Vitamin B\textsubscript{12}-binding Protein of Leukocytes as a Possible Major Source of the Third Vitamin B\textsubscript{12}-binding Protein of Serum

By Ralph Carmel and Victor Herbert

Much evidence suggests that granulocytes are a major source of serum vitamin B\textsubscript{12}-binding protein (BBP). The latter has three components: \(\alpha\)-1-globulin BBP (Transcobalamin I, TC I), \(\beta\)-globulin BBP (TC II), and a recently described third BBP. Granulocytic BBP has appeared to be identical to TC I except in electrophoretic mobility. In the present study, the dominant BBP of leukocyte extracts from subjects with and without myeloproliferative disease behaved like the third serum BBP. With a few exceptions, more than half the leukocytic binder eluted with the "\(\beta\) globulins" on rapid DEAE-cellulose chromatography. At pH 8.6, electrophoretic mobility of the leukocytic BBP was always \(\alpha_2\) or \(\beta\). At pH 4.5, normal and chronic myelogenous leukemia leukocytic BBP, unlike TC I and TC II, showed little electrophoretic migration. These findings suggest that leukocytic BBP is probably heterogeneous and that its major component resembles the third serum BBP more than it does TC I. The third serum BBP, levels of which are elevated in some states of leukocytic proliferation, may derive directly from mature granulocytes. TC I may arise by addition of sialic acid to the third (granulocytic) BBP under certain circumstances or be released from other cells, such as less mature granulocytes. Much of the confusion in the literature regarding source and significance of serum BBP may relate to separating it into only two fractions (\(\alpha\) globulin and "\(\beta\) globulin," or "TC I" and TC II) instead of into three fractions.

THREE VITAMIN B\textsubscript{12}-BINDING PROTEINS (BBP) are presently identifiable in serum: "Transcobalamin (TC) I," or \(\alpha\)-1-globulin BBP; TC II, or "\(\beta\)-globulin BBP,"\(^{1}\) and a third BBP that shares different characteristics with each of the other two.\(^{4-9}\) The latter has been shown to elute with the "\(\beta\)-globulin" fraction on rapid chromatography or batch separation with DEAE-cellulose.\(^{3,8-10}\)

From the MRC Experimental Hematology Unit, St. Mary's Hospital Medical School, London, England; The Mount Sinai School of Medicine, New York, N.Y.; The Veterans Administration Hospital, Bronx, N.Y.; and Columbia University College of Physicians & Surgeons, New York, N.Y.

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Ralph Carmel, M.D.: Assistant Professor of Medicine, Department of Medicine, Wayne State University School of Medicine, Detroit, Mich.; formerly held a Wellcome Research Fellowship, and was supported during the beginning of this study by USPHS Post-doctoral Research Fellowship 5-F2-AM-32, 518-02. Victor Herbert, M.D.: Clinical Professor of Pathology, Columbia University College of Physicians and Surgeons, New York, N.Y., and Medical Investigator, Veterans Administration Hospital, Bronx, N.Y.; recipient of Health Research Council of the City of New York Career Scientist Award 1-683, and a Veterans Administration Medical Investigatorship for the support of this study.
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While the source of TC II is unknown, “TC I” appears to be closely related to granulocytes. “TC I” levels are greatly elevated in chronic myelogenous leukemia and other myeloproliferative disorders,¹ ¹⁰ ¹¹ and in some cases of leukocytosis.¹ ¹² ¹³ Experiments have suggested that “TC I” may be synthesized¹⁴ and secreted¹⁵ by granulocytes. Granulocytic BBP resembles “TC I” immunologically,¹⁶ ¹⁷ ¹⁸ in molecular weight,¹⁴ ¹⁷ ¹⁸ in behavior on CM-cellulose chromatography,¹⁸ and in transferring vitamin B₁₂ poorly to reticulocytes¹⁷ and HeLa cells.¹⁹ The only difference has been in electrophoretic mobility at pH 8.6,¹⁸ which has been attributed to possible sialic acid content difference.

