

Anti-avian antibodies and rheumatoid factor in pigeon hypersensitivity pneumonitis

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Summary

Background Although several immunological abnormalities may be present in pigeon hypersensitivity pneumonitis (HP), few specific hallmarks have been described.

Objective To determine whether the presence of rheumatoid factor (RF) could be useful to discriminate pigeon HP from asymptomatic breeders (AB) and other interstitial lung diseases.

Methods Fifty-three patients with pigeon HP, 47 AB, 31 idiopathic pulmonary fibrosis (IPF) patients and a rheumatoid arthritis (RA) group were studied. IgM RF was determined through enzyme-linked immunosorbent assay (ELISA) and western blot using human IgG and IgG Fc fragment as antigens. IgG and IgA anti-avian antibodies (AA) against pigeon serum antigen were also measured. The use of F(ab')₂ fraction of peroxidase-labelled anti-human immunoglobulins prevented endogenous interferences. Possible cross-binding of RF with avian antigens and the reactivity against human IgG by AA were studied.

Results RF tests were frequently positive in HP (52.8%) in comparison to AB (4.2%) and IPF (12.9%; $P = 2.6 \times 10^{-10}$ and 4.1×10^{-5}). Therefore, the presence of RF in pigeon HP showed a sensitivity of 52% and was highly specific considering the results of AB and IPF (95 and 87%, respectively). The RA group revealed positive RF but negative AA tests. RF activity was confirmed through western blot using purified IgG Fc fragment. Overlapping levels of IgG and IgA AA were found in HP and AB. The frequency of AA was low in IPF. The cross-reaction of RF with avian antigens was excluded, and no reactivity against human IgG by AA was detected. Other endogenous interferences were ruled out.

Conclusion No single immunological test may definitively distinguish pigeon HP from AB and other interstitial lung disorders; however, positive RF, together with high AA levels, seems to be useful in differentiating the diagnosis.

Keywords anti-avian antibodies, bird lung disease, IgG, IgM, interstitial pulmonary disease, lung hypersensitivity, pigeon breeder's disease, rheumatoid arthritis, rheumatoid factor

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Introduction

Hypersensitivity pneumonitis (HP) secondary to pigeon exposure is an inflammatory lung disorder in which detection of several humoral and cellular immune abnormalities have been useful in evaluating its pathogenesis, diagnosis and disease evolution [1].

Determination of anti-avian antibodies (AA), in particular high values of IgG, and their specific binding to some glycoproteins [2–4] might be helpful for the diagnosis of HP. Nevertheless, overlapping levels of anti-avian immunoglobulins in asymptomatic fanciers [2, 4–6] and the presence of positive tests in other interstitial lung disorders [7, 8] have limited their use as reliable markers of the disease.

Hypergammaglobulinemia, complement activation, and circulating immune complexes (IC) [9–14] have not been considered as specific findings of HP. The report of other laboratory abnormalities including high values of acute phase reactants, β_2 microglobulin and angiotensin-converting enzyme [10, 13, 15–17] did not reveal a definitive association with the clinical features of HP.

Although the occurrence of additional serological findings has previously been recorded in HP there are only a few reports of lung reactive immunoglobulins, antinuclear antibodies, and rheumatoid factor (RF) associated with this group of pulmonary disorders [13, 18–21].

The presence of RF has been described in different HP subsets including humidifier's disease and farmer's lung [13, 18, 22–24]. In HP secondary to pigeon exposure, positive RF by latex agglutination tests was first described in anecdotal reports [25, 26] and similar results were also found during the immunological assessment of the disease [14, 27]. Recent studies in sera with positive AA revealed that high values of RF may be

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detected through enzyme-linked immunosorbent assay (ELISA) [28], although unfortunately the clinical value of these serological findings has received scant attention, in particular for the differential diagnosis of HP.

This study was conducted to extend previous observations, which suggested that positive RF tests might be present in a high frequency of HP, including the pigeon breeder's group [4, 24]. The purpose was to evaluate whether this immunological hallmark could differentiate HP secondary to avian exposure from asymptomatic fanciers. Comparison with other interstitial lung disorders that may show positive RF tests was also performed.

