The Clinical and Physiological Implications of Hepatoma B$_{12}$-binding Proteins

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SUMMARY

A serum B$_{12}$-binding protein with increased sialic acid content (termed hepatoma B$_{12}$-binding protein) that causes elevations of serum B$_{12}$ and unsaturated B$_{12}$-binding capacity has been found in some patients with hepatocellular carcinoma (hepatoma). We now report another patient with hepatoma with initial near-normal, unsaturated B$_{12}$-binding capacity that increased 400-fold as the disease progressed and then fell 50% with response to chemotherapy. A perfusate of the tumor in the liver had 5 times more B$_{12}$-binding protein than did the serum and was immunologically the same as the serum hepatoma B$_{12}$-binding protein isolated from previous cases. A cell line derived from hepatoma produced significant amounts of B$_{12}$-binding protein similar to hepatoma B$_{12}$-binding protein, whereas cell lines from normal liver and other neoplasia did not. The hepatoma sera, perfusate, and media from the hepatoma cell line contained elevated sialyltransferase activity. These data suggest that some hepatomas produce increased hyperglycosylated B$_{12}$-binding protein that is cleared slowly from the plasma and accumulates there as hepatoma B$_{12}$-binding protein.

INTRODUCTION

It has gradually become clear that there are many conditions in which measurements used to assess vitamin B$_{12}$ metabolism indicate abnormalities that are manifested by alterations in serum vitamin B$_{12}$ content, UB$_{12}$BC, total vitamin B$_{12}$-binding capacity (endogenous B$_{12}$ level plus UB$_{12}$BC), and percentage of unsaturated and total binding capacity due to various types of vitamin B$_{12}$-binding proteins. The vitamin B$_{12}$-binding proteins present in serum have been characterized and designated as transcobalamin I (or a-globulin vitamin B$_{12}$-binding protein), transcobalamin II (or b-globulin vitamin B$_{12}$-binding protein), and transcobalamin III (or polycythemia vera binder) (13). Studies of serum vitamin B$_{12}$ and vitamin B$_{12}$ binders have revealed alterations not only in vitamin B$_{12}$ deficiency states but also in pregnancy, liver disease, myeloproliferative disorders, chronic myelocytic leukemia, chronic leukopenia, chronic leukocytosis, and uremia (6).

A recent report (14) described 3 adolescents who presented with hepatocellular carcinomas (hepatoma), normal leukocyte counts, extraordinary elevations of serum vitamin B$_{12}$, and serum-unsaturated B$_{12}$-binding capacity. The serum B$_{12}$-binding protein elaborated by these patients appeared to be tumor related since it increased in amount as the disease progressed. Determination of these vitamin B$_{12}$ parameters in several other children with hepatoma suggests that a similar serum B$_{12}$-binding protein was present in 7 other children but to a lesser amount. Studies (2) indicated that the elevations of serum B$_{12}$ and of unsaturated B$_{12}$-binding capacity were due to the presence of a B$_{12}$-binding protein that belonged to the R-type class of immunologically related B$_{12}$-binding proteins normally present in a number of human tissues and body fluids (4). These studies also demonstrated that the hepatoma-related B$_{12}$-binding protein differed from the R-type B$_{12}$-binding proteins that are found in increased amounts in the sera of patients with chronic myelocytic leukemia in that the hepatoma B$_{12}$-binding protein appeared more acidic during gel electrophoresis and anion-exchange chromatography and had a smaller apparent weight when studied by gel filtration (15).

Hepatoma B$_{12}$-binding protein has been isolated in homogeneous form from the plasma and pleural fluid of 2 of these patients by the use of vitamin B$_{12}$ Sepharose affinity chromatography (2). The hepatoma B$_{12}$-binding protein is essentially indistinguishable from the recently isolated human milk and saliva R-type B$_{12}$-binding proteins except that it contains more sialic acid and less fucose than do the milk and saliva B$_{12}$-binding proteins. To further elaborate the source, mechanism of production, and possible clinical significance of hepatoma B$_{12}$-binding protein, we now report another case of an adolescent with hepatoma studied over a 12-month period as well as the characteristics of the B$_{12}$-binding proteins produced by a cell line established from a human hepatoma. Some of these studies were reported previously in abstract form (16, 17).

