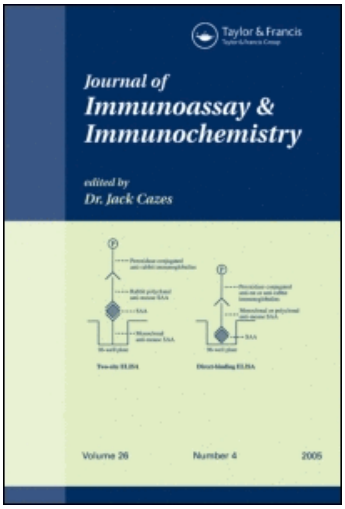

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A Competitive Enzyme Immunoassay Subclass® for the Determination of Total IgG—Subclass Levels in Human Serum

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A COMPETITIVE ENZYME IMMUNOASSAY SUBCLASS® FOR THE DETERMINATION OF TOTAL IgG - SUBCLASS LEVELS IN HUMAN SERUM

Comparison with Single Radial Immunodiffusion

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ABSTRACT

A quantitative immunoassay has been developed for the determination of the total IgG subclass levels in human serum. The method is based on an enzyme immunoassay in which IgG subclass proteins IgGx in an unknown serum sample compete with a known quantity of peroxidase (PO)-labelled IgGx in fluid phase for subclass-specific monoclonal antibodies coated to the solid phase of microtiter wells. Using a series of human blood samples an excellent correlation was observed with the IgG-subclass levels determined by single radial immunodiffusion.

Key Words: Competitive EIA, IgG subclasses, Subclass®, Radial Immunodiffusion.

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INTRODUCTION

The IgG immunoglobulin class, which comprises about 75 % of the total human serum immunoglobulin protein pool, is known to encompass four distinct subclasses or isotypes which occur in a concentration ratio of IgG1 > IgG2 > IgG3 > IgG4 [1]. The normal mean absolute concentrations for each of these subclasses differ considerably, ranging from about 7-8 g/L for IgG1 to around 1,0 g/L for the IgG4 isotype, which impedes the design of single-dilution quantitative assays establishing all four subclasses in a single step. Furthermore, total or partial deficiencies described for each individual subclass require test systems covering the entire range of concentrations from zero to above the average values for the normal population. The need for rapid and reliable quantitative methods for each of the four subclasses is underlined by reports of selective IgG-subclass deficiencies being associated with recurrent infections of the upper and lower airways and in asthmatic conditions [2-6].

The design and sensitivity of enzyme immunoassays in general precludes the utilization of methods based on the direct capture of the IgGx subclass proteins from the serum by anti-IgGx coated microtiter plates followed by development with an enzyme-labelled anti-IgGx, because the serum dilutions to be employed in this case must be excessively high. Also, the magnitude of dilution varies among the distinct subclasses. We have attempted to circumvent this problem by developing a competitive immunoassay, whereby the IgGx in the unknown sample competes with a known quantity of enzyme-conjugated and partially purified IgGx for the anti-IgGx on the plates. The present paper details the methodology and compares the results with the determination of IgG subclass concentrations by single radial immunodiffusion.

MATERIALS AND METHODS

Single radial immunodiffusion for the determination of total IgG subclass levels was performed using a commercial test kit, following the manufacturer's instructions for use (The Binding Site, Birmingham, England).Briefly, samples were diluted 1 :10 in bovine serum albumin 7% for IgG1 and IgG2

but were used undiluted for IgG3 and IgG4 determination. Five µl of each sample was pipetted into the well of anti IgGx gel plate . After 72 hours of diffusion the ring diameters were measured with 0,1 mm precision. Finally, concentrations were calculated by a ring diffusion diameter *versuss* concentration conversion table. Detection limits in mg / ml under the normal recommended dilutions were: 1.18 - 21.9 for IgG1, 0.676 - 12.5 for IgG2, 0.079 - 1.94 for IgG3, and 0.033 - 0.808 for IgG4.

