Circulating Antibody to Transcobalamin II Causing Retention of Vitamin B\textsubscript{12} in the Blood

By Ralph Carmel, Basil Tatsis, and Lynn Baril

A patient with recurrent pulmonary abscess, weight loss, and alcoholism was found to have extremely high serum vitamin B\textsubscript{12} and unsaturated vitamin B\textsubscript{12}-binding capacity (UBBC) levels. While transcobalamin (TC) II was also increased, most of his UBBC was due to an abnormal binding protein which carried $>80\%$ of the endogenous vitamin B\textsubscript{12} and was not found in his saliva, granulocytes, or urine. This protein was shown to be a complex of TC II and a circulating immunoglobulin (IgG\textsubscript{K} and IgG\textsubscript{\lambda}). Each IgG molecule appeared to bind two TC II molecules. The reacting site did not interfere with the ability of TC II to bind vitamin B\textsubscript{12}, but did interfere with its ability to transfer the vitamin to cells in vitro. The site was not identical to that reacting with anti-human TC II antibody produced in rabbits. Because of this abnormal complex, $^{57}$Co-vitamin B\textsubscript{12} injected intravenously was cleared slowly by the patient. However, no metabolic evidence for vitamin B\textsubscript{12} deficiency was demonstrable, although the patient initially had megaloblastic anemia apparently due to folate deficiency. The course of the vitamin B\textsubscript{12}-binding abnormalities was followed over 4 yr and appeared to fluctuate with the status of the patient’s illness. The IgG–TC II complex resembled one induced in some patients with pernicious anemia by intensive treatment with long-acting vitamin B\textsubscript{12} preparations. The mechanism of induction of the antibody formation in our patient is unknown.

Vitamin B\textsubscript{12} transport involves various vitamin B\textsubscript{12}-binding proteins, as recently reviewed.\textsuperscript{1,2} Two of the serum proteins, transcobalamin (TC) I and third binder (TC III) are closely related. Their roles in vitamin B\textsubscript{12} transport are not established yet, and their absence does not seem to cause metabolic difficulty.\textsuperscript{3} TC II, the protein primarily involved in delivery of vitamin B\textsubscript{12} to cells, is the more crucial serum transport protein. Its absence causes severe vitamin B\textsubscript{12} abnormality.\textsuperscript{4,5}

Patients with abnormally elevated TC levels have been frequently described. Often, it is TC I that is elevated\textsuperscript{6–13} and, since TC I normally carries endogenous vitamin B\textsubscript{12}, serum vitamin B\textsubscript{12} levels are also high. High serum TC III levels have been described\textsuperscript{14–16} and, despite the major problem of in vitro artifact,\textsuperscript{17,18} often appear to be elevated in plasma as well.\textsuperscript{19,20} High TC II levels\textsuperscript{13,21} have been described less often and less systematically and, as with TC III elevation, have usually not been accompanied by elevated vitamin B\textsubscript{12} levels. Some earlier
reports may have overestimated TC II elevations because TC III was not adequately separated from TC II.

In this paper we present studies on a unique patient with serum vitamin B\textsubscript{12} levels and unsaturated vitamin B\textsubscript{12}-binding capacity (UBBC) approximately 40 times normal. The cause was found to be different from the previously mentioned TC disorders. A circulating antibody to his own TC II apparently resulted in retention of both TC II and vitamin B\textsubscript{12} in the blood. In many respects the antibody resembled one induced in some patients with pernicious anemia by repeated injections of long-acting preparations of vitamin B\textsubscript{12}.

In the following presentation the antibody–TC II complex will be referred to as “abnormal binder.” It is important to keep in mind that, as in most studies of vitamin B\textsubscript{12}-binding proteins, the quantity and fate of binding of \textsuperscript{57}Co-B\textsubscript{12} is assumed to reflect the quantity and the fate of the protein in question.

