—used as the gold standard in this study—immunological diagnosis based on the presence of arcs IgAp and/or P2 was highly reliable, with a sensitivity, specificity, and accuracy of 95.5%, 98.7%, and 98%, respectively. These values compare favorably with those of ELISA (Sandoval et al., 1990) (Se 93%, Sp 89%), counter-immunoelectrophoresis (Siested and Hansen, 1990) (Se 73% and 86% in smokers and nonsmokers; Sp 98%), and immunoelectrophoresis (Reynaud et al., 1990) (Se 86%, Sp 93%, accuracy 92%). The results of these latter three methods are based on the number of precipitating arcs and, therefore, on the antigen preparations used. In our study, the use of arcs IgAp and P2 yielded only four false-positive and four false-negative immunological diagnoses. One of the latter patients was retested with an appropriate antigen preparation (Ag-Ck-Du), yielding nine precipitating arcs but no IgAp- or P2-like arcs. Homologous antigens can thus be useful only in small number of cases, as cross-reactions are frequent among bird antigens (feather dust, sera, secretions, excreta, etc.) both within a given species and among species (Tauer-Reich et al., 1994). In our experience with Ag-SCM and Ag-SEPE, arcs IgAp and/or P2 are found in the serum of patients who develop BBD after contact with pigeons and also parrots, doves, turtledoves, canaries, and serins, but not after exclusive contact with chickens, ducks, geese, or turkeys.

The presence/absence of arcs IgAp and/or P2 appears to be a more reliable diagnostic criterion than is a particular number of arcs or a hemagglutinin cutoff titer. The pigeon IgA antigen, which is abundant in crop milk extract, has previously been tested (Goudswaard et al., 1978; Pinon et al., 1982; Todd et al., 1991). The P2 constituent (Ag-SEPE) forms a precipitating arc that stains positive with the Schiff's reagent (reflecting the presence of carbohydrates or glycoproteins) and is also detected with a MAb anti-mucin by Co-IED. Other authors (Todd et al., 1991; Baldwin et al., 1998a, 1999) have described antibodies recognizing the carbohydrate epitopes of intestinal mucins, which are also found in pigeon excreta

and secreta. Several of our patients had large numbers of precipitating arcs, and co-migration of their sera showed that the specificity of their antibodies may differ from one patient to the next. Different bands reactive to BBD sera have been found by Western blotting (Sandoval et al., 1990). Some (29–32 kDa) were considered to have major diagnostic value, and were also described as "disease-associated antigens" in 9/9 of symptomatic patients in a study of 16 exposed subjects (de Beer et al., 1990). Western blot method is not so simple, rapid, and easy to do compared with Co-IED, but it could be interesting in the described cases of patients with typical BBD without any precipitating antibodies (Sandoval et al., 1990).

The detection in serum, BAL fluid, or saliva of specific isotypes or Ig subclasses has been assessed as a diagnostic criterion and as a sign of medium-term persistence of BBD. This approach mainly concerns IgG antibodies (IgG1–IgG4), IgA (IgA1, IgA2) (Yoshizawa et al., 1994, 1995b; Mendoza et al., 1996; Baldwin et al., 1998a), and more rarely IgM and IgE (Reynolds et al., 1991; Rodriguez de Castro et al., 1993). ELIFA detection of IgG, IgM, and IgA specific for Ag-SEPE in our study yielded no supplementary diagnostic information relative to the presumed disease-associated precipitating antibodies, simply showing that most patients had antibodies of these three isotypes.

