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Autoantibodies against intrinsic factor (IF) measured with an ELISA using recombinant human IF as both catching and detecting reagent

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Abstract

The aim was to develop and validate an assay for measuring autoantibodies against human intrinsic factor (IF). For this purpose 1.25 pmol of recombinant IF was coupled to each well. Samples (100 μL of plasma diluted 1:1) were incubated for 1 h, followed by 30 min with the detection reagent (biotinylated IF, 3.3 pmol/mL). Samples were obtained from healthy blood donors (n=141) and patients with suspected vitamin B_{12} deficiency (n=355). The initial assay results (n=99 samples) were in reasonable agreement with those obtained using a commercial assay (Diagnostic Products Corporation). All donors but one showed a negative result. For the patient populations the fraction of positive results decreased with increasing levels of serum vitamin B_{12}: 0.67 for <100 pmol/L (n=13), 0.17 for 100–150 pmol/L (n=23), 0.06 for 151–200 pmol/L (n=65) and <0.01 for >200 pmol/L (n=254). The imprecision was 11% as judged by repeat analyses. All samples remained positive when the catching reagent was exchanged with vitamin B_{12}-saturated IF, suggesting that the samples contained both blocking (type I) and non-blocking (type II) antibodies. Results obtained with IF as the detection reagent correlated to those obtained when anti-immunoglobulin G was used instead. In conclusion, an ELISA using recombinant IF as both catching and detection reagent seems suitable for the detection of IF autoantibodies in plasma.

Keywords: autoimmune disease; blocking antibodies; cobalamin; non-blocking antibodies; pernicious anaemia.

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Introduction

Measurement of autoantibodies against intrinsic factor (IF) is considered a helpful diagnostic tool in the investigation of patients with suspected pernicious anaemia, with autoantibodies reported to occur in up to 70% of such patients (1).

Unfortunately, the methodology for detecting autoantibodies is far from optimal. The first assays available relied on the ability of autoantibodies to inhibit the uptake of vitamin B_{12} in the Schilling test (2). Later, several in vitro tests were developed. Most rely on the ability of autoantibodies to inhibit the binding of labelled vitamin B_{12} to IF (2–4). These assays are only able to identify type I autoantibodies, most often referred to as blocking antibodies because they are able to block the uptake of vitamin B_{12} by IF. The main drawback of this assay type is the inability to detect non-blocking antibodies, also referred to as type II autoantibodies (5). In addition, false-positive results have been reported in patients with high serum vitamin B_{12} levels (6). Later, numerous other designs have been suggested, including assays employing iodine-labelled IF (7). More recently, ELISA employing IF as both the catching and the detection reagent was also reported (8). The drawback of these assays has been the lack of a source of pure human IF of a well defined quality.

In the present paper we further developed the concept to use IF as both catching and detection reagent (8) and we present an assay based on commercially available recombinant human IF.

Materials and methods

Samples from donors and patients

Four sets of samples were included.

For the proof of concept study, 48 sera testing positive and 51 sera testing negative for IF autoantibodies with a commercial assay were selected from the routine laboratory during the period July 2002 to August 2003.

As a reference population we employed EDTA plasma samples from 141 healthy blood donors (70 females and 71 males) collected in May 2000.

To analyse the occurrence of autoantibodies in patients suspected of vitamin B_{12} deficiency, we used 219 serum samples received in the routine laboratory for measurement of serum vitamin B_{12} during the period 2000–2004 and 136 EDTA plasma samples from patients participating in a vitamin B_{12} intervention study (9). Repeat samples were obtained from the latter group of patients 3 months later.

All patients included for the main study had serum vitamin B_{12} below the upper limit of the reference interval (600 pmol/L).

ELISA assay

Reagents Recombinant human IF not saturated with vitamin B_{12} was obtained from Cobento Biotech A/S (Aarhus, Denmark).

For the proof of concept study we coated plastic microplate strips (Duo Strips; Dynatech, Billinghurst, West Sussex, UK) with 50 μL of human IF semi-purified from gastric juice (8).