The third serum BBP is elevated in polycythemia vera (and had been designated “PV” binder⁴) and in most cases of leukocytosis.⁸ Since its α₂-β electrophoretic mobility at pH 8.6⁴ ⁵ ⁸ more closely resembles that of granulocytic BBP than does “TC I,” and since it resembles both the latter binders in all the other characteristics listed above,⁴ ⁸ the possibility arose that in fact granulocytic BBP is identical to, and granulocytes may be the source of, third BBP. This report presents further evidence that granulocytic BBP may be a major source of the third BBP of serum.

In this report, quotation marks around “TC I” and “β globulin” indicate that they contain the third binder, as well as TC I and β-globulin B₁₂-binding protein, respectively.

MATERIALS AND METHODS

The 16 subjects included four without hematologic disease, two of whom were healthy volunteers. Leukocytes were obtained, and extracts were prepared as described previously²⁰ according to the method of Gilbert et al.²¹ Leukocytes counts of the suspensions, obtained just before lysis and sonication, ranged from 18 to 425 × 10⁶/ml. The great majority of leukocytes in each case were mature granulocytes, though some contaminating mononuclear cells were present. The latter, however, contain little BBP.¹⁴ ¹⁷ ²² ²³ The extracts were stored at −20°C. Thawing and refreezing had little effect on binding capacity. Their vitamin B₁₂-binding capacity and, when possible, that of concurrent sera from the subjects was determined by coated charcoal radioassay.²⁴

After excess radioactive cyanocobalamin (⁵⁷CoB₁₂) had been added (“excess” determined by exceeding binding capacity by at least 20%) and final volume brought to 1–2 ml with 0.9% NaCl, the extracts were dialyzed overnight at room temperature in Visking casing against 0.02 M phosphate buffer, pH 6.3. Separation into α-globulin and “β-globulin” fractions was then done by rapid DEAE-cellulose chromatography.³

Three extracts were saturated with ⁵⁷CoB₁₂ for 1 hr and filtered through Sephadex G-200 gel, using 0.1 M Tris buffer, pH 8.5, containing 1 M NaCl. The single, high-molecular weight peak of one of these extracts was subsequently dialyzed and chromatographed on DEAE-cellulose as above.

Several extracts were saturated with excess ⁵⁷CoB₁₂ (specific activity of 15–19 [Merck, Sharp and Dohme Research Lab.] or of 118 µCi/µg [The Radiochemical Centre, Amersham, England]). In most cases, the free ⁵⁷CoB₁₂ was not removed. Electrophoresis was done on cellulose acetate in barbital buffer, pH 8.6. The strips were exposed, unstained, to x-ray film for 6 wk, or for 3 wk if concentrated samples or the highest specific activity ⁵⁷CoB₁₂ were used.

In three separate experiments, 0.2 ml aliquots of extract were applied, alongside various serum samples, to 14 × 18 × 0.8 cm Pevikon (Pevikon C-870, Shandon Scientific, London, England) blocks. The specimens had been previously incubated with ⁵⁷CoB₁₂ (specific activity 118 µCi/µg), free ⁵⁷CoB₁₂ being removed with coated charcoal pellets, and bromphenol blue added as a marker. Electrophoresis was done in acetate-sodium chloride buffer, pH 4.5, ionic strength 0.1,²⁵ at 38 mA for 18 hr at 6°C. The block was cut into 1 cm
segments, whose radioactivity was counted. Distal anodal and cathodal segments were used as control blanks.

RESULTS

Fractionation of leukocytic BBP by rapid DEAE-cellulose chromatography showed that, in most cases, more than half the BBP eluted with "β-globulin" fraction (Table 1). The exceptions were both patients with chronic myelogenous leukemia, one of the two with myeloid metaplasia, and the Sephadex-filtered sample from one healthy subject. While the mean (±1 standard deviation) "β-globulin" fraction of the chronic myelogenous leukemia leukocyte BBP was only 21%, that of patients without hematologic disease was 55% (±27) and of polycythemic patients was 64% (±9). The two subjects with myeloid metaplasia had divergent results, though their serum patterns on DEAE-cellulose were virtually identical (Table 1). Even so, there was significant correlation ($r = 0.63; p < 0.05$) between the leukocyte and serum BBP "β-globulin" fraction levels in the entire series.

