Vitamin B₁₂ binding protein as a tumour marker for hepatocellular carcinoma

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SUMMARY Grossly raised levels of tumour related vitamin B₁₂ binding protein, reflected by rises in serum vitamin B₁₂ and unsaturated vitamin B₁₂ binding capacity (UBBC), were found in three of 44 patients with hepatocellular carcinoma. All three were HBsAg negative and had normal serum alpha fetoprotein levels. The patients did not have underlying cirrhosis and the tumours contained characteristic intracellular inclusions. In the first patient the UBBC level fell during a partial remission induced by adriamycin therapy and in the second patient UBBC levels rose with progression of her disease. In the third patient serum B₁₂ binding protein levels fell after tumour resection. Assay and subsequent monitoring of serum vitamin B₁₂ and UBBC may prove valuable in the assessment and follow-up of some patients with hepatocellular carcinoma whose alpha fetoprotein levels are normal.

Alpha fetoprotein (AFP) is a well-established tumour marker for primary hepatocellular carcinoma, the serum levels being greatly raised in 70-90% of cases (Alpert, 1976). However, a suitable serum marker for diagnosis and follow-up during treatment has been lacking for those cases in which AFP levels are normal or only slightly raised. Recently, three adolescents (Waxman and Gilbert, 1973; Waxman *et al.*, 1977) and one adult patient (Nexø *et al.*, 1975a) with hepatocellular carcinoma and remarkable rises in serum vitamin B₁₂ binding protein, have been described in detail. In three of these cases the tumour was shown to contain excessive amounts of the binding protein (Burger *et al.*, 1975; Nexø *et al.*, 1975a; Waxman *et al.*, 1977). This closely resembled transcobalamin I (TCI), the alpha-globulin granulocyte-secreted binder of normal serum, but differed from TCI in its amino-sugar content (Burger *et al.*, 1975; Nexø *et al.*, 1975b).

The vitamin B₁₂ binding 'protein' of serum is made up of a number of separate components. All the endogenous circulating vitamin B₁₂ is protein bound, but the binders with the exception of TCI are largely unsaturated. Their unsaturated portions are

assayed together by their ability to bind exogenous radioactive vitamin B₁₂, this being the 'unsaturated B₁₂ binding capacity' (UBBC). The sum of the serum B₁₂ and the UBBC is the 'total vitamin B₁₂ binding capacity' (TBBC) and reflects the total level of serum vitamin B₁₂ binding protein.

We have examined the sera of a group of patients with hepatocellular carcinoma for the presence of increased levels of UBBC, and report in detail the effects of therapy in three cases with greatly raised levels.

Methods

In this study, UBBC was assayed by charcoal radioassay (Gottlieb *et al.*, 1965) using ⁵⁷Co-cyanocobalamin (Radiochemical Centre, Amersham) as described previously (Kane *et al.*, 1974). Serum samples were stored at -20°C until the time of assay when they were diluted in distilled water as appropriate.

SUBJECTS

The normal range of serum UBBC was established in 15 healthy medical and laboratory staff aged 19 to 43 years. The sera of a group of 20 adult patients with non-neoplastic chronic liver diseases, consisting of alcoholic cirrhosis (nine), alcoholic hepatitis (two), cryptogenic cirrhosis (one), primary

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biliary cirrhosis (four), chronic active hepatitis (three), and sclerosing cholangitis (one) were also studied. UBBC was measured in sera from 44 patients aged 15 to 71 years with hepatocellular carcinoma. Six cases had characteristic histological appearances but AFP levels within the normal range, 27 had typical histology and AFP levels above 1000 $\mu\text{g/l}$, five cases displayed typical arteriographic appearances with serum AFP levels exceeding 1000 $\mu\text{g/l}$, and six cases were diagnosed on clinical evidence of primary hepatic carcinoma and markedly increased serum AFP levels (above 3000 $\mu\text{g/l}$). Sera from four children aged 9 months to 3 years with biopsy-proven hepatoblastoma were also studied and three of these cases had grossly raised AFP levels ($>120\,000$ $\mu\text{g/l}$).

Results

The range of UBBC levels in the 15 normal subjects was 0.7-1.6 $\mu\text{g/l}$, while the levels in the 20 patients with non-neoplastic chronic liver diseases ranged from 0.9-2.8 $\mu\text{g/l}$. All four children with hepatoblastoma had UBBC levels below 0.7 $\mu\text{g/l}$. Of the 38 patients with malignant hepatoma and high AFP levels, 35 had serum UBBC levels which were within or below the range for the chronic liver disease group (<2.8 $\mu\text{g/l}$), and three had UBBC levels which were moderately raised above this range (3.7, 4.2, and 6.1 $\mu\text{g/l}$). Of the six patients with hepatoma and normal AFP levels, three had normal or low UBBC levels (0.5, 0.6, and 1.4 $\mu\text{g/l}$), while the remaining three had greatly raised values of serum UBBC (>79 $\mu\text{g/l}$). They are described in greater detail below.