For the later part of the study we incubated microtitre plates (F96 Maxisorp, Nunc, Copenhagen, Denmark) overnight at 4°C with 100 μL of 50 mmol/L sodium carbonate buffer, pH 9.6 containing 1.25 pmol of recombinant human IF. The plates were blocked by adding 100 μL of 1 mol/L ethanolamine (Riedel de Häen, Seetze, Germany) and stored at −20°C until use.

To prepare biotinylated recombinant human IF, 0.2 mg of the protein was dissolved in 0.2 mL of distilled water. After dialysing against 0.1 mol/L sodium bicarbonate, pH 8.3 for 24 h, 10 μL of a solution of 2 mg of biotin-amido-caproate-N-hydroxy-succinamide (Sigma, Steinheim, Germany) dissolved in 1 mL of dimethyl sulfoxide (Merck, Darmstadt, Germany) was added. After 4 h of incubation at room temperature, 10 μL of 10 mmol/L lysine HCl (Fluka 62930, Sigma) was added. After 15 min of incubation, 10 μL of γ-globulin, 1 mg of rabbit γ-globulin (Calbiochem-Novabiochem, La Jolla, CA, USA) and 2 mg of bovine γ-globulin (Sigma) in 20 mL of dilution buffer (10 mmol/L sodium phosphate, 0.154 sodium chloride, pH 7.4) were added. The sample was then dialysed against dilution buffer for 3 days with three changes of dialysis fluid, aliquoted and stored at −20°C until use. The stock solution of the biotinylated recombinant IF contained 10 nmol/L (0.5 mg) protein.

The assay buffer was 0.1 mol/L phosphate buffer, pH 8.0 containing 0.1% human albumin (ICN, Aurora, OH, USA). The washing buffer was 10 mmol/L sodium phosphate, 145 mmol/L sodium chloride, 0.1% Tween 20, pH 7.4 (Bie and Berntsen, Copenhagen, Denmark). POD-Avidin reagent contained 6 μL of POD avidin (DakoCytomation, Copenhagen, Denmark) and 120 μL of 20 mg/mL hydrazine (Sigma) diluted in 12 mL of 10 mmol/L sodium phosphate, 400 mmol/L sodium chloride, pH 7.4. 3,3′,5,5′-Tetramethyl-benzidine (TMB) was obtained from Kem-En-Tec (Copenhagen, Denmark). The γ-globulin fraction of rabbit anti-human IF (F2832-0994, custom-produced towards native pure human IF by DAKO A/S, Copenhagen Denmark) at a dilution of 100 mg/L was used as the stock solution for preparation of the calibrators. Biotinylated immunoglobulin (Ig)G and biotinylated IgA (Sigma) diluted 1:9000 in assay buffer was used in place of biotinylated IF to characterise the nature of the autoantibodies. Cyanocobalamin (Sigma) diluted to 1 nmol/mL in assay buffer was used to saturate IF coupled to the microtitre plates.

Procedure

Proof of concept study A 100-μL aliquot of serum (diluted 1:1 with assay buffer) and positive and negative control were incubated for 90 min at 37°C. After washing three times with washing buffer, 100 μL of biotinylated recombinant human IF was added and the plate was incubated for a further 90 min at 37°C. After five washes, 100 μL of streptavidin peroxidase conjugate diluted 1:1000 with assay buffer was added and the plates were incubated for 45 min at room temperature. After three washes the TMB solution was added and the colour reaction was allowed to develop for 20 min. This was terminated by the addition of 100 μL of 100 mmol/L hydrochloric acid. The positive control was serum from a patient with pernicious anaemia and the negative control was that included with a DPC kit (see below). Results are expressed as the ratio between OD at 450 nm for the sample and OD at 450 nm for the negative control.

