Measurement of Vitamin B₁₂-binding Proteins of Plasma. II. Interpretation of Patterns in Disease

By James A. Begley and Charles A. Hall

The technique described in the preceding paper was applied to 12 abnormal sera selected for their increase in one or more B₁₂-binding proteins. Even in the presence of large amounts of R-type binder, the ammonium sulfate technique gave a reliable separation of R binding proteins from TC II. Measurement of the TC II in abnormal sera gave results identical to those obtained by the more standard gel filtration. The R binders of four subjects with myeloproliferative disease were further separated into α₂-R and α₁-R. The pattern of B₁₂ binding of polycythemia vera (PV) was an exaggeration of the normal pattern. Binding to α₂-R was three to four times that to α₁-R, although the total amounts bound to both were increased. In chronic myelogenous leukemia (CML), both α₂-R and α₁-R were also increased, but in contrast to binding in normal sera, α₁-R predominated. In order to interpret the findings, either whole serum R or α₁-R and α₂-R from patients with myeloproliferative disease were subject to isoelectric focusing. α₁-R consisted principally of components isoelectric at pH 2.9, 3.0, and 3.1. These components were present in only minor amounts in normal serum and were somewhat increased in the serum of PV. These components were very much increased in the serum of CML and predominated. α₂-R consisted of those components isoelectric at pH 3.4, 3.6, and 4.0. These components predominated in the unsaturated binding capacity of normal sera and that of PV. It was concluded that the division of plasma R binders into α₁-R and α₂-R by the technique described provided information useful in the study of myeloproliferative diseases.

THE FOLLOWING is an application and interpretation of the technique described and evaluated in the preceding paper.

RESULTS

Sera from 12 patients with various types and stages of myeloproliferative disease were studied by steps 1 and 2a as applied to normal sera in Part I. As shown in Fig. 1, the technique gave TC II levels close to those of parallel gel filtration.

When the precipitate of the (NH₄)₂SO₄, TC II, was examined by gel filtration there was a 10⁺₀ ± 8⁺₀ contamination with plasma R. This value would seem quite acceptable in the presence of such large amounts of plasma R. The values for plasma R as determined by both methods are shown in Fig. 2. The (NH₄)₂SO₄ method performed well over a wide range of plasma R; the expected high levels of R in myeloproliferative states were observed.

Four abnormal sera were carried through the entire technique, steps 1, 2a, and 3. The results are shown in Table 1. Combining the data from Fig. 2 and

From the Hematology Research Laboratory, Veterans Administration Hospital and the Department of Medicine, Albany Medical College of Union University, Albany, N. Y. 12208.
Submitted June 18, 1974; accepted August 8, 1974.
Supported in part by V. A. research project number 3390-01.
Address for reprint requests: James A. Begley, Research Service (151E), Veterans Administration Hospital, Albany, N. Y. 12208.
"1975 by Grune & Stratton, Inc.

Blood, Vol. 45, No. 2 (February), 1975 287
Fig. 1. Comparison of the TC II UBBCs obtained by (NH₄)₂SO₄ precipitation and gel filtration (steps 2a and b, Fig. 1, Part I) for 12 abnormal sera. The line is drawn as if correlation were perfect.

Fig. 2. Comparison of the plasma R UBBCs obtained by (NH₄)₂SO₄ precipitation and gel filtration (steps 2a and b, Fig. 1, Part I) for 12 abnormal sera.

Table 1. Binding Capacities Expressed as Picograms $^{57}$Co B₁₂ per Milliliter of Serum

<table>
<thead>
<tr>
<th>Subject</th>
<th>UBBC</th>
<th>TC II</th>
<th>Total R</th>
<th>$\sigma_2$-R</th>
<th>$\sigma_1$-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± 1 SD of 10 normals</td>
<td>1092 ± 211</td>
<td>969 ± 204</td>
<td>167 ± 48</td>
<td>127 ± 42</td>
<td>40 ± 12</td>
</tr>
<tr>
<td>G.W. (PV*)</td>
<td>1305</td>
<td>666</td>
<td>853</td>
<td>694</td>
<td>159</td>
</tr>
<tr>
<td>P.L. (PV1)</td>
<td>4224</td>
<td>396</td>
<td>4396</td>
<td>3270</td>
<td>1126</td>
</tr>
<tr>
<td>T.K. (CML*)</td>
<td>1876</td>
<td>792</td>
<td>1155</td>
<td>463</td>
<td>694</td>
</tr>
<tr>
<td>R.B. (CML)</td>
<td>6072</td>
<td>819</td>
<td>5085</td>
<td>2022</td>
<td>3063</td>
</tr>
</tbody>
</table>

* Treated.
† Some features of myeloid metaplasia.
Fig. 3. Isoelectric focusing (IEF) of total R from normal serum. The cells were removed from the plasma by immediate centrifugation before clotting took place, and then the serum was allowed to clot and the fibrin removed. $^{57}Co B_{12}$ was added to the whole serum to capacity.