CASE HISTORY

A.S., a 38-yr-old black man, was first hospitalized in April 1972 with a right apical pulmonary cavity associated with fever and early clubbing of fingernails, 30-lb weight loss on a diet of milk, crackers, and soup over a 5-mo period, and alcoholism. There was no history of vitamin B\textsubscript{12} therapy, and no neurologic symptoms were elicited. Abnormal laboratory data were folate deficiency with megaloblastic anemia (hemoglobin 6.1 gm/dl), hypocholesterolemia, polyclonal hypergammaglobulinemia, and low erythrocyte glucose-6-phosphate dehydrogenase activity. At no time during this admission or in his subsequent course did his neutrophil count exceed 8800/\mu{l}. All liver chemistry tests were normal although the liver was palpable 2 cm below the right costal margin; the spleen was not palpable. A very high serum vitamin B\textsubscript{12} level prompted further evaluation. The vitamin B\textsubscript{12} data during this hospitalization and subsequently are all shown in Fig. 1. Evaluation of the pulmonary abscess included tomography and bronchoscopy. All cytologic and bacterial studies were unrevealing, except for the finding of “atypical cells of bronchial and histiocytic origin” on one of the many sputum samples examined cytologically.

Fig. 1. Serum vitamin B\textsubscript{12}, UBBC, and unsaturated binder levels of the patient. Normal serum vitamin B\textsubscript{12} (150–900 pg/ml), UBBC (600–1600 pg/ml), and TC II (500–1400 pg/ml) ranges are dwarfed by the patient's levels. Abnormal binder and TC II in this graph refer to unsaturated binding only.
CIRCULATING ANTIBODY TO IC II

A second-strength tuberculin skin test was positive and the patient was treated empirically with isoniazid, streptomycin, ethambutol, and pyridoxine after a brief course of penicillin. During the following month the patient gained weight, his hemoglobin level rose to 11.9 gm/dl and the pulmonary lesion improved rapidly. He left the hospital against advice, and his outpatient course was punctuated by very irregular antituberculous and folic acid therapy, binges of wine drinking, and intermittent mild elevations of serum GOT, GPT, lactic acid dehydrogenase, bilirubin, and alkaline phosphatase.

In December 1973 (day 582 in Fig. 1) the patient was readmitted with relapse of his earlier problems, further accompanied by diarrhea and dyspnea. He had taken no antituberculous therapy for 6 mo, but had stopped his heavy wine drinking in November 1973 following a convulsion. Although his liver was greatly enlarged, biochemical tests were normal except for minimal abnormalities of SGOT and prothrombin time. Liver biopsy showed only minimal fatty metamorphosis. There was moderate anemia of chronic disease, hypoalbuminemia, and hypcholesterolemia. The bone marrow showed 11%, plasmacytosis but was normoblastic. There was now a large abscess in the left lung, although all cultures and smears were again repeatedly negative. The patient was treated with penicillin, isoniazid, ethambutol, and pyridoxine. Again he showed excellent improvement. Since the course of the pneumonia was deemed not to be that of tuberculosis, he was discharged with only penicillin.

The patient was next admitted in August 1975 (day 1210) with recurrence of pneumonia in the right lung, which now showed an air fluid level, and of diarrhea. All cultures remained negative and bronchial biopsy showed only acute and chronic inflammatory changes. The liver was not enlarged and tests of liver function were normal. Isoniazid, ethambutol, rifampin, and penicillin were given. Upper gastrointestinal x-ray studies to investigate the diarrhea were negative except for a suggestion of chronic gastritis. The patient was discharged in January 1976 without antituberculous therapy. His right pulmonary lesion has shown continued improvement, though the diagnosis remains unclear.

MATERIALS AND METHODS

All venous blood samples were allowed to clot at room temperature and were centrifuged. Plasma was also obtained once, using EDTA NaF anticoagulant (Vacutainer No. 4601, Becton Dickinson & Co., Rutherford, N.J.). Saliva was collected by direct, unstimulated spitting. Random urine samples were collected without preservative. Granulocyte extracts were prepared as previously stated. All specimens were stored at -20°C. Some sera were thawed and refrozen several times while preparing aliquots for experiments. Only after thawing more than 6 times was there deterioration of the vitamin B12 binders. The proteins were otherwise not affected by simple storage at -20°C, even after 6 mo. All experiments, unless otherwise stated, were done on minimally manipulated specimens. Depending on the experiment, cyanocobalamin labeled with cobalt-57 (57Co-B12) of either 15 µCi/µg or 167 µCi/µg specific activity (Amer- sham Searle Corp., Arlington Heights, Ill.) was used.