Main study The coated microtitre plates went through the washing procedure (three washes with 300 μL of washing buffer) prior to incubation for 1 h with 100 μL of sample (serum or plasma diluted 1:1 with assay buffer) or calibrators (rabbit anti-human IF diluted to contain between 100 and 1.6 mg/L protein and assigned values of 100–1.6 arbitrary units). Then followed a washing procedure, incubation with 100 μL of biotinylated IF for 30 min, another washing procedure, incubation for 30 min with 100 μL of POD-avidin reagent, a washing procedure, and finally incubation for approximately 5 min with 100 μL of TMB. The reaction was terminated with the addition of 100 μL of 1 mol/L phosphoric acid. All incubations were performed at room temperature using a shaking platform (SLT LAB Instruments, Grodig, Austria). The plates were read on an ELISA reader (Multiscan Lab System MCC 340, Helsinki, Finland) at 620 and 450 nm.

Other methods

The DPC radioassay kit for measurement of autoantibodies against human IF (Diagnostic Products Corporation, Los Angeles, CA, USA) was used according to the manufacturer's instructions. In brief, the principle of this assay is that serum is incubated with insolubilised unsaturated IF. If antibodies are present they will bind to the IF, thereby decreasing its ability to bind added labelled vitamin B_{12}. The result is expressed as the amount of labelled vitamin B_{12} bound in the negative control divided by the amount of label bound in the patient sample.

Measurement of serum vitamin B_{12} was performed on an ACCESS immunoassay analyser (Beckman Coulter, High Wycombe, Buckinghamshire, UK) for the proof of concept study and on a Centauer analyser (Bayer, Terrytown, NY, USA) for the main study. The reference interval was 200–600 pmol/L.

Results

We used an ELISA using IF as both the catching and detection reagent for measurement of IF autoantibodies in human samples.

Proof of concept

In the first part of the study, we compared results obtained using our prototype assay with those obtained using a commercially available assay. Out of 51 samples negative by the commercial assay, 3 samples were positive by the ELISA, and out of the 48 samples positive by the commercial assay, 44 samples also showed a positive result by the ELISA (Figure 1). Two of the negative ELISA samples had levels of serum vitamin B_{12} exceeding 2000 pmol/L and probably produced false-positive results for the commercial assay.

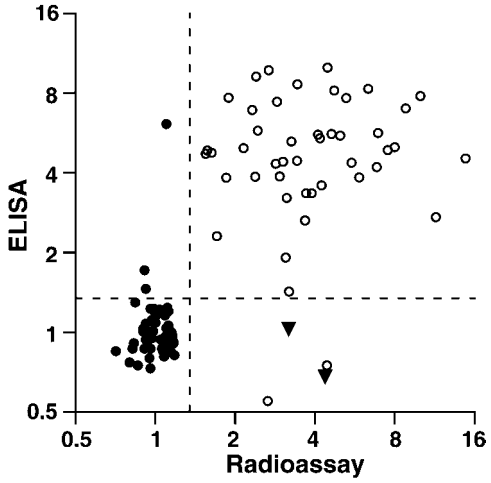

Figure 1 Autoantibodies against IF in samples testing positive (n = 48, open symbols) or negative (n = 51, closed symbols) when measured with a commercial assay (DPC radioassay kit, X-axis) were measured by the prototype ELISA assay employing human IF as both catching and detection reagent (Y-axis). Values on the X-axis are indicated as ^{57}Co-B_{12} counts bound to IF in the negative control divided by ^{57}Co-B_{12} counts bound to IF in patient samples. The Y-axis represents OD at 450 nm for patient samples divided by OD at 450 nm for the negative control. Two samples with increased levels of serum vitamin B_{12} are indicated (triangles). The dashed lines indicate the cut-off values for the two methods.

Despite the reasonable agreement of the two methods for the identification of patients with autoantibodies against IF, there was no consensus concerning the amount of antibodies present in individual samples (Figure 1).

We considered that the initial results were promising and therefore we decided to develop and standardise an ELISA assay using recombinant human IF not saturated with vitamin B_{12} as both catching and detection reagent. This assay design would allow us to detect both type I and type II autoantibodies independent of the γ-globulin class.

