CONCISE REPORT

An Assay for Intrinsic Factor Based on Blocking of the R Binder of Gastric Juice by Cobinamide

By James A. Begley and Alan Trachtenberg

An in vitro assay for measurement of gastric juice intrinsic factor (IF) was developed based on the ability of the cobinamide (Cbi) [(CN, OH) Cbi] to bind to the gastric juice R-type binders of cobalamin (Cbl) and not to the IF binder. Subsequently added radioactive Cbl, CN-[57Co] Cbl, binds only to the IF binders and allows for direct measurement of this Cbl binding protein.

INTRINSIC FACTOR (IF), a glycoprotein secreted by the glandular mucosa of the stomach, binds cobalamin (Cbl) and facilitates its physiologic absorption across the intestinal mucosa. Glass has written an excellent review of this subject. The most common cause of Cbl deficiency is pernicious anemia (PA) characterized by deficient secretion of IF. Although measurement of the output of IF can be used to make or exclude the diagnosis of PA, this approach has not enjoyed wide clinical application.

Various physiochemical methods have been used to measure the IF content of gastric juice in vitro. Gel filtration accurately separates IF from gastric R-type binders by molecular size, but the procedure is time-consuming and requires column chromatography. Paper and starch gel electrophoresis have been used, but they have inherent limitations. The most common methods are based on immunologic principles using the blocking or binding type of antibody to IF found in the serum of some patients with PA. These techniques are easy to perform, but they rely on a reliable and continually available source of the respective antiserum.

Kohlhouse and Allen9 and Allen et al.10 recently showed that the cobinamide (Cbi) [(CN, OH) Cbi] bound to human R-type binders with high affinity but not to IF. We report here an in vitro assay measurement of IF that employs [(CN, OH) Cbi] to block R-type binders of gastric juice without affecting the capacity of gastric IF to bind subsequently added CN-[57Co] Cbl. The binding capacity for Cbl becomes, therefore, a direct measure of IF. We have compared the results of this assay with two independent methods: gel filtration and blocking antibody assay. This Cbi blocking assay is easy to perform; it does not rely on an antibody that is difficult to obtain, and it gives results comparable to those of other techniques.
MATERIALS AND METHODS

Gastric Juice and Saliva Collection

The “control” gastric juice was obtained principally from 9 participants in a study of the treatment of peptic ulcer. Most of these aspirates were collected prior to any surgery. In all instances the subject was capable of releasing gastric HCl, although the sample collected from subject 9 post surgery contained minimal acid. Five additional subjects had diseases that reduced the amount of one of the two binders of Cbl in their gastric juice. Gastric juice and saliva were collected and processed as previously described[1] and frozen at −20°C. Multiple timed samples were collected after stimulation with Histalog, but only the sample with the highest binding capacity for Cbl was used in the present study.

CN-[125]Co Cbl

Working stocks of CN-[125]Co Cbl (7500 pg/ml, specific activity 10 µCi/µg) were prepared by diluting radioactive [125]Cyanocobalamin (Phillips-Duphar, Petten, Holland) with nonradioactive cyanocobalamin (CN-Cbl) (Sigma Chemical Company). These final stocks were checked for Cbl content and purity by bioassay[2] and column chromatography,[3] respectively, and stored at 4°C.

Preparation of [(CN, OH) Cbl]

[(CN, OH) Cbl] was prepared by the method of Armitage et al.[4] and purified according to Kolhouse and Allen.[5] Ten milligrams of CN-Cbl were dissolved in 1 ml of concentrated HCl and heated for 6 min at 65°C. Ten milliliters of cold (4°C) deionized H2O were then added, and the sample was applied to a 2-× 30-cm column of AG 1 × 8 acetate ion-exchange resin (Bio-Rad Laboratories) equilibrated with deionized H2O. After the sample had completely entered the resin bed it was eluted with 0.05-M acetic acid, lyophilized, dissolved in 2 ml of deionized H2O, and applied to a 0.6 × 4-cm column of phosphocellulose (Cellex-P, Bio-Rad Laboratories) equilibrated with 0.01-M acetic acid. The column was washed with four column volumes of 0.01-M acetic acid, and the sample was eluted with 1-M NaCl. All columns were run at 4°C. Two-milliliter aliquots of the Cellex-P eluate were desalted by extraction into 8 ml of phenol (containing 25% H2O). The phenol phase was washed six times with 2-ml aliquots of deionized H2O, and the red material was extracted back into H2O by addition of 7 ml of acetone and 21 ml of ether. The lower aqueous phase was then washed three times with equal volumes of ether to remove residual phenol and evaporated to dryness. This final material was redissolved in deionized H2O and further purified by paper chromatography[6] for 24 hr. The material with the highest R value [(CN, OH) Cbl] was eluted from the dried paper with deionized H2O, and the concentration was determined on an aliquot spectrophotometrically using a molar extinction coefficient of 30,800/mole-cm at 367.5 nm in 0.1-M KCN.9 The final [(CN, OH) Cbl] was frozen at −20°C.

