Cobalamin (Vitamin B₁₂) and B₁₂ Binding Proteins in Hypereosinophilic Syndromes and Secondary Eosinophilia

By Jacqueline Zittoun, Jean Pierre Farcet, Jeanine Marquet, Claude Sultan, and Robert Zittoun

Serum cobalamin (vitamin B₁₂) and unsaturated B₁₂ binding capacity (UBBC) have been measured in 24 cases of hypereosinophilia: 16 were cases of hypereosinophilic syndrome (HES) and 8 of secondary eosinophilia. The two groups were similar with respect to absolute eosinophil counts. Serum cobalamin and UBBC were found to be markedly increased in most cases of HES and normal in secondary eosinophilia. This elevation of UBBC was mainly related to the increase of R binders (transcobalamins I and III). The elevated serum cobalamin and R binders in HES were due neither to a higher intracellular content of R binders nor to an increased release of these binders from eosinophils of HES. Pure fractions of eosinophils obtained from HES and secondary eosinophilia did not exhibit any difference in vitamin B₁₂ binders. On the other hand, neutrophil-rich fractions from the same patients showed a higher content of intracellular B₁₂ binding proteins than pure eosinophil fractions, irrespective of the cause of eosinophilia. These findings suggest that the increased serum vitamin B₁₂ and UBBC could reflect an expanded pool of both eosinophils and neutrophils in HES and, thus, provide an additional argument for the inclusion of this syndrome in the group of myeloproliferative disorders.

Among the hematologic abnormalities found in HES, an elevation of serum vitamin B₁₂ has been reported, but the mechanism underlying this increased serum vitamin B₁₂ is not clear. The present study was undertaken to investigate the possible mechanism responsible for this increased serum vitamin B₁₂ and to see if this could help in clarifying the nature of HES. Serum cobalamin and unsaturated B₁₂ binding capacity (UBBC) were determined in 24 patients with eosinophilia, of which 16 cases were HES and 8 eosinophilia secondary to an identified cause. In some of these patients, intracellular B₁₂ binding proteins (B₁₂BP) and release of vitamin B₁₂ binders were also analyzed.

MATERIALS AND METHODS

Patients

Twenty-four patients with hypereosinophilia were studied: 16 had HES, defined according to the criteria proposed by Chusid, 6 had parasitic infestation, 1 had Hodgkin's disease, and 1 had angioimmunoblastic lymphadenopathy (AILD).

A search for Ph' chromosome in 10 patients with HES revealed that it was present in one case (case 5). Total white blood cells and absolute neutrophil and eosinophil counts were done. Bone marrow aspirate was performed in all except 2 patients with HES.

Serum Cobalamin, UBBC, and Separation of Transcobalamins

Serum samples were separated within 1 hr of venipuncture. Cobalamin was assayed microbiologically using L. leichmannii.
UBBC was determined by saturating serum with an excess of $^{31}$Co-cyanocobalamin (Amersham, Arlington Heights, IL; specific activity 300 μCi/μg) and removing the unbound $^{31}$Co-cyanocobalamin by dialysis.10

Fractionation of serum transcobalamin (R binders and transcobalamin II) was performed by two techniques: gel filtration and adsorption of transcobalamin II (TC II) on activated charcoal. Gel filtration was performed through 2 x 45 cm columns of Sephadex G200; this column was calibrated with 3 markers of known molecular size. The buffer used was 0.1 M Tris—1 M CINA, pH 8. A quantity of 0.1 ml of saturated serum was applied and eluate fractions of 1 ml were collected at a flow rate of 30 ml/hr.

The percentage of R binders in serum was also obtained after adsorption of TC II on activated charcoal according to the method outlined by Lawrence.11 Of saturated and dialyzed serum, 0.1 ml was added with 0.5 ml of 0.06 M phosphate buffer, pH 6.3, and 0.2 ml of a solution of charcoal in the same buffer (150 mg/ml), mixed thoroughly for 15 min, and centrifuged. Radioactivity of the supernatant containing R binders was determined. The two techniques used gave very close results.