The serologic outcome of BBD is highly variable (Bourke et al., 1989; Grammer et al., 1990). There appears to be a self-regulation of antigen exposure, with a gradual loss of antibodies despite persistent exposure (McSharry et al., 1984). Even after avoidance of direct exposure, the time course of antibody detection in both serum and BAL fluid is variable, and appears to be influenced by many factors such as the duration of antigen exposure, the concentration and chemical nature of the inhaled antigens, and the patient's age and genetic predisposition (Boyd, 1990; Yoshizawa et al., 1994). In our study, after 10 to 52 months of presumed antigen eradication, the number of arcs generated with Ag-SEPE fell in most cases (to zero in one young child). In contrast, the hemagglutination titer may fall or remain stable; a rapid reincrease points to renewed exposure. Specific IgM antibodies remain detectable for several months, but they fall more rapidly than IgA, which persisted throughout follow-up in several of our patients. This time course of the different antibody isotypes detected by ELIFA concords with that observed with ELISA methods (Reynolds et al., 1991; Yoshizawa et al., 1994, 1995b).

In conclusion, although immunological tests can contribute the diagnosis of bird breeders' disease, they suffered from the drawback of being unable to distinguish active disease patients from simple contact subjects. This large study shows that the presence/absence of precipitating arcs IgAp and/or P2 (presumed disease-associated precipitating antibodies) by Co-IED contributes largely to the intrinsic credibility of immunological test results with excellent sensitivity-specificity. Moreover, Co-IED on cellulose acetate membrane is simple, rapid, and low-cost. While not contributing to the diagnosis, hemagglutination provides useful complementary quantitative information. The detection and time course of specific IgG, IgM, or IgA antibodies do not appear to have greater diagnostic value than anti-IgAp and/or P2 antibody or to provide supplementary prognostic arguments to judge disease activity after antigen eradication. The latter can be difficult, as exposure often results from a hobby. In the occupational setting, routine immunological screening tests, including anti-IgAp and/or P2 antibody detection, should be used for personnel at risk.

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References

Allen, J.T., Spiteri, M.A., 1996. Pigeon breeder's disease. J. Lab. Clin. Med. 127, 10–12.

Andersen, P., Christensen, K.M., Jensen, B.E., Axel, K., Laursen, J.C., Geday, H., Lundsgaard, A., Andersen, H.K., 1982. Antibodies to pigeon antigens in pigeon breeders. Detection of anti-

bodies by an enzyme-linked immunosorbent assay. Eur. J. Respir. Dis. 63, 113–121.

Aubert, D., Foudrinier, F., Kaltenbach, M.L., Guyot-Walser, D., Marx-Chemla, C., Geers, R., Lepan, H., Pinon, J.M., 1995. Automated reading and processing of quantitative IgG, IgM, IgA and IgE isotypic agglutination results in microplates: development and application in parasitology-mycology. J. Immunol. Methods 186, 323–328.

Baldwin, C.I., Todd, A., Bourke, S.J., Allen, A., Calvert, J.E., 1998a. IgG subclass responses to pigeon intestinal mucin are related to development of pigeon fanciers's lung. Clin. Exp. Allergy 28, 349–357.

Baldwin, C.I., Steven, B., Connors, S., Todd, A., Bourke, S.J., Calvert, J.E., Allen, A., 1998b. Pigeon fanciers' lung: the mucin antigen is present in pigeon droppings and pigeon bloom. Int. Arch. Allergy Immunol. 117, 187–193.

Baldwin, C.I., Todd, A., Bourke, S.J., Allen, A., Calvert, J.E., 1999. Pigeon fanciers's lung: identification of disease-associated carbohydrate epitopes on pigeon intestinal mucin. Clin. Exp. Immunol. 117, 230–236.

Bourke, S., Banham, S.W., Carter, R., Lynch, P., Boyd, G., 1989. Longitudinal course of extrinsic allergic alveolitis in pigeon breeders. Thorax 44, 415–418.

Bourke, S., Boyd, G., 1997. Pigeon fancier's lung: antigen avoidance and respiratory protection are the mainstays of management. Br. Med. J. 315, 70–71.

Boyd, G., 1990. Pulmonary function changes in pigeon fancier's lung. Respir. Med. 84, 5–7.

Calvert, J.E., Baldwin, C.I., Allen, A., Todd, A., Bourke, S.J., 1999. Pigeon fancier's lung: a complex disease? Clin. Exp. Allergy 29, 166–175.

de Beer, P.M., Bouic, P.J., Joubert, J.R., 1990. Identification of a 'disease-associated' antigen in pigeon breeder's disease by western blotting. Int. Arch. Allergy Appl. Immunol. 91, 343–347.

