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Clinical Significance of Serum Vitamin B_{12} Measured by Radioassay Using Pure Intrinsic Factor

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Serum vitamin B_{12} (B_{12}) levels of 53 patients (15 with pernicious anemia) and 42 healthy volunteers were determined using crude intrinsic factor (IF), pure IF, and a mixture of crude IF + R-protein blocking agent (block IF). The radioassay using pure IF showed less sample-to-sample variation in nonspecific binding than the radioassay using block IF. The mean B_{12} levels in 42 healthy subjects were significantly higher with crude IF (499 ± 23 pg/ml, 1 s.e.m.) than with pure IF (408 ± 29 pg/ml) or with block IF (407 ± 22 pg/ml). B_{12} levels were abnormally low in all 15 patients with pernicious anemia by pure IF (<100 pg/ml), in 14 patients by block IF (<150 pg/ml), and in only seven patients by crude IF (<200 pg/ml). Our data confirm previous reports that B_{12} deficiency can be diagnosed more reliably by measuring serum B_{12} levels with either pure IF or block IF.

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Studies by Kolhouse et al. (*1*) show that commercially available concentrates of intrinsic factor (IF) used in radioassays of vitamin B_{12} (B_{12}) contain 51–85% of R-proteins, which can also bind biologically inactive B_{12} analogs, and that the use of such IF concentrates would result in spuriously high serum B_{12} concentrations. Cooper and Whitehead (*2*) also reported that 10–15% of patients with pernicious anemia (PA) had normal levels of serum B_{12} as measured by a radioassay using crude IF, whereas their serum B_{12} levels were abnormally low when measured microbiologically using the *Euglena gracilis* assay.

Kolhouse et al. (*1*) suggested that the accuracy of B_{12} radioassays could be increased either by using pure IF as a binder or by presaturating the binding sites of R-protein contained in crude IF with B_{12} analogs, such as cobinamide. Since late 1978, many commercial producers of B_{12} radioassay kits have modified their binding agents to eliminate the nonspecific effects of R-proteins. The present study investigated the clinical significance of serum B_{12} levels measured by commercial radioassay kits using either pure IF or crude IF + R-protein blocking agent.

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MATERIALS AND METHODS

Crude-IF assay. The modified method of Raven (*3*) was used. Briefly, this involves boiling of a mixture containing 0.1 ml of serum sample or standard, 0.1 ml of 7% human serum albumin, 0.05 ml of 0.013% NaCN, 0.2 ml of 0.25 *N* HCl, and 0.6 ml of saline. This is followed by the addition of 0.2 ml of Co-57-B_{12} and 0.2 ml of crude hog IF solution,* and incubation at room temperature for 30 min. A charcoal suspension (0.8 ml) is added to each tube and the supernate obtained after centrifugation is counted for radioactivity. B_{12} standards were prepared in 7% human serum albumin. The minimum serum B_{12} concentration detectable by this assay is 50 pg/ml. The reference range determined among 60 healthy subjects was 200–900 pg/ml, with the range between 200 and 290 pg/ml considered borderline. The normal range is the tolerance limits for the coverage of at least 95% of the population with 90% confidence (*4*). The borderline range was determined by a hematologist

(EBS) based on the B_{12} values obtained from more than 100 patients with proven and suspected B_{12} deficiency who had (a) megaloblastoid or megaloblastic marrow pictures but with Schilling-test urinary excretion between 7 and 8% over 48 hr, and/or (b) whose urinary methylmalonic acid levels were low normal or at the borderline between normal and abnormal (*5*).

Assay with crude IF + R-protein blocking agent (block IF). This commercial kit† was designed to assay serum B_{12} and folate concentrations simultaneously. The sample is heated in a boiling-water bath for 15 min in borate buffer (pH 9.3) containing Co-57-B_{12}, cobinamide as a blocking agent (~190 ng/ml), dithiothreitol as an antioxidant, and KCN. The heat-treated sample is then incubated with crude IF, and dextran-coated charcoal is used for the separation of the bound from free ligand. The procedure suggested by the kit manufacturer was followed exactly except that volumes of all reagents were reduced to half the suggested volumes. The minimum detectable serum B_{12} concentration by this assay is 100 pg/ml.