MATERIALS AND METHODS

Serum samples, liver perfusates, and tissue culture supernatants were kept frozen at $-10^\circ$ and thawed before use. Repeated freezing and thawing was avoided. Serum vitamin B$_{12}$ and UB$_{12}$BC were determined by coated-charcoal assay (8). The diluent used was 0.9% NaCl solution.
Studies of serum-unsaturated vitamin B₁₂-binding proteins were performed on serum labeled in vitro with radioactive vitamin B₁₂ in the form of cyanocobalamin. [³²P]Vitamin B₁₂ (10 mCi/mg) and [³⁵S]Vitamin B₁₂ (1.06 mCi/mg) were purchased from E. R. Squibb & Sons, Inc., New Brunswick, N. J., and Amersham/Searle Corp., Arlington Heights, Ill., respectively.

The molecular weights of the B₁₂-binding proteins were estimated by gel filtration on Sephadex G-200 in a column 2.6 x 86 cm at 23°C. A phosphate buffer (0.1 M, pH 7.2) with 0.15 M NaCl was used at a flow rate of 25 ml/hr, and 5-ml fractions were collected. Protein content was measured by absorbance at 280 nm in a Gilford spectrophotometer, and radioactivity was measured by scintillation spectrometry in a Searle analytic gamma counter to a counting error of 1% or less. The gel filtration data are expressed in terms of Kₐᵥ as defined by Laurent and Killander (9). Blue Dextran 2000 was used to determine the value of Kᵥ. Phenol red was used to determine the value of Vᵥ. The column was calibrated with proteins of known molecular weight: RNase (13,700), chymotrypsin A (25,000), ovalbumin (45,000), bovine serum albumin (67,000), and aldolase (158,000).

Test fluids were subjected to fractionation on DEAE-cellulose [capacity, 0.95 mEq/g (Schleicher & Schull, Inc., Keene, N. H.)], with phosphate buffers of increasing molarity (0.025 to 0.2 M) at pH 6.2 (15). Elutions were performed at room temperature on a 0.7 x 20.5-cm column. Fourteen ml of column effluent (in 2-ml fractions) were collected during elution with each buffer.

Isoelectric focusing studies of R-type B₁₂-binding proteins were done by a modification of the method of Stenman (13). Samples were submitted to isoelectric focusing in an LKB Model 8100 Ampholine column with a capacity of 110 ml. Ampholytes [1%; Ampholine, Batch 7 (LKB Instruments Inc., Rockville, Md.)] with a pH range of 2.5 to 4 were used to stabilize with sucrose in a linear gradient of 5 to 50%. Electrofocusing was started with an initial output of 350 V and an initial power output of 3 watts. Focusing was complete within 5 days at which time the column was emptied with 3-ml fractions collected.

Immunological identification of vitamin B₁₂-binding proteins was performed by the method of Finkler et al. (3) by incubating hepatoma serum with rabbit antibody to human salivary "R" binder and hepatoma B₁₂-binding protein. Equal volumes of serum and antibody were incubated for 30 min at 37°C and refrigerated for 18 hr before gel filtration on Sephadex G-200.

Sialyltransferase activity was determined by a modification of the method of Grimes (5). The incubation mixture had a total volume of 0.075 ml and contained 0.2 M potassium phosphate, (pH 7.0), 1 mM MgCl₂, 0.1% Triton X-100, 0.4 nmole CMP-[(⁺⁴C)acetylneuraminic acid (100 mCi/ nmole (New England Nuclear, Boston, Mass.)), 0.5 mg desialylated fetuin, and 0.2 to 0.6 mg enzyme preparation. The samples were incubated at 37°C for 1 hr, and then the reaction was stopped by adding 1 ml of 10% TCA. Millipore filters were used to retain the 10% TCA-precipitated sialylated protein products and, after washing 3 times with 10% TCA, the radioactivity of the filters was counted with 10 ml Aquasol in a Beckman Model LS 250 liquid scintillation system to a counting error of 2% or less.