Leukocytic vitamin B₁₂-binding capacity did not correlate with diagnosis, serum vitamin B₁₂-binding capacity, or any other BBP parameter.

Table 1. Vitamin B₁₂-Binding Capacities of Subjects' Leukocyte Extracts and Sera and Their Fractionation by Rapid DEAE-Cellulose Chromatography

<table>
<thead>
<tr>
<th>Subject</th>
<th>Leukocyte</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α⁻-Globulin (ng/10⁸ cells)</td>
<td>α⁻-Globulin (pg/ml)</td>
</tr>
<tr>
<td>Without hematologic disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1†</td>
<td>2.6</td>
<td>2248</td>
</tr>
<tr>
<td>2†</td>
<td>16.3 (78)</td>
<td>721</td>
</tr>
<tr>
<td>3</td>
<td>36.6</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>5.8</td>
<td>—</td>
</tr>
<tr>
<td>Polycythemia vera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17.7</td>
<td>3512</td>
</tr>
<tr>
<td>6</td>
<td>22.1</td>
<td>1942</td>
</tr>
<tr>
<td>7</td>
<td>21.1</td>
<td>2134</td>
</tr>
<tr>
<td>8</td>
<td>20.9</td>
<td>1808</td>
</tr>
<tr>
<td>9</td>
<td>35.2</td>
<td>2366</td>
</tr>
<tr>
<td>10</td>
<td>20.4</td>
<td>—</td>
</tr>
<tr>
<td>11</td>
<td>15.1</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>20.9</td>
<td>—</td>
</tr>
<tr>
<td>Chronic myelogenous leukemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>19.8</td>
<td>4640</td>
</tr>
<tr>
<td>14</td>
<td>16.0</td>
<td>4298</td>
</tr>
<tr>
<td>Myeloid metaplasia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>64.0</td>
<td>3668</td>
</tr>
<tr>
<td>16</td>
<td>23.9</td>
<td>2218</td>
</tr>
<tr>
<td>Mean ± 1 SD</td>
<td>22.4 ± 14.0</td>
<td>46 ± 21</td>
</tr>
<tr>
<td>Normal mean ± 1 SD</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* UBBC, unsaturated vitamin B₁₂-binding capacity.
† Subject is a healthy volunteer.
‡ Results of DEAE-cellulose fractionation of the single, vitamin B₁₂-binding peak obtained from Sephadex G-200 gel filtration.
Fig. 1. Radioautograph of cellulose acetate electrophoresis at pH 8.6 of two leukocyte extracts labeled with $^{57}$CoB$_{12}$. Note slightly faster mobility of leukemic leukocyte BBP, as compared to $\beta$ mobility of leukocytic BBP of subject without hematologic disease.

The BBP of leukocytes from both healthy subjects and a patient with chronic myelogenous leukemia was homogeneous on Sephadex gel filtration, despite heterogeneity on DEAE-cellulose fractionation (18%–83% "$\beta$-globulin").

Electrophoresis at pH 8.6 showed leukocytic BBP to have the same $\beta$-globulin mobility as transferrin (sera of two subjects without hematologic disease) or slightly faster (sera of one patient with polycythemia vera and three with chronic myelogenous leukemia) (Fig. 1). At pH 4.5, normal leukocyte BBP showed virtually no migration, whereas TC I migrated anodally and TC II cathodally (Fig. 2). Thus, normal leukocytic BBP resembled "PV binder," the third serum BBP that predominates in polycythemia vera serum. Chronic myelogenous leukemia leukocyte BBP behaved similarly, although in one of the three experiments it moved slightly more anodally, at pH 4.5.

