IgM, IgG, and IgA Rheumatoid Factors in Pigeon Hypersensitivity Pneumonitis

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The association of rheumatoid factor (RF) and lung disease in several immunologically mediated conditions has suggested that it may be physiopathologically relevant. Since previous reports in hypersensitivity pneumonitis (HP) have dealt mainly with the immunoglobulin M (IgM) RF measurement, we studied such antibody activity in other immunoglobulins, to determine the IgG and IgA RF levels in pigeon-HP, and in asymptomatic breeders (AB) and rheumatoid arthritis (RA) as controls. RFs were measured in 35 HP patients, 41 AB, 31 RA controls, and 55 healthy donors by enzyme-linked immunosorbent assay (ELISA) using human or rabbit immunoglobulin G (IgG), anti-IgM, F(ab')₂ of IgG, and IgA F(ab')₂ conjugates. An affinity chromatography, fragment crystallizable (Fc) preparations of IgG, pepsin digestion, and

Western blots were used to confirm RF specificity. We also evaluated anti-avian antibodies (AA) and cross-reacting antibodies. The HP group revealed positive IgM (51.4%), IgG (31.4%), and immunoglobulin A (IgA) (34.2%) RF tests, and these antibody values exceeded the AB reference levels ($P < 0.02$). HP and RA showed a similar frequency and distribution of RFs. Possible immunoassay interferences were excluded. As in other immunologically mediated diseases, IgG and IgA RFs may play a pathogenic role in HP, amplifying the inflammatory reaction, immune-complex formation, and complement activation. IgM-RF producing cells that have been implicated in the presentation of self and foreign antigens, and T-cell activation might induce the isotype switching of RFs. *J. Clin. Lab. Anal.* 21:315–321, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

Hypersensitivity pneumonitis (HP) or extrinsic allergic alveolitis defines an heterogeneous group of lung disorders secondary to the exposure of a variety of environmental antigens. Pigeon fancier's disease has been recognized as one of the most common HP subsets, and is caused by the inhalation of organic dusts derived mainly from feathers and/or avian droppings (1). The clinical manifestations, functional respiratory tests, imaging studies, and histopathological findings are consistent with a predominant interstitial lung involvement (2,3). A diversity of immunological abnormalities may also distinguish the disease, and they have been useful to define the diagnosis and/or approach its pathogenesis (1–4).

A prominent humoral involvement has been reported in pigeon fancier's disease, as revealed by the interstitial and alveolar infiltrates of B lymphocytes and/or plasma cells; a marked development of bronchus-associated lymphoid tissue (BALT), or the appearance of germinal centers in the lung with proliferation and maturation of

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centroblasts (5,6). In addition, the presence of elevated levels of immunoglobulins in bronchoalveolar lavage, and a number of serum abnormalities have pointed out that a significant B-cell response may occur in HP (7,8).

The serological evaluation of pigeon fancier's disease has showed the frequent occurrence of hypergammaglobulinemia, high levels of anti-avian antibodies (AA), complement fixation by these antigen-specific immunoglobulins, circulating immune-complexes (IC), and other findings that have received less attention, such as rheumatoid factor (RF) (1,9–13).

Since RF was originally found in rheumatoid arthritis (RA) and characterized as an immunoglobulin M (IgM) antibody, several reports have demonstrated the broad distribution of such serologic hallmarks, and that different immunoglobulin classes may show this anti-immunoglobulin G (IgG) activity (14,15).

An interesting association between RF and lung involvement has been described in an heterogeneous group of immunologically mediated conditions, which include a variety of autoimmune, infectious, and occupational diseases, as well as some specific respiratory abnormalities, like cystic fibrosis, chronic eosinophilic pneumonia, or HP (2,16–19).

RF was initially reported in humidifier's lung (10), and has been further described as a common and clinical relevant finding in other HP groups. A high incidence and elevated levels of this immunoglobulin may occur in farmer's hypersensitivity and pigeon fancier's disease: RF was detected as an interfering antibody during the immunological evaluation of these respiratory disorders; the serologic study of chronic extrinsic allergic alveolitis has revealed the frequent occurrence of RF; and other reports have shown increasing values of IgM RF tests in HP secondary to pigeon exposure and farmer's lung in comparison to asymptomatic controls (2,11,12,20–29).

