Studies on Transcobalamin (TC). 1. Detection of TC II Isoproteins in Human Serum

By Marjke Fräter-Schröder, W. H. Hitzig, and R. Büter

Vitamin B\textsubscript{12} is transported in blood by three functionally different carrier proteins, the transcobalamins (TC), designated TC I, II, and III. TC II is of particular interest because it delivers vitamin B\textsubscript{12} to the cells. Chemically TC II lacks sialic acid residues, which are components of TC I and III. A method to evaluate structural variants of TC II was needed for further biochemical and genetic studies in a case of congenital TC II deficiency. The system reported here utilizes polyacrylamide gel electrophoresis (PAGE) after neuraminidase degradation to separate TC II from TC I and III. Unsaturated and neuraminidase-treated serum proteins were labeled with radioactive vitamin B\textsubscript{12} (\textsuperscript{57}Co-B\textsubscript{12}) and subsequently evaluated in autoradiographs. Of the unsaturated TC II fraction, 70%-75% appeared as discrete bands. TC II exhibited two or three, or rarely four, bands. The remainder (25%-30%), obscured by overlapping desialylated TC I and III fractions, was detected after isolation of TC II by gel filtration or by ammonium sulfate precipitation and appeared as a diffuse radioactive band with reduced electrophoretic mobility when compared to the sharp-banded pattern.

In contrast to the multiple forms exhibited by TC I and III, which vary during disease, the clearly delineated TC II bands reflect individual person to person differences that are not influenced by disease. Family studies are compatible with a genetic polymorphism of TC II. The ten TC II patterns observed are unequivocally explained by the assumption of a five-allele system.

THREE SPECIFIC transport proteins for vitamin B\textsubscript{12} called transcobalamins (TC) I, II, and III are known as trace components in blood.\textsuperscript{1,2} TC I and III, also called R binders or cobalophilines, are sialoglycoproteins and are immunologically identical.\textsuperscript{3} TC II, devoid of sialic acid,\textsuperscript{4} is an immunologically different protein. It makes up 80%-90% of the B\textsubscript{12} binding capacity of serum. The biologic role of TC II, transport of B\textsubscript{12} to the cells,\textsuperscript{5,8} is clearly demonstrated in patients suffering from its congenital absence: they present with a life-threatening disease with severe impairment of hematopoietic, immunologic, and gastrointestinal functions.\textsuperscript{9,12}

Several methods for separating the TC have been reported.\textsuperscript{13-17} Various techniques were applied to determine the TC II content of serum: fractionated ammonium sulfate precipitation, gel filtration, selective pH-dependent B\textsubscript{12} binding, and selective absorption to charged cellulose or silicate. However, a more versatile method to measure TC II was wanted for biochemical studies in the case of congenital TC II deficiency observed in our hospital. Electrophoretic separation of TC in a polyacrylamide gel, as reported by three groups,\textsuperscript{18-20} was revised and modified. By the technique finally elaborated the bulk of TC II was clearly...
separated from TC I and TC III. Surprisingly, multiple TC II bands were visualized that express a new genetic isoprotein system.\textsuperscript{21,22}

**MATERIALS AND METHODS**

Vitamin B\textsubscript{12} labeled with \textsuperscript{57}Co (\textsuperscript{57}Co-B\textsubscript{12}) with a specific activity of 200 mCi/mg and radioactivity of 1 \muCi/ml (Philips-Duphar, Petten, Holland) was employed as a radioactive label. Neuraminidase 0.2 IU/ml was obtained from Behring-Werke, Marburg/Lahn, Germany. The neuraminidase buffer contained 0.05 M sodium acetate, 0.1% CaCl\textsubscript{2}, and 0.95% NaCl at pH 5.5. Acrylamide and N,N'-methylene-bis-acrylamide were purchased from Senva, Heidelberg. A Packard gamma counter (model 3002) was used to count the \textsuperscript{57}Co-B\textsubscript{12} samples. Densitometric scanning of the autoradiographs was performed on a Chromoscan (Joyce-Loebl, Kalkofen, Germany). Specific anti-TC II antisera was a gift from Professor C. A. Hall, Albany, N.Y.

**Patients and probands.** Serum samples were collected from laboratory personnel, volunteers, and hospital patients. For genetic studies 510 nonrelated individuals and 57 members of ten families were tested. For evaluation of the influence of disease, 20 patients with various diseases were sampled repeatedly, at least twice at half-yearly intervals. From these groups 37 sera were selected for quantitative studies using three fundamentally different TC II determinations.