Serum UBBC was determined by saturating 0.1 ml of serum with excess 57Co-B12 and, after 30 min of incubation, chromatographing in 0.1 M Tris 1 M NaCl buffer, pH 8.6, on Sephadex G-200 or G-150 gel (Pharmacia Fine Chemicals, Piscataway, N.J.) as previously described. The amount of 57Co-B12 carried in the various binder peaks was calculated, the sum being the UBBC. Results agreed closely with coated charcoal radioassay. In a few experiments, different buffers were substituted in order to assess their effect on the binder; in each case the gel was also first swollen in that buffer. In some experiments, serum unlabeled with 57Co-B12 was chromatographed and UBBC was subsequently determined on each eluate fraction by radioassay. In other studies, each such fraction was instead assayed for vitamin B12 content, in order to determine which peaks carried the endogenous vitamin B12 of the serum.

Vitamin B12-binding proteins were also fractionated by 2 M ammonium sulfate precipitation, DEAE-cellulose separation, and autoradiography (by exposure to x-ray film for 6 wk of cellulose acetate strips on which samples had been subjected to electrophoresis at pH 8.6). TC fractions labeled with 57Co-B12 were obtained by pooling gel filtration eluates, and they were concentrated by centrifuging in CF 25 Centriflo membrane cones (Amicon Corp., Lexington, Mass.). The absence of other contaminating TC in each product was confirmed by chro могaphing on the Sephadex gel. Use of such TC fractions is identified specifically below. Otherwise, whole serum was used, which, depending on the nature of the study, was sometimes first satu-
rated with nonradioactive vitamin B₁₂ (the excess being removed by treating with a hemoglobin-coated charcoal pellet) in order to prevent possible undesirable transfer of $^{57}$Co-B₁₂ from components of the experiment to unsaturated binding proteins of the test serum.

Anti human saliva "R binder" antiserum was prepared in rabbits.²¹ It reacted only with R binder (TC I, TC III, or granulocytic and salivary R binders) and not with TC II. Rabbit anti human TC II antiserum* was shown to react only with TC II. Precipitating anti intrinsic factor antibody was obtained from the serum of a patient with pernicious anemia and was shown to react only with intrinsic factor. Results obtained with rabbit anti-IgG, anti-IgA, anti-IgM, anti $\kappa$ chain, and anti $\lambda$ chain antisera (Behring Diagnostics, Woodbury, N.Y.) were confirmed with purified anti-IgG, anti-IgM, and anti-Fab antiserum prepared in rabbits.⁴

Immune reactions were identified by change in Sephadex gel elution of the $^{57}$Co-B₁₂ bound to the binding protein in question, always comparing the pattern to a control run in which the antigen was incubated with inactive serum instead, and by autoradiography of immunoelectrophoretic or Ouchterlony immunodiffusion plates to detect radioactive arcs. In these studies, free $^{57}$Co-B₁₂ was first removed by treating the sera with hemoglobin-coated charcoal pellets. Antibody absorption studies used pure IgG² and TC fractions prepared as above. Incubation at room temperature and then at 4°C was followed by centrifugation to remove precipitated complexes before use.

In studies of pH effect, sera were acidified to pH 3 with 0.2 N HCl, or alkalinized to pH 10 with 0.2 N NaOH, and reneutralized after 1 hr. To test temperature sensitivity, sera were heated to 56°C for 1 hr and then cooled. In all cases, sera were not saturated with $^{57}$Co-B₁₂ until afterward in order to avoid misinterpreting $^{57}$Co-B₁₂ release from binders as denaturation. Gel chromatography was then done to ascertain the effect of the procedures on the individual binding proteins.

The various TC fractions were incubated with neuraminidase, type VI (Sigma Chemical Co., St. Louis, Mo.) or in 5 M guanidine HCl to determine their effect. After dialysis at 4°C, the samples were saturated with $^{57}$Co-B₁₂ and subjected to gel chromatography and cellulose acetate electrophoresis.