The reagents employed in this investigation for the competitive immunoassay of the IgG subclasses were those supplied with the commercial test kits Subclass ® as specified by the manufacturer (Laboratorios LETI S.A., Barcelona, Spain). In this assay system, the source of the individual IgG-subclass proteins is the isolated and purified gamma-globulin fraction of pooled human plasma subsequently labelled with horse radish peroxidase according to Nakane and Kawaoi [7] with some slight modifications. Microtiter strips of 8 flat-bottom wells each were individually coated with specific mouse monoclonal antibodies against human IgG1, IgG2, IgG3 and IgG4 (Janssen Pharmaceutica, Belgium) as previously described [8]. The commercial Subclass ® kit also includes a lyophilized calibrating serum with known concentrations for each IgG subclass determined separately, as well as freezedried samples of positive and negative controls.

The procedure proposed by the manufacturer for the determination of total IgG subclass levels by competitive immunoassay was adopted throughout these investigations. Briefly, sera to be investigated were diluted 1 : 50 in neutral buffer solution and 200 µl aliquots were pipetted into a series of small plastic test tubes. To each tube a volume of 200 µl of a 1 : 25 dilution in the same buffer of a standard PO-IgGx conjugate solution at a concentration adjusted for each subclass by the manufacturer was then added and the mixture was allowed to stand at ambient temperature for 10 min. Subsequently, 100 µl aliquots were transferred into the wells of 4 separate microtiter strips coated with anti IgG1, anti IgG2, anti IgG3 and anti IgG4, respectively. The same procedure was followed using the reconstituted calibrating serum. After a 30 min incubation period at room temperature, the strips were washed with phosphate buffered

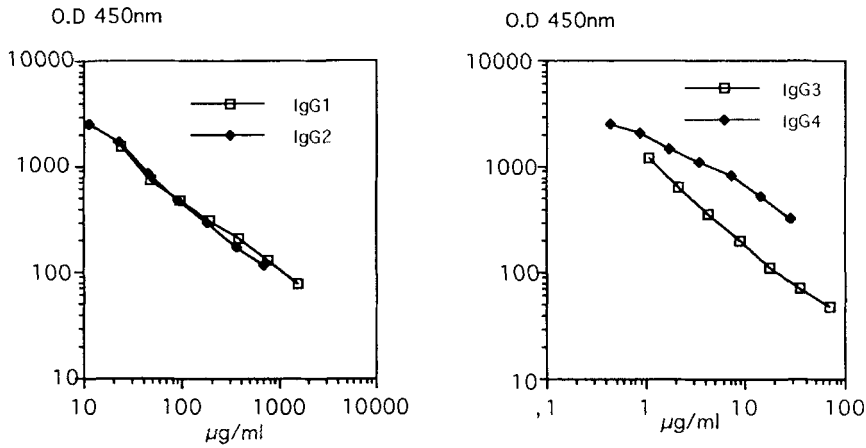

Figure 1. Calibration curves obtained by linear regression of double logarithmic transformed values. On the left IgG1 and IgG2 calibration curves with correlation coefficients from the regression lines r = 0,996 (P = 0,0001) and r = 0,997 (P = 0,0002).On the right IgG3 and IgG4 calibration curves with correlation coefficients r = 0,995 (P = 0,001) and r = 0,993 (P = 0,0001).

saline-Tween 20 (PBS-T) and subsequently developed with 100 µl per well of a development solution containing a mixture of 3,3'-4,4'-tetramethylbenzidine - H_2O_2 in 0.1 M acetate buffer [9]. The enzymatic reaction was terminated by adding 100 µl 0.5 N sulphuric acid per well and optical densities were finally read at 450 nm in an automatic microplate reader against the appropriate blanks and controls. All determinations were performed in duplicate.

For evaluating the results, a double-log standard curve was constructed for each subclass by plotting the optical densities obtained with the calibrating serum in a series of 2-fold sequential dilutions against the actual IgGx protein concentrations expressed in mg/ml (Figure 1). IgG subclass concentrations of unknown serum samples were found by interpolating the observed optical densities at the standard dilution on the standard curves. Detection limits expressed in mg / ml under the normal recommended dilutions were: 1.1 - 76.6 for IgG1, 0.55 - 35.2 for IgG2, 0.053 - 3.45 for IgG3 and 0.02 - 1.4 for IgG4.