G-100 Column Chromatography

One-tenth milliliter of gastric juice was incubated with a 50% excess (as determined by UBBC[7]) of CN-[125]Co Cbl for 30 min at 37°C and applied to 2.5-× 100-cm columns of Sephadex G-100 (Pharmacia Fine Chemicals). All columns were equilibrated and eluted at 4°C with 0.05-M sodium phosphate buffer (pH 7.5) containing 0.5-M NaCl and 0.02% Na azide. Five-milliliter fractions were collected, and the radioactivity of each was determined in a well-type gamma scintillation spectrometer. With this method of fractionation,2 gastric R binder elutes 2–3 tubes past the void volume, followed by IF. Only the radioactive peak eluting as IF of molecular weight 120,000 d reacts with antibody to R binder.17 The amount of radioactivity in each peak was converted to picograms of CN-[125]Co Cbl bound using the appropriate specific activity.

IF Blocking Antibody Assay

IF was measured in gastric juice samples by the method of Gottlieb et al.[5] using serum from a single PA patient with high titer of the blocking type of IF antibody.

Cbi Blocking Assay for IF

Table 1 shows the scheme for the assay of gastric juice IF by the Cbi blocking technique. The bovine serum albumin (BSA) was from Sigma Chemical, fraction V powder. After the addition of the Cbi or
Table 1. Scheme of Cbi Blocking Assay for IF*

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Buffer†</th>
<th>Sample</th>
<th>Cbi‡</th>
<th>H2O</th>
<th>CN-[35Co] Cbl§</th>
<th>Ch/Alb‖</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>1.9</td>
<td>0.1</td>
<td>—</td>
<td>0.025</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>0.1</td>
<td>0.025</td>
<td>—</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*All volumes in milliliters; all tubes in duplicate.
†0.01-M Tris-HCl, pH 8.0, containing 0.15-M NaCl and 50 μg/ml BSA.
‡Containing 33,750 pg Cbi.
§Containing 7500 pg CN-[35Co] Cbl.
‖Charcoal-albumin.

deionized H2O, all tubes were incubated at 37°C for 30 min, followed by the addition of 1 ml CN-[35Co] Cbl (7500 pg) and an additional incubation for 30 min at 37°C. Unbound CN-[35Co] Cbl and Cbi were removed by addition of 2.0 ml of albumin-coated charcoal. After mixing for 2–3 min, the charcoal was pelleted by centrifugation, the supernatant was decanted, and the radioactivity was determined in each, with the duplicates being averaged. The counts of tube number 2 (supernatant control) were subtracted from all tubes except number 1. This gives essentially any radioactivity not taken out by the addition of the charcoal-albumin and usually represents 2%–4% of the total counts. This percentage was unchanged in the presence or absence of the added Cbi. The picograms of CN-[35Co] Cbl bound were calculated as follows:

\[
\text{sample counts} - \text{supernatant control} \times 7500 \text{ pg} \\
\text{total counts}
\]

The picograms of CN-[35Co] Cbl bound in the sample without Cbi represent total UBBC, whereas those in tubes containing Cbi are the picograms bound to IF only. The amount of R binder, if desired, can be calculated by subtracting the binding capacity in the presence of Cbi (IF) from the total binding capacity.

RESULTS

Figure 1A shows the effects of preincubating increasing amounts of the Cbi with essentially equal amounts (with respect to Cbl binding ability) of salivary R binder and IF. The saliva was from a healthy laboratory worker, and the gastric juice was from a patient with a congenital absence of R-type binders and therefore contained only IF as a Cbl binder. Preincubation of IF (5655 pg of CN-[35Co] Cbl binding ability) with 0–37,750 pg of Cbi had no effect on the subsequent binding of the radioactive Cbl. However, the Cbi was capable of completely inhibiting binding of subsequently added CN-[35Co] Cbl to R binder at concentrations of 13,500–33,750 pg Cbi. Figure 1B shows the ability of 33,750 pg of Cbi to inhibit binding to R-type binder when it is premixed with an equal amount of IF. These data show that Cbi is capable of completely inhibiting binding to R-type binders without affecting IF binding ability.

Table 2 shows the results obtained by three IF assays of the 14 gastric juices. Analysis by gel filtration was assumed to be the most analytical. In general, there was good agreement among methods, and where there was not, as in the IF level of No. 7 and No. 8, the level by the Cbi method correlated best with that by gel filtration.