Materials and methods

Patients and controls

A total of 118 consecutive patients were studied. The serum samples of all subjects were referred to the laboratory for their serological study as part of the diagnostic approach in our institute.

Fifty-three patients (48 women, five men; mean age 40.1 years) were classified as definite pigeon HP according to earlier studies [4]. The diagnosis in all these cases was based on: (i) previous history of pigeon exposure and cause-effect relationship; (ii) clinical, radiological, and respiratory functional abnormalities compatible with an interstitial lung disease, including dyspnea, end-inspiratory rales, restrictive functional pattern, and diffuse micronodular, nodular, or reticulonodular images on chest film, without hilar adenopathy; (iii) positive AA tests by Ouchterlony or ELISA using pigeon serum as antigen; and (iv) lung biopsy with morphological features that met the internationally accepted criteria for HP diagnosis.

A group of 31 patients with idiopathic pulmonary fibrosis (28 women, three men; mean age 41.3 years) in whom the diagnosis had been made following international criteria [29] was analysed.

We also studied 34 rheumatoid arthritis (RA) patients (30 women, four men; mean age 42.2 years), including 12 of them with interstitial lung fibrosis. All these subjects fulfilled the classification criteria for RA according to the American College of Rheumatology [30].

Forty-seven asymptomatic pigeon breeders with no HP (43 women, four men; mean age 38.2 years) were studied as controls. Healthy volunteers from the Blood Bank, members of a local pigeon-racing club, and first-degree relatives of pigeon HP patients were included in this group. The history of exposure to pigeons in these subjects was comparable to the HP group according to previous studies [4]. All these asymptomatic fanciers revealed negative data of pulmonary diseases by history and physical examination and showed normal pulmonary function tests and chest X-ray studies.

Eighty healthy blood donors with no history of bird contact (71 women, nine men; mean age 36.7 years) were also included. These healthy volunteers from the Blood Bank were previously studied to exclude any pulmonary disease [4]. Clinical evaluation for respiratory abnormalities was negative, and all of them showed normal pulmonary function tests and negative X-ray changes suggestive of lung impairment.

The study was approved by our Institutional Review Board and written consent was obtained from all patients and healthy volunteers.

Anti-avian antibodies

IgG and IgA AA were measured through ELISA according to previous studies using optimal conditions selected after standardization of these tests in our laboratory [4, 31]. Microtiter plates (Maxisorb, Nunc, Naperville, IL, USA) were coated with 100 µL of 12 µg/mL pigeon-pooled serum in NaHCO₃ (0.05 mol/L, pH 9.6). After incubation for 12 h, the wells were washed with phosphate-buffered saline (PBS)-Tween-20 (0.05%) and quenched with 1% bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St Louis, MO, USA). Decomplemented sera (56 °C, 20 min) were added to the wells for 1 h at 37 °C. Binding of AA was detected using optimal dilution of peroxidase label F(ab')₂ fragment of anti-human IgG or IgA (Sigma). Orthophenylenediamine (OPD) prepared in citrate buffer (0.1 mol/L, pH 5.0) was used as substrate and the reaction was stopped after 15 min at room temperature with 50 µL of 2.5 N sulphuric acid. Absorbance was read in a micro ELISA minireader (Labsystem, Helsinki, Finland) and the results were reported in optical density (OD; 492 nm) units (U). The sera were assayed in duplicate and positive and control reference samples were included in each plate. Reference values were established as the mean + 2 standard deviations (2SD) of the control groups.

The specificity of ELISA for the detection of AA was analyzed to exclude possible cross-reactivity of avian-pooled sera antigen with human sera as described [4]. For this objective, untreated patient sera and samples incubated with avian-pooled antigen, as well as sera from patients previously combined with hypogammaglobulinemic human samples, were used.