TABLE 1

Statistical values for intra-assay reproducibility.

subclass	mean mg/ml	S.D.	coefficient of variation	number of tests
IgG1	7,88	1,54	19,61	54
IgG2	6,18	1,36	22,02	54
IgG3	1,28	0,23	17,79	54
IgG4	1,6	0,23	14,53	48

TABLE 2

Statistical values for inter-assay reproducibility.

subclass	mean mg/ml	S.D.	coefficient of variation	number of tests
IgG1	8,59	1,43	16,67	37
IgG2	7,52	1,04	13,77	37
IgG3	1,04	0,14	13,79	37
IgG4	0,37	0,06	17,65	37

RESULTS AND DISCUSSION

In the proposed competitive mode of immunoassay for the IgGx subclasses, the quantity of captured enzyme-labelled IgGx will in each case vary inversely with the amount of IgGx present in the unknown serum sample. Hence, the optical density readings decrease with an increasing level of IgGx in the sample. In order to check the reproducibility of the results in this set-up, we first determined the intra-assay reproducibility (within-day) by repeated determinations on a thawed serum sample in one single day. Second, the inter-

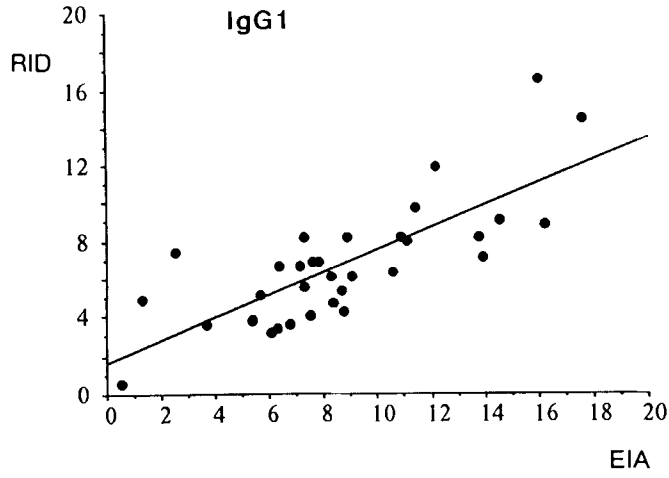

Figure 2. Scatter diagram showing the relationship between the IgG1 values obtained by competitive EIA and by RID in 34 individual serum samples. Concentrations are given in mg/ml. The coefficient of correlation from linear regression was r = 0,77 (P < 0,0001).

assay reproducibility (between-day) was established by repeated determinations on 37 consecutive days using freshly thawed aliquots daily of a single serum pool. The results, shown in Tables 1 and 2, demonstrate that the standard deviations and coefficients of variation were in the range acceptable for enzyme immunoassays.

The analytical results obtained for each of the four IgG subtypes with the Subclass ® kit were then compared in a series of 39 individual human serum samples with the concentrations established by means of radial immunodiffusion. The sera were selected to cover the range of high, medium and low IgG-subclass concentrations. The results are shown in the plots and legends to figures 2 - 5 for each of the IgG-subclass isotypes, respectively.

In view of the significant correlation coefficients for each of the four subclasses, it appears from these results that the EIA method provides higher values for IgG1, IgG2 and IgG4 than RID, as confirmed by t - test analysis (table 3) This discordance may be explained on the basis of inaccurate

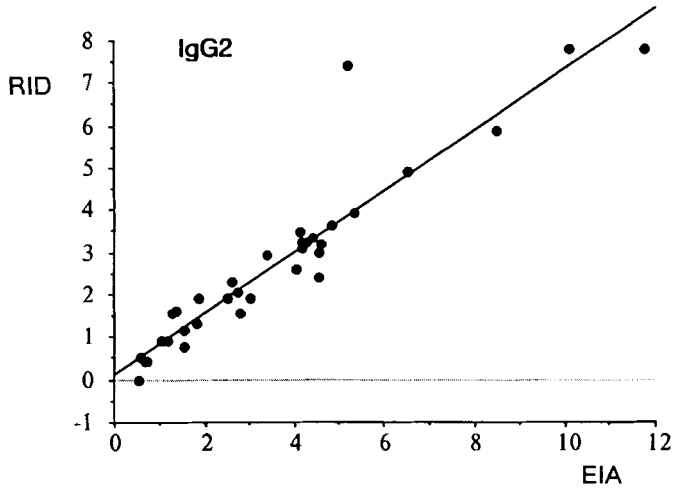

Figure 3. Scatter diagram showing the relationship between the IgG2 values obtained by competitive EIA and by RID in 34 individual serum samples. Concentrations are given in mg/ml. The coefficient of correlation from linear regression was r = 0,94 (P < 0,0001).