Optimising the assay

To determine the amount of catching and detection IF required, we used rabbit anti-human IF as a source of antibodies.

Since autoantibodies are likely to have low affinity for IF, we decided to use a relatively high amount of catching IF (1.25 pmol/well). Next we optimised the biotinylated recombinant IF used for detection (Figure 2A). The addition of 3.3 pmol/mL biotinylated IF allowed a sufficient signal but gave virtually no non-specific background (mean OD 0.014, range 0.09–0.023, n = 14 runs over 2 months). Finally, we designed a calibration curve using rabbit anti-human IF (Figure 2B). The samples were considered positive if they had an OD above the lowest calibrator. The lowest calibrator had a mean OD that was six-fold (range 4–10, n = 14) higher than the non-specific binding.

Assay of samples from reference and patient populations

Three groups of samples were analysed. First, a reference population consisting of 141 blood donors aged between 21 and 65 years (69 below 50 years) was tested. All but one of these samples were negative. The positive sample, however, was strongly positive (Figure 4A). It came from a 36-year-old female. Due to ethical rules, it was not possible to obtain further information concerning this patient. Second, we analysed 355 patients referred for measurement of serum vitamin B_{12} including samples from a population of 136 patients participating in a vitamin B_{12} treatment trial (9). The proportion of patients in whom autoantibodies were detected decreased with increas-

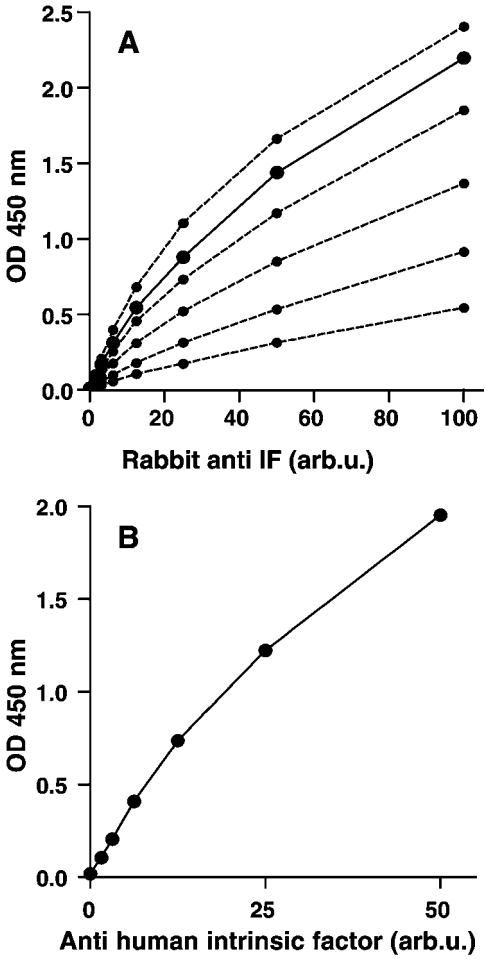

Figure 2 Optimising the ELISA assay for measurement of autoantibodies against IF employing human recombinant IF as both catching and detection reagent. (A) Titration of detection reagent. Plates coated with 1.25 pmol of recombinant unsaturated IF were incubated with anti-rabbit IF followed by incubation with biotinylated recombinant unsaturated IF in a two-fold dilution from the starting dilution of 6.6 pmol/mL biotinylated recombinant IF. A dilution of 3.3 pmol/mL biotinylated IF was employed for further studies (bold line). (B) A typical calibration curve employing rabbit anti-human IF as calibrator. The measurement range covers 1.6–50 arbitrary units.

