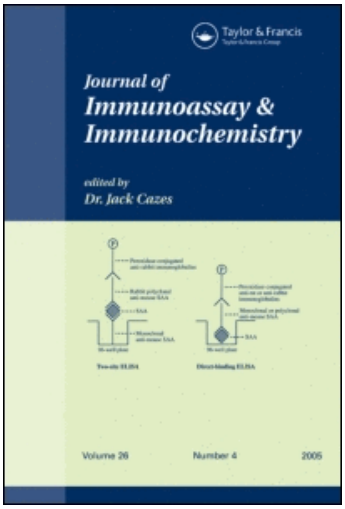

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ENZYME IMMUNOASSAY FOR PIGEON BREEDERS' DISEASE WITHOUT THE USE OF EXTRANEOUS ANTIBODY

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ABSTRACT

A rapid enzyme immunoassay technique is described for the detection in human sera of antibodies of any class to pigeon antigens. The method is based on binding human antibodies in excess to polystyrene-fixed antigens from pigeon droppings or pigeon serum, followed by capture of the same antigens coupled to peroxidase. A very good correlation was found of titers obtained by means of this 60-min assay with traditional sandwich immunoassay and with precipitation- and complement - fixation tests.

INTRODUCTION

The antibodies in human blood sera associated with Type III hypersensitivity pneumonitis due to the inhalation of antigens from bird droppings are known to be predominantly of the IgG - (precipitating and non-precipitating) isotype. In the acute and subacute forms of the disease the IgG - antibody titers are impressively elevated, giving rise to immune complexes in the presence of the corresponding multivalent antigen(s). One of the major groups of multivalent and very high-molecular weight antigens in the bird droppings is composed of the heteropolysaccharides also involved in the phenomenon of complement-fixation [1-3]. However, pigeon blood serum proteins have also been found useful in

serological diagnosis. If the multivalent antigens from either source are physically adsorbed to a polystyrene surface and are subsequently brought into contact with the patient's serum in appropriate dilution, antibody may be bound in excess, leaving unoccupied F(ab) sites available for capturing (enzyme-labelled) antigen in the second step of an immunoassay procedure. In this situation, rapid assessment of the antibody titer in the patient's serum might be achieved without using a second antibody. The present investigation was initiated to evaluate this technique for application in routine serological analysis.

MATERIALS AND METHODS

Blood serum samples of known patients with acute, subacute or chronic pigeon breeders lung (PBL) were obtained from the Allergy Laboratory, University Hospital, Utrecht (The Netherlands). After analysis of the freshly collected samples for precipitating and complement-consuming antibodies and for IgG-class antibodies by second - antibody mediated enzyme-immunoassay (4), aliquots were freezedried and transported to Spain for analysis by Rapid Antigen Capture Enzyme-immunoassay (RACE). Dialysed and lyophilized extracts from pigeon droppings were separated on 94 x 3 cm columns of Sephadex G150 eluted with 1 % NaCl as described (1,2); the heteropolysaccharide fraction PDA emerging immediately after the void volume was pooled and used in the present studies. Pigeon serum proteins as antigen source were separated by centrifugation of coagulated blood samples obtained by heart puncture. Antigen coupling to horse radish peroxidase (HRP, Boehringer, Mannheim) was carried out by the method of Nakane and Kawaoi (5). Analysis of the patients' sera was performed using the unconjugated pigeon antigens for double immunodiffusion (1,2), complement fixation and second antibody enzyme-immunoassay as reported before (3,4). Rapid antibody capture enzyme-immunoassay (RACE) without second antibody was carried out on microtiter plates, using the following procedure :

Antigen fraction PDA from pigeon droppings was coated overnight at 37 ^{o}C in a concentration of 10 µg/ml to the wells of flat-bottom microtiter plates. With pigeon serum as antigen source, whole serum was coated at a dilution of 1 : 25000 v/v in 0.1 M sodium bicarbonate buffer pH 9.5. The plates were washed 3x with phosphate-buffered saline containing 0.05 % Tween-20 (PBS-T) and dried at room

temperature. Dilutions of human serum (100 µl) to be investigated were incubated in the wells for 15 min at room temperature, washed 3x with PBS-T, and subsequently incubated for 15 min with 100 µl (0.2 µg) HRP-conjugated PDA antigens in PBS-T, or with 100 µl of a 1 : 100 diluted stock solution (50 µg / ml) of HRP-labelled pigeon serum proteins. After a final wash cycle, the wells were incubated for 10 min with o-phenylenediamine substrate solution, the reaction was terminated with 0.5 N sulphuric acid, and the colour was read at 491 nm in an automatic microplate reader. The average time span for a complete analysis ranged from 45 - 60 min. In the RACE-test, the antibody titer was expressed as the reciprocal of the serum dilution producing a limiting O.D.reading of 0.50. The best starting serum dilution for routine application was found to be 1 : 10.

