Serum Vitamin B₁₂-Binding Capacity and Muramidase Changes With Cyclic Neutropenia Induced by Cytosine Arabinoside

By RALPH CARMEL AND CHARLES A. COLTMAN, JR.

A patient with acute leukemia in sustained complete remission was studied for 8 months. Periodic maintenance chemotherapy with cytosine arabinoside regularly induced granulocytopenia, with granulocytosis upon marrow recovery. Changes in serum unbound B₁₂-binding capacity (UBBC) occurred simultaneously with peripheral granulocyte fluctuations. Both α- and β-globulin UBBC were affected. Serum muramidase rose and fell out of phase with the granulocyte and UBBC fluctuations, preceding them by several days. These data support the concept that serum UBBC and serum muramidase reflect different aspects of granulocyte activity.

Serum B₁₂-binding protein abnormalities are regularly found in myeloproliferative disorders. Serum muramidase abnormalities occur in various myelocytic and monocytic leukemias. However, serial changes in either of these proteins in granulocyte suppression and proliferation have not been described in man, other than a study of B₁₂-binding changes in polycythemia during remission and relapse.

A patient received periodic myelosuppressive doses of cytosine arabinoside during the complete remission of his acute leukemia. He thus provided the opportunity of studying both B₁₂-binding and muramidase patterns during the cyclic neutropenia resulting from periodic marrow suppression.

Case Report

The patient, a 21-year-old man, presented with acute myelomonocytic leukemia 1 year before the beginning of this study. Initial treatment with a combination of 6-mercaptopurine and 6-methylmercaptopurine riboside was unsuccessful. A complete remission was induced with cytosine arabinoside 7 months prior to our study. The remission has been maintained throughout the study and beyond with 125–190 mg. cytosine arabinoside/M² given intravenously for 5 days every 3–4 weeks. Complete blood counts were done 1–3 times weekly, and a bone marrow examination was done prior to each course of maintenance therapy. No more than 4 per cent blasts were identified in his marrow at any time, with the brief exception of days 154–159 of the study when 12 per cent of cells of a hypocellular marrow were blastic. At no time were blastic cells seen in peripheral blood.

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He had been on a normal diet throughout. Serial liver function studies and serum electrophoreses were normal.

**MATERIALS AND METHODS**

Serum was obtained in the morning, 1–3 times weekly. Sixty-two samples were collected over 8 months, and studied fresh or after storage at −20°C.

**B₁₂-binding Studies**

Unbound B₁₂-binding capacity (UBBC) was determined by hemoglobin-coated charcoal assay.¹² UBBC was separated into α-globulin (or Transcobalamin I) and β-globulin (or transcobalamin II) fractions by the DEAE-cellulose batch method.¹³ Normal values in our laboratory are 1759 pg./ml. ± 269 (SD) UBBC and 25 per cent ± 3 (SD) α-globulin fraction.

In several instances, fractionation of UBBC was also done by the following:

- **Cellulose acetate electrophoresis and autoradiography.** Serum was incubated with a slight excess of ⁵⁷Co B₁₂ (11.5 μCi./μg.), and subjected to electrophoresis alongside ⁵⁶Fe-labeled serum for 20 minutes at 250 v. in 0.075 ionic strength barbital buffer, pH 8.6. The unstained strips were then exposed to Cronex 4 X-ray film (DuPont de Nemours, Wilmington, Del.) for 6 weeks.

- **Sephadex G-200 gel filtration.** One ml. serum, containing ⁵⁷Co B₁₂ (unbound ⁵⁷Co B₁₂ having been removed with a pellet of hemoglobin-coated charcoal) and 20 per cent sucrose, was applied to a 1.4 × 100 cm. column. Samples of 100 drops were collected and counted for radioactivity. The buffer was 0.1 M Tris buffer, pH 8.5, containing 1 M NaCl.

