

New enzyme immunoassay for detecting total, type I, and type II intrinsic factor antibodies

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SUMMARY A method for the detection of total, type I, and type II intrinsic factor antibodies was devised. The technique comprises a two-site solid phase enzyme linked immunosorbent assay (ELISA), with human intrinsic factor conjugated with horseradish peroxidase as label and attached to polystyrene tubes as solid phase. One conjugation provides sufficient material to assay more than 10 000 patient samples. The label proved stable during the course of this evaluation and was still in use more than 12 months after preparation. When applied to 45 serum samples from cases of pernicious anaemia, intrinsic factor antibodies were shown in 30 (67%). Simplicity, high capacity, low cost and label stability, combined with relatively high clinical sensitivity make the method suitable for cost effective screening of large numbers of samples. Simple modifications to the basic assay reagents permitted type I and type II intrinsic factor antibodies to be differentiated.

Despite being available for almost 30 years,¹ application of tests for circulating antibodies to gastric intrinsic factor has been limited, because the tests have not been readily available to all routine laboratories, and secondly, they have been capable of showing the presence of antibodies in only about 50% of patients with pernicious anaemia. It has been proposed, however, that the demonstration of intrinsic factor antibodies in patients with megaloblastic anaemia and low serum vitamin B12 concentrations could be regarded as diagnostic of pernicious anaemia and may avoid the need for B12 absorption tests.²

Two types of intrinsic factor antibody are known to exist,³ one of which reacts with the intrinsic factor-B12 binding site (type I); the other (type II) detects an antigen site unrelated to the B12 binding activity. Methods of detection have concentrated on type I, making use of the ability of the antibody to prevent the uptake of ⁵⁷Co-B12 by intrinsic factor, combined with a charcoal separation procedure.⁴⁻⁶ Recently, a method that used ¹²⁵I-labelled intrinsic factor has been described: this focuses on the ability of the antibody to bind to intrinsic factor directly⁷ instead of showing a type I antibody blocking effect.⁴⁻⁶ This method has also been modified to detect and quantify both type I and type II antibody.^{8,9} A commercial intrinsic factor antibody method [Walker Laboratories Ltd, Ely, Cambridgeshire, England] based on the ELISA technique using polystyrene pins

coated with purified hog intrinsic factor as solid phase and an anti-IgG-peroxidase label has recently been introduced.

We report a new two-site solid phase ELISA capable of detecting total, type I, and type II intrinsic factor antibodies in patients' serum. The method uses polystyrene tubes coated with human intrinsic factor as solid phase (S-IF) and purified human intrinsic factor conjugated to horseradish peroxidase as label. In a simple two-stage addition procedure a complex of S-IF:antibody:intrinsic factor/horseradish peroxidase is formed. When intrinsic factor antibodies are present in the patients' serum, a colour reaction is produced when a suitable substrate is added. No such complex is formed in the absence of antibody, as indicated by the absence of a coloured end point. When native serum is used in this system, total intrinsic factor antibody (type I and type II) may be detected. Addition of a B12-intrinsic factor complex to the serum absorbs out type II antibody and only type I antibody reacts in the assay; prior addition of B12 to the intrinsic factor/horseradish peroxidase conjugate occupies its B12 binding sites and only type II antibody will be detected.

Material and methods

GASTRIC JUICE

A pool of human gastric juice stimulated by pentagastrin was used for the preparation of assay reagents. All individual samples had a pH of less than 1.5 before

depepsinisation¹⁰ and storage at -20°C . This gastric juice was used as the source of intrinsic factor in the preparation of the solid phase intrinsic factor and enzyme labelled intrinsic factor test reagents.

SOLID PHASE INTRINSIC FACTOR

Polystyrene tubes were coated with intrinsic factor using the glutaraldehyde pre-treatment method of Boenisch.¹¹ Five hundred microlitres of a 0.1% solution of glutaraldehyde (Sigma Chemical Company Ltd, Poole, Dorset, England) in 0.1 M carbonate/hydrochloric acid buffer (pH 9.0) were dispensed into 75 mm \times 11 mm polystyrene tubes (Sarstedt Ltd, Beaumont Leys, Leicester, England). These were then incubated at 56°C for three hours, washed twice with distilled water, and dried. Two hundred microlitres of ammonium sulphate precipitated human gastric juice¹² containing 100 ng intrinsic factor were dispensed into all tubes, which were then stored at 4°C until required.