Pure-IF assay. This commercial kit‡ was also designed for the simultaneous measurements of serum B_{12} and folate. The procedure involved is very similar to that of the block-IF assay except that pure intrinsic factor is used in place of crude IF, and no blocking agent is required. The procedure suggested by the kit manufacturer was followed exactly. The minimum detectable serum B_{12} concentration by this assay is 50 pg/ml.

Study subjects. Forty-two healthy volunteers from our laboratory and 53 patients sent to our laboratory for the B_{12} assay and/or Schilling tests were investigated. All serum samples were fasting samples. Criteria used for the diagnosis of B_{12} deficiency included megaloblastic bone marrow, abnormal Schilling tests, and hematologic response to B_{12} therapy.

RESULTS

Radioassay method comparison. Composite standard curves of three B_{12} radioassays are shown in Fig. 1. Coefficients of variation (CV) of each standard point ranged from 6.0–18.5% for the crude-IF assay, 3.8–6.8% for the block-IF assay, and 2.4–6.1% for the pure-IF assay. Precision of the assays expressed as within-assay and between-assay CVs ranged from 3.5–5.9, 4.4–14.5, and 4.1–5.6%, respectively, with B_{12} concentrations ranging from 132–1315 pg/ml.

During the course of this study, we found that nonspecific binding (NSB), expressed as percentage of radioactivity present in the bound fraction in the absence of added IF (*7*), of the matrix used for preparing B_{12} standards (assay NSB) was considerably lower than NSB of patients' sera (sample NSB). Therefore, effects of sample NSB on the B_{12} values obtained by the pure-IF and block-IF methods were investigated. As shown in Table 1, the mean assay NSB of the pure-IF assay was about the same as its sample NSB, whereas the mean assay NSB was about one fifth of the sample NSB in the block-IF assay. The difference between the assay NSB and the sample NSB was also reflected in the B_{12} values obtained with and without sample NSB correction. The difference between the mean B_{12} value with and without sample NSB correction was 5.9% for the pure-IF assay, whereas the difference was 12.1% for the block-IF assay. Therefore, in the pure-IF assay, the assay NSB was used

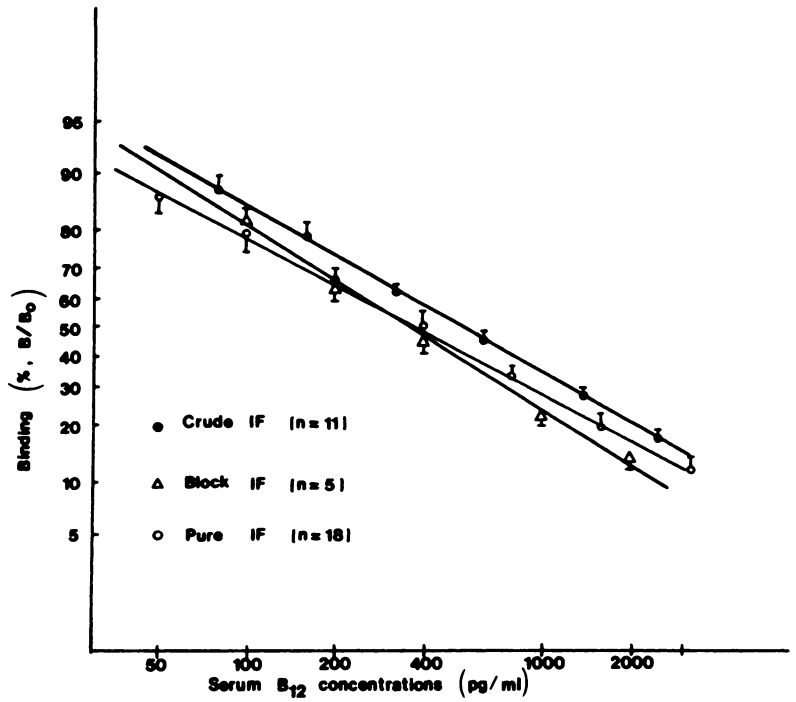

FIG. 1. Composite standard curves (logit-log plot). Vertical bars indicate one standard deviation; n denotes number of runs for each composite standard curve.