Interestingly, a number of clinical and experimental observations have pointed out that the presence of RF activity may play a significant immunoregulatory role, particularly during the humoral response, or contribute in the development of disease through different pathogenic mechanisms (14,15).

Several reports have described that the naturally occurring nonpathogenic RFs are mainly of the IgM isotype, and according to some evidences their presence in healthy subjects and even in some pathological conditions appears to modulate the antibody response, IC handling, and antigen presentation (14,15). In contrast, the appearance of IgG and/or immunoglobulin A (IgA) RFs in several immunological diseases characterized by lung involvement, has suggested that these antibodies may play a pathogenic role. Their ability to form IC, activate complement, and/or amplify the inflammatory reaction has previously been documented,

and a direct participation in lung damage has been proposed by some groups.

Because not only IgM but IgG and IgA RFs have been associated to lung involvement in several immunologically mediated disorders, and since these antibodies may play a significant role in the pathophysiology of the disease, we extended previous HP studies to determine whether the activity of RF involves different classes of immunoglobulins.

MATERIALS AND METHODS

Patients and Controls

The study included the following: 35 consecutive HP patients (33 women, two men; mean age: 39.8 years) who had previous pigeon exposure and presented respiratory involvement by clinical, radiological, and spirometric tests, positive AA, and the distinctive histopathological features of the disease (26,28); 41 asymptomatic pigeon breeders (39 women, two men; mean age 39.1 years), including members of a local pigeon-racing club and healthy blood donors with antecedents of avian contact similar to the HP group (26,28); 31 definite RA patients (29 women, two men; mean age 41.2 years) who met the American College of Rheumatology criteria (28,30); and 55 healthy subjects with no previous history of bird contact (51 women, four men; mean age 37.2 years). The healthy controls did not have evidence of pulmonary disease by history, physical examination, lung functional tests, and imaging studies. Our Institutional Review Board approved the study, and written consent was obtained from all patients and controls.

RF Immunoassays

The IgM, IgG, and IgA RF activity was measured through enzyme-linked immunosorbent assay (ELISA). Polystyrene plates (Maxisorb; Nunc, Rochester, NY) were coated with purified and heat-aggregated (63°C for 20 min) human (IgM RF test) or rabbit IgG (IgA and IgG RF determinations) (40.0 µg/mL) in NaHCO₃ (0.05 mol/L, pH 9.6) according to previous studies (31–35), and quenched with 3% bovine serum albumin (BSA) fraction V in phosphate buffered saline-Tween 20 0.05% (PBS-T) (Sigma, St. Louis, MO). An appropriate dilution of serum samples was incubated in the antigen-coated wells. The enzyme conjugates included peroxidase labeled F(ab')₂ fractions of goat antibodies specific for human IgM, IgA (Sigma), and F(ab')₂ preparations of monoclonal antibodies that specifically bind the F(ab')₂ portion of human IgG (Jackson ImmunoResearch, West Grove, PA) (IgG RF measurement) (28,31). PBS-T, 3% BSA was the dilution buffer, and the ELISA plates were washed with PBS-T. The

chromogen substrate contained *o*-Phenylenediamine (OPD) in 0.1 M citrate buffer pH 5.0, and the stop reagent was 2.5 M sulfuric acid.

An affinity chromatography was standardized in our laboratory (26) to appropriately measure the presence of RF activity by ELISA. Sera samples were separated through agarose bead columns containing recombinant protein G (Mini-Spin-columns; Pierce, Rockford, IL) (26), and an optimal dilution of the fractions containing IgG or depleted of this immunoglobulin was used in order to prevent the interference by IgM RF and eliminate the binding of RF classes with endogenous immunoglobulins (26,31–35).

The specificities of RF tests were corroborated using ELISA plates coated with purified fragment crystallizable (Fc) preparations of human or rabbit IgG (Chemicon, Temecula, CA), a pepsin digestion method that was performed following the manufacturer instructions (ImmunoPure; Pierce, Rockford, IL), and Western blots (26,28).