ENZYME LABELLED INTRINSIC FACTOR

Human gastric juice was concentrated by ultrafiltration (Amicon Ltd, Woking, Surrey, England) through a 10 000 molecular weight cut off membrane. Following overnight dialysis against three \times one litre changes of 0.05 M phosphate buffered saline (PBS), pH 7.4, the preparation was centrifuged at 30 000 \times g for 30 minutes and any sediment discarded. The supernatant was further purified by gel filtration on a 50 cm \times 2.5 cm column of Sephacryl S200 (Pharmacia Ltd, Central Milton Keynes, Buckinghamshire, England) equilibrated in 0.05 M PBS (pH 7.4). Two main protein peaks (A280) were obtained within 80 fractions, each of 2 ml volume. The intrinsic factor, as measured by the method of Chanarin,⁴ was located within the second of these peaks. The fractions containing intrinsic factor were then pooled, further concentrated by ultrafiltration as before (Amicon Ltd, Woking, Surrey, England), and divided into 5 ml amounts containing 5 mg of protein before storage at -20°C .

A 5 mg portion of the purified gastric juice was conjugated to horseradish peroxidase (Sigma Chemical Company Ltd) by the periodate-aldehyde method of Nakane and Kawoia.¹⁴ The resulting preparation was applied to a 50 cm \times 2.5 cm column of Sephacryl S200 (Pharmacia Ltd) equilibrated in 0.05 M PBS (pH 7.4). Fractions of 2 ml volume were collected and the individual absorbances measured at 280 nm (total protein) and 403 nm (horseradish peroxidase). The fractions containing intrinsic factor associated with horseradish peroxidase were pooled. Bovine serum albumin (BSA) was added at a concentration of 10 g/l before being stored in aliquots of 100 μl at -20°C .

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Assay diluents Buffer A: 0.02 M PBS (pH 7.4), containing 40 g/l BSA (for serum dilution). Buffer B: 0.02 M PBS (pH 7.4), containing 10 g/l BSA (intrinsic factor/horseradish peroxidase conjugate dilution). Buffer C: 0.2 M citrate phosphate (pH 5.5) (enzyme substrate preparation).

Enzyme substrate This was prepared immediately before use and consisted of 20 ml buffer C, 75 mg O-phenylenediamine (dihydrochloride) (Sigma Chemical Company Ltd); 20 μl of 20 volume hydrogen peroxide.

Serum samples were obtained from the following subjects:

- 1 One hundred and fifteen samples on which intrinsic factor antibody testing was requested on clinical or laboratory grounds. Pernicious anaemia was subsequently diagnosed in 45 of these patients.
- 2 Eighty requiring vitamin B12 measurement all of whom had concentrations greater than 170 ng/l (laboratory reference range = 120-900 ng/l). Assays were performed by the method of Matthews, Gunasegaram, and Linnell.¹⁵
- 3 Ten from blood donors.
- 4 Two from cases 1 and 2 with pernicious anaemia, previously known to have intrinsic factor antibodies, which were used for the comparison of antibody titres.

TEST PROTOCOL FOR TOTAL ANTIBODY DETECTION

The requisite number of polystyrene tubes coated with intrinsic factor were loaded into numbered centrifuge multitube carriers (MSE Scientific Instruments, Crawley, Sussex, England). From this point all manipulations and timings were carried out within these carriers in strict numerical order. The tubes were washed three times with a 0.05% solution of Tween 20 in distilled water. After the final wash they were briefly centrifuged and aspirated to remove the last drops of wash solution.

One hundred microlitres of the appropriate control or test sample were added. The tubes were centrifuged and incubated at 37°C for 60 minutes. At the end of this time the tubes were again washed three times as before.

Two hundred microlitres of intrinsic factor/horseradish peroxidase conjugate, freshly diluted in buffer B, were then added. The tubes were centrifuged and incubated at 37°C for a further 60 minutes and washed three times.