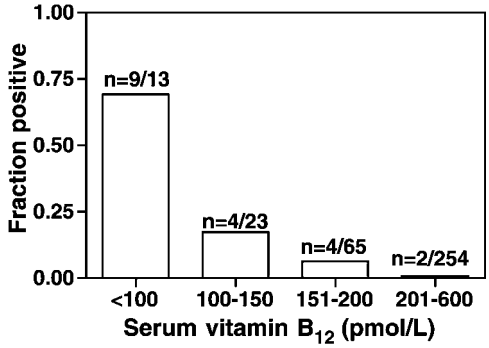

Figure 3 Fraction of patient samples positive for autoantibodies against IF as a function of serum vitamin B_{12} (n = 355). Indicated above each column are the positive and total number of samples.

ing levels of serum vitamin B_{12} (Figure 3). Amongst those with serum vitamin B_{12} below 100 pmol/L, the proportion of positive samples was 0.69, while this figure was <0.01 in those with serum vitamin B_{12} above 200 pmol/L. The association between serum vitamin B_{12} levels and positivity for autoantibodies against IF was highly significant ($p<0.0001$) as tested by comparing patients with vitamin B_{12} levels ≤ 150 pmol/L with those having a serum vitamin B_{12} >150 pmol/L (χ^2 test).

Characterisation of the autoantibodies

All samples with more than 1.6 arbitrary units autoantibodies against IF in a serum dilution of 1:1 were further evaluated (n = 20).

First we examined whether it was possible to measure the antibody level in samples. Serial dilutions of positive samples were compared with each other and with the anti-rabbit human IF employed as calibrator (Figure 4AA). The results indicated that it is not possible to use a common calibrator, since samples showed considerable variation in their dilution profiles.

We then examined whether results obtained using the standard serum dilution of 1:1 could be reproduced by repeating all of the positive samples (n = 20). All samples remained positive with a value range of 2–83 arbitrary units (mean 34). Imprecision based on repeat measurement of the 20 samples was 11%. We analysed paired samples, taken 3 months apart, from 136 patients. All individuals testing negative based on the first sample (n = 128) remained negative and those positive (n = 6) remained positive. Limited variation was observed between the values obtained on the two occasions (3, 54, 62, 18, 68 and 48 vs. 3, 38, 57,20, 59 and 42 arbitrary units, respectively).

Next we examined whether any of the antibody-positive samples were able to recognise IF saturated with vitamin B_{12} by running the assay in parallel with the catching IF not saturated or saturated with vitamin B_{12}. Although most samples gave a slightly lower response when IF saturated with vitamin B_{12} was employed as the catching reagent, all remained positive (Figure 4B).

Finally, we replaced biotinylated IF with biotinylated IgG or IgA (Figure 4C). A positive correlation ($p=0.03$) was observed between results obtained when biotinylated IF and IgG were used, while that was not the case when anti-IgA was used.