Cell Lines. SK-H-MA* was derived from a malignant pleural effusion of an infant with disseminated hepatoblastoma (7). This line has been maintained in vitro for 3 years. The cells were grown at 37°C in 25-ml plastic flasks in complete media containing Eagle's minimal essential medium supplemented with 15% fetal calf serum, glutamine, and nonessential amino acids. The cells attach to the plastic readily and are present morphologically as monolayers of small round cells with occasional giant cells. Chromosome preparation reveals both aneuploid and diploid cell populations. On electron microscopy they contain a minimum of ultrastructural features and lack viral particles. The population-doubling time of cells under these conditions is 44 hr, and flask inoculated with 100,000 cells are confluent by 7 days. Cells inoculated s.c. into nude mice develop into 1-cm tumors that are histologically similar to the primary hepatoblastoma. Conditioned media from 7-day cultures lack α-fetoglobulin and carcinoembryonic antigen but contain relatively large amounts of B₁₂-binding protein, which was used for our studies. The cells contain 5'-nucleotidase by both biochemical and cytochemical analyses. Complete medium is used as the control when a culture medium is assayed for the presence of B₁₂-binding protein.

SK-N-BE, SK-N-SH, and SK-N-SH-SY are continuous cell lines established in culture from metastatic neuroblastoma tissue (12). Growth and biochemical properties confirm that they are malignant cells of neurogenic origin. Normal human liver (Chang), cell repository number CCL 13, was purchased from the American Type Culture Collection, Rockville, Md., and was grown in Eagle's minimal essential media with 10% fetal calf serum.

Case History. Patient M. S. was a 16-year-old boy who was found to have hepatocellular carcinoma and underwent excision of the tumor mass and resection of the left lobe of his liver in January 1975. Three small tumor masses at the apex of the right lobe of the liver were not removed. Evaluation at Memorial Hospital on February 5, 1975, revealed no hepatomegaly; liver and gallium scans were normal, but arteriogram revealed metastatic hepatoma involving the right lobe of the liver. Chemotherapy was instituted with cyclophosphamide, vincristine, and trifluorodeoxyxymethidine followed by 5-azacytidine and Adriamycin.

Hepatomegaly became evident on April 15, 1975, and, by May 4, 1975, liver and gallium scans and arteriography confirmed tumor progression. After an exploratory laparotomy, the patient underwent isolated liver perfusion with dactinomycin, which caused a brief decrease in tumor mass. The patient continued on combination chemotherapy (5-fluorouracil, dactinomycin, cyclophosphamide, and methotrexate) but experienced further tumor growth. In September, chemotherapy was changed to Velban, bleomycin, hydroxyurea, and Adriamycin. On October 13, 1975, the patient was clinically improved and the liver scan decreased to 10.5 cm from 13 cm.

The clinical course was complicated by a duodenal fistula that went through the abdominal wall in February 1976. Thereafter, the patient deteriorated, and further chemotherapy...
S. Waxman et al.

apy was withheld. The patient expired 1 month later, and autopsy revealed that the tumor was confined to the liver, which was infiltrated with multiple small tumor nodules.

RESULTS

Serial measurements from Patient M. S. of serum vitamin B12, UB12BC, and the results of fractionation of serum B12-binding proteins with the use of DEAE-cellulose chromatography are summarized in Table 1. The initial findings included an elevated serum B12 level, which did not change significantly in several months although the unsaturated B12-binding capacity increased from a normal level almost 400-fold. Late in the course of disease, there was a fall in the serum B12 level although the UB12BC remained markedly elevated. The total UB12BC from the onset was more then 95% α-globulin B12-binding protein; it was that type of ease progressed. Themewas clinical stabilization of disease aphy was withheld. The patient expired 1 month later, and autopsy revealed that the tumor was confined to the liver, which was infiltrated with multiple small tumor nodules.

The relationship of UB12BC to the patient’s clinical course is expressed in Chart 1. The UB12BC increased as the disease progressed. There was clinical stabilization of disease following i.a. dactinomycin from April 1, 1975, to June 2, 1975, and this was reflected by a plateau in the UB12BC level. Thereafter, the tumor increased in size, as did the UB12BC, until September 11, 1976, when the combination of Velban, bleomycin, hydroxyurea, and Adriamycin was administered. This resulted in measurable tumor regression, clinical improvement and, concomitantly, a 50% fall in UB12BC.