RESULTS

Time-curve for Antibody-binding to Antigen-coated Tubes.

A PBL-positive serum (++ precipitation score with pigeon dropping purified B-fraction, 80% huC-consumption with antigen mixture PDA, detectable circulating immune complexes) and a negative control serum of a healthy pigeon breeder were used. Serum aliquot dilutions in PBS-T were incubated in the standard assay system for 0, 2, 4, 8, 10, and 14 min at 37 °C. The plates were emptied after the incubation period and washed 3 x with PBS-T. The HRP-conjugated PDA was then added, incubated for 10 min at 37 °C and removed. The tubes were finally washed 3 x in rapid succession with PBS-T, whereupon 1 ml substrate solution was added. After 10 min at room temperature, the reaction was terminated with 0.5 N H_2SO_4. The results of the O.D.readings are shown in figure 1, which demonstrates that human antibody binding is virtually complete in a little over 10 minutes. Complete and quantitative antibody assay therefore proved possible with this system in about 45 minutes.

Statistical comparison with other serological methods.

From introductory experiments a good correlation was established in particular with the complement-fixation test, as has been reported before with a classical enzyme-immunoassay using HRP-labelled goat anti human IgG (4). The coefficient

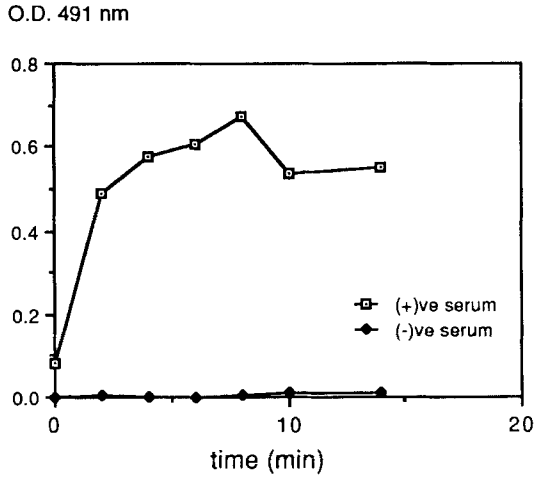

FIGURE 1. Time curve for the binding of antibodies from a PBL-positive and a PBL-negative human serum to microtiter wells precoated with polyvalent heteropolysaccharide antigen fraction PDA from pigeon droppings (1,2). For conditions of assay see text.

of correlation between the C-fixation test and the present method in this restricted series of 11 patients sera was 0.83, χ^2 analysis $P < 0.001$.

Under the standard conditons described under Materials and Methods, a series of 39 serum samples of patients suspected of, or known to have, pigeon breeders' disease was investigated in relation to the ELISA-titer determined in a regular second antibody enzyme-immunoassay against pigeon serum antigens (4). The data were also compared with the results of complement-fixation assays (3) and with the visually appraised precipitation scores from double diffusion in agar gel (2,3). Figure 2 shows the comparative data for the classical immunoassay using pigeon serum-coated plates and anti-IgG HRP-labelled second antibody, and the RACE-test using HRP-conjugated pigeon serum proteins. There was an excellent statistical correlation between both techniques (Spearman rank coefficient of correlation $r = 0.90$ ($N = 28$), $P < 0.001$). The RACE-test also correlated quite well with the complement-consumption test for pigeon breeder s lung described elsewhere (3), as depicted in figure 3 (Spearman rank coefficient of correlation $r = 0.87$ ($N = 46$), $P < 0.001$).