All tests included previously characterized positive and negative RF controls, and the sample reactivity was corrected for nonspecific binding considering the absorbance values of BSA-coated wells (32,34). The results were recorded using an ELISA plate reader (LabSystem, Helsinki, Finland), and the net absorbance at 492 nm (A_{492}) of wells was calculated subtracting the blank values (34,35). In addition, a competitive method with increasing concentrations of pigeon antigen was used to evaluate the possibility of cross-reacting antibodies during the RF assays according to previous reports (26,28).

AAs

The levels of IgG and IgA AA were measured as previously reported (9,28). Pigeon serum, 10 μ g/mL in NaHCO₃ buffer was used to prepare the microtiter plates (Maxisorb; Nunc). The ELISA wells were quenched with PBS-T, 3% BSA (Sigma). An appropriate dilution of serum samples was prepared and added to the plates. Peroxidase-labeled goat F(ab')₂ fragments specific for human IgG or IgA (Sigma) were included as conjugates in the respective AA assays. These tests included PBS-T, 3% BSA as dilution buffer, and PBS-T as washing solution. OPD in citrate buffer was the chromogen substrate, and the stop reagent contained 2.5 M sulfuric acid. As in previous studies, the determination of AA included internal controls with purified IgG and IgA preparations, and parallel tests using hypogammaglobulinemic human serum for the assessment of a possible cross-reactivity (9).

Statistical Analysis

The results of RF and AA by ELISA were statistically compared using the nonparametric U Mann-Whitney

test, and the relationship between the RF isotypes, as well as their association with AA, were evaluated determining their correlation coefficients through the nonparametric Spearman's rank test.

RESULTS

Rheumatoid Factor

Our group with pigeon breeder's disease showed IgM, IgG, and IgA RF-positive tests in 51.4%, 31.4%, and 34.2%, respectively. The values of these antibodies exceeded the reference values (mean $\pm$ 2 standard deviation [SD]) of healthy donors with no bird exposure and asymptomatic breeders (AB) (mean $\pm$ SD, IgM 0.30 ± 0.12 vs. 0.21 ± 0.04 and 0.22 ± 0.03 ; IgG 0.38 ± 0.25 vs. 0.20 ± 0.11 and 0.19 ± 0.12 , and IgA 0.28 ± 0.15 vs. 0.19 ± 0.05 and 0.17 ± 0.09 A_{492} , respectively) ($P = 0.0045$ and 0.026 for IgM; 0.00008 and 0.00007 for IgG; and 0.001 and 0.0004 for IgA) (Fig. 1). A low percentage of positive IgM (4.8%), IgG (4.8%), and IgA (9.1%) RF tests were found in asymptomatic fanciers, considering the reference limits of healthy controls with no pigeon exposure ($P = 0.339$ for IgM; 0.378 for IgG; and 0.179 for IgA). As expected the values of IgM, IgG, and IgA RF in RA (mean $\pm$ SD; IgM $= 0.34 \pm 0.13$; IgG $= 0.36 \pm 0.26$; and IgA $= 0.36 \pm 0.13$ A_{492} , respectively) ($P = 2.1 \times 10^{-6}$ and 0.00003 for IgM; 0.0009 and 0.0003 for IgG; and 1.6×10^{-10} and 2.2×10^{-8} for IgA) were above the reference levels of healthy subjects and asymptomatic fanciers.

The simultaneous presence of different classes of RF in HP and RA showed that a similar distribution. IgM, IgG, and IgA RFs occurred in six and seven serum samples of the corresponding studied groups; IgM and IgG RF were positive in five and seven patients with these clinical entities, and the combination of IgM and IgA RF tests involved four HP and seven RA serum samples. IgM RF with no other positive tests occurred in three RA controls, and IgA RF was the only abnormality in two HP samples and one RA patient. In addition, the statistical evaluation revealed that there was a significant correlation between the IgM and IgA RF values in pigeon fancier's disease and RA ($r = 0.632$, $P = 0.00004$, and $r = 0.604$, $P = 0.0003$, respectively).