Table 1, it appears that total R was increased in myeloproliferative states, and both components participated in the increase in all variants of the group. In chronic myeloid leukemia $\alpha_1$-R predominated over $\alpha_2$-R. In polycythemia vera $\alpha_2$-R predominated, more of an exaggeration of the normal pattern.

The products of the fractionations were examined by isoelectric focusing (IEF). In all instances either the total R, as isolated by (NH$_4$)$_2$SO$_4$, or the $\alpha_1$ or $\alpha_2$ components of R were focused. Figure 3 shows the IEF of plasma R, labeled to capacity, from a normal subject. The main components were isoelectric at pH 3.57 and 3.38 with lesser components at pH 3.12 and 4.01 and a small peak at pH 2.89. Thirteen focussings of various sera showed six consistent components, a–f, isoelectric at the respective pHs: a, 2.9; b, 3.0; c, 3.1; d, 3.4; e, 3.6; and f, 4.0. Components c, d, and e were always present, with d predominating in the R of normal serum. Components a and b were either small or absent from normal serum. When pools of normal $\alpha_1$-R and $\alpha_2$-R were studied by focusing (Part I), components a, b, and c were in $\alpha_1$-R and d, e, and f in $\alpha_2$-R.

Figure 4 shows at the top the IEF of the total R from subject R.B. with CML. Components d and e were increased but remained in the same proportion as in normal serum. The greatest deviations from normal were in peaks a, b, and c, especially a and b. When the $\alpha_2$-R was focused (Fig. 4), components a, b, and c were shown to be absent for the most part, having been retained in the DEAE cellulose. Components a and b-c combined were eluted with the strong buffer to form $\alpha_1$-R. Some of components d and e which should have been eluted to form $\alpha_2$-R had not been removed by the 0.075 $M$ buffer and were contaminants of the $\alpha_1$-R. The component focusing at a pH of above 6 probably represented free B$_{12}$, since B$_{12}$ alone was found at this point upon focusing.

The $\alpha_2$-R of a sample of PV serum consisted principally of components d and e with some f, while the $\alpha_1$-R held a and c plus 5%–10% of the total e–f. Combining all of the observations, it would appear that absorption of plasma R to DEAE-cellulose with subsequent elution with phosphate buffer 0.075 $M$, pH 6.2, left in the DEAE those components of pI of 2.9, 3.0, and 3.1. The eluted
The serum from subject R.R., Table 1, was labeled at 5000 pg B12 per ml. The pH gradient of the studies of a2-R and a1-R are not illustrated; they were almost identical to that of the top. Because of differences in size of sample and losses in processing, the IEF of total R can be compared only semiquantitatively with the IEF of a1-R and a2-R. a1-R and a2-R can, however, be compared quantitatively as well as qualitatively. It should be noted that the total plasma R of this patient was 60% a1-R and 40% a2-R (Table 1).

**DISCUSSION**

The entire evaluation of plasma R in myeloproliferative states is pointless unless it can be related to events and conditions in vivo.

Mendelsohn et al.\(^1\) showed that the natural binder of most of the B12 in blood had a\(_1\) mobility, although a\(_2\) binding was almost equal to a\(_1\) in one of his three cases and was substantial in another. The B12 in these studies became attached to the binder through normal metabolism, but when B12 was added to serum in vitro to detect the unsaturated portion of the binder, a\(_1\) binding was greater than a\(_2\).\(^2,3\) This concept was challenged by Gizis et al.\(^4\) who, by a technique different from those used previously, showed that the dominant unsaturated R binding was to a protein of a\(_2\) mobility. Moreover, in an unreported study of unsaturated R binding in six normal subjects by a technique similar to that of Gizis et al., we found a mean a\(_2\)-R binding of 130 pg/ml and a\(_1\)-R binding of 39 pg/ml. The studies reported here confirm that distribution and show that with IEF of normal serum there was little unsaturated R binding isoelectric at pH less than 3.4. Probably these changes in observation of R binders are a direct consequence of development of superior methods of separa-
tion of proteins that differ in charge. It must be realized that techniques based on $^{57}$CoB$_{12}$ added in vitro do not measure binding of the native B$_{12}$ of plasma. It may be that once current techniques of analysis can be adapted to permit measurement of unlabeled B$_{12}$ by bioassay or isotope-dilution assay other facts will emerge.