Buffy Coat Preparations and Fractionation of Cells

Buffy coat and fractionated cells were obtained from peripheral blood collected into heparin. The technique of separation has been thoroughly described elsewhere.12 The separation of the various cell populations on a discontinuous albumin gradient exhibited enriched or pure eosinophilic populations in the light density (upper) layers, whereas pure or enriched eosinophils and enriched neutrophil populations were obtained in the layers of higher density or in the pellets. The unfractionated buffy coat and the cells from each layer of the gradient were collected, washed, and resuspended in Hanks’ balanced salt solution (HBSS) and counted. The cells were frozen, thawed twice, and sonicated before the measurement of intracellular $B_{12}$ binding proteins. An aliquot of the cells was cytocentrifuged and smeared for differential counts. The intracellular unsaturated $B_{12}$ was determined by filtration on Sephadex G200, as described above.

Release of $B_{12}$ Binding Proteins by Leukocytes

A quantity of 40–60 ml of heparinized blood was mixed with one-fourth volume of dextran and sedimented for 1 hr. The supernatant was suspended in vitamin $B_{12}$-free RPMI medium in a final suspension of 5 x 10⁶ cells/ml. The release was carried out at 37°C and compared to control values at 0°C each half hour over a 3-hr period. The cells were then centrifuged, and fractions of 0.5 ml of supernatant were analyzed for $B_{12}$ binding proteins by filtration on Sephadex G200.

RESULTS

Hematologic Data, Serum Cobalamin, UBBC, and R Binders

The results of the studies on 24 patients are shown in Table 1. The white blood cell count and the absolute eosinophil count in the HES group (cases 1–16) and in the secondary eosinophilia group (cases 17–24) were not statistically different. However, the HES group presented a marked elevation of serum cobalamin and transcobalamin (R binders and transcobalamin) compared to control values at 0°C each half hour over a 3-hr period. The cells were then centrifuged, and fractions of 0.5 ml of supernatant were analyzed for $B_{12}$ binding proteins by filtration on Sephadex G200.

### Table 1. Hematologic Data, Serum Vitamin $B_{12}$, UBBC of Total Serum and of R Proteins ( transcobalamin I and III) in 16 Patients With HES and 8 Patients With Secondary Eosinophilia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>WBC (10⁵/Liter)</th>
<th>Eosinophils (10⁵/Liter)</th>
<th>Neutrophils (10⁸/Liter)</th>
<th>R Binders</th>
<th>UBBC (NI = 200–500 ng/Liter)</th>
<th>R Binders</th>
<th>UBBC (NI = 950–2,000 ng/Liter)</th>
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<td>1</td>
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<td>4,600</td>
<td>4,000</td>
<td>3,680</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>14</td>
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</tr>
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<td>1.7</td>
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<td>2,395</td>
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<tr>
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<td>1,480</td>
<td>680</td>
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<td>Parasitic infestation</td>
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<td>15.5</td>
<td>2.8</td>
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<td>2,015</td>
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<td>21</td>
<td>Parasitic infestation</td>
<td>12</td>
<td>6</td>
<td>4.6</td>
<td>340</td>
<td>1,900</td>
<td>580</td>
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<tr>
<td>22</td>
<td>Parasitic infestation</td>
<td>19</td>
<td>12</td>
<td>3.4</td>
<td>460</td>
<td>1,750</td>
<td>490</td>
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<td>23</td>
<td>AILD</td>
<td>24.7</td>
<td>20.5</td>
<td>3.2</td>
<td>300</td>
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<td>350</td>
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<td>24</td>
<td>Hodgkin’s disease</td>
<td>20</td>
<td>4.8</td>
<td>14</td>
<td>440</td>
<td>3,300</td>
<td>1,350</td>
<td></td>
</tr>
</tbody>
</table>

HES, hypereosinophilic syndrome; AILD, angioimmunoblastic lymphadenopathy.
UBBC mainly due to R proteins compared to the levels observed in the secondary eosinophilia \((p < 0.001)\). This elevation in HES, however, was not constant, as shown by case 12, who exhibited a normal serum cobalamin with increased UBBC and R binders, whereas case 4 showed markedly increased serum cobalamin associated with normal UBBC and R binders. On the other hand, case 19, with parasitic infestation, presented an elevation of serum cobalamin with normal UBBC and R binders and the patient with Hodgkin’s disease (case 24) exhibited a net increase of UBBC and R proteins, associated with an excess of neutrophils.