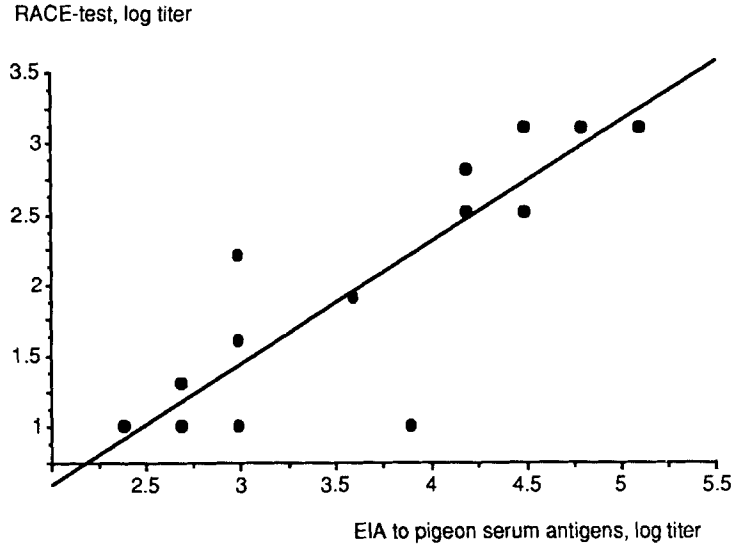

FIGURE 2. Scatter diagram of enzyme-immunoassay results in a collection of 28 sera of patients exposed to pigeon-derived antigens obtained by traditional sandwich immunoassay using pigeon serum-coated plates and HRP-labelled anti human IgG antibody (horizontal axis), and by rapid antigen capture test not using extraneous antibody but HRP-conjugated pigeon serum proteins (vertical axis). Linear regression : $y = 0.86x - 1.12$, $r = 0.93$.

The boxed grey area in figure 3 defines the cut-offs for selecting those patients known to have pigeon breeders disease in the clinically manifest acute form, with complement-consumption values at over 55% and detectable precipitating antibodies (3). The shaded area therefore defines the titers (at over 1000) for establishing a positive serological diagnosis by means of the RACE-test for patients with pigeon breeders' disease.

Finally, a good correlation was obtained in 46 serum samples between the results of the RACE-test and classical analysis for precipitating antibodies to purified pigeon dropping antigens PDB (1,2): $r = 0.84$, χ^2 - analysis significant at $P < 0.001$.

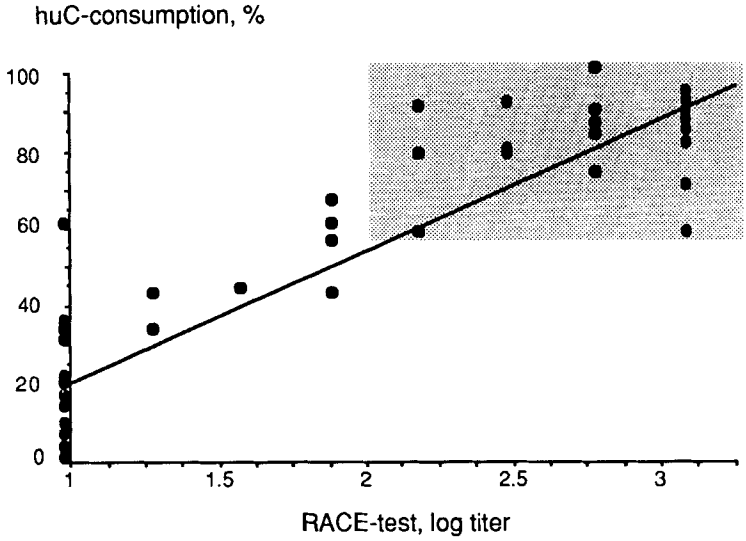

FIGURE 3. Comparison of antibody estimates based on a human complement - fixation test using pigeon-derived antigen fraction PDA (1) and the titers obtained by rapid antibody capture test using the same antigens coated to microtiter plates. Number of investigated serum samples : 46, linear regression : y = 33.98 x - 13.89, r = 0.91. Samples in the shaded area were from patients with PBL in the clinically acute form.

DISCUSSION

The clinical symptomatology of pigeon breeders' lung (PBL) as a manifestation of hypersensitivity pneumonitis in its acute form usually is quite clear, but the final diagnosis needs *in vitro* confirmation for the ultimate identification of the causative antigens (8). Of the various serological methods proposed for this purpose over the years, analysis by double immunodiffusion for precipitating antibodies has proved to be the least reliable, because the titers drop below the level of detection in the equally suspect cases of subacute or chronic PBL, and because healthy pigeon fanciers exposed to appreciable antigen loads may likewise have precipitating antibodies in the circulation. This problem was largely overcome by the development of complement-fixation tests which detected "non-precipitating" antibodies as well; the fairly constant level of such antibodies even in the absence of

exposure or clinical symptoms was shown to be of value as a prognostic parameter and for follow-up studies (3). Several investigators have subsequently demonstrated the usefulness of traditional enzyme-immunoassays in the serodiagnosis of PBL (4,6,7). The antibody isotype evaluated with these techniques has usually been of the IgG-class; the present RACE-test measures antibodies of other classes as well, although both the published data and the results of figure 2 support the conclusion that IgG is the predominant antibody class in the blood serum of patients with PBL (3,4). The correlation with the results of complement-fixation tests depicted in figure 3 confirms that the RACE-test is a fast and easy screening method for the detection and quantitation of antibodies to pigeon-derived antigens as an aid in the diagnosis of acute PBL (figure 3, shaded area). However, the complement-fixation test is more sensitive and will identify subjects with subacute or chronic PBL as well, even though in those cases the antibody level may be too low for detection by either immunodiffusion or RACE-test analysis (figure 3).

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