The increase in B$_{12}$ binding of either endogenous or added B$_{12}$ seen in chronic myeloid leukemia has always been considered to be a consequence of an increase in the same R binder dominant in normal serum. This concept, too, seems challenged by developments in techniques. The data of Table 1 show that whereas there was in increase in binding to $\alpha_2$-R, the major deviation in binding pattern from normal was an increased binding of B$_{12}$ to $\alpha_1$-R. The abnormality was better illustrated by the IEF which shows the prominence of the R components more acidic than pI 3.4 (Fig. 4). The explanation and source of these components are unknown. Stenman et al. postulated that these $\alpha_1$ components are created when sialic acid is added to the $\alpha_2$-R binder of leukocytes by a mechanism yet to be observed. It is interesting that R components isoelectric at less than pH 3.3-3.4 are uncommon or minor in all R binders studied to date. Natori found a lesser component of pI 2.9 in both normal and leukemic leukocytes with a similar distribution of the components of R binders in cells of both origins. We found a consistent distribution of the added $^{57}$Co B$_{12}$ in the IEF of the binding protein of granulocytes from two normal subjects, two with CML in relapse, and one with a leukemoid reaction. There was only trace labeling of substances isoelectric at pH less than 3.3. It should be noted that the gradient used by Natori did not extend as adequately into the low pH range as the one used here. One IEF of normal granulocyte R is shown in Fig. 5. Granulocytes seem, then, to be an unlikely source of $\alpha_1$-R unless, as Stenman et al. postulated, sialic acid is added to their $\alpha_2$-R as it is excreted.

The pattern of unsaturated R binding in polycythemia vera (PV) resembled that of normal serum R but with greatly increased amounts of binding; in other words, $\alpha_2$-R binding predominated. This observation was not surprising, since the increased binding protein in PV sera was known to have $\alpha_2$ mobility.

R binders differ from each other in carbohydrate constituents with the ex-
Table 2. R Binders in Disease*

<table>
<thead>
<tr>
<th>Condition</th>
<th>TC II</th>
<th>R</th>
<th>α2-R</th>
<th>α1-R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombocytaphagia</td>
<td>1600</td>
<td>833</td>
<td>551</td>
<td>394</td>
</tr>
<tr>
<td>Polycythemia due to hepatoma</td>
<td>1665</td>
<td>1451</td>
<td>172</td>
<td>141</td>
</tr>
<tr>
<td>Myeloproliferative state and hemorrhage</td>
<td>3286</td>
<td>2179</td>
<td>919</td>
<td>687</td>
</tr>
<tr>
<td>Leukemoid reaction from carcinoma-WBC &gt; 100,000</td>
<td>4186</td>
<td>1541</td>
<td>2029</td>
<td>1294</td>
</tr>
<tr>
<td>Severe myelofibrosis but scant normal hematopoiesis</td>
<td>855</td>
<td>630</td>
<td>104</td>
<td>104</td>
</tr>
<tr>
<td>Myeloid metaplasia—cellular marrow with little fibrosis</td>
<td>2376</td>
<td>1747</td>
<td>594</td>
<td>371</td>
</tr>
<tr>
<td>Myelophthisic from carcinoma—peripheral myeloblasts</td>
<td>2087</td>
<td>1921</td>
<td>144</td>
<td>86</td>
</tr>
<tr>
<td>Acute myeloblastic leukemia</td>
<td>2502</td>
<td>1786</td>
<td>732</td>
<td>277</td>
</tr>
</tbody>
</table>

*Binding capacities expressed as pg $^{57}$CO B$_{12}$ per ml of serum and measured by simplified protocol of Part I.

pression of these differences in charge. Individually they show isoelectric heterogeneity.$^{6,9}$ Isoelectric focusing is then a superior means of analyzing these proteins but is too complex and time consuming for widespread and routine use. The technique presented here gave a separation of plasma R into $\alpha_1$ and $\alpha_2$ components which reflected well the patterns of plasma R observed by IEF. Should biological facts yet to be uncovered so suggest, a buffer other than 0.075 M might divide the plasma R at a more appropriate place. The use of two or more buffers might even give a separation of plasma R more closely resembling the IEF pattern. For the present it seems advisable to apply the technique used here in the exploration of binding patterns in the myeloproliferative states, leukemoid reactions, polycythemias, and other states where the B$_{12}$ binding pattern is disturbed. Some such uses are illustrated in Table 2, and practical applications become apparent.

Little has been said here about the measurement of TC II, a primary objective of the study. Precipitation by (NH$_4$)$_2$SO$_4$ proved to be a good measure of TC II in the blood of normal subjects and of those with abnormal binding patterns. The term transcobalamln I (TC I) was avoided in the present discussion. In light of recent observations some revision of its definition may be necessary, but it would be premature to do so at this time.

REFERENCES

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