Rheumatoid factor

The presence of RF was studied through ELISA using purified human IgG (Sigma). Microtiter plates (Maxisorb, Nunc) were coated with 100 µL of 10 µg/mL aggregated human IgG (63 °C, 30 min) in NaHCO₃ as described [21, 32]. After incubation for 12 h, the wells were washed five times with PBS-Tween-20 (0.05%) and quenched with 1% BSA (Sigma). The samples to be tested were added to the wells for 1 h at 37 °C. The use of optimal dilution of peroxidase-labelled F(ab')₂ fragment of anti-human IgM prevented the interaction of RF with the Fc portion of endogenous antibodies. The substrate, ODP, was prepared in citrate buffer containing hydrogen peroxide and the reaction was stopped at room temperature with sulphuric acid. Absorbance was read in a micro ELISA minireader (Labsystem) and the results were recorded in ODU (492 nm). The reference values were defined in healthy subjects. A purified IgM RF was included as positive control in these tests. This antibody was obtained by affinity chromatography using activated sepharose CL-4B (Pharmacia, Uppsala, Sweden) coupled to purified human IgG. A pool of sera from RA patients was the source of this antibody. A purified IgM (Sigma) with no RF activity was included as negative control in these assays. The specific binding of IgM was evaluated using aggregated human and rabbit IgG (Sigma) as RF absorbents, and confirmed with a purified human IgG Fc fragment (Chemicon, Temucula, CA, USA).

Cross-reactivity tests and interaction of immunoglobulins

To assess the possible binding of RF to pooled serum avian antigen the samples with positive RF results were tested for AA through ELISA and western blot. We used all sera from the RA patients who showed positive RF levels above the mean + 2SD of healthy controls. A purified IgM fraction with RF activity obtained from a pool of the RA group was also included in the AA tests. The possible cross-reaction of AA with human IgG was studied using the sera from pigeon HP patients with positive AA results but negative RF tests. A purified serum fraction obtained by affinity chromatography using sepharose 4B (Pharmacia) coupled with pooled serum avian antigen was also used to assess the presence of RF activity.

Because the reactivity to IgG could be affected by endogenous IgG–IgM binding, the presence of IgG with anti-IgM function was also studied. In brief, purified IgM (Sigma; 10 µg/mL in NaHCO₃) was immobilized in polystyrene plates (Maxisorb, Nunc) for 12 h. After addition of 1% BSA (Sigma) and washing, the samples were diluted in PBS–Tween-20 (0.05%) and incubated for 1 h at 37 °C. Immunoglobulin binding in tested sera was detected by adding optimal dilution of peroxidase-labelled F(ab')₂ fragment of anti-IgG antibodies (Sigma). ODP prepared in citrate buffer was used as substrate, and the reaction was stopped with sulphuric acid. The results were recorded in ODU (492 nm). Sera from healthy subjects were included as controls. A purified serum fraction with AA and normal IgG with no anti-avian activity obtained through affinity chromatography were also tested.

Western blot

RF binding and IgG antibody reactivity against IgM were confirmed by western blot. The separation of purified human IgM (Sigma) or IgG Fc fragment (Chemicon; 10 µg/well) was performed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) under reducing conditions [33]. These proteins were transferred onto nitrocellulose sheets [34] and after quenching with PBS containing 1% BSA (Sigma), the samples were incubated for 3 h and washed with PBS–Tween-20 (0.1%). The immune reaction was detected using optimal dilution of peroxidase labelled F(ab')₂ fragment of goat anti-human immunoglobulins (Sigma) and revealed with 4-chloronaphthol prepared in methanol–PBS. Results were compared to molecular weight markers (Bio-Rad, Richmond, CA, USA). Sera with positive RF from the RA group and purified IgM RF were included as controls.

Statistics

The statistical analysis to compare the levels of AA and RF tests in the different groups was performed using the Student's *t*-test (Statistica v. 4.3). Sensitivity and specificity values and positive and negative predictive values for RF test were determined using the appropriate 2 × 2 table.

Results

Anti-avian antibodies

The results of AA through ELISA in the studied groups disclosed several interesting findings. The frequency of positive tests in pigeon HP differed when AA values of healthy subjects

with no history of avian exposure and asymptomatic fanciers were considered in the analysis (Fig. 1).