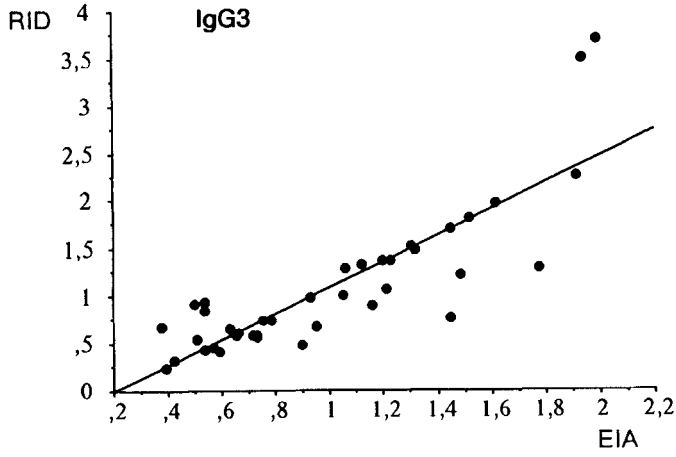

Figure 4. Scatter diagram showing the relationship between the IgG3 values obtained by competitive EIA and by RID in 39 individual serum samples. Concentrations are given in mg/ml. The coefficient of correlation from linear regression was r = 0,84 (P < 0,0001).

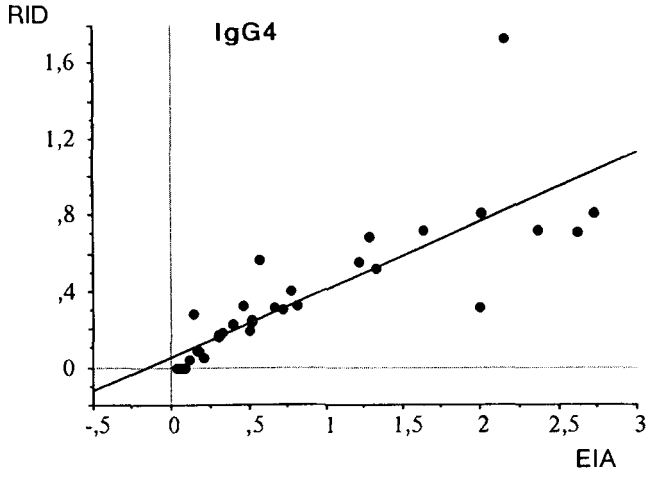

Figure 5. Scatter diagram showing the relationship between the IgG4 values obtained by competitive EIA and by RID in 36 individual serum samples. Concentrations are given in mg/ml. The coefficient of correlation from linear regression was r = 0,82 (P < 0,0001).

TABLE 3

t-Test for EIA versus RID concentration values

Subclass	D.F	Mean EIA-RID	Paired t value	Prob (2-tail)
IgG1	33	1,9356	4,3139	0,0001
IgG2	33	0,875	4,9801	0,0001
IgG3	38	-0,088	-1,2402	0,2225
IgG4	35	0,4389	4,6961	0,0001

DF = degree of freedom

calibration. In fact, slight differences between the results of both methods were found when exchanging the two calibrators (data not shown). This problem is not uncommon whenever international standards are unavailable.

This type of competitive enzyme immunoassay method provides a number of distinct practical advantages over other existing methodologies. The recommended serum dilution of only 1 : 50 entails less experimental errors than those inherent to the dilution range of 1: 40 000 to 1 : 80 000 required for a non-competitive sandwich EIA system for these IgG subclass proteins.

Setting the serum dilution for competitive EIA at 1 : 50 leaves open the possibility of retesting samples with a low IgGx concentration by choosing a lower dilution range. By contrast, the RID dilution range precludes the retesting of samples for IgG3 and IgG4.

The procedure for the competitive EIA method was found to be easy and fast to perform, enabling the handling of at least 40 duplicate determinations in a time span of less than 2 h. The speed and simplicity of the method should therefore make it well-suited for automation.

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