DISCUSSION

The present assay of gastric juice IF is based on the relationship between vitamin B₁₂ structure and binding to IF. Studies have shown that IF is the most selective
Fig. 1. A. Effects of preincubating R binder (5970 pg Cbl binding ability) and IF binder (5655 pg Cbl binding ability) with increasing amounts of [(CN, OH) CblJ (0–33,755 pg) on subsequent binding of 7500 pg CN-[57Co] Cbl. B. Binding abilities of R and IF (approximately 2450 pg Cbl binding ability each) and an equal mixture of both (R + IF) with and without preincubation with 33,750 pg [(CN, OH) Cbl]. In both A and B the preincubations were performed for 30 min at 37°C, followed by addition of 7500 pg CN-[57Co] Cbl and an additional 30 min of incubation at 37°C. Conditions were the same as outlined in Table 1.

Table 2. IF and Gastric R Binder by Three Systems of Assay*

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>IF G-100 Antibody</th>
<th>Cbl</th>
<th>R Binder G-100 Antibody</th>
<th>Cbl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1†</td>
<td>69.5</td>
<td>62.7</td>
<td>68.5</td>
<td>8.9</td>
</tr>
<tr>
<td>2</td>
<td>19.4</td>
<td>14.6</td>
<td>16.5</td>
<td>0.8</td>
</tr>
<tr>
<td>3†</td>
<td>89.9</td>
<td>74.8</td>
<td>77.3</td>
<td>4.9</td>
</tr>
<tr>
<td>4†</td>
<td>73.8</td>
<td>78.5</td>
<td>66.4</td>
<td>3.0</td>
</tr>
<tr>
<td>5</td>
<td>53.6</td>
<td>49.7</td>
<td>49.4</td>
<td>2.8</td>
</tr>
<tr>
<td>6†</td>
<td>129.3</td>
<td>106.7</td>
<td>115.6</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>30.0</td>
<td>12.3</td>
<td>42.3</td>
<td>0.1</td>
</tr>
<tr>
<td>8</td>
<td>59.3</td>
<td>44.0</td>
<td>52.1</td>
<td>0.3</td>
</tr>
<tr>
<td>9</td>
<td>1.1</td>
<td>0.8</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Possible pernicious anemia†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>8.4</td>
<td>5.6</td>
<td>7.9</td>
<td>5.5</td>
</tr>
<tr>
<td>Well-documented pernicious anemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.0</td>
<td>0.0</td>
<td>2.6</td>
<td>41.9</td>
</tr>
<tr>
<td>12</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>20.8</td>
</tr>
<tr>
<td>13</td>
<td>4.6</td>
<td>5.5</td>
<td>4.3</td>
<td>16.2</td>
</tr>
<tr>
<td>Congenital deficiency of R binder††</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>42.3</td>
<td>36.7</td>
<td>33.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*All values in nanograms per milliliter.
†Because of the high Cbl binding capacity, these samples were diluted 1:1 prior to the antibody and Cbl assays.
‡Severe Cbl deficiency owing to some reduction in IF and to multiple high intestinal diverticula.

791
Cbl binding protein. Removal of the dimethylbenzimidazole moiety of CN-Cbl (CN-Cbl – (CN, OH) Cbi) renders the derivative incapable of competing with CN-Cbl for binding to IF. Mathan et al. showed that [(CN, OH) Cbi] did not bind to IF at concentrations as high as $10^{-3} M$. Allen et al. showed that [(CN, OH) Cbi] bound to R-type binders with an affinity equal to that of CN-Cbl at pH 8.0. [(CN, OH) Cbi] was bound to IF with an affinity 600,000-fold lower than that of CN-Cbl at pH 8.0. Owing to the inability of IF to bind [(CN, OH) Cbi], preincubation of gastric juice with [(CN, OH) Cbi] effectively blocks the unsaturated sites of gastric R-type binders. Subsequently added CN-[57Co] Cbl, therefore, binds to IF binder only.

We believe that this method of measuring IF is at least as reliable as other current methods, and it eliminates the need for a source of antibody or elaborate forms of protein separation. The Cbi could be made and distributed commercially, although it is simple enough to be made and stored by the user.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Dr. Charles A. Hall in the performance of the study and Dr. Herbert D. Gullick in obtaining the gastric juice.

REFERENCES

8. Jacob E, O’Brien HAW, Mollin DL: A radioisotope dilution assay for unlabeled vitamin B12-intrinsic factor complex employing the binding intrinsic factor antibody: Probably evidence
15. Hogenkamp HPC: The chemistry of cobal-
COBINAMIDE BLOCKING ASSAY FOR IF


16. Hippe E, Haber E, Olesen H: Nature of vitamin B₁₂ binding. II. Steric orientation of vitamin B₁₂ on binding and number of combining sites of human intrinsic factor and transcobalamin.

Biochim Biophys Acta 243:75, 1971

An assay for intrinsic factor based on blocking of the R binder of gastric juice by cobinamide

JA Begley and A Trachtenberg