TC separations were performed in serum (if not specified, serum was centrifuged 1-3 hr after sampling; a "normal" increase\textsuperscript{23} of TC III was acceptable), quick-spun serum, EDTA plasma, EDTA + LiCl plasma,\textsuperscript{24} leukocytes (isolated by Ficoll-Hypaque technique), and tears.

**Established Methods**

Established methods were used to verify results obtained by the newly elaborated techniques reported in this paper: (1) Determination of unsaturated B\textsubscript{12} binding capacity (UBBC);\textsuperscript{20} (2) gel filtration to separate TC I and III from TC II;\textsuperscript{26} (3) ammonium sulfate precipitation to separate TC I and III from TC II;\textsuperscript{13} (4) the acid-resistant binding capacity (ARBC) determines TC I + TC III quantitatively, and the difference between UBBC and ARBC corresponds to TC II;\textsuperscript{15} and (5) polyacrylamide gel electrophoresis (PAGE).\textsuperscript{18,20,27}

**Newly Developed Methods**

PAGE of serum and transcobalamin fractions was performed in a 9% polyacrylamide slab gel (1.5 mm × 7.0 cm × 12.5 cm) with a 3.5% concentrating top gel (1.5 cm high) and Tris-glycine electrode buffer at pH 8.3.\textsuperscript{27} Serum (0.1 ml, diluted if necessary) was saturated with 50 \mu g radioactive B\textsubscript{12} containing 250 pg B\textsubscript{12}, which corresponds to twice the UBBC of a normal sample. After 5 min at room temperature 50 \mu l of 65% (w/v) sucrose solution was added with bromphenol blue as an albumin and frontmarker. Ten 15-\mu l samples were applied to one slab gel (or 20 samples of 8 \mu l). Electrophoresis was done at 5°–10°C, 25 mA, 5 hr. The gel was stained with amido black and/or dried immediately (infrared lamp, reduced pressure). Since staining of proteins and subsequent washings led to loss of 10%–20% of the \textsuperscript{57}Co-B\textsubscript{12} activity, these steps were usually omitted. Transcobalamin fractions TC II and TC I + III respectively, separated by gel filtration with a G-150 Sephadex column,\textsuperscript{26} were reconstituted and processed as serum samples in PAGE. Ammonium sulfate (2 M) was used to precipitate TC II.\textsuperscript{15} The precipitate, redissolved in and dialyzed against 0.1 M Na-K phosphate buffer pH 7.4, and the reconstituted supernatant containing R binders were treated similarly to the TC fractions separated by gel filtration and applied to PAGE.

Neuraminidase degradation of R binders in serum, tears, and leukocyte lysates with subsequent electrophoresis. Neuraminidase degradation of TC I and III in serum, tears, or leukocytes, as described by Stenman et al.,\textsuperscript{26} was slightly modified: The sample (0.4 ml serum, tears diluted 1:10, or leukocyte lysate) in 5.0 ml neuraminidase buffer (see above) was treated with 0.4 ml neuraminidase (0.2 IU/ml) for 30 min at 37°C. Subsequently, the sample was concentrated by lyophilization or by ultrafiltration over a 10,000 dalton cut off membrane. After reconstitution of the lyophilized samples with 0.8 ml distilled water and saturation with 200 \mu l (1000 pg) radioactive B\textsubscript{12} solution, the samples processed as above were applied to the slab gel and submitted to PAGE.

Autoradiographic evaluation and quantitation of TC II and TC 1 + III. Autoradiography was performed by attaching the dried polyacrylamide gel to an x-ray film (Okum T4, Agfa-Gevaert) for 10 days. Neuraminidase-treated samples were evaluated by densitoscans, thus yielding relative concentra-
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Absolute values of UBBC were determined according to the method of Gottlieb et al. Multiplication of relative values determined by integration and UBBC yielded absolute binding capacities of R binders and of TC II.

Immunochemical determination of unsaturated TC II was based on the precipitation of TC II (saturated with radioactive B12) by insolubilized anti–TC II antiserum (radioimmunosorbent technique).