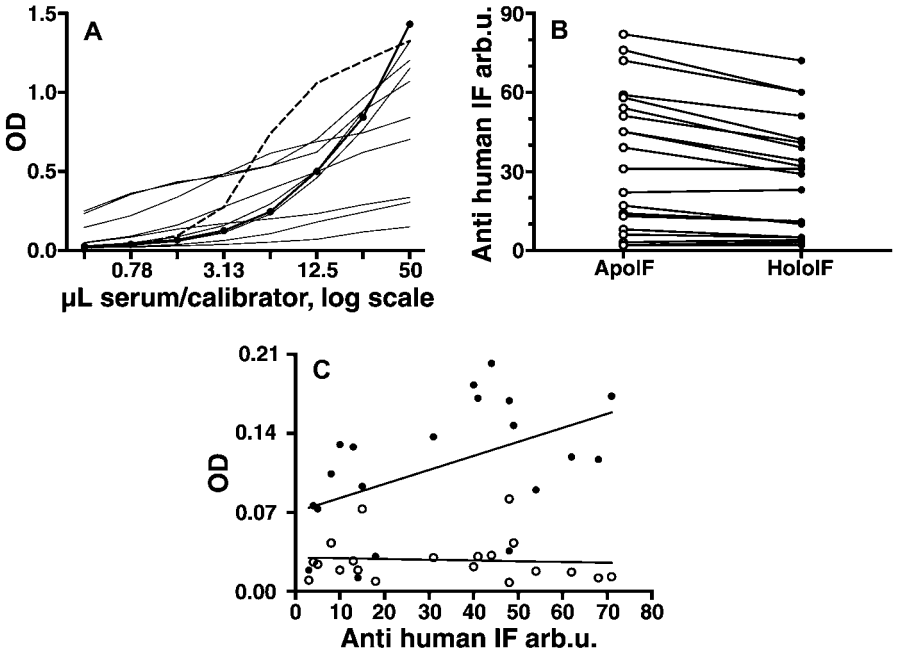

Figure 4 Characterisation of autoantibodies against IF. (A) Dilution curves for nine samples (thin lines) and the calibrator (closed symbols). The result for the donor sample showing a positive result is indicated as a dashed line. (B) Results obtained employing IF unsaturated with vitamin B_{12} (ApoIF) as opposed to employing IF saturated with vitamin B_{12} (HoloIF) as the catching reagent. (C) Results obtained using anti-IgG (closed symbols) or -IgA (open symbols) (Y-axis) or -IF (X-axis) as the detection reagent. The regression lines are indicated ($r^2=0.25$, $p=0.03$ for IgG, ns for IgA).

Discussion

In the late 1950s it was realised that patients treated with hog IF may show an immunological reaction, leading to the presence of antibodies against hog IF in the circulation (10). This led to the development of assays able to detect antibodies directed against hog and human IF (11). With such assays available, it soon became evident that the presence of autoantibodies against IF was a common feature in patients with pernicious anaemia (12).

While it is generally agreed that autoantibodies against IF are rare or absent in patients not suffering from pernicious anaemia (the specificity is close to 1) (13, 14), less consensus exists concerning the sensitivity for detecting autoantibodies against IF, with figures ranging between 30 and 70% (1, 4, 15). These discrepancies may partly relate to differences in sensitivity caused by race or age (1), but a major factor may well relate to differences in the assays used for detection of the autoantibodies, as revealed in a systematic evaluation of such tests (16).

In the present paper we describe a simple method for detecting autoantibodies that uses commercially available reagents. Our data clearly demonstrate that it was not possible to measure the amount of antibody present in samples, since we observed non-parallel dilution curves for the individual samples and the calibrator used. This is in agreement with previous studies (17). However, using a standard assay design it was possible to obtain reproducible results with a coefficient of variation of approximately 10%.

The classification of antibodies as blocking or non-blocking types has been extensively discussed in the past. In our assay design there seems to be no major difference between the results obtained employing not saturated or saturated IF as the catching reagent. Similar results have been obtained in other assay designs using labelled IF rather than those based on the binding of vitamin B_{12} to IF (7, 18). We believe that these results reflect the fact that the antibody will bind to IF independent of the presence of vitamin B_{12} on IF. However, if the antibody is added to IF prior to vitamin B_{12}, this will interfere with binding of the vitamin.

Our assay design allows simultaneous determination of autoantibodies independent of the immunoglobulin type. At the same time, the assay design makes it easy to carry out supplementary studies to classify the type of antibody in question. In agreement with previous work, we found that most of the antibodies identified belonged to the IgG class (19), with a few also containing antibodies of the IgA class.

Most studies performed so far have mainly included patients with proven pernicious anaemia and small cohorts of normal individuals. In the main part of our study, we included a reference population, as well as patient populations with low levels of serum vitamin B_{12} and with serum levels well within the normal range. There was a strong relation between the fraction of patients positive for autoantibodies and the level of serum vitamin B_{12}, and very few positive samples were observed amongst those with a normal level of serum vitamin B_{12}.

In conclusion, we have presented a semiquantitative assay for the measurement of autoantibodies against IF based on commercially available reagents. The assay analyses the autoantibodies independent of the immunoglobulin type and independent of blocking nature. Furthermore, the assay design is well suited for the analysis of large series of samples.

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