**Intracellular Unsaturated B\(_{12}\) Binding Protein Levels \((B_{12}BP)\)**

The \(B_{12}BP\) are shown in Table 2. These \(B_{12}BP\) levels measured on buffy coat of 4 cases (cases 2, 3, 4, 5) of HES and 5 cases (cases 17, 18, 20, 22, 24) of secondary eosinophilia presented a broad range. The \(B_{12}BP\) capacities in pure eosinophilic fractions were much lower than those observed in neutrophil-rich fractions, but were similar in the two groups of eosinophilia; however, in fractions where eosinophils were contaminated by neutrophils, \(B_{12}BP\) increased along with the proportion of neutrophils.

Figure 1 shows the \(B_{12}BP\) in pure eosinophils, lymphocytes, and neutrophil-rich fractions of 3 patients [(A) case 20 with parasitic infestation, (B) case 24 with Hodgkin’s disease, (C) case 3 with HES]. Intracellular \(B_{12}BP\) was very low in lymphocytes; it was higher in pure eosinophil fractions, but there appeared to be no major difference in the intracellular \(B_{12}BP\) according to the cause of eosinophilia. The enriched neutrophil fractions (respectively, 65% in case A, 92% in case B, and 83% in case C) showed very high \(B_{12}BP\). The \(B_{12}BP\) level in the patients is of the same order of magnitude as that observed in pure populations of neutrophils obtained from 5 normal subjects (PN). Whatever the cells, R binders accounted for more than 95% of this binding.

**Release of \(B_{12}\) Binders by Leukocytes**

The release of \(B_{12}\) binders by leukocytes from the buffy coats of four patients with eosinophilia is shown in Fig. 2. Case 4 had HES, with 66% eosinophils; case 20 had parasitic infestation, with 76% eosinophils; case 23 had AILD, with 83% eosinophils, and case 24 had Hodgkin’s disease, with 24% eosinophils and 72% neutrophils. The leukocytes of the 3 patients with a high percentage of eosinophils (cases 4, 20, and 23) released very small amounts of \(B_{12}\)BP at \(37^\circ\)C (constituted almost exclusively by R binders); moreover, there

Table 2. Intracellular Unsaturated B\(_{12}\) Binding Proteins \((B_{12}BP)\) in Buffy Coat, Pure Eosinophil Fractions, and Neutrophil-Rich Fractions of 4 Patients With HES and 5 Patients With Secondary Eosinophilia

<table>
<thead>
<tr>
<th></th>
<th>Buffy Coat (pg/10(^6) Cells)</th>
<th>Pure Eosinophil Fractions (pg/10(^6) Cells)</th>
<th>Neutrophil-Rich Fractions (pg/10(^6) Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES</td>
<td>2,040 (1,590–2,825)</td>
<td>640 (520–785)</td>
<td>2,465 (2,130–2,670)</td>
</tr>
<tr>
<td>Secondary eosinophilia</td>
<td>1,690 (460–2,820)</td>
<td>845 (430–1,060)</td>
<td>3,370 (1,820–4,360)</td>
</tr>
</tbody>
</table>
with Hodgkin's disease also presented the highest elevation of vitamin B₁₂. We also found significant elevation of the most common abnormality in this disease is an increase in serum UBBC of the patients with HES, Flaum et al. observed serum vitamin B₁₂ levels in idiopathic eosinophilia and secondary eosinophilia, reveal a significant difference between the 2 groups: the mean serum B₁₂BP in his neutrophils (Fig. 1, case B) and the highest serum UBBC in the group of secondary eosinophilia. Whatever the composition of the buffy coat, the release of B₁₂ binders at 0°C in the medium was undetectable.

**DISCUSSION**

Serum cobalamin and UBBC measured in 24 patients with hyper eosinophilia, of which 16 had HES and 8 secondary eosinophilia, reveal a significant difference between the 2 groups: the mean serum cobalamin is markedly increased in HES, whereas it is normal in symptomatic eosinophilia. These results are in agreement with a previous study in which elevated serum vitamin B₁₂ levels in idiopathic eosinophilia and normal levels in secondary eosinophilia were reported. More recently, in a retrospective blind study of 32 patients with HES, Flaum et al. observed that the most common abnormality in this disease is an elevation of vitamin B₁₂. We also found significant increase in serum UBBC of the patients with HES, mainly related to R binders of vitamin B₁₂, e.g., transcobalamin I and III.