In vitro mediation of $^{57}$Co-B₁₂ uptake by reticulocyte-rich red cell suspensions was tested by the method of Retief et al.³⁷ When $^{57}$Co-B₁₂ saturated TC fractions were used, the fraction volumes were adjusted so that each presented the same amount of bound $^{57}$Co-B₁₂ to the cells.

In vivo clearance of vitamin B₁₂ was determined after injection of a subsaturating dose of 0.21 µg of sterile $^{57}$Co-B₁₂ into a forearm vein. Informed consent was obtained from the patient for the study. Venous blood was drawn from the opposite arm at timed intervals. The clearance curve may be compared to a normal one obtained in a previous study by Carmel and Herbert and by others.³⁸,³⁹ Some of the timed serum samples were also chromatographed on Sephadex gel to determine which binders carried the $^{57}$Co-B₁₂.

### RESULTS

**Serum Vitamin B₁₂ and UBBC Levels (Fig. 1)**

The patient's initial vitamin B₁₂ level was 21,980 pg/ml and UBBC was 49,530 pg/ml. The bulk of his UBBC (71%, or 35,400 pg/ml) consisted of an abnormal binder. The patient's UBBC and abnormal binder levels were the same in plasma anticoagulated with EDTA NaF as in serum. The extremely high serum levels fell with clinical improvement, but at no time became normal. The lowest vitamin B₁₂ level recorded was 1222 pg/ml (day 262) and the lowest UBBC was 2889 pg/ml (day 1480). With each clinical relapse vitamin B₁₂ and UBBC values rose, though peak levels were progressively lower each time. While TC II was also elevated and its rise and fall adhered to the above pattern, the most dramatic fluctuations were in levels of the abnormal binder. TC II level exceeded that of the abnormal binder only during clinical remissions.

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*Provided by Dr. Robert H. Allen, Washington University School of Medicine, St. Louis, Mo.

*Provided by Dr. Donald I. Feinstein, USC School of Medicine, Los Angeles, Calif.
Vitamin $B_{12}$ was also assayed in one serum microbiologically.* The result was comparable to that of our radioassay $^3$ (5056 versus 4162 pg/ml), demonstrating that the high levels were not due to radioassay artifact.

Characterization of the Abnormal Vitamin $B_{12}$-binding Protein

Sephadex gel chromatography (Fig. 2). The binder eluted between void volume and TC I, giving a calculated Stokes radius of 7.8 nm. Comparable to the findings of others, $^{40}$ TC I had a Stokes radius of 5.2 nm and TC II of 2.8 nm. The abnormal binder was present even in serum chromatographed without $^{57}$Co-$B_{12}$ (Fig. 2), indicating that it did not represent aggregation induced by vitamin $B_{12}$. However, its binding capacity was thereby reduced 58%, suggesting the binder’s partial stabilization by vitamin $B_{12}$. TC II was unchanged by chromatography without $^{57}$Co-$B_{12}$.

Cellulose acetate electrophoresis. The binder migrated as a fast $\gamma$-globulin, coming just behind TC II ($\beta$-globulin). Free $^{57}$Co-$B_{12}$ migrated as a slow $\gamma$-globulin.

Reaction with anti-binder antisera. Anti-TC II antiserum caused the binder to shift to the void volume on Sephadex G-200 gel chromatography (Fig. 3). The reaction was confirmed by autoradiography of Ouchterlony plate immunodiffusion, the abnormal binder giving a radioactive precipitin line of identity with TC II against anti-TC II antiserum. The abnormal binder showed no reac-

* Euglena gracilis assay performed in the laboratory of Dr. Charles A. Hall, V.A. Hospital, Albany, N.Y.
Fig. 3. Effect of rabbit anti-TC II antiserum on the patient's binders. Both TC II and abnormal binder are shifted to void volume on Sephadex G-200 gel filtration after incubation with anti-TC II.

DEAE-cellulose separation and (NH₄)₂SO₄ precipitation. The abnormal binder eluted from DEAE-cellulose with 0.06 M phosphate buffer, pH 6.3, and was precipitated in 2 M (NH₄)₂SO₄. In both these screening methods of fractionation it resembled TC II and would therefore not have been differentiated from normal TC II.