- **Ammonium sulfate precipitation.** To 0.5 ml. serum and 1 ml. 0.4 M K₂H PO₄, pH 8.6, 0.5 ml. ⁵⁷Co B₁₂ (10 ng./ml.) was added. Unbound ⁵⁷Co B₁₂ was removed as above. By adding 4 ml. 3 M (NH₄)₂SO₄, a 2 M (NH₄)₂SO₄ concentration resulted. After incubation at 37°C for 30 minutes the sample was centrifuged at 3500 rpm for 20 minutes. The supernate and precipitate were counted for radioactivity. Per cent radioactivity in the supernate, which corresponded primarily with per cent α-globulin, was calculated.

The effect of cytosine arabinoside on UBBC was studied by incubating 0.1 ml. of the drug (10 mg./ml.) (Ben Venue Laboratories, Bedford, Ohio) with 0.5 ml. serum, comparing the resulting UBBC to that of serum alone.

Serum vitamin B₁₂ levels were assayed by coated charcoal radioassay.¹⁴

**Muramidase Studies**¹⁵

A fresh substrate suspension of 10 mg. lyophilized Micrococcus lysodeikticus in 100 ml. 0.06 M sodium phosphate buffer, pH 6.2, was prepared. Fresh standards were made from lyophilized crystalline egg-white lysozyme (Worthington Biochemical Corp., Freehold, N.J.) for each assay. The assay was initiated by adding 0.3 ml. serum or standard to 3 ml. substrate. Change in turbidity was recorded at 640 μ in a Beckman DB ratio-recording spectrophotometer for 3 minutes, beginning 30 seconds after mixing of the reagents. These recordings were linear over a range from 4 to 15 μg. egg-white standard/ml. Because the tracings were significantly nonlinear outside this range, appropriate dilutions or increased amounts of sera were used when needed. The standard egg-white solutions contained 5.0, 5.7, 8.0 and 10.0 μg./ml. When change in turbidity was plotted against logarithm of the muramidase concentration of the standards, the relationship was always linear. Normal level in our laboratory is 7.5 μg./ml. ± 1.6 (SD).

**RESULTS**

**Hematologic Changes**

The patient's changes are shown in Fig. 1. The granulocyte cycle averaged 25 days, and counts ranged between 100 and 9600/cu. mm. On several occasions a biphasic fall in granulocyte count was seen.
**Fig. 1.**—Hematologic changes during maintenance chemotherapy. Complete remission was induced 7 months before day 1 of the study. Each arrow represents a 5-day course of cytosine arabinoside.

Monocyte fluctuations were not striking. A slight rise usually appeared during granulocytopenia, with another at peak granulocytosis. Counts ranged between 100 and 2400/cu. mm.

The platelet counts fell and rose out of phase with leukocytic fluctuations.

**B12-binding Changes**

Figure 2 shows the parallelism of UBBC changes with granulocyte fluctuations. In virtually every instance their nadirs coincided. Simultaneous rises occurred, in 1 to 2 weeks both parameters reaching a peak. The close parallelism was evident even in the biphasic falls.

**Fig. 2.**—Comparison of UBBC and granulocyte changes during maintenance chemotherapy. The shaded area represents normal mean ± 2 SD. The vertical solid and dotted lines denote UBBC peaks and nadirs, respectively. Each arrow represents a 5-day course of cytosine arabinoside.
In every instance UBBC rose above normal levels at peak granulocytosis, even though the granulocyte counts were not abnormally high. Abnormally low UBBC was not seen. UBBC values plotted against simultaneous granulocyte levels (Fig. 3) showed a statistically significant correlation ($r = 0.47$), although there was some scatter about the regression line.

In contrast, UBBC fluctuation did not correlate with monocyte ($r = 0.24$), platelet ($r = 0.30$), or hemoglobin changes ($r = 0.18$). Marrow aspirations were done just before each course of chemotherapy. Unfortunately, serial marrow examinations during a single cycle, to allow exact correlation with actual granulocytic proliferation, were not done.

DEAE-cellulose fractionation of all sera produced $\alpha$-globulin levels of 18–30 per cent of the UBBC. This is within the normal range in our laboratory. Although slightly higher percentages were sometimes seen during granulocytosis, the changes were neither consistent nor significant ($r = 0.0$). The granulocyte changes were reflected, thus, by $\alpha$-globulin ($r = 0.40$) and $\beta$-globulin binding changes ($r = 0.46$) equally.