CASE 1

A 15 year old girl presented in February 1977 with a six month history of weight loss and upper abdominal pain. On examination she had a hard and irregular liver extending into the right iliac fossa. Selective hepatic arteriography demonstrated a vascular tumour involving both lobes and a needle biopsy of the liver revealed a hepatocellular carcinoma. Other laboratory investigations included haemoglobin 10.3 g/dl, WBC $8.8 \times 10^9/\text{l}$ (neutrophils 68%), serum AFP <6 $\mu\text{g/l}$. HBsAg was not detected in the serum. Her serum vitamin B₁₂ was 15 500 ng/l (normal 160-750 ng/l) and her serum UBBC was 414 $\mu\text{g/l}$ (normal 0.7-1.6 $\mu\text{g/l}$). Over the next three months she was given five doses of intravenous adriamycin (60 mg/sq.m). She failed to improve clinically and there was a progressive deterioration in her liver function, along with a steady rise in her serum UBBC levels (Fig. 1), which reached 828 $\mu\text{g/l}$ by May 1977.

Fig. 1 Case 1 (M.M., female, aged 15 years, malignant hepatoma). Serial measurement of UBBC during therapy with adriamycin. Values for serum bilirubin and alkaline phosphatase are also shown.

CASE 2

A 16 year old boy was first seen in February 1977 with a two year history of weight loss and abdominal pain associated with an upper abdominal mass. At laparotomy a huge tumour was found to be replacing the left lobe of the liver. The right lobe was normal. The tumour was closely adherent to the pancreas and colon and was, therefore, considered unsuitable for resection. A wedge biopsy showed the tumour to be a hepatocellular carcinoma. Investigations included haemoglobin 11.4 g/dl and WBC $3.8 \times 10^9/\text{l}$ (neutrophils 52%). His AFP was <6 ng/l and his serum was negative for HBsAg. His serum vitamin B₁₂ was 3500 ng/l and his serum UBBC level was 144 $\mu\text{g/l}$. After three doses of intravenous adriamycin given at three weekly intervals his UBBC had fallen to 79 $\mu\text{g/l}$ (Fig. 2). He gained weight and the liver tumour became measur-

Fig. 2 Case 2 (M.K., male, aged 16 years, malignant hepatoma). Serial values of UBBC during treatment with adriamycin. The fall in levels was accompanied by a partial clinical remission.

ably smaller. From May to July 1977 he was given four further injections of adriamycin, the doses being reduced because of myelosuppression. UBBC levels in serum rose again to reach 136 µg/l by mid-August 1977 when a repeat laparotomy was performed in the hope that the tumour would now be resectable but the findings were comparable to those of six months previously. Over the period of treatment there was no change in the standard tests of liver function

CASE 3

This 30 year old woman presented with multiple spider naevi, palmar erythema, and hepatomegaly. Her haemoglobin was 12.9 g/dl, WBC 10×10^9 /l (neutrophils 57%). Her serum contained no detectable AFP and HBsAg was also absent. Serum vitamin B₁₂ was 13 000 ng/l and UBBC was 79 µg/l. Arteriography showed a highly vascular tumour in the right lobe of the liver. A right hepatic lobectomy was performed on 9 September 1977 (Mr K. Reynolds). Histological examination of the tumour confirmed a highly malignant hepatocellular carcinoma which was invading the portal vein and infiltrating the diaphragm. The left lobe of the liver was congested but otherwise normal. Follow-up chest radiography showed a few small rounded shadows suggestive of pulmonary metastases. Three weeks post-operatively her serum vitamin B₁₂ was 38 000 ng/l, but her UBBC had fallen to 1.6 µg/l. Thus her TBBC was 92 µg/l preoperatively and 39.6 µg/l postoperatively.

HISTOLOGICAL FEATURES

The tumour in case 2 was a well-differentiated (grade II) hepatocellular carcinoma and in cases 1 and 3 moderately differentiated (grade III of Edmondson). Bile was conspicuous in cases 1 and 2 but rare in case 3. PAS-positive diastase-resistant autofluorescent globular bodies were seen intra- and extracellularly in all cases. In cases 2 and 3 there was also PAS-positive diastase-resistant and alcian blue-positive granular material within vacuoles. Electron-microscopy in cases 2 and 3 showed the globular

bodies as electron dense granular inclusions of variable size surrounded by a unit membrane. In both cases there was abundant rough endoplasmic reticulum with dilatation of cisternae which contained granular material. The electronmicroscopic features are similar to those described in a previously reported liver tumour associated with high UBBC (Nexø *et al.*, 1975a).