These findings suggest that the determination of serum cobalamin, UBBC, and R proteins may be very useful in the diagnosis of HES, although this elevation is not constant (as evident in one case with a normal serum B₁₂ and another case with a normal UBBC).

Fledelius has suggested that the elevated vitamin B₁₂ levels in idiopathic and persistent eosinophilia presented an argument for the diagnosis of the eosinophilic variant of chronic myeloid leukemia. In three cases of eosinophilic leukemia with immature forms in peripheral blood, a concomitant increase of vitamin B₁₂ and UBBC, mainly TC I, has also been reported. The elevation of serum cobalamin and R proteins observed in patients with HES is like that observed in myeloproliferative disorders, especially in chronic myeloid leukemia. In these diseases, the elevation of vitamin B₁₂ parameters is due to the excessive proliferation of granulocytes. The R binders, especially transcobalamin I, which carries most of the endogenous cobalamin, originate from the granulocyte. A close relationship between the UBBC and blood neutrophils on the one hand and with total blood granulocytic pool on the other hand has been reported.

The abnormalities of serum cobalamin and UBBC found in HES lead us to reconsider the pathophysiology of this disease. Increased serum cobalamin and UBBC cannot be explained in these patients by the absolute number of circulating eosinophils or neutrophils, as these are not significantly different from those of patients with secondary eosinophilia who have no elevation of vitamin B₁₂ parameters. Two hypotheses that are not mutually exclusive could explain the increased vitamin B₁₂ parameters in HES: first, an overproduction and/or an excessive release of B₁₂ binding proteins by eosinophils in HES compared to that of symptomatic eosinophilia; second, an increased granulocytic tissue pool reflected in the serum by the elevation of cobalamin and UBBC.

Some workers have suggested that the eosinophilic granulocytes have the capacity to produce R binders in amounts similar to those by the neutrophilic granulocytes; however, this conclusion has been drawn on indirect arguments. The intracellular content of B₁₂ binding proteins in pure populations of eosinophils isolated from our patients with HES or secondary eosinophilia has shown that eosinophils may contribute to the production of R binders, in quantities much higher than lymphocytes but far lower than neutrophils of the same patients or from the neutrophils obtained from normal subjects. Moreover, we have found no significant difference in B₁₂ binding protein content between pure eosinophil fractions of HES and of secondary eosinophilia. In addition, the amount of B₁₂ binding proteins released from the buffy coat of
patients with eosinophilia is similar, irrespective of the origin of eosinophilia (HES or secondary eosinophilia). On the contrary, the amount of B₂₂ binding proteins released is higher when the buffy coat is constituted mainly of neutrophils; in both cases, the B₂₂ binding proteins released are constituted by R proteins.

Despite the smaller content and lower release of B₂₂ binding proteins by eosinophils than by neutrophils, it can be assumed that the increased vitamin B₂₂ parameters in HES are partly due to an increased tissue pool of eosinophils, which is probably greater in this disease than that in secondary eosinophilia. Many reports with histologic studies²⁰,²¹ have described the prominent infiltration of various organs by large numbers of eosinophils. Most of the clinical manifestations in HES are directly related to the degree of organ infiltration by eosinophils and the tissue damage induced by their contents.

Besides the expanded eosinophilic tissue pool in HES, it is likely that the neutrophil pool is also increased. In our series, some cases of HES also have a mild neutrophilia associated with the eosinophilia, without any obvious evidence of infection. Bone marrow aspirates and biopsies that have been performed in most of our 16 patients with HES have shown hypercellularity with an excess of both eosinophils and neutrophils; skin lesion biopsies performed in HES have also shown eosinophil and neutrophil infiltrates.²² The excess of neutrophils that produce and release large amounts of vitamin B₂₂ binders should also contribute greatly to the elevation of serum cobalamin and UBBC in HES.

The expanded eosinophilic and neutrophilic pool and the absence of an identifiable cause in HES is an additional argument to include this syndrome in myeloproliferative disorders.

Despite a lack of specific marker, our findings, added to some other data, such as cyto genetic abnormalities²³ and absence of eosinophilopoietin in sera of HES,²⁴ suggest that most of HES results from a clonal proliferation of a granulocyte precursor with a peculiar propensity to differentiate into the eosinophilic lineage.

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