Other studies showed absence of α-fetoprotein, normal thyroglobulin level, and moderately elevated α1-antitrypsin in the serum. The serum ceruloplasmin level was 50 mg/100 ml, which was above the normal range (20 to 40 mg/100 ml) but common in neoplastic conditions. There was no difference in mobility of the ceruloplasmin in the patient’s serum compared to that in a simultaneously run control sample on acrylamide gel electrophoresis at pH 8.6.

Six additional children (ages 6 months to 18 years) with hepatoma were studied. In 5 cases with absence of α-fetoprotein, there was a modest (2- to 3-fold) elevation of UB12BC and total α-globulin B12-binding protein. The remaining case had normal B12 parameters but positive α-fetoprotein. Serial studies over 1 year (3 cases) showed a significant rise in UB12BC and total α-globulin B12-binding protein in the 2 patients proven to have progressive disease, whereas there was no increase in the 3rd patient who lacked evidence of tumor recurrence.

Table 1

<table>
<thead>
<tr>
<th>Source</th>
<th>Date</th>
<th>Serum B12 (pg/ml)</th>
<th>UB12BC</th>
<th>Total</th>
<th>α-Globulin* (%)</th>
<th>β-Globulin (%)</th>
<th>Total α-globulin</th>
<th>B12BP*</th>
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<td>Normal*</td>
<td>386</td>
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<td>12.1-29.1</td>
<td>70.9-87.9</td>
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<td>1,978</td>
<td>66.8</td>
<td>33.2</td>
<td>97.1</td>
<td>97.4</td>
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<tr>
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<td>3/4/75</td>
<td>23,500</td>
<td>6,400</td>
<td>90.2</td>
<td>9.8</td>
<td>97.5</td>
<td>97.4</td>
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<tr>
<td></td>
<td>3/11/75</td>
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<td>17,250</td>
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<td>3.4</td>
<td>98.4</td>
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<td>1.8</td>
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<td>6/2/75</td>
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<td>40,525</td>
<td>98.6</td>
<td>1.4</td>
<td>99.1</td>
<td>99.1</td>
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<td>99.8</td>
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<td></td>
<td>9/11/75</td>
<td>13,900</td>
<td>764,000</td>
<td>99.7</td>
<td>0.3</td>
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<td>9/29/75</td>
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<td>620,000</td>
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<td>10/13/75</td>
<td>9,350</td>
<td>323,000</td>
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<td>0.4</td>
<td>99.7</td>
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<td>11/4/75</td>
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<td></td>
<td>12/19/75</td>
<td>9,365</td>
<td>403,000</td>
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<td>99.6</td>
<td>99.6</td>
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<tr>
<td></td>
<td>1/25/76</td>
<td>10,350</td>
<td>428,900</td>
<td>99.6</td>
<td>0.4</td>
<td>99.7</td>
<td>99.7</td>
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</tr>
<tr>
<td>0.9% NaCl solution perfusate of liver</td>
<td>5/1/75</td>
<td>2,016 (44,795)</td>
<td>9,023 (200,491)</td>
<td>99.4</td>
<td>0.6</td>
<td>99.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Transcobalamin I.

b Transcobalamin II and III.

c Represents:

\[
\text{endogenous serum } \text{B}_{12} + \alpha\text{-globulin UB}_{12}\text{BC} \\
\text{total B}_{12}\text{-binding capacity}
\]

d Values obtained from Herbert (6).

e Calculated to serum protein equivalents.

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Twelve children with various forms of cancer other than hepatoma were studied: 6 with neuroblastomas, 2 with poorly differentiated carcinomas in the liver, 2 with rhabdomyosarcomas, and 2 with Wilms' tumors. None of these patients had abnormalities of UB12BC, increase in percentage of \( \alpha \)-globulin B12-binding protein, or measurable \( \alpha \)-fetoprotein. The 2 cases of poorly differentiated carcinoma in the liver were initially thought to be hepatoma but at postmortem were found to be adrenal carcinoma. In support of previously reported data (14), additional studies of 36 adults with hepatoma did not reveal abnormalities of UB12BC or percentage of \( \alpha \)-globulin B12-binding protein.