TABLE 1. EFFECTS OF NONSPECIFIC BINDING (NSB) OF SAMPLES ON SERUM B_{12} VALUES

Methods	Assay NSB (%) mean ± 1 s.d.	Sample NSB (%) mean ± 1 s.d.	B_{12} values, mean ± 1 s.d. (pg/ml) Without sample NSB correction	With sample NSB correction	CV* of difference
Pure IF	2.26 ± 0.08 (n = 18)	1.96 ± 0.08 (n = 20)	190 ± 23 (n = 10)	199 ± 26 (n = 10)	5.9%
Block IF	0.88 ± 0.03 (n = 8)	4.37 ± 0.21 (n = 20)	206 ± 19 (n = 10)	242 ± 22 (n = 10)	12.1%

* CV = coefficient of variation; assay NSB = NSB of the matrix used for preparing B_{12} standards.

only for the calculation of percent bindings of both standard and sample tubes, whereas in the block-IF assay, the assay NSB was used for percent bindings of standard tubes, and the NSB of pooled serum was used for sample tubes. In both assays, NSBs of each individual patient were statistically not different.

Figure 2 shows least-squares regression lines obtained from the regression analysis of serum B_{12} values obtained from serum samples, with B_{12} levels ranging from 92–2319 pg/ml. The correlations between the crude-IF and block-IF assay, and between the crude-IF and pure-IF assay, were poor, but there was a general trend that the B_{12} values obtained by the crude-IF assay were considerably higher than those obtained by either the block-IF or pure-IF method throughout a wide range of serum B_{12} concentrations. In contrast, the B_{12} values obtained by the pure-IF assay tended to be lower than those obtained by the block-IF when the values were below 400 pg/ml, and the opposite trend was observed when the values were greater than 400 pg/ml.

Serum B_{12} concentrations. B_{12} concentrations in fasting serum samples from 42 healthy subjects and 53 patients were measured by the three B_{12} assay methods. Fifteen of 53 patients were subsequently found to have pernicious anemia (PA); in the remaining patients, PA could be ruled out. Data obtained are plotted in Fig. 3. The shaded areas indicate a borderline region that was determined from serum B_{12} levels obtained from both healthy subjects and patients, and also from clinical findings in patients. In the borderline region, the serum B_{12} levels alone may be suggestive but not conclusive in diagnosing B_{12} deficiency. The borderline regions were 200–290, 145–225, and 100–180 pg/ml for the crude-, block-, and pure-IF assays, respectively.

The mean value of 499 ± 23 pg/ml (±1 s.e.m.) of healthy subjects (Fig. 3, dotted line) obtained by the crude-IF assay was significantly higher than the value of 407 ± 22 pg/ml obtained by the block-IF assay (paired t-test, $p < 0.001$, $n = 42$) and also than the value of 407 ± 29 pg/ml obtained by the pure-IF assay ($p < 0.001$). The last two values were not different statistically. The serum B_{12} levels of healthy subjects ranged from 260–812 pg/ml for the crude-IF assay, 138–830 pg/ml for the block-IF assay, and 92–960 pg/ml for the pure-IF assay.

Mean serum B_{12} levels in hospital patients suspected of pernicious anemia are shown in Table 2. Again, the mean serum B_{12} levels of 499 pg/ml, obtained from patients without PA by the crude-IF method, were statistically higher than the values obtained by the other two methods (paired t-test, $p < 0.001$). The mean value of 238 pg/ml obtained from 14 PA patients with detectable serum B_{12} levels by the crude-IF assay was also significantly higher than the mean value of 139 pg/ml obtained from four PA patients by the block-IF assay. The serum B_{12} levels of all 15 PA patients were undetectable (<50 pg/ml) by the pure-IF assay.

Table 3 shows the numbers of subjects with serum B_{12}

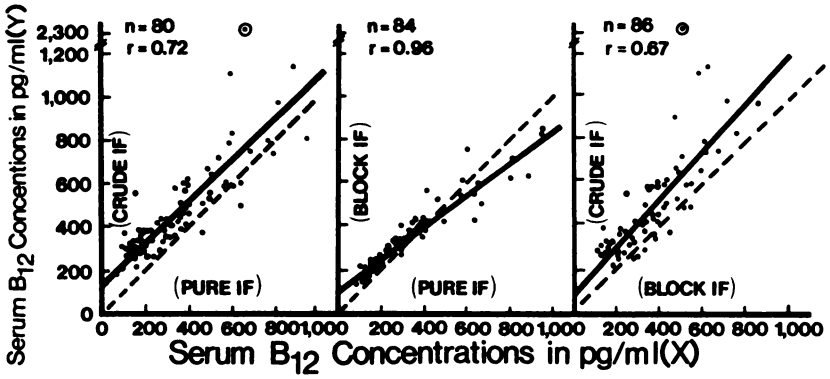

FIG. 2. Correlations between B_{12} values obtained by three methods. Least-squares regression lines (solid) are obtained from linear regression analysis of B_{12} values obtained by three methods. r = correlation coefficient; n = number of samples; dashed line is for Y = X. ⊙ indicates a value above break in ordinate.