Other physicochemical and biochemical properties. The binding capacity of the abnormal binder was destroyed by heating and by acidification but was unaffected by alkalinization, generally resembling TC II in all of these features. Dialysis against 5 M guanidine·HCl converted most of the abnormal binder to TC II, TC II itself being unaffected by such treatment. Sephadex gel elution in low ionic strength buffer (0.01 M phosphate, pH 6.3) decreased both the abnormal binder and TC II greatly. The decrease was partly due to the shifting of both into the void volume fraction and partly to their retention within the gel. TC II from normal serum behaved similarly, as also found by Hom, whereas TC I was unaffected. Freezing and thawing of serum with use over several months caused a progressive decrease of the abnormal binder and a slight increase of TC II, suggesting partial dissociation to TC II and partial destruction of the abnormal binder. Normal serum, similarly handled, had only a slight decrease in binding capacity. Neuraminidase did not affect the electrophoretic mobility or Sephadex gel elution of the abnormal binder or of TC II (in contrast...
Fig. 4. Autoradiograph of immunoelectrophoretic reaction of the patient’s serum with various rabbit anti-immunoglobulin antisera. The arcs represent $^{57}$Co-B$_{12}$ radioactivity, which serves as a marker for the reaction with binders. The troughs, which are very faint, contain from top to bottom: anti-whole serum antiserum (Wh), anti-IgG antiserum (G), anti-IgA antiserum (A), anti-IgM antiserum (M), anti-$\kappa$ chain antiserum (K), and anti-$\lambda$ chain antiserum (L). The wells (outlined circles) contain patient’s serum (s) and normal control serum (n) in an alternating pattern. Normal serum reacts only with anti-whole serum, probably representing a TC II–anti-TC II reaction.

Immunoglobulin characterization. The abnormal binder reacted only with rabbit anti-IgG, anti-$\kappa$ chain, and anti-$\lambda$ chain antisera on immunoelectrophoresis (Fig. 4), but not with anti-IgM or anti-IgA antisera. Identical results were demonstrated with Sephadex gel filtration. In addition, the binder reacted with anti-Fab antiserum in the latter system, whereas unimmunized rabbit serum had no effect on the binder. Identical results were demonstrated in serum provided to us by Dr. Henrik Olesen (Bispebjerg Hospital, Copenhagen, Denmark) from a patient with antibody to his own TC II due to therapy with depot preparations of vitamin B$_{12}$.

In a preliminary report of our findings, we had mistakenly interpreted our patient’s binder as not reacting with anti-IgG antiserum. The reasons for that initial impression were (1) presaturating anti-IgG antisera with nonradioactive vitamin B$_{12}$ in order to saturate their own vitamin B$_{12}$-binding proteins and then treating with a coated charcoal pellet greatly diminished their subsequent reaction with the abnormal binder, and (2) reaction with the abnormal binder could be blocked not only by absorbing the anti-IgG antiserum with human IgG, but also by absorbing with TC II. The explanation for the first set of findings remains unclear but was due entirely to the charcoal treatment. Whatever the cause, anti-Fab reaction was not similarly blocked, suggesting that the prob-
lem was peculiar to only some antisera. The block of anti-IgG reactivity by TC II absorption turned out to be due to the unexpected contamination of the normal TC II fraction by IgG fragments. Thus, TC II prepared from an agammaglobulinemic patient did not block anti-IgG reaction with the abnormal binder. Furthermore, TC II itself did not react with the anti-IgG antiserum, indicating that the antiserum was not contaminated with anti–TC II.

**Binding of endogenous vitamin B₁₂.** More than 80% of the patient's endogenous vitamin B₁₂ was carried in the abnormal binder peak on gel chromatography. TC I, which normally carries most vitamin B₁₂, carried only 15%-20%. The rest was bound to TC II. DEAE-cellulose fractionation confirmed these findings.