Further Studies of Hepatoma B12-binding Protein. A 0.9% NaCl perfusate of the tumor bed, in preparation for isolated chemotherapy perfusion, was obtained from Patient M. S. in May 1975. The perfusate contained B12-binding protein of the type present in the serum and, based on protein concentration (2.2 mg protein per ml perfusate), the UB12BC level in the perfusate was 5 times more than it was in the serum at that time (Table 1). In contrast, the haptoglobin, fibrinogen, and transferrin levels of the perfusate were similar in amount to the serum level when corrected for protein concentration.

The behavior of serum and liver perfusate from Patient M. S., labeled with [\( ^{57} \text{Co} \)]B12, on Sephadex G-200 gel filtration is shown in Chart 2. The [\( ^{57} \text{Co} \)]B12 in the serum eluted in 1 sharp peak with an estimated molecular weight of 92,000, whereas the liver perfusate also demonstrated a peak at a M.W. 92,000, but in addition contained a significant shoulder of [\( ^{57} \text{Co} \)]B12 radioactivity at M.W. 120,000. In comparison, a normal serum labeled with [\( ^{57} \text{Co} \)]B12 exhibited 2 peaks (a minor peak at M.W. 120,000 and a major peak at M.W. 60,000). Serum or liver perfusate from Patient M. S. that was labeled with [\( ^{57} \text{Co} \)]B12 and incubated with rabbit antiserum to hepatoma B12-binding protein resulted in the recovery of the [\( ^{57} \text{Co} \)]B12 activity in the void volume of the Sephadex G-200 column.

The B12-binding protein in the serum and liver perfusate of Patient M.S. eluted from DEAE-cellulose predominantly in 0.15 to 0.2 M phosphate buffer, which is similar to that of the hepatoma B12-binding protein previously reported but is different from normal serum in which the B12-binding protein eluted predominantly in 0.05 to 0.075 M phosphate buffer (Chart 3).

The isoelectric focusing pattern of the B12-binding proteins in serum from Patient M. S. labeled with [\( ^{57} \text{Co} \)]B12 and liver perfusate labeled with [\( ^{57} \text{Co} \)]B12 are shown in Chart 4. The serum B12-binding proteins are in 1 predominant peak (PI 2.65), whereas the liver perfusate B12-binding proteins have 2 components (PI 2.65 and PI 3.65).

Cell Line Studies. The cell line derived from hepatoma (SK-H-MA) produced significant amounts of B12-binding protein compared to other established, growing lines and a line derived from normal liver (Chang) (Table 2). The B12-binding protein produced by the hepatoma cell line was predominantly the \( \alpha \)-globulin B12-binding protein type, whereas the small amount of B12-binding protein from the normal liver cell line was mainly of the \( \beta \)-globulin B12-binding protein type. DEAE-cellulose elution of the hepatoma cell line media B12-binding protein labeled with [\( ^{57} \text{Co} \)]B12 showed the \( ^{57} \text{Co} \) activity mainly in the 0.10 to 0.15 M phosphate buffer fraction. There was no B12-binding protein in this fraction in similarly studied complete media (Chart 5).

The media produced by the hepatoma cell line when passed through a Sephadex G-200 column contained B12-binding proteins, which eluted in 1 peak at M.W. 120,000 and was recovered in the void volume when preincubated with rabbit anti-hepatoma B12-binding protein serum (Chart 6).

Sialyltransferase Studies. Sialyltransferase activity was measured in various conditions (Table 3). A normal range was established with an average of 175 cpm/hr/ml \( \times 10^{-3} \). Patients with neoplastic disease had a 2-fold increase above normal, whereas patients with juvenile hepatoma and abnormal B12-binding protein were 4 times higher than normal. The perfusate from the tumor bed of Patient M. S., when corrected for protein, had 20 times greater sialyltransferase activity than did normal serum. The sialyltransferase activity was highest in the media obtained from the hepatoma cell line and appeared to be directly related to the amount of B12-binding protein present in the media (Table 2).
S. Waxman et al.