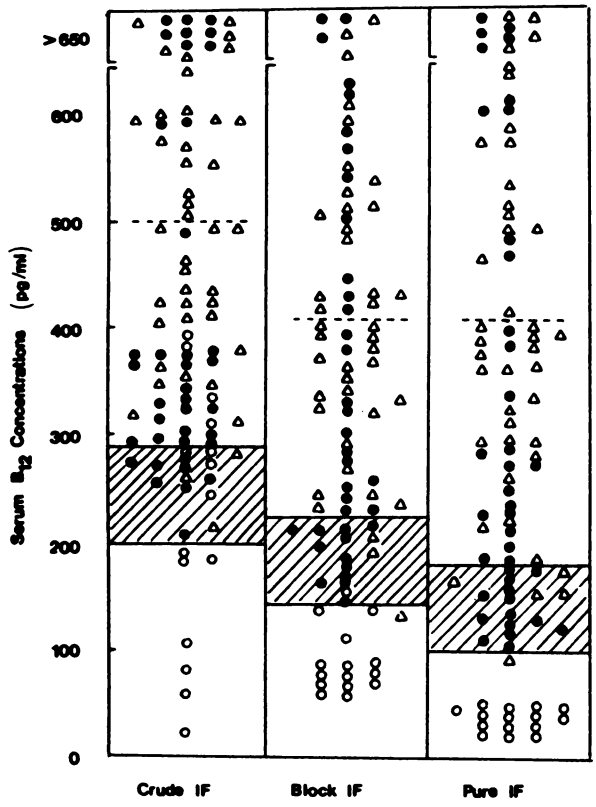

FIG. 3. Serum B_{12} concentrations for 42 healthy subjects (△), 38 patients without pernicious anemia (●), and 15 patients with pernicious anemia (○). --- shows mean B_{12} values for healthy subjects. Shaded areas indicate borderline regions.

in the deficient, borderline, and normal or high regions. The pure-IF assay was able to diagnose correctly all 15 patients with PA (100% sensitivity). If the borderline cases were considered to be false diagnoses, the diagnostic sensitivity of the block-IF assay was 93% and that with the crude-IF was only 46%. PA was correctly ruled out in 61 of 80 subjects by the pure-IF assay (76% specificity) and 62 of 80 by the block-IF assay (78% specificity). The crude-IF assay showed a better diagnostic specificity of 86%; none of the subjects was in the deficient region.

DISCUSSION

After it was reported that the crude-IF concentrates used in all 1978 commercial B_{12} radioassay kits contained R-protein, which would bind not only B_{12} but also biologically inactive B_{12} analogs, and that patients with B_{12} deficiencies determined by these radioassay kits might falsely show B_{12} levels in the normal range (*1*), the Food and Drug Administration asked the National Committee for Clinical Standards (NCCLS) to carry out a thorough investigation of the problem. Recently, NCCLS has established guidelines for binders used in B_{12} radioassay kits. The guidelines include the following methods of evaluating binders.

1. They should not measure cobinamide (B_{12} analog) up to concentrations of 10 ng/ml in serum.
2. B_{12} binding should be more than 95% inhibited by specific anti-IF blocking antibody, and/or
3. the IF-B_{12} complex should be more than 95% precipitated by specific IF-precipitating antibody (*6*).

Manufacturers of the radioassay kits used in the present study claimed that their binders had been evaluated according to the NCCLS guidelines.

The assay pH of the crude-IF method was about 2, compared with about 9 for the other two methods. According to Kolhouse et al., IF could lose more than 98% of its B_{12}-binding capacity at pH 2.0, whereas R-proteins could retain their full B_{12}-binding capacity (*1*). It is possible, therefore, that the binders used in our crude-IF method were essentially the R-proteins.