**Binding avidity.** The abnormal binder appeared to bind vitamin B₁₂ more avidly than did TC II. When subsaturating amounts of $^{57}$Co-B₁₂ were added to serum in which the abnormal binder constituted 79% and TC II 21% of the UBBC, the abnormal binder bound 96% of the $^{57}$Co-B₁₂ and TC II only 4%.

**Effect of the patient’s serum on other binders.** The patient's serum was presaturated with nonradioactive vitamin B₁₂ to prevent simple transfer of $^{57}$Co-B₁₂ from test sera to his abnormal binder. Incubation of the patient's serum with various sera or with normal TC II consistently shifted the TC II to the abnormal binder peak (Fig. 5). His serum had no such effect on TC I, salivary R binder, or intrinsic factor. Nor, incidentally, did his serum react with rabbit “TC II,” the predominant vitamin B₁₂-binding protein in rabbit serum which behaves like human TC II on Sephadex gel filtration. Other sera, used as controls, had no effect on TC II or TC I, nor did the patient's granulocyte extract.

In another set of experiments, the patient's serum was first filtered through Sephadex G-150 gel. Each eluate fraction was then incubated with normal TC II labeled with $^{57}$Co-B₁₂. The mixtures were each rechromatographed and the amount of TC II shifted to the abnormal binder position was quantitated.

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Fig. 5. Effect of the patient's serum on normal TC II (Sephadex G-150 gel chromatography). Lack of effect of normal serum on TC II is shown as a control. Both sera were first saturated with nonradioactive vitamin B₁₂ in order to avoid the possibility of transfer of the $^{57}$Co-B₁₂ from TC II to their binding proteins.
Table 1. Mediation of Vitamin B\(_{12}\) Uptake by Reticulocytes

| Whole serum | Normal serum | 5.4 |
| Chronic myelogenous leukemia serum | 2.8 |
| Patient's serum | 2.0 |

| TC fractions | Experiment A | Experiment B* |
| Patient's TC II | 9.0 | 3.6 |
| Patient's abnormal binder | 2.4 | 2.4 |
| Chronic myelogenous leukemia TC I | 1.7 | 1.2 |
| Saline | 2.7 |

*In this set of experiments, the differences in endogenous content of (nonradioactive) vitamin B\(_{12}\) among the various TC fractions was neutralized by prior addition of nonradioactive vitamin B\(_{12}\) to the fractions. Thus the vitamin B\(_{12}\) contents as well as the UBBC of TC II and abnormal binder in these experiments were identical. The result was a decrease in TC II-mediated uptake, yet TC II still promoted uptake better than did the abnormal binder. (The values given represent the average of three experiments.)

†In a separate experiment normal TC II was shown to behave identically.

Peak-shifting ability was found within that elution area of the patient's serum corresponding to his abnormal binder. The shifting effect was less in serum obtained during clinical remission when vitamin B\(_{12}\) and UBBC were also lower and appeared to parallel those levels. Serum repeatedly frozen and thawed lost its ability to shift normal TC II.

Mediation of cell uptake (Table 1). The patient's serum mediated uptake of \(^{57}\)Co-B\(_{12}\) by human reticulocyte-rich red blood cells in vitro poorly. Using binder fractions isolated by gel chromatography, his TC II was shown to behave normally, whereas his abnormal binder did not. This difference was only partly due to the difference in endogenous vitamin B\(_{12}\) content between the two binders (Table I, experiment B). The patient's serum mediated \(^{57}\)Co-B\(_{12}\) uptake by HeLa cells poorly too.

Other Vitamin B\(_{12}\)-binding Proteins of the Patient

Other than its quantitative increase, the patient's TC II behaved normally electrophoretically, immunologically, on Sephadex gel chromatography, in mediating cell uptake of \(^{57}\)Co-B\(_{12}\), and by carrying virtually no endogenous vitamin B\(_{12}\). TC I levels were never elevated and on gel filtration TC I was often obscured by the other two peaks. Only 15–20% of the patient's vitamin B\(_{12}\) was carried by TC I in a serum whose vitamin B\(_{12}\) content was 3027 pg/ml.