DISCUSSION

We and others (10, 14) have reported 10 cases of adolescents with hepatoma who produced a specific hepatoma B$_{12}$-binding protein that accumulated in the serum and resulted in the elevation of vitamin B$_{12}$ and UB$_{12}$BC. Serial measurements of serum vitamin B$_{12}$ levels, UB$_{12}$BC, and fractionation into $\alpha$- and $\beta$-B$_{12}$-binding proteins are helpful in the diagnosis and the following of therapy in adolescents with hepatoma. The presence of hepatoma B$_{12}$-binding protein or $\alpha$-fetoprotein appears to be diagnostic of hepatoma in this age group and can be useful in the differential diagnosis of poorly differentiated liver tumors. In contrast, measurements of other sialoglycoproteins such as haptoglobin, $\alpha$-antitrypsin, ceruloplasmin, thyroglobulin, and transferrin were either normal or slightly elevated in these patients.

Hepatoma B$_{12}$-binding protein appears to be tumor related since hepatoma tissue contains 10 times the amount of B$_{12}$-binding protein found in adjacent normal liver tissue (2), and the level of hepatoma B$_{12}$-binding protein clearly reflects the clinical tumor burden. The higher levels of hepatoma B$_{12}$-binding protein found in the 0.9% NaCl solution perfusate of the tumor compared to that in the serum lends further support to the idea that the hepatoma B$_{12}$-binding protein was tumor derived in Patient M. S. Finally, that there is a cell line derived from hepatoma that produces a B$_{12}$-binding protein immunologically and biochemically similar to hepatoma B$_{12}$-binding protein, whereas other neoplastic cell lines and normal liver do not produce significant B$_{12}$-binding protein, gives further evidence for the tumor origin of hepatoma B$_{12}$-binding protein.

Hepatoma B$_{12}$-binding protein is a variant of several nor-
Implications of Hepatoma $B_{12}$-binding Proteins

Table 3

<table>
<thead>
<tr>
<th>Serum sialyltransferase activity</th>
<th>cpm/hr/ml $\times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range</td>
<td>Avg.</td>
</tr>
<tr>
<td>Normal</td>
<td>150-200</td>
</tr>
<tr>
<td>Neoplastic</td>
<td>220-900</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>330-2315</td>
</tr>
<tr>
<td>Hepatoma perfusate from Patient M. S.</td>
<td>4900</td>
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</tbody>
</table>

sialyltransferase. Thereafter, the plasma levels of hepatoma $B_{12}$-binding protein would further accumulate because of its prolonged plasma survival time in comparison to the lesser sialylated R-type $B_{12}$-binding proteins (1). The finding of 2 isoelectric peaks of $B_{12}$-binding protein in the liver perfusate and of only 1 (the more acidic) in the serum of Patient M. S. supports the importance of selective hepatic clearance of sialoglycoproteins as another mechanism for the accumulation of hepatoma $B_{12}$-binding protein. However, it is difficult to explain why other sialoglycoproteins are not similarly elevated in the adolescents with hepatoma unless they, in contrast to the R-type $B_{12}$-binding proteins, are destroyed during passage through the liver or are so rapidly cleared by the liver that they do not accumulate in the plasma.

ACKNOWLEDGMENTS

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ADDITIONUM

We have had the opportunity to examine the following sera obtained from Dr. Elliot Alpert, Massachusetts General Hospital. These sera had been stored for varying amounts of time at $-70^\circ$. Despite prolonged serum storage, 3 adolescents with hepatoma which were $\alpha$-fetoprotein negative demonstrated elevations of hepatoma $B_{12}$-binding protein, causing elevations of serum vitamin $B_{12}$, $UB_{12}BC$, and the $\alpha$-globulin fraction of the $UB_{12}BC$ (Patients 1 through 3). Hepatoma $B_{12}$-binding characteristics were found in Patient 4, although the $\alpha$-fetoprotein was detectable in small amounts. Sera from 18 additional young patients with $\alpha$-fetoprotein-positive hepatoma (3) or non-hepatocyte primary tumors of the liver (15) did not demonstrate these abnormalities. Therefore, hepatoma $B_{12}$-binding protein is helpful in the differential diagnosis of the various primary liver tumors and usually is found in the absence of $\alpha$-fetoprotein.

REFERENCES


\[\text{a-Fetoprotein, } 82 \text{ ng/ml.}\]


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