The slopes of standard curves obtained by the three methods are similar, with the standard curve for block-IF being slightly steeper (Fig. 1). In all three methods, crystalline B_{12} was used to construct the standard curve. Kolhouse et al. showed that the standard curve obtained with IF and crystalline B_{12}, and the standard curve obtained with R-proteins and crystalline B_{12} in their radioassay (pH 9.0), were superimposable. The slight shift in the standard curves observed in our study may reflect the difference in the assay systems used in each method.

The marked difference between the assay NSB and the sample NSB observed in the block-IF method (Table 1) was probably due to the matrix used in preparing the standards. According to the manufacturer of this kit, the matrix is synthetic. In spite of several requests to the

TABLE 2. MEAN SERUM B_{12} LEVELS IN PATIENTS SUSPECTED OF PERNICIOUS ANEMIA (PA)

Patients	n	Mean serum B_{12} levels ± 1 s.e.m. (pg/ml)		
		Crude IF	Block IF	Pure IF
Without PA	38	499 ± 64	348 ± 30	382 ± 62
		(251–2319)*	(149–854)	(105–960)
With PA†	15	238 ± 39	139 ± 14	<50
		(<50–385)	(<100–159)	(all <50)

* Values in parenthesis denote ranges.

† Mean ± 1 s.e.m. of detectable values (one undetectable by crude IF, 11 by block IF, and all 15 by pure IF).

TABLE 3. NUMBERS OF PATIENTS IN EACH REGION OF SERUM B_{12} LEVELS

Method	Patients / Region	With pernicious anemia			Without pernicious anemia		
		Deficient	Borderline	Normal or high	Deficient	Borderline	Normal or high
Crude IF		7	4	4	0	11	69
		(46%)	(27%)	(27%)		(14%)	(86%)
Block IF		14	1	0	1	17	62
		(93%)	(7%)		(1%)	(21%)	(78%)
Pure IF		15	0	0	1	18	61
		(100%)			(1%)	(23%)	(76%)

manufacturer, the composition of this synthetic matrix was not made available to us. The difference between the assay NSB and the sample NSB makes it necessary to determine the NSB for the samples (*7*). Since there were no significant differences among NSBs of numerous individual samples tested, the NSB of a serum pool was determined in each run and was used to make the NSB correction for all samples. Without the sample-NSB correction, the B_{12} values obtained were significantly lower than those obtained with sample-NSB correction, and this discrepancy was greater in samples with higher B_{12} concentrations. For example, one sample gave a B_{12} value of 770 pg/ml without the sample-NSB correction, whereas the value was 920 pg/ml with the sample-NSB correction, a deviation from the mean of about 18%. The former value is within the normal range, whereas the latter is abnormally high.

The difference between the sample NSB and the assay NSB was not significant in the pure-IF assay. Therefore, it was not necessary to make the sample-NSB correction. According to the manufacturer of this assay kit, the matrix used for the standard is B_{12}-free human serum.

Regression analysis of serum B_{12} values obtained by the three methods revealed that those obtained by the crude-IF assay were considerably higher than those obtained by the other two methods (Fig. 2). This is reflected in the higher mean B_{12} values obtained by the crude-IF assay from normal subjects (Fig. 3) and from patients with or without PA (Table 2).

The pure-IF assay gave the highest sensitivity, but its specificity is somewhat lower than that of the crude-IF assay for the diagnosis of B_{12} deficiency (Table 3). Of 53 patients studied, abnormally high serum B_{12} levels were detected in five patients by the crude-IF assay, but only one each by the block-IF and pure-IF assays. All five patients were subsequently diagnosed to have liver disease, which might have caused the elevated serum B_{12} levels by the crude-IF assay. One of them had a serum B_{12} level as high as 2320 pg/ml by the crude-IF assay, but by the block-IF and pure-IF assays his serum B_{12} levels were only 502 and 671, respectively. Further studies are needed to determine whether there is an increase in B_{12} analogs in patients with liver disease, and whether the measurement of B_{12} analogs is of clinical value in the evaluation of liver disease.

A recent study by Kubasik et al. (*8*) showed good agreement between B_{12} values obtained by purified intrinsic factor or cobinamide-blocked R-protein binders, and B_{12} values obtained by a microbiological assay. Our data also confirm previous reports that B_{12} deficiency can be diagnosed more accurately by measuring serum B_{12} levels with use of either pure IF or block IF.

FOOTNOTES

* Nutritional Biochemical Co.

† Becton Dickinson Simul TRAC Kit.

‡ Diagnostic Products Corp., Dualcount with Purified B_{12} Binder.

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