Discussion

The three cases of hepatocellular carcinoma with very high UBBC levels described here and the four cases previously reported in detail in the literature have similarities which are summarised in the Table. None of the patients had pre-existing cirrhosis, which may produce a modest rise in UBBC, as is well established (Rachmilewitz *et al.*, 1956; Herbert, 1968), and was apparent in the present series of patients with chronic liver diseases. None had raised AFP levels and the six patients tested were negative for HBsAg. Neutrophil leucocytosis is associated with some rise in vitamin B₁₂ binding protein levels (Carmel, 1972), but none of the present patients or those reported previously had a leucocytosis. Of the six in whom histological details were available, five had inclusions within the malignant hepatocytes. Intracytoplasmic PAS-positive diastase-resistant globular inclusions are found in about 15% of all hepatomas, but their frequency appears to be higher in patients with high AFP levels (Cohen, 1976). It has been suggested that they may represent hepatocyte-produced glycoproteins; and in some cases AFP (Chu *et al.*, 1974) and α -1-antitrypsin (Palmer and Wolfe, 1976) have been found in these bodies by immunofluorescence or immunoperoxidase methods. No specific antisera have yet been used to demonstrate the presence of vitamin B₁₂ binding protein in hepatomas but the protein extracted from previously reported cases had a very high sialic acid content and it would be reasonable to expect positive staining with methods such as alcian blue specific for acid mucopolysaccharides. In our cases alcian blue and PAS-positive material was found within intracellular

Table Details of three patients in present series, and cases previously reported in detail in literature

Authors	Age (y)	Sex	Rise in AFP	Intracellular inclusions	Serum UBBC (µg/l, normal 0.7-1.6 µg/l)	Serum B ₁₂ (ng/l, normal 160-750 ng/l)
Waxman and Gilbert (1973)	13	M	Absent	Pos.	243	18 200
Nexø <i>et al.</i> (1975a)	15	F	Absent	Neg.	480	53 100
	47	F	Absent	Pos.	4 400-25 800	13 800
Waxman <i>et al.</i> (1977)	16	M	Absent	—	2.0-784	8 000-35 200
Present series	15	F	Absent	Pos.	414	15 5000
	16	M	Absent	Pos.	144	3 500
	30	F	Absent	Pos.	79	13 000

vacuoles and might represent B₁₂ binding protein, but the well formed PAS positive globular bodies were alcian blue-negative. Serum alpha-1-antitrypsin levels were raised in our three cases (7.1-10.6 g/l), making it most unlikely that the inclusions were related to alpha-1-antitrypsin deficiency (Sharp, 1971). Raised plasma levels have been reported previously in a variety of liver diseases (Murray-Lyon and Williams, 1974).

The studies of Nexø *et al.* (1975a) and Burger *et al.* (1975) have shown increased levels of hepatoma vitamin B₁₂ binding protein in tumour tissue compared with normal liver, and Waxman *et al.* (1977) demonstrated similar rises in a perfusate from the tumour of their patient. The perfusate contained increased amounts of sialyltransferase, prompting their suggestion that the vitamin B₁₂ binder is granulocyte-derived but is sialylated during transit through the malignant hepatocytes. The carbohydrate-containing material seen on light microscopy in all our cases, together with the very prominent dilated rough endoplasmic reticulum demonstrated on electron microscopy in two cases, are features consistent with a role of the tumour cells in production or alteration of the glycoprotein. The considerable fall in TBBC after excision of the tumour in case 3 points to the hepatoma as the source for the great rise in vitamin B₁₂ binding protein found before operation.

All the patients with high UBBC values also had considerable rises in serum vitamin B₁₂, although in most instances this was very much less than the UBBC. In one case in the literature high serum vitamin B₁₂ levels preceded the appearance of a high UBBC, but the hepatoma B₁₂ binder increased in concentration some 400-fold and became progressively desaturated as the disease progressed (Waxman *et al.*, 1977). Waxman and colleagues (Waxman and Gilbert 1973; Waxman *et al.*, 1977) mention nine additional children and adolescents with hepatocellular carcinoma who had a two to 10-fold rise in serum alpha-globulin B₁₂ binding protein and four of them had a considerable rise in serum vitamin B₁₂. Eight had no detectable serum AFP, and AFP was slightly raised (82 µg/l) in one case. In a survey of 36 adult patients with liver cell cancer they found none with abnormal B₁₂ binding protein but the case of Nexø *et al.*, (1975a) and our case 3 show that the abnormality is not confined to children and adolescents. The similarities between the patients reported so far with hepatomas and high B₁₂ binding protein levels suggest that they represent a separate sub-group within the spectrum of hepatocellular cancer. However, high serum levels of abnormal vitamin B₁₂ binding protein are not entirely specific to hepatocellular car-

cinoma and, as with AFP, have been described in cases of disseminated carcinoma, particularly with tumour metastatic to the liver (Carmel, 1975; Carmel and Eisenberg, 1977).

Our survey of patients with malignant hepatoma showed that gross rises in UBBC did not occur in those who had substantial rises in serum AFP. On the other hand, three of the six patients with normal AFP levels had high UBBC levels. In these cases the relatively simple assay for UBBC proved useful for monitoring the response to chemotherapy or for follow-up after surgical excision, though the rise which occurred in vitamin B₁₂ in case 3 in spite of the fall in UBBC suggests that, for a true reflection of B₁₂ binding protein levels, the TBBC should be determined by assaying both serum vitamin B₁₂ and UBBC. An awareness of this alternative tumour marker is of increased importance now that effective chemotherapeutic agents are available for the treatment of hepatocellular carcinoma (Johnson *et al.*, 1978).

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