The specificity of IgM, IgG, and IgA tests was confirmed through ELISA and Western blot, using purified Fc preparations from rabbit and human IgG as antigens, and enzyme conjugates that specifically recognized the corresponding purified human IgM, IgG, and IgA immunoglobulins, or previously characterized RF samples. F(ab')₂ fractions of conjugates lead us to detect the different RF classes eliminating endogenous interferences (26). Other sources of false-positive or false-negative results including the presence of indicator and

Fig. 1. Scattergrams showing the levels of IgM (A), IgG (B), and IgA (C) RF tests by ELISA in HP secondary to pigeon antigen, rheumatoid arthritis, in asymptomatic breeders and healthy controls with no bird exposure. Horizontal lines represent the mean $\pm$ SD of the studied groups.

matrix effects and heterophilic immunoglobulins were ruled out (34,35), as revealed by the ELISA values of serum samples using BSA coated wells and competitive tests with pigeon antigen that excluded the possibility of cross-reacting antibodies. In addition, the method of pepsin digestion and the use of F(ab')₂ conjugates specific for F(ab')₂ of human IgG, was particularly useful to corroborate the RF activity of IgG.

In clinical terms, we did not find a statistical relationship between the HP features and the levels of IgM, IgG, and IgA RF, nevertheless the last two tests were more frequently positive in patients with chronic disease.

AAs

The levels of IgG and IgA AA were high in the pigeon HP group vs. the reference limits of healthy subjects with no avian contact (mean $\pm$ SD vs. mean $\pm$ 2 SD; for IgG: 0.69 ± 0.26 vs. 0.11 ± 0.06 ; and for IgA: 0.44 ± 0.44 vs. 0.05 ± 0.03 A₄₉₂; $P = 1.6 \times 10^{-15}$ and 1.3×10^{-12} ,

respectively). The AA results and RF values in pigeon fancier's disease did not reveal a statistically significant relationship.

In addition, although the HP patients and AB (mean $\pm$ SD; IgG: 0.39 ± 0.40 ; and IgA: 0.17 ± 0.18 A₄₉₂) revealed overlapping results, the respective IgG and IgA AA levels were higher in the first group ($P = 0.0007$ and 3.0×10^{-11}). As expected, the AB revealed positive IgG and IgA tests in 41.4% and 53.6%, considering the reference limits of healthy controls with no avian exposure. It was of interest that the simultaneous presence of IgG and IgA AA was predominantly found in patients with pigeon fancier's disease (31 out of 35 tested sera) in comparison to the group of AB (14 out of 41 samples) ($P = 0.0001$). The levels of IgG and IgA AA in RA (mean $\pm$ SD; 0.11 ± 0.04 and 0.06 ± 0.03 , respectively) including those patients with interstitial lung disease, did not differ from those results found in healthy controls with no previous history of bird contact ($P = 0.120$ and 0.159 , respectively), and were below the reference values of AB.

DISCUSSION

Pigeon fancier's disease is one of the most common HP subsets characterized by different humoral and T cell abnormalities. The presence of AA, hypergammaglobulinemia, circulating IC, complement activation, and other serological findings including RF has not only been clinically useful, but may provide important clues to approach its pathogenesis (4,8,11). This study describes the appearance of different immunoglobulin classes with RF activity, and intends to support the possibility that some of these antibodies may participate in the physiopathology of HP.

The association between high levels of IgM RF, intermediate IC, and lung disease was initially reported in idiopathic pulmonary fibrosis, RA, and Sjögren's syndrome (SS) (36). In subsequent studies, several groups have described the presence of IgG and/or IgA RFs in high frequency in patients with RA and SS characterized by prominent respiratory manifestations (37,38). A similar relationship between the antibodies with RF activity and pulmonary involvement has been observed in other immunologically mediated disorders, including cystic fibrosis, chronic eosinophilic pneumonia, asbestosis, sarcoidosis, tuberculosis, systemic sclerosis, and some vasculitis (16–18,38–40).