The fractionations were corroborated in several of the patient’s low- and high-UBBC sera by:

Cellulose acetate electrophoresis. Most of the $^{57}$Co $B_{12}$ radioactivity was always found in the $\alpha$-2 to $\beta$-globulin region, where $\beta$-globulin $B_{12}$-binding protein normally migrates.

Sephadex G-200 gel filtration. Peak radioactivity was always eluted around tube 31. This is where $\beta$-globulin $B_{12}$-binder (MW = 36,000) appears under these conditions of filtration. $\alpha$-Globulin $B_{12}$-binder (MW = 121,000), a much smaller peak, appeared around tube 23. No abnormal peaks were seen. Hemoglobin (MW = 68,000) appears around tube 27 in the column.

Ammonium sulfate precipitation. Mean supernate percentages of 26 per cent in three high-UBBC sera and 23 per cent in three low-UBBC sera resulted, compared with 25 per cent and 24 per cent $\alpha$-globulin, respectively, obtained by DEAE-cellulose separation of the same sera.
Cytosine arabinoside had no in vitro effect on either UBBC level or fractionation by DEAE-cellulose.

Serum B₁₂ levels varied during the 8-month period between 180 and 589 pg./ml. There was no correlation with granulocytes (r = 0.22), and the fluctuations followed no significant pattern.

**Muramidase Changes**

The patient’s levels varied between 1.7 and 16.4 µg./ml. Although the levels did not correlate with simultaneous granulocyte (r = 0.0) or monocyte counts (r = 0.22, n = 43), muramidase rose above normal with leukocytosis and fell below normal with leukopenia. Muramidase fluctuation varied with granulocyte fluctuation, its cycle usually preceding the granulocyte cycle by 5–8 days. The pattern can best be appreciated by comparing the muramidase nadirs with granulocyte nadirs in Fig. 4.

Poor correlation with monocytosis was evident. However, in a few cycles in Fig. 4, slight monocytosis appeared to coincide with the muramidase peaks.

**DISCUSSION**

Striking UBBC elevations, primarily of the α-globulin B₁₂-binder, have been found consistently in chronic myelogenous leukemia since the original observation of Beard et al.¹ Mature granulocytes have subsequently been shown to be the richest cellular source of the α-globulin binder.¹⁷-¹⁹ Simons and Weber suggested the protein was synthesized in leukocytes.²⁰ It has further been reported that UBBC correlated with the size of the granulocyte pool, but not with granulocyte turnover, suggesting that intact intravascular cells released the binder.²¹

Elevated UBBC has also been found in polycythemia vera and myeloid metaplasia³,⁵ and in acute myelogenous leukemia.²,⁴ However, the elevations

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**Fig. 4.**—Comparison of muramidase, UBBC and leukocyte fluctuations during maintenance chemotherapy. Shaded areas represent normal mean ± 2 SD. The days are numbered from onset of the study period; each arrow denotes a course of cytosine arabinoside.
were not as high nor as consistently seen as in chronic myelogenous leukemia, and \( \beta \)-globulin UBBC was usually involved to as great an extent as \( \alpha \)-globulin. Similar patterns have been reported in leukocytosis unrelated to myeloproliferative disease.\(^2,4,22\) In leukopenic states high,\(^2\) normal\(^3\) and low UBBC\(^4\) have been reported, but little detail of the leukopenia was given. Serial studies have not been reported.

Our patient presented the opportunity to study UBBC changes related to the cyclic neutropenia of intermittent chemotherapy. Complete remission of leukemia was induced 7 months prior to the study, and maintained during routine periodic administration of cytosine arabinoside, a primarily myelosuppressive drug.\(^23,24\) The only hematologic changes seen were the typical cyclic changes reported with cytosine arabinoside.\(^23,24\) The drug had no demonstrable effect on serum UBBC in vitro.

The UBBC paralleled granulocyte fluctuations and correlated with no other parameter. With rebound granulocytosis, even though abnormally high counts were not reached, UBBC almost always rose to levels above normal. There was a statistically significant correlation between UBBC and granulocyte levels in spite of scatter about the regression line.