RESULTS

Identification and quantification of TC II. Serum was separated by gel filtration into three transcobalamin fractions (Fig. 1). The eluted peaks 1, 2, and 3 were quantitated by densitoscanning after PAGE and autoradiography, as shown in the inset of Fig. 1: peak 1 (TC O) was a small, unidentified fraction that eluted in the void volume as described by England et al. and that showed no electrophoretic mobility in PAGE. Peak 2 (fivefold concentrated) was made up of TC I + TC III, and peak 3 contained TC II, which formed three narrow bands and a fourth, diffuse, broader one in PAGE.

PAGE of serum fractions separated by an ammonium sulfate precipitation yielded nearly identical results: The supernatant that contained TC I + III, and the precipitate with TC II, appeared in the same electrophoretic positions. Both experiments showed that TC II and TC I + III overlapped in the transferrin region. This indicated that TC II cannot be separated from TC I + III in PAGE under these conditions.

Neuraminidase removes sialic acid residues from sialoproteins, and as a consequence the electrophoretic mobility of such a molecule is slowed down. This treatment changed the migration distance of the sialoglycoproteins TC I + III but left TC II unchanged (Figs. 2 and 3). The R binders in leukocytes (10⁶ lysed cells/ml) exhibited a similar change in electrophoretic mobility after neuraminidase treatment (Fig. 2) as the R binders in serum (Fig. 3), and the same was demonstrated in tears, which contain a high concentration of R binders and no TC II. For complete degradation of R binders and other sialoproteins in 0.4 ml of serum, 0.08 IU of neuraminidase was required.
A serum sample with elevated TC I + III (TC II of 1200 ng/liter, TC I + III of 2041 ng/liter) was treated with neuraminidase. The sample was (1) incompletely and (2) completely saturated with $^{57}$Co-B$_{12}$ to investigate possible selective binding properties of different TC fractions. Densitoscopy showed that the relative B$_{12}$ binding capacity of the TC fractions remained approximately identical in cases (1) and (2).

Figure 3 presents individual sera selected for increased concentration of TC I, II, or III before (odd numbers) and after (even numbers) neuraminidase treatment.

In Fig. 4 the densitogram of a serum with an elevated TC I + III fraction is evaluated quantitatively: Integration of the radioactive zones, unchanged by neuraminidase treatments, presented a TC II fraction that corresponds to 43% of the B$_{12}$ binding capacity in this sample. However, we know from Fig. 1 and the corresponding experiments that 70(-75)% of TC II moves as discrete bands in a
ISOPROTEIN PATTERNS OF TC II

Fig. 4. Densitogram of PAGE and autoradiogram from serum saturated with radioactive B₁₂ before and after neuraminidase treatment. Arrow, marker (transferrin). Integration is presented as relative percentage of the B₁₂ binding capacity of this sample, which contained 64% TC II and 36% TC I + III as determined by UBBC-ARBC (UBBC, 1597 ng/ml).

region electrophoretically in front of the slower desialylated R binders and that (25–30%) of TC II moves as a diffuse band, which merges with the desialylated R binders in serum. Therefore 43% in Fig. 4 represents only 70(–75)% of the original TC II content of the sample. Thus TC II contributes 43/70 × 100 = 61% and the R binders 100 – 61 = 39% to the UBBC of this particular sample. This calculation was verified by the method of Gilbert,¹⁵ which determines TC II and R binder levels by measuring UBBC and ARBC (acid-resistant B₁₂ binding capacity): UBBC was 1597 ng/liter, made up of 64% TC II and 36% R binders.

Absolute binding capacities (UBBC × percentage TC) of TC II and R binders of normal and abnormal sera (also those shown in Figs. 3 and 6) were similarly determined in 37 serum samples. Measurements with the two methods correlated significantly (Fig. 5; r = 0.96, y = 0.91x + 88.61).

Furthermore, TC II values measured by PAGE of 25 sera were compared with the values of an immunochemical assay (radioimmunosorbent technique) for unsaturated TC II.²⁰ The correlation was significant if the overlapping of 30% TC

![Diagram](image-url)
II with TC I + III in PAGE was taken into account (Fig. 5), with $r = 0.98$, $y = 0.91x + 109.88$.

The paired-sample $t$ test was used to evaluate the differences between the results of (1) electrophoresis and UBBC-ARBC and (2) electrophoresis and radioimmunosorbent technique. There was no significant difference between values obtained in procedures used in (1) or in (2).