DISCUSSION

With 0.06 M phosphate buffer, pH 6.3, DEAE-cellulose separates, among other protein, $\beta$-globulins from $\alpha$-1 globulins. This separates two serum vitamin B$_{12}$ binders, but the third BBP is generally also eluted with the "$\beta$-globulin" fraction. There is no evidence that granulocytes contain TC II, although it is possible the latter is a monomeric variant of TC I or of
granulocytic BBP. None of the leukocytic BBP was of the low molecular weight of TC II (32,000–36,000). In this context, elution of more than half the leukocytic BBP with "β-globulin" in most cases is significant. Gilbert has recently suggested that the third Bs-binding protein may also be easily eluted on DEAE-cellulose chromatography of white cell lysates by addition to the lysate of a carrier protein, rather than by the overnight dialysis vs. buffer that we used. Either procedure would correct for low ionic strength.

Therefore, the findings indicate that the bulk of leukocytic BBP is identical or very similar to the third BBP of serum. This conclusion is further supported by electrophoretic results at pH 8.6 (confirming a previous report of mobility different from TC I) and at pH 4.5, at which mobility unlike that of either TC I or TC II occurred.

Identity of the third serum BBP with leukocytic BBP would be consistent with the elution with "β-globulin" on rapid DEAE-cellulose chromatography of the serum "R" binder of Grässbeck, which is the serum equivalent of leukocytic BBP, and would fit with the characteristics of the "R" binder that led Simons to speculate initially that "R" binder was identical with TC II. As previously suggested and as supported by the third binder's immunologic identity with other "R" binders, the third BBP appears to be the serum...
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“R” binder. While TC I may be a variant, it does not appear to be, in strict terms, the latter binder.

The significant correlation between the DEAE-cellulose “β-globulin” BBP fraction levels of leukocytes and serum among our subjects may reflect release of third BBP into serum by leukocytes. This possibility does not invalidate previous reports suggesting leukocytes synthesize¹⁴ and secrete¹⁵ serum “TC I,” as the reports’ characterization of the protein as “TC I” could apply equally to third BBP. If leukocytes are indeed the source of both TC I and the third BBP, some transformation of the protein must occur inside or outside the cell, as previously postulated.¹⁸ Alternatively, only certain cells, perhaps the more immature ones, may contain TC I, the rest containing third BBP.

Chronic myelogenous leukemia leukocytes, unlike normal leukocytes, had high α-globulin and low “β-globulin” BBP fractions on DEAE-cellulose separation, paralleling their serum fractionation patterns. Surprisingly, however, although their electrophoretic behavior at both pH levels may have been slightly more like TC I than was that of normal leukocyte BBP, their mobility remains clearly more similar to that of third BBP. It is uncertain whether the slight difference in mobility at pH 8.6 between the two normal and four abnormal leukocyte extracts, as exemplified in Fig. 1, is coincidental or not. Stenman et al.¹⁸ found no difference between leukemic and normal leukocyte BBP. It is possible that some of the electrophoretic differences observed here may be due to in vitro changes, as shown by the effect chromatographic purification or even simply prolonged incubation at 37°C can have on leukocytic BBP mobility at pH 8.6.¹⁸ However, this is unlikely to affect the conclusion that leukocytic BBP is identical to third BBP of serum.

The fact that leukocytic BBP is neither purely α globulin nor “β globulin” on DEAE-cellulose chromatography and the slight electrophoretic variations suggest that the protein is not completely homogeneous. Such is also suggested by its behavior on CM-Sephadex¹⁸ and biphasic binding curve.²² Heterogeneity has been demonstrated in “R” binders from other sources²³–²⁸ and may be due to sialic acid content differences. Indeed TC I and “R” binder may mark the extreme ends of a spectrum of isoelectric variants of a single protein.

The present findings support the concept that leukocytes are a major source of serum BBP. Third BBP is elevated in leukocytosis⁸ and is probably identical with “PV binder.”⁴ It may also be the elevated “β-globulin” BBP of myeloproliferative disorders,¹⁰ though this awaits further study. It is of historic interest that the earliest studies of serum vitamin B₁₂ binders located them mainly in the α₁ and α₂ globulins.³²–³⁴ Much of the confusion in the literature regarding source and significance of serum BBP may relate to separating it chromatographically into only two fractions instead of three.

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