Five hundred microlitres of freshly prepared substrate were added and the tubes centrifuged, after which the reaction was allowed to proceed in the dark at room temperature for 60 minutes. Reagent blanks containing 500 μl of substrate alone were treated in the same way. The reaction was stopped by the addition of

2 ml 2M hydrochloric acid. This was also added to the reagent blanks. Absorbance at 492 nm (A492) was measured in a spectrophotometer [Beckman-RIIC Ltd, High Wycombe, Buckinghamshire, England] zeroed on reagent blank.

TEST PROTOCOL FOR TYPE I ANTIBODY

DETECTION

A complex of B12 and intrinsic factor was prepared by adding 1 ml of ammonium sulphate precipitated gastric juice¹² containing 200 ng units intrinsic factor¹³ to 1 ml of a 700 ng/ml solution of cyanocobalamin. The mixture was left at room temperature for 30 minutes and unbound B12 was removed by means of albumin-coated charcoal.

Type II intrinsic factor antibodies were then absorbed by adding 100 µl of the prepared B12/intrinsic factor complex to 100 µl of control and patients' sample. The mixture was left at room temperature for 30 minutes before testing. The resulting preparation was then subjected to the test protocol for total antibody detection.

TEST PROTOCOL FOR TYPE II ANTIBODY

DETECTION

The test protocol followed that for the detection of total antibody; but B12-blocked intrinsic factor/horseradish peroxidase conjugate was used in this case to ensure that only type II antibodies could react with the label. Vitamin B12-blocked intrinsic factor/horseradish peroxidase conjugate was prepared by adding excess cyanocobalamin (10 ng) to conjugate (1 ng unit intrinsic factor). The mixture was left at room temperature for 30 minutes before being diluted for use.

The radioassay method used for comparative purposes was that of Ardeman and Chanarin 1963.⁴ All volumes used were proportionately decreased.

Non-specific binding was determined using buffer A instead of serum in the total antibody test. The procedure was performed 59 times in 12 assays.

Negative serum response was assessed using a serum pool from the 10 blood donors. The pool was tested for total intrinsic factor antibodies 59 times in 12 assays.

TOTAL, TYPE I, AND TYPE II ANTIBODY DOSE RESPONSES

These were assessed by diluting sera from two patients (cases 1 and 2) known to contain antibodies to intrinsic factor. The dilutions were carried out in buffer A and were tested on four occasions at weekly intervals by the methods outlined above. Dilutions of the same two sera were also tested by the radioassay method.⁴

Four different batches of polystyrene tubes (Sarstedt Ltd), were used to prepare solid phase intrinsic factor. Total antibody dose responses for two intrinsic factor antibody positive sera were then determined

Table 1 Measured and calculated data for enzyme labelled intrinsic factor

Measured data		(mg)
1	Total protein ¹⁶	3.314
2	Total horseradish peroxidase protein	2.102
1 minus 2	Intrinsic factor protein	1.212
Calculated data		Intrinsic factor/horseradish peroxidase
Mass ratio (intrinsic factor/horseradish peroxidase)		0.58:1
Molecular weight ratio (intrinsic factor/horseradish peroxidase)		1.50:1
Molecular equivalent (mass ratio/molecular weight ratio)		0.39:1

Assuming that: intrinsic factor molecular weight = 60 000¹²
 horseradish peroxidase molecular weight = 40 000
 (Sigma Chemical Company Ltd)

using each tube batch on four occasions at weekly intervals.

Results

Measured and calculated data for an enzyme labelled intrinsic factor preparation are shown in table 1. An average of 2.56 horseradish peroxidase molecules were calculated to be attached to one intrinsic factor molecule.

Table 2 shows the mean absorbances and standard deviations obtained using buffer A (non-specific binding) and the negative serum pool. An unpaired Student's *t* test was performed to investigate differences between the two groups. As shown in the table, no significant difference was shown. Consequently, the results for the negative serum pool were used to establish 95% confidence limits of 0.043 ± 0.03, to indicate the range of absorbances expected for negative intrinsic factor antibody sera. Non-specific binding and negative serum pool responses, expressed as percentages of those obtained for sera from the two cases of intrinsic factor antibody positive pernicious anaemia in the same assay are shown in table 3. The background to signal relation ranged from 5.4% to 13.2%, and the lack of trend in these figures indicates that the assay reagents were stable during the evaluation period.