In normal quick-spun serum about 90% of the UBBC corresponds to unsaturated TC II.21 Several such sera were evaluated before and after neuraminidase treatment (Fig. 6). As expected, 90% of the B,2 binding fraction showed unchanged electrophoretic mobility in PAGE, since the polypeptide chain of TC II is not degraded by neuraminidase.

Isoprotein patterns of TC II. It was then noted that after neuraminidase treatment the TC II region separated into well-defined bands of different mobility, as shown in the even-numbered samples of Fig. 6. This led to the suspicion that several isoproteins of TC II might exist. Quick-spun EDTA plasma and quick-spun

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**Table 1. TC II Isoprotein Patterns in 10 Families**

<table>
<thead>
<tr>
<th>Large families</th>
<th>Father</th>
<th>Mother</th>
<th>Children*</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Heterozygote</td>
<td>Homozygote</td>
<td>Heterozygote</td>
</tr>
<tr>
<td>1</td>
<td>1-3</td>
<td>1-3</td>
<td>4</td>
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<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1-1</td>
<td>3-3</td>
<td>4</td>
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</tbody>
</table>

Families with rare alleles

| 7              | 3-3 | 3-4 | 1 | 3-4 |
| 8              | 1-3 | 3-4 | 1 | 1-4 |
| 9              | 1-2 | 1-3 | 5 | 1-3 2-3 3+1 1 |
| 10             | 1-5 | 1-1 | 1 | 1-5 |

* Data support autosomal inheritance according to the mendelian law.

TC II types in italics represent rare alleles.
ISOPROTEIN PATTERNS OF TC II

serum samples taken simultaneously from the same blood donor yielded identical results. EDTA blood allowed to stand overnight and supplemented with LiCl exhibited—as expected—a considerable increase of the TC I + III fraction, degradable by neuraminidase.

Sera of five patients with various clinical disorders (acute infection, inguinal hernia, candidiasis, malabsorption, and hemolytic anemia) were tested during active disease with treatment. The TC II patterns were compared to data obtained at least 6 mo later, when patients were in a state of clinical health without therapy. No change was noted. A second group of five patients with malignant diseases and ten patients with lupus erythematosus under immunosuppressive and antibiotic

Fig. 7. Sera from three families were separated by PAGE as described in Materials and Methods. (A) Serum of the daughter is shown in 1 and 2, the mother in 3 and 4, and the father in 5 and 6, each before and after neuraminidase treatment (family 1 in Table 1). (B) Neuraminidase-treated sera of the father (no. 1), mother (no. 2), and five children (nos. 3–7) (family 5 in Table 1). (C) Neuraminidase-treated sera of the father (no. 5), mother (no. 6), and four children (nos. 1–4) (family 1 in Table 1). A, albumin as in Fig. 2.
treatment in active and inactive phases of disease showed no change of the position of the TC II bands for each individual. Only the intensity of bands differed according to TC II serum levels.30,31

An uncommon TC II pattern (Fig. 7) consisting of four TC II bands was found in a father and his daughter. Three blood samples of the child, taken at monthly intervals, demonstrated that the four-banded pattern was reproducible.

Observations in ten families, including four with rare TC II patterns, are compatible with the assumption of isoprotein patterns presenting polymorphic variants of TC II (Table 1 and Fig. 7). Ten different variants were observed in 510 nonrelated individuals (Figs. 8 and 9). All of the observed variants are compatible with a five-allele system. The corresponding tentative allele frequencies are shown in Table 2. Three variants are frequent in a heterogenous European population: TC II 3-3 occurs in 38%, TC II 1-3 in 41%, and TC II 1-1 in 16% of the population.