All pigeon HP patients revealed IgG (mean ± SD, 0.640 ± 0.271 ODU) and 86.7% showed IgA AA (mean ± SD, 0.428 ± 0.411 ODU) above the reference limits of healthy subjects with no avian contact (mean ± SD, IgG 0.105 ± 0.066 and IgA 0.054 ± 0.026 ODU; $P = 5.0 \times 10^{-20}$ and 2.7×10^{-13}).

As expected, the comparison between HP and asymptomatic fanciers revealed overlapping levels of AA. Although the values of these immunological tests were higher in the HP group (IgG, $P = 4.0 \times 10^{-5}$; IgA, $P = 5.6 \times 10^{-5}$) a low frequency of positive IgG (7.5%) and IgA (22.6%) results above the mean + 2SD of asymptomatic breeders (mean ± SD, 0.353 ± 0.390 and 0.156 ± 0.173 ODU) was found. Indeed, the analysis of AA in the 47 asymptomatic controls showed that 17 (36.1%) and 22 (46.8%) samples presented IgG and IgA levels over the mean + 2SD of healthy controls with no bird exposure.

No positive AA tests were detected in our IPF group. The levels of IgG and IgA AA in these patients with interstitial lung

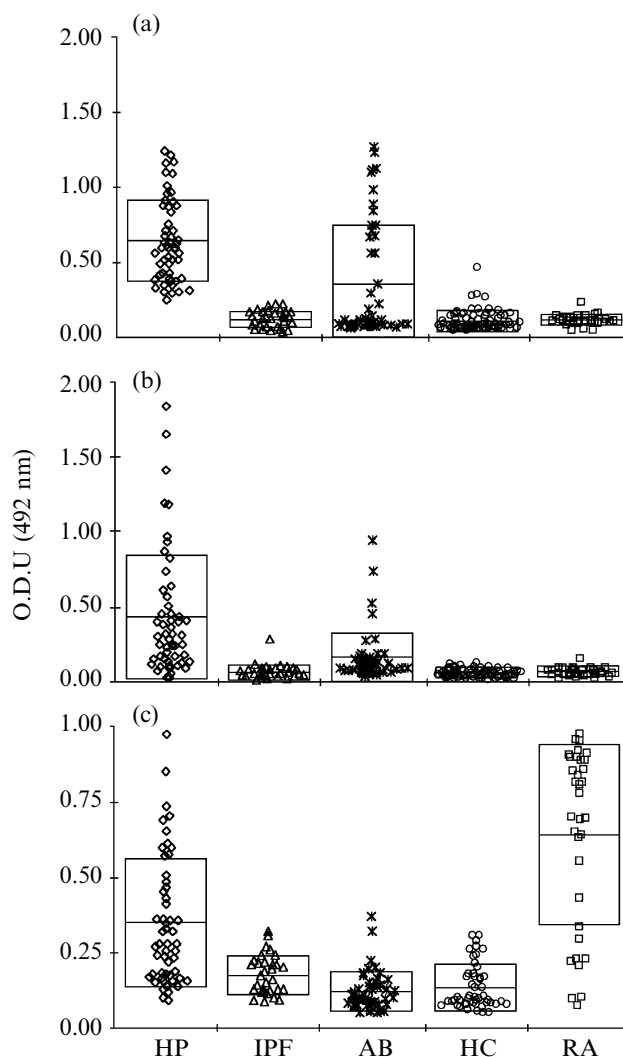


Fig. 1. Levels of IgG (a) and IgA (b) anti-avian antibodies through ELISA in pigeon HP (HP), idiopathic pulmonary fibrosis (IPF), asymptomatic breeders (AB), healthy controls (HC) and rheumatoid arthritis (RA). Horizontal lines represent the mean ± SD of optical density (OD) units in each group. (c) Comparison of IgM RF values against IgG through ELISA in the respective groups.

disease (mean $\pm$ SD, 0.123 ± 0.055 and 0.068 ± 0.048 ODU) did not show substantial differences in comparison to healthy subjects ($P = 0.14$ and 0.13 ; see Fig. 1), and these AA values were lower than the IgG and IgA levels of asymptomatic fanciers. The patients with RA including those with interstitial lung disease showed negative AA tests. The IgG and IgA AA results in this group (mean $\pm$ SD, 0.110 ± 0.034 and 0.057 ± 0.026 , respectively) did not differ from those levels detected in healthy controls ($P = 0.68$ and 0.51) and were below the reference IgG and IgA AA limits of asymptomatic breeders. A further comparison of AA between the IPF and RA groups disclosed similar values of IgG and IgA ($P = 0.23$ and 0.26).