The UBBC of two saliva specimens was 75.8 and 67.5 ng/ml (normal is 21–110 ng/ml) and was composed entirely of the normal R binder, as demonstrated immunologically, electrophoretically, and by gel filtration. The UBBC of two leukocyte extracts was 2.6 and 2.9 ng/10\(^6\) cells (normal is 2.6–36.6 ng/10\(^6\) cells). The binder behaved like R binder by the same criteria as above. Urine

*Performed in the laboratory of Dr. Charles A. Hall, V.A. Hospital, Albany, N.Y.
Clearance of $^{57}$Co-B$_{12}$ In Vivo

The patient cleared an intravenous tracer dose of $^{57}$Co-B$_{12}$ slowly (Fig. 6). The pattern was comparable to that seen in some patients with chronic myelogenous leukemia.\textsuperscript{38,39} After 4 hr, 49.3\% of the original dose was circulating; after 3 days, 15.6\% was still left. Normally the values are $<10\%$ and $<5\%$, respectively. Furthermore, this study was done at a time when his binder abnormalities were in partial remission, suggesting that clearance may have been even more abnormal during relapse. The timed serum samples were also chromatographed on Sephadex gel (Table 2). His abnormal binder carried 45\% of the $^{57}$Co-B$_{12}$ initially and declined slowly; $^{57}$Co-B$_{12}$ bound to his TC II, surprisingly, also declined slowly. There was a relative increase in his TC I binding of the tracer dose after the first hour, but TC I still carried only 21\% of the remaining $^{57}$Co-B$_{12}$ even after 7 days.

Table 2. Sephadex G-200 Gel Fractionation of Timed Serum Specimens Obtained During the In Vivo Clearance Study

<table>
<thead>
<tr>
<th>Time of Sample</th>
<th>$^{57}$Co-B$_{12}$ (cpm in sample)</th>
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<tbody>
<tr>
<td></td>
<td>Abnormal Binder</td>
</tr>
<tr>
<td>1 hr</td>
<td>360</td>
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<tr>
<td>4 hr</td>
<td>196</td>
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<tr>
<td>24 hr</td>
<td>131</td>
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<td>7 days</td>
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CIRCULATING ANTIBODY TO TC II

Miscellaneous

No evidence for vitamin B₁₂ deficiency was found by testing the patient’s urinary excretion of methylmalonic acid on two occasions. The megaloblastic morphological changes, seen only initially, were apparently due to nutritional folate deficiency as suggested by low serum folate levels.

The patient’s mother, the only relative available for study, had normal serum vitamin B₁₂ and UBBC levels. Her serum was unable to shift normal TC II to form abnormal binder. Sera from five patients with tuberculosis (before or during antituberculous therapy), cachexia, and liver disease, and from two patients with hypergammaglobulinemia were also tested. Although most had moderate elevations of serum vitamin B₁₂ and some of TC II, none had the abnormal binder seen in our patient and none could shift normal TC II.

DISCUSSION

The great elevation of UBBC and attendant retention of large amounts of vitamin B₁₂ in our patient’s serum were found to be due to his elaboration of a circulating antibody to his own TC II. The resultant complex—the “abnormal binder”—was found only in his serum, and was absent from secretions such as saliva and from granulocyte extract.

The fact that the binder contained TC II was demonstrated immunologically and by its dissociating to TC II and no other TC. In addition, the binder behaved like TC II in its DEAE-cellulose elution, precipitation in 2 M ammonium sulfate, acid and heat denaturation, behavior on gel filtration in low ionic strength buffer, and in not being affected by neuraminidase. Many of these properties are noteworthy because they indicate that routine screening of sera by various commonly used methods would not reveal the presence of this abnormal binder.

Several authors have described apparent complexing and dissociation of TC II with various manipulations in vitro. This phenomenon would not explain our patient’s binder since its molecular size was quite different from those reported, and in reproducing one of these studies we could not alter the binder’s behavior with heparin or EDTA. Most importantly, the abnormal binder was obviously present in vivo, and its extent may even have been underestimated due to dissociation in vitro. Such dissociation (even a single pass of the complex through a Sephadex gel column produced a small TC II peak), incidentally, may explain the apparently prolonged persistence of ⁵⁷Co-B₁₂ binding by “free” TC II in the clearance study in vivo.