**Homozgygotes**

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| origin | 1-1  | 2-2  | 3-3  | 4-4  | (5-5) |
```

**Heterozygotes**

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| 1-2  | 1-3  | 2-3  | 1-4  | 1-5  | 3-4  |
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Fig. 9. Schematic survey demonstrating ten different variants of TC II, observed in 510 unrelated individuals. Division into homozgygous and heterozygous types was made on the basis of family studies (Table 1). TC II** has not yet been observed in the homozygous form.
Table 2. A Comparison of the Observed and Expected Distribution of Transcobalamin II Phenotypes in 510 Nonrelated Individuals

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Observed</th>
<th>Percentage</th>
<th>Expected</th>
<th>Percentage</th>
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<td>15.7</td>
<td>73.6</td>
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<td>0.1</td>
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<td>3-3</td>
<td>191</td>
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<td>177.5</td>
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<td>4-4</td>
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<td>0.09</td>
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<tr>
<td>5-5</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>-</td>
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<td>0.2</td>
</tr>
<tr>
<td>4-5</td>
<td>-</td>
<td>-</td>
<td>0.03</td>
<td>0.005</td>
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<td>510</td>
<td>100.2</td>
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*Calculation on the basis of the Hardy Weinberg equilibrium observed allele frequencies: TCII1, 0.590; TCII2, 0.380; TCII3, 0.017; TCII4, 0.013; TCII5, 0.002.

DISCUSSION

Identification and Quantification of TC II

We described an electrophoretic-autoradiographic method for separation of TC II and R binders. PAGE-autoradiography clearly separates TC II into several zones of different electrophoretic mobility (Figs. 1, 3, 4, and 6–8). That the bands obtained are indeed TC II is proven by the following findings:

1. Figure 5 demonstrates that very similar results are obtained in selected samples with a wide range of TC II concentrations, using (a) a method based on different pH-dependent B12 binding properties of TC I + III and TC II,15 (b) an immunochemical determination of TC II,26 and (c) the electrophoretic-autoradiographic transcobalamin separation reported here. This indicates that the discrete bands actually correspond to TC II.

2. Neuraminidase treatment has no effect on the electrophoretic mobility of the TC II bands, but it clearly changes the mobility of the TC I and III fractions (Figs. 2, 3, and 6). This indicates that TC II is free of sialic acid, which is in accordance with the results reported by Allen and Majerus4 and Stenman et al.26

3. The third factor that confirms that we are really looking at multiple forms of TC II is demonstrated in Fig. 1, where a TC II fraction from a Sephadex G-150 gel filtration was separated electrophoretically and was shown to contain three discrete bands and one broad band. The TC II–containing ammonium sulfate precipitate yielded nearly identical results. Neuraminidase treatment of these fractions was without effect on the electrophoretic pattern and again confirms that we are actually dealing with TC II and not with either TC I or TC III. These data probably confirm the observations made by Daiger et al.,32 who recently reported the occurrence of similar isoprotein patterns of TC II after ammonium sulfate fractionation of serum.
The relative percentage of B₁₂ binding fractions remains approximately identical when bound radioactive B₁₂ is either less than or equal to the UBBC of the sample. Thus we have shown that the B₁₂ binding properties of various TC fractions in serum are indistinguishable in our system.

**Isoprotein Patterns of TC II.** In contrast to the previously described disease-dependent multiple forms of TC I and III, obtained by isoelectric focusing, the multiple forms of TC II reflect characteristic person-to-person differences that are not influenced by disease. Normal sera (Figs. 6 and 7) exhibit consistent and repeatedly observed differences in their TC II patterns.

Family studies (Fig. 7 and Table 1) demonstrate that homozygotes exhibit two and heterozygotes two + two isoproteins, yielding three- (when overlapping) or four-banded patterns. The fact that the distance between the two bands of the various homozygotes is identical (Figs. 8 and 9) suggests that one-point mutation might be responsible for changing the electrophoretic mobility of both components simultaneously. This observation indicates that the two forms of TC II might represent a postsynthetic alteration. The relationship of the double bands has not yet been evaluated.

According to the IUPAC-IUB nomenclature recommendations of 1976–77 the term isoprotein can be applied to multiple forms of a protein arising from genetically determined differences in primary structure.

The term isoprotein is applicable to the multiple forms of TC II because data from family studies (Fig. 7 and Table 1) support genetic determination of the multiple TC II patterns. The diffuse TC II fraction (25%–30% TC II) with reduced electrophoretic mobility, which seems to be a regular component of all serum samples tested, as indicated by the quantitative evaluation of electrophoretic data (Fig. 5), apparently does not contribute to genetic variation.

Evidence presented by our family studies, confirmed in additional extensive investigations to be reported elsewhere, indicates that the different TC II isoprotein patterns described above belong to a new system of human genetic polymorphism. These patterns may be identical to the TC II polymorphic system reported by Daiger et al.

The electrophoretic and enzymatic procedure presented here can be used to quantitate unsaturated TC II and TC I + III in serum. The results are comparable to those of other methods. This separation procedure reveals isoprotein patterns of TC II and provides information concerning genetic variation of TC II in human serum.

**ACKNOWLEDGMENT**

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Studies on transcobalamin (TC). 1. Detection of TC II isoproteins in human serum

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