Figs 1 and 2 show total, type I, and type II antibody

Table 2 Comparison of non-specific binding and negative serum responses

	Non-specific binding	Negative serum pool
n	59	59
Mean (A492)	0.039	0.043
SD (A492)	0.017	0.015
<i>t</i>	1.252 NS	

Table 3 *Non-specific binding and negative serum responses expressed as percentages of those obtained for two intrinsic factor antibody positive sera (cases 1 and 2)*

Week	Non-specific binding		Negative serum control	
	Case 1	Case 2	Case 1	Case 2
1	9.3	8.3	7.8	6.9
2	7.1	5.4	13.2	10.0
3	8.8	10.4	10.4	12.2
4	7.3	7.7	8.1	8.6

dose responses. The points shown represent the mean absorbance values obtained from four tests carried out at weekly intervals. Type I antibody titres obtained for the same sera using the radioassay method⁴ and ELISA are included for comparative purposes (table 4).

Fig 3 shows the total antibody dose responses for one of the intrinsic factor antibody positive sera, measured with solid phase intrinsic factor prepared using four different batches of polystyrene tubes (Sarstedt Ltd). The points shown represent the mean absorbance values obtained from four tests carried out at weekly intervals. Essentially similar results were obtained for the second of the two sera tested.

Results obtained by the radioassay and ELISA methods for samples from patient groups 1 and 2 are shown in table 5. All control sera gave negative results.

Discussion

The method presented was sensitive, of high capacity, and non-isotopic, capable of detecting total, type I, and type II antibodies to human intrinsic factor. Intrinsic factor/enzyme conjugate was prepared by the method of Nakane and Kawaoi.¹⁴ These authors reported that a maximum of five to six horseradish peroxidase molecules may be attached to one IgG molecule and that for immunoassay purposes, conju-

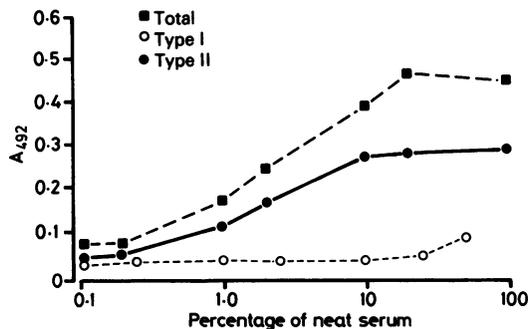


Fig 1 *Total, type I, and type II intrinsic factor antibody dose responses for case 1.*

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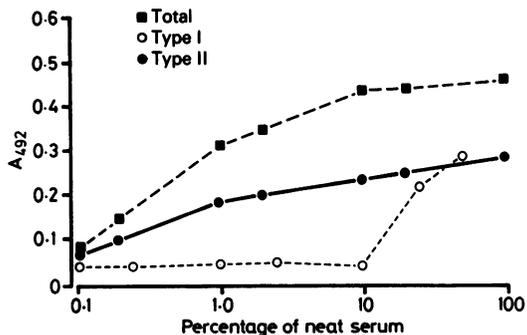


Fig 2 *Total, type I, and type II intrinsic factor antibody dose responses for case 2.*

gates with a horseradish peroxidase:protein molecular ratio of greater than 1 produced enhanced sensitivity.^{14,17} The optimal ratio quoted was 2:3 horseradish peroxidase:1 protein molecule. The horseradish peroxidase:intrinsic factor molar ratio of 2.56:1 produced in our first conjugation was in good agreement with this, and in a second conjugation a ratio of 4.0:1 was obtained. The assay reagents were stable over four weeks and we have continued to use the original conjugate label without signs of serious deterioration for more than 12 months.