Eade, O.E., Hodges, J.R., Berill, W.T., Lang, C., Llyod, R.S., Wright, R., 1978. Immunofluorescent antibodies in patients with bird fancier's lung. Clin. Exp. Immunol. 32, 259–262.

Gari, M., Pinon, J.M., Soudre, R., Suinat, J.L., Caulet, T., Pluot, M., 1983. A report of a fatal case of bird fancier's disease. Rev. Fr. Allergol. 23, 87–89.

Goudswaard, J., Vaerman, J.P., Heremans, J.F., 1977. Three immunoglobulin classes in the pigeon (*Columbia livia*). Int. Arch. Allergy Appl. Immunol. 53, 409–419.

Goudswaard, J., Noordzij, A., Stam, J.W., 1978. Pigeon IgA: a major antigen in pigeon breeder's disease. Immunol. Commun. 7, 661–668.

Grammer, L.C., Roberts, M., Lerner, C., Patterson, R., 1990. Clinical and serologic follow-up of four children and five adults with bird-fancier's lung. J. Allergy Clin. Immunol. 85, 655–660.

Longbottom, J.L., 1989. Pigeon breeder's disease: quantitative immunoelectrophoretic studies of pigeon bloom antigen. Clin. Exp. Allergy 6, 619–624.

McSharry, C., Banham, S.W., Lynch, P.P., Boyd, G., 1984. Antibody measurement in extrinsic allergic alveolitis. Eur. J. Respir. Dis. 65, 259–265.

Mendoza, F., Baltazares, M., Ramirez, A., Sansores, R., Nava, A., Banales, J.L., Selman, M., 1996. Detection of salivary and seric IgG and IgA antipooled pigeon sera activities in patients with pigeon breeder's disease. J. Clin. Lab. Anal. 10, 149–154.

Patterson, R., Wang, J.L., Fink, J.N., Calvanico, N.J., Roberts, M., 1979. IgA and IgG antibody activities of serum and bronchoalveolar fluid from symptomatic and asymptomatic pigeon breeders. Am. Rev. Respir. Dis. 120, 1113–1118.

Pinon, J.M., Smets, P., Gari, M., Recco, P., Seguela, J.P., 1982. Pigeon IgA is a major antigen involved in bird fancier's lung disease: the diagnosis value of its detection by co-immuno-electrodiffusion. Rev. Fr. Allergol. 22, 207–210.

Pinon, J.M., Thoannes, H., Gruson, N., 1985. An enzyme-linked immuno-filtration assay used to compare infant and material antibody profiles in toxoplasmosis. J. Immunol. Methods 77, 15–23.

Pinon, J.M., Puygauthier-Toubas, D., Lepan, H., Boulant, J., Marx-Chemla, C., Dupont, H., 1990. Enzyme-linked immuno-filtration assay applied to accelerated immunodetection of gel transferred proteins on immobilizing matrices. J. Immunol. Methods 131, 143–145.

Reynaud, C., Slosman, D.O., Polla, B.S., 1990. Precipitins in bird breeder's disease: how useful are they? Eur. Respir. J. 3, 1155–1161.

Reynolds, S.P., Edwards, J.H., Jones, K.P., Davies, B.H., 1991. Immunoglobulin and antibody levels in bronchoalveolar lavage fluid from symptomatic and asymptomatic pigeon breeders. Clin. Exp. Immunol. 86, 278–285.

Rodrigo, M.J., Benavent, M.I., Cruz, M.J., Rosell, M., Murio, C., Morell, F., 2000. Detection of specific antibodies to pigeon serum and bloom antigens by enzyme-linked immunosorbent assay in pigeon breeder's disease. Occup. Environ. Med. 57, 159–164.