Other findings of interest were that the IgA AA values showed a better degree of discrimination between HP and asymptomatic breeders and that, considering our healthy controls, the simultaneous presence of IgG and IgA AA occurred in a high percentage of pigeon HP (46 out of 53 patients) as compared to asymptomatic breeders (14 out of 47 samples; $P = 1.1 \times 10^{-51}$).

In this study as in previous reports [4], the tests used to determine the specificity of ELISA revealed a decrease in AA over 95% after incubation with avian-pooled sera with respect to their untreated samples. The use of hypogammaglobulinemic human sera did not reveal substantial differences in comparison to untreated samples.

Rheumatoid factor

A high frequency of positive RF exceeding the mean $+ 2$ SD of healthy controls was detected through ELISA in pigeon HP sera (28 out of 53 patients; 52.8%; Fig. 1). The statistical analysis revealed significant differences between these two groups (mean $\pm$ SD, 0.345 ± 0.210 vs. 0.132 ± 0.073 ODU; $P = 7.8 \times 10^{-10}$).

In contrast, a low percentage of asymptomatic breeders and IPF were RF-positive (4.2 and 12.9%). The levels of this antibody in the asymptomatic fanciers (mean $\pm$ SD, 0.123 ± 0.064 ODU) were similar to those obtained in healthy donors with no bird exposure ($P = 0.50$) and the IPF group showed high RF values in comparison to these healthy controls (mean $\pm$ SD, 0.177 ± 0.064 ODU; $P = 0.005$).

The RF results in pigeon HP were also above the reference levels of asymptomatic breeders and exceeded the IPF values, showing statistically significant differences ($P = 2.6 \times 10^{-10}$ and 4.1×10^{-5}).

The patients with RA included as a representative group with elevated RF values showed the highest levels of these serological markers (mean $\pm$ SD, 0.637 ± 0.296 ODU). As expected, the values of these immunoglobulins in RA exceeded the reference limits of healthy controls and asymptomatic fanciers ($P = 6.5 \times 10^{-19}$ and 1.0×10^{-18}). The comparison between HP and RA revealed that some patients with pigeon lung disease have RF values comparable to those detected in the RA group (see Fig. 1).

With regard to the diagnostic value, the presence of positive RF in pigeon HP showed a sensitivity of 52%, and high specificity values of this test were found considering the results of asymptomatic breeders and IPF groups (95 and 87%, respectively). Positive and negative predictive values were similar taking into account the comparison of HP with asymptomatic

fanciers (0.93 and 0.64, respectively) and IPF (0.87 and 0.51, respectively),

Cross-reactivity and interaction of immunoglobulins

A cross-reactivity of RF with avian antigens was ruled out as the measurement of AA was consistently negative through ELISA and western blot using sera with positive RF from RA patients. Additionally, the use of the purified IgM RF obtained from the RA group revealed negative AA tests.

The cross-reaction of AA with human IgG was also excluded. Positive AA sera with no RF activity revealed negative results through ELISA and western blot using purified IgG Fc fragment as antigen. An additional purified serum fraction with positive AA results did not show RF activity.

The use of F(ab')₂ fragment of peroxidase-labelled anti-human immunoglobulins prevented endogenous interferences by IgG Fc fragments in the AA and RF tests. The interaction of other immunoglobulins as possible endogenous interferences was also excluded. The samples from HP patients compared to healthy controls showed negative IgG anti IgM results through ELISA. The comparison of these groups did not reveal statistical differences (IgG with anti-IgM activity $P = 0.09$). The use of a purified serum fraction with positive AA and normal IgG with no anti-avian activity did not show anti IgM activity. All these results were confirmed through western blot.