The sensitivity of the method was confirmed by the low level of non-specific binding and by the fact that no significant difference could be shown between this and the response produced by an intrinsic factor antibody negative serum pool. Dose responses were obtained for total, type I, and type II intrinsic factor antibodies (figs 1 and 2). Total antibody was detectable to a serum dilution of 1/100 in case 1 and 1/1000 in case 2. Although the curve produced by type II antibody reflected the sigmoid shape produced by total antibody in both cases, differences were observed in the responses obtained for type I antibody. For case 1, a positive type I response could be shown to a dilution of 1/2; a positive result was obtained for the 1/4 dilution in case 2. These results are both in agreement with those obtained using the radioassay

Table 4 *Antibody titres for cases 1 and 2 by radioassay and ELISA methods*

Serum dilution	Case 1		Case 2	
	Radioassay	ELISA	Radioassay	ELISA
Neat	+	NT	+	NT
1/2	+	+	+	+
1/4	-	-	+	+
1/8	-	-	-	-
1/16	-	-	-	-

+ = Positive antibody response
 - = Negative antibody response
 NT = Not tested at this dilution

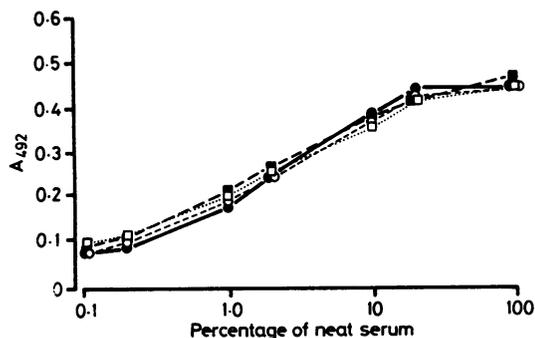


Fig 3 Total intrinsic factor antibody dose responses obtained using four different batches of polystyrene tubes as solid phase support.

method (table 4). It seems that the method is less sensitive for type I antibody than for type II. If technical effects are responsible for the apparent discrepancy these are most likely to be associated with antibody uptake by the solid phase intrinsic factor. Type I antibody may have a restricted access to the B12 binding site of the solid phase intrinsic factor, thus giving the appearance of an enhanced type II and a decreased type I antibody reaction. These features may be important to understanding the nature of intrinsic factor antibodies and merit further investigation. At present, however, the method is used only to show the presence of antibodies to intrinsic factor in serum and as such has potential advantages over present methodology.

When applied to the group of 45 patients with pernicious anaemia, 62% were found to be positive for type I antibody by the radioassay method of Ardeman and Chanarin 1963.⁴ This was in good agreement with previously reported clinical sensitivity figures for type I intrinsic factor antibody methods.^{7 13 18} Our ELISA showed increased sensitivity—the proportion of positive reactions rising to 67%. Both methods were specific for pernicious anaemia as no known false positive results were detected in the control group of 80 patients. Two patients in group 1, however, gave discrepant results, one being positive by the radioassay and negative by the ELISA method; the converse being true in the second. Unfortunately, further information was unavailable on these patients and we

were unable to reach a conclusive diagnosis. By increasing the serum:intrinsic factor antigen ratio by up to 1000-fold, Nimo and Carmel recently improved the sensitivity of their radioassay method for type I antibody.¹⁹ We intend to investigate the effect of different solid phase intrinsic factor concentrations on the sensitivity of our ELISA system.

The intrinsic factor antibody ELISA described here uses a non-isotopic label which has proved stable for a minimum period of 12 months. One enzyme/intrinsic factor conjugation procedure is capable of producing sufficient label to test in excess of 10 000 patient samples. The assay method is quick, results being produced within the working day, and the procedural steps are straightforward. The method is cost effective when compared with other, particularly commercial intrinsic factor, assay methods which cost approximately £3.00 a patient. These factors, combined with increased clinical sensitivity, constitute an ideal method for a screening test in the investigation of B12 deficiency. The technique has the added potential for the quantitative measurement of type I and type II intrinsic factor antibodies and may contribute to the further understanding of their importance in relation to pernicious anaemia.

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Table 5 Incidence of positive results in patient groups

	n	Positive results	
		Radioassay	ELISA
Group 1	115	29	31
Pernicious anaemia	45	28	30
Group 2	80	0	0

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