The antibody component of the abnormal binder was a polyclonal IgG. Interestingly, the antibody differed from that produced in rabbits by immunization with human TC II in several respects: their sites of attachment to TC II were different, and rabbit anti TC II formed a larger complex with TC II which eluted with the void volume fraction on Sephadex G-200 gel chromatography.

*Performed in the laboratory of Dr. Lewis A. Barness, University of South Florida College of Medicine, Tampa, Fla.
Our patient's antibody bound TC II whether or not the latter was carrying vitamin B$_{12}$, although whether avidity was greater for saturated or unsaturated TC II was not clear. The binding site for antibody on TC II was competitive neither with its ability to bind vitamin B$_{12}$, since the isolated complex could bind further vitamin B$_{12}$, nor with its ability to react subsequently with anti-TC II made in rabbits. However, ability to deliver vitamin B$_{12}$ to cells in vitro appeared impaired by the antibody and clearance in vivo of vitamin B$_{12}$ was abnormal. These findings suggest that the antibody interfered with the TC II site for attachment to cells.

If one calculates molecular size from gel filtration, the patient's binder complex corresponded to 230,000-250,000 daltons, suggesting that each IgG molecule bound two TC II molecules, the molecular weight of TC II being 38,000 by gel filtration. The apparent dissociation of TC II from antibody in 5 M guanidine-HCl suggests that antibody bound TC II noncovalently.

The antibody in our patient appears to be identical to that found in 29% of Danish patients with pernicious anemia treated with long-acting vitamin B$_{12}$ preparations. Our patient is unique in that his antibody appeared to arise de novo since he at no time received even cyanocobalamin injections, let alone the long series of injections of the special preparations given the Danish patients. The antibody level and the vitamin B$_{12}$ and UBBC levels rose with each exacerbation of his infectious illness, the nature of which remains unclear, and the concomitant malnutrition. The infection may have stimulated the general production of antibodies, or possibly antibody to the invading organism cross-reacted with TC II. It is interesting that in one of the Danish patients a secondary rise in vitamin B$_{12}$ and UBBC levels coincided with a severe urinary tract infection. Our patient's hepatic dysfunction did not correlate directly with the course of his abnormal binder. Nevertheless, relation of liver disease, and possibly the hypergammaglobulinemia associated with it, to this antibody must also be considered. While the complex contains immunoglobulin, its nature, of course, may not be that of true antibody formation and interaction with an antigen. Alternatively, specific autoantibody may have been stimulated by a subtle alteration of TC II or by impairment of normal vitamin B$_{12}$ metabolism or transport.

Interestingly, despite evidence of abnormal transport, no evidence of vitamin B$_{12}$ deficiency could be found in our patient, though subtle or selective cellular deficiency may have remained undiscovered. The closest analogy may be to patients with chronic myelogenous leukemia who, because of TC I accumulation, also have large amounts of vitamin B$_{12}$ circulating which are poorly delivered to tissue. Yet cellular depletion of vitamin B$_{12}$, as measured by the "deoxyuridine suppression" test, has not been found in one such randomly chosen leukemic patient (R. Carmel, unpublished data). The Danish patients have also had no apparent evidence of vitamin B$_{12}$ deficiency resulting from their antibody. The presence of some TC II unbound by antibody may be sufficient for adequate vitamin B$_{12}$ delivery to cells.

Olesen et al. have suggested that everyone may have minute amounts of anti-TC II antibody in the serum. However, such a finding would be unexpected in the normal state. We have been unable to demonstrate even small
amounts of antibody in several normals, treated and untreated pernicious anemia patients, and other subjects tested by gel filtration and by effect of their serum on TC II. However, careful search in our laboratory has uncovered other antibody-TC complexes. Two patients were found to have antibody to their TC I, apparently arising de novo. It is clear that immunoglobulin complexes with various TCs may be much more common than heretofore suspected. Since most screening methods of vitamin B₁₂-binding protein fractionation may be misleading, as we have demonstrated, identification will obviously depend on the use of appropriate techniques.

ACKNOWLEDGMENT

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