Rodriguez de Castro, F., Carrillo, T., Castillo, R., Blanco, C., Diaz, F., Cuevas, M., 1993. Relationships between characteristics of exposure to pigeon antigens. Clinical manifestations and humoral immune response. Chest 103, 1059–1063.

Salvaggio, J.E., 1990. Recent advances in pathogenesis of allergic alveolitis. Clin. Exp. Allergy 20, 137–144.

Salvaggio, J.E., 1997. Extrinsic allergic alveolitis (hypersensitivity pneumonitis): past, present and future. Clin. Exp. Allergy 27, 18–25.

Sandoval, J., Banales, J.L., Cortes, J.J., Mendoza, F., Selman, M., Reyes, P.A., 1990. Detection of antibodies against avian antigen in bronchoalveolar lavage from patients with pigeon breeder's disease: usefulness of enzyme-linked immunosorbent assay and enzyme immunotransfer blotting. J. Clin. Lab. Anal. 4, 81–85.

Siested, H.C., Hansen, N.C., 1990. Precipitating antibodies against pigeon bloom antigens as a specific and sensitive measure of pigeon exposure. Allergy 45, 293–297.

Simpson, C., Shirodiaria, P.V., Evans, J.P., Simpson, D.I., Stanford, C.F., 1992. Comparison of immunodiffusion and enzyme linked immunosorbent assay in the detection of abnormal antibodies in pigeon breeder's disease. J. Clin. Pathol. 45, 490–493.

Smets, P., Gari, M., Recco, P., Seguela, J.P., Pinon, J.M., 1981. Intérêt de l'IgA de pigeon dans le diagnostic de la maladie du poumon d'Eleveurs d'Oiseaux. Recherche des anticorps spécifiques par immuno-electro-diffusion sur acétate de cellulose,

hémagglutination passive et immunofluorescence indirecte. Bull. Soc. Mycol. Med. 10, 105–112.

Smets, P., Gari, M., Pinon, J.M., Seguela, J.P., 1983. Diagnosis of Pigeon Breeder's disease by indirect immunofluorescence on sections of pigeon oviduct. Clin. Allergy 13, 57–60.

Tasaka, S., Kanazawa, W., Kawai, C., Soejima, K., Yamagucki, K., Takata, A., Torikata, C., Hata, J., 1997. Fatal diffuse alveolar damage from bird fancier's lung. Respiration 64, 307–309.

Tauer-Reich, I., Fruhmann, G., Czuppon, A.B., Baur, X., 1994. Allergens causing bird fancier's asthma. Allergy 49, 448–453.

Todd, A., Coan, R., Allen, A., 1991. Pigeon breeders' lung: pigeon intestinal mucin, an antigen distinct from pigeon IgA. Clin. Exp. Immunol. 85, 453–458.

Todd, A., Coan, R., Allen, A., 1993. Pigeon breeders' lung; IgG subclasses to pigeon intestinal mucin and IgA antigens. Clin. Exp. Immunol. 92, 494–499.

Ward, A.M., White, P.A.E., 1997. Current methodology is not sensitive enough to monitor effectiveness of avoidance measures. Br. Med. J. 315, 1311.

Yoshizawa, Y., Miyashita, Y., Inoue, T., Sumi, Y., Miyazaki, Y., Sato, T., Ohtsuka, M., 1994. Sequential evaluation of clinical and immunological findings in hypersensitivity pneumonitis: serial subclass distribution of antibodies. Clin. Immunol. Immunopathol. 73, 330–337.

Yoshizawa, Y., Ohtani, Y., Inouet, T., Miyake, S., Ikeda, A., Tanba, M., Kurup, V.P., 1995a. Immune responsiveness to inhaled antigens: local antibody production in the respiratory tract in health and lung diseases. Clin. Exp. Immunol. 100, 395–400.

Yoshizawa, Y., Miyake, S., Sumi, Y., Hisauchi, K., Sato, T., Dirup, V.P., 1995b. A follow-up study of pulmonary function tests, bronchoalveolar lavage cells, and humoral and cellular immunity in bird fancier's lung. J. Allergy Clin. Immunol. 96, 122–129.