The UBBC fluctuations occurred in \( \alpha \)-globulin and \( \beta \)-globulin components equally. The involvement of both components was interesting, in view of the evidence that granulocytes contain only \( \alpha \)-globulin binder.\(^26\) It confirms, however, previous observations where both components were elevated in leukocytosis.\(^2,4,22\) When the data of Gottlieb and associates\(^4\) in leukopenia is evaluated, it is evident that \( \beta \)-globulin UBBC was low. The alpha-globulin UBBC was also low, but total \( \alpha \)-globulin \( B_12 \)-binding (calculated by adding \( \alpha \)-globulin UBBC and serum \( B_12 \) level) was normal or perhaps elevated.

Unlike the \( \alpha \)-globulin \( B_12 \)-binder, the source of the \( \beta \)-globulin is unknown. In the mouse, the liver is the source of a \( B_12 \)-binding protein resembling human \( \beta \)-globulin binder.\(^28\) However, specific human data is lacking. Patients with cirrhosis occasionally have mild UBBC elevations,\(^27,28\) whereas those with acute liver disease have low UBBC.\(^28\)

It is unlikely that cytosine arabinoside effected nongranulocytic (e.g., hepatic) release of \( \beta \)-globulin \( B_12 \)-binding protein, although this cannot be ruled out. Liver toxicity does not usually occur with the drug,\(^23,24\) and high \( \beta \)-globulin UBBC has been seen in leukocytosis with no chemotherapy or liver disease.\(^22\) Both \( \alpha \)- and \( \beta \)-globulin binder fluctuations followed the granulocyte pattern remarkably closely in our patient. Even the biphasic falls in granulocyte count, which commonly occur following cytosine arabinoside and have not been observed in cells other than granulocytes,\(^23,24\) were frequently reflected. Finally, \( \beta \)-globulin binder changes were identical in degree and time with \( \alpha \)-globulin changes.

The abnormal “polycythemia vera” binder of Hall and Finkler,\(^29\) which may also occur in some patients with leukocytosis,\(^22\) elutes with \( \beta \)-globulin on DEAE-cellulose separation.\(^22,30\) This protein, however, behaves as an \( \alpha \)-globulin binder on Sephadex G-200 filtration and \( (NH_4)_2SO_4 \) precipitation in our laboratory, and was not present in our patient.
Thus, the data support the hypothesis that granulocytes are the source of the UBBC elevations and suggest that \( \beta \)-globulin \( B_{12} \)-binder may be related to these cells as well, as proposed by Retief and associates.\(^{31}\)

The evidence for leukocytic origin of serum muramidase in many ways resembles that of UBBC. It has been established that granulocytes and monocytes are the only blood cells containing the enzyme.\(^{32,33}\) Cell content of muramidase increases with maturity, although little increase occurs beyond the myelocyte stage.\(^{34}\) While abnormal muramidase elevation in leukemia is well documented, changes in normal subjects have not been studied. Finch, Lamphere and Jablon,\(^{35}\) studying random muramidase and granulocyte levels in normal subjects, reported a weak correlation between the two parameters. Fink and Finch\(^{36}\) induced granulocytopenia in rabbits with nitrogen mustard and observed that muramidase fell with granulocytopenia, returning 1–2 days before granulocyte return. Monocyte changes were not reported. Dale et al.\(^{37}\) recently reported that muramidase changes in gray collie dogs with cyclic neutropenia preceded granulocyte changes. The dogs manifested granulocyte fluctuations strikingly similar to these in our patient, including the biphasic granulocyte fall. The findings presented here in man corroborate the animal data to a large extent.

At no time were the exceedingly high muramidase levels of acute myelomonocytic leukemia seen. In every instance the muramidase rise preceded granulocyte return by 5–8 days, suggesting that muramidase may have indicated early myeloid recovery. The out-of-phase relationship explains the lack of significant correlation between simultaneous muramidase and granulocyte levels. Similarly, it may explain the poor correlation in random subjects.\(^{38}\)

While no correlation existed between muramidase and monocyte levels, it appeared in a few instances that muramidase peaks coincided with the slight monocytosis during granulocytopenia. The poor statistical correlation may have been partially due to the slight degree of monocytic change and its inconstant pattern. Thus, the contribution of monocytes to muramidase elevation could not be entirely ruled out.

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