Western blot

The presence of RF activity in pigeon HP sera was confirmed through western blot (Fig. 2). A positive binding activity against a 29.3-kDa component was found using IgG Fc fragment (Chemicon) previously transferred into nitrocellulose strips and peroxidase label F(ab')₂ fragments of anti-human IgM (Sigma) [4]. All pigeon HP sera with positive RF through ELISA revealed an anti-IgG binding activity using the purified Fc fraction as the antigen source. The results of positive RF through western blot were comparable in HP, IPF, and RA.

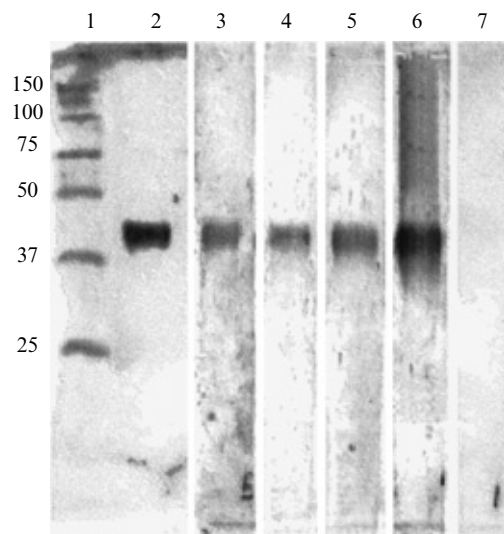


Fig. 2. Molecular weight markers (lane 1), separation of purified IgG Fc fragment through electrophoresis (lane 2) (Coomassie blue staining) and representative IgM reactivity against this IgG fraction (29.3 kDa) through Western blot in pigeon HP, idiopathic pulmonary fibrosis and rheumatoid arthritis (lanes 3, 4, and 5). A purified IgM with rheumatoid factor activity (lane 6) and a control sample with no RF (lane 7) are also shown.

Clinical evaluation

Positive RF tests were found in our pigeon HP patients irrespective of their clinical features. Patients with positive RF did not differ from those subjects with negative tests in age, smoking habit, length of time, and intensity of antigen exposure. Disease onset was also similar in both groups. Clinical, functional respiratory changes, and radiographic findings in the HP patients did not reveal any significant relationship with the RF results. None of the HP patients showed the simultaneous occurrence of other disorders that could explain the presence of RF, in particular the co-occurrence of RA or other connective tissue diseases.

Discussion

Previous studies have emphasized that no single immunological test may definitively distinguish between antigen exposure and pigeon breeder's disease. Besides, no pathognomonic laboratory features appear to separate the HP group from other pulmonary disorders [2–8].

In this report, AA and RF tests were useful for the study of pigeon lung disease as both serological abnormalities occurred in a high frequency of this HP subset as opposed to asymptomatic fanciers. The comparison with IPF and other lung disorders secondary to RA, in which circulating RF may be present, disclosed negative AA results.

Quantitative differences in the immunoglobulin levels with anti-avian activity have been useful to discriminate the HP group from asymptomatic fanciers [2–4, 35], and only few studies support that specific binding of these antibodies may have a diagnostic value [3, 35]. Although we detected a certain degree of overlapping in the AA tests, the HP group revealed higher IgG and IgA levels than asymptomatic controls.

Comparison of pigeon lung with other pulmonary disorders through AA tests has only been assessed in few reports [36]. Diagnostic evaluation of AA through ELISA revealed that the IgG levels in pigeon breeder's disease exceeded the values of other respiratory disorders different from HP [7, 36–38] and no significant overlapping AA results have been described in HP and idiopathic pulmonary fibrosis [7, 8]. In the present study, high values of IgG and IgA were useful to distinguish HP from IPF and RA.

With regard to RF, no previous observations have pointed out the possible relationship of this antibody with the diagnosis of HP. In this report a high frequency of patients with pigeon lung disease showed positive RF, whereas only few asymptomatic controls revealed high RF levels. The IPF group had also a low percentage of positive RF tests. As expected, the patients with RA revealed the highest RF values.