Since RF was initially found in pigeon fancier's disease in anecdotal reports (22,23) the frequent occurrence of this antibody has been recognized by several groups (11–13, 27–29). These studies have dealt primarily with the IgM measurement through agglutination tests, nephelometry, or using some enzyme immunoassays, whereas the evaluation of other immunoglobulins with RF activity has received less attention (11,27,28).

The demonstration of different classes of RF in pigeon fancier's disease suggest that the immunoregulatory properties or pathogenic potential of these serological abnormalities may be physiopathologically relevant, as occurs in other immune-mediated disorders. The IgM RF activity has showed a striking physiological role, as it may participate in the clearance of IC, amplify the antibody response to some foreign antigens, stabilize the binding of low-affinity IgG during the early immune reaction, or modulate antigen presentation and B cell function (14,15,41). In contrast, the appearance of IgG and IgA RFs has been implicated in several immunopathogenic mechanisms that appear to be significant for lung disease. IgG RF may participate in the formation of small and intermediate size IC and complement activation; this antibody has revealed the ability to amplify the inflammatory reaction through the stimulation of platelets and phagocytic cells; and the binding of IgG RF to Fc γ III receptors may induce

the release of proinflammatory cytokines, including interleukin-1 (IL-1) and/or tumor necrosis factor α (TNF α) from monocytes and probably from alveolar macrophages (42–44).

In the same context, the association of IgA RF and lung disease has suggested that it may have a pathogenic value (45). This antibody has showed the ability to recognize the Fc portion of IgG with or without RF activity, and contribute to IC formation; another effect of polymeric IgA RF include complement activation through the alternate pathway, the interaction of IgA and possibly IgA RF with membrane CD89 on myeloid cells seems to have a proinflammatory role, and this immunoglobulin may increase the lysosomal release by phagocytic cells, or stimulate T lymphocytes via Fc α receptors (46,47). Other studies have suggested that the presence of circulating IgA RF may be a result of mucosal stimulation by foreign antigens or reflect an increasing number and/or activation of IgA RF-producing B cells in the lung (48,49).

The clinical significance of RF in HP has not been defined. This antibody may occur in the acute state of farmer's lung, humidifier's hypersensitivity, and pigeon fancier's disease (10,11,24,28); recent reports have found positive RF tests in patients with the recurrent and insidious subsets of chronic HP secondary to avian antigen and it has been suggested that the presence of IgM RF antibody may distinguish the disease from AB (12,21–24,28). In the present study, IgG and IgA RF tests occurred predominantly in HP in comparison to asymptomatic pigeon contacts. Although these abnormalities were particularly frequent in patients with chronic HP, it was difficult to establish a definitive relationship between the RF classes and the clinical features of the disease, considering the retrospective analysis of the present study.

The predisposing factors and illness conditions that may explain the synthesis of RFs in HP have not been studied. As in other immunologically-mediated disorders, the immunogenetic antecedents, antigenic characteristics of organic dusts, or the duration of avian exposure, and the intensity of antibody production, among several characteristics, may contribute to the appearance of different RF classes (41,50–53). In this context, some clinical and experimental observations have suggested that IgM RF may arise as a consequence of IC formation (35,54,55), and the production of monospecific, somatically mutated IgG or IgA RFs may be associated with chronic antigenic stimulation, as occurs in some infectious lung diseases (18). In addition, since IgM RF was a frequent finding in HP patients who presented IgG and/or IgA RF activity, it is possible that the appearance of IgM RF producing B cells may activate a T cell response and induce the antibody

differentiation and isotype switching of immunoglobulins with RF activity, considering that these lymphocytes have been implicated in the endocytosis of IC containing xenoantigens and self IgG via B cell receptor (BCR) and subsequent antigen presentation (56). Other mechanisms that may participate in the appearance of RF classes with pathogenic potential in pigeon fancier's disease include the upregulation of this humoral response by stimulating B cell factors; a predominant synthesis of transforming growth factor (TGF) β 1 derived from different sources including T_{reg} lymphocytes, an abnormal expression and/or function of membrane or soluble Fc receptors, and a polarized differentiation of some B cell subsets (41,57,58).

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