When RF tests were analyzed in parallel to AA it was evident that both serological changes occurred in pigeon lung disease and not in asymptomatic fanciers and IPF. In comparison the RA group showed positive RF but negative AA.

The appearance of RF in pigeon breeder's disease is not an unusual finding. This antibody has been detected in some HP subsets like farmer's lung and humidifier's hypersensitivity [3, 18, 22–24]. In pigeon fancier's disease, positive RF was initially described in anecdotal studies [25, 26] and similar results were also found in immunological reports [14, 27]. Interestingly,

some patients showed high titers of latex agglutination tests together with positive levels of AA [22, 26]. Although the clinical value of RF has not specifically been analyzed, the association with acute episodes or chronic disease was reported in farmer's lung and humidifier's HP [22, 23]. In pigeon breeder's lung we did not find a consistent association with the clinical features of the disease, however, further studies are required before any conclusion can be drawn.

Previous studies have revealed a low frequency of RF in interstitial lung disorders excluding the group associated with connective tissue diseases [39] as occurred in RA in the present report. Specific records in IPF and some subsets of interstitial pneumonitis have revealed that positive RF tests may occur in 5–12%, which are not too different from the results obtained in the general population [39, 40].

The scant attention given to the possible relation between RF and HP might be explained because the detection of this antibody through ELISA, a highly sensitive method, has rarely been used for the clinical assessment of the disease [13, 32]. Indeed, previous observations that describe positive RF results in farmer's lung and humidifier's disease have used agglutination tests [24].

The presence of RF in HP and not in asymptomatic fanciers agrees with previous immunological studies that support that specific differences may distinguish these groups. The elevation of some immunoglobulins like IgG in sera and bronchoalveolar fluid (BALF) has been reported in pigeon breeder's disease in comparison to asymptomatic controls [15, 41]. High levels and persistent synthesis of AA, as well as sustained formation of IC, may distinguish pigeon HP from healthy contacts [35, 42, 43]. Increased numbers of neutrophils in BALF after antigen challenge has been observed in HP but not in healthy exposed subjects [44, 45]. In addition, different cell-mediated reactivity to mitogens and pigeon antigens, including the production of soluble IL-2 receptors seems to separate pigeon breeder's disease and asymptomatic fanciers [46, 47]. In this sense, the appearance of RF could be a reflection of a distinctive inflammatory response that occurs in HP [35] but not in asymptomatic breeders.

Besides the clinical value of RF, it might be related to the pathogenesis of interstitial lung diseases. The participation of RF in the development of pneumonitis and pulmonary fibrosis with or without an associated vasculitis has been suggested in RA [48]. This antibody may have a direct or indirect contribution during the appearance of lung disease. Complement fixation by RF may trigger an inflammatory reaction with macrophages activation, neutrophil recruitment, and the subsequent T cell reactivity [49]. In HP, no definitive evidences support a physiopathological role of RF, however, the elevation of plasma cells, IgM, and IC in BALF [15, 41, 50–53], particularly during the acute disease, suggests that RF synthesis may occur in the lung.

In pigeon fancier's disease, different mechanisms must be considered as responsible for the appearance of RF. Immunogenetic factors in HP may be associated with the presence of RF [31, 54, 55]. The antigen exposure may induce a polyclonal B cell activation with RF synthesis [56]. Additionally, a Th2 cell polarization in HP may play a role in this antibody production [57, 58].

The experimental evidence that RF appears as the result of hypergamaglobulinemia and IC formation [59, 60] suggest that

a similar mechanism may occur in diseases like HP. Indeed, induction of RF with IC has shown that not all IC have comparable stimulatory activities and effectiveness in cross-linking IgM receptors and Fc RIIB receptors [60], a situation that might explain the serological differences in pigeon breeder's disease and asymptomatic fanciers. Alternatively, the RF synthesis in HP might be the result of a T helper activity triggered after the presentation of pigeon antigen by RF producing B cells in the context of an IC via membrane immunoglobulin [59].

We are presently studying the relation of RF with IC formation, as well as complement activation, and other serological changes that have been related with pigeon HP but are not considered specific findings of the disease [9–17].

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