Pergamon

European Journal of Cancer, Vol. 34, No. 12, pp. 1935–1940, 1998

Printed in Great Britain
0959-8049/98 $19.00+0.00

PII: S0959-8049(98)00196-8

Original Paper

Serum γ-Glutamyltransferase and Alkaline Phosphatase During Experimental Liver Metastases. Detection of Tumour-specific Isoforms and Factors Affecting their Serum Levels

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Tumour-specific isoenzymes and tumour markers in serum are potentially useful in the detection and monitoring of liver metastases. An experimental rat model was used in the search for such isoenzymes and to study factors affecting their serum levels. Splenic injection of CC531 colon carcinoma cells in syngeneic WagRij rats caused liver metastases after 3 weeks with concomitant and significant increases in serum levels of γ-glutamyltransferase (GT) and alkaline phosphatase (ALP). The presence of tumour-specific isoforms of both enzymes, as well as increased amounts of the liver isoform of ALP, were demonstrated in serum. The serum levels of the tumour variants were clearly related to their elimination rates from the circulation. Thus, the slow clearance of the tumour ALP resulted in high serum levels of this isoform, compared with the more rapid elimination of tumour GT and its lower serum level. When using another colon carcinoma cell line (DHD/K12), metastatic to liver in BD IX rats, no increases in serum GT were detected. This was related to the rapid elimination from the circulation of the GT variant from the DHD/K12 metastatic tissue. The relatively high amount of the tumour ALP isoform detected in serum during growth of the CC531 liver metastases indicated that this isoform could be useful as a marker of tumour growth. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: γ-glutamyltransferase, alkaline phosphatase, cultured tumour cells, experimental liver neoplasms, enzymology liver neoplasms
Eur J Cancer, Vol. 34, No. 12, pp. 1935–1940, 1998

INTRODUCTION

SERUM γ-GLUTAMYLTRANSFERASE (GT) and alkaline phosphatase (ALP) are widely used as diagnostic parameters of liver diseases. The activities of both enzymes are frequently elevated in patients with liver neoplasia, but their low sensitivity and specificity as tumour markers have resulted in a search for more tumour-specific isoforms. Both enzymes are glycoproteins showing large variations and heterogeneity in their carbohydrate chains when isolated from various organs and tissues. GT belongs to a multigene family in humans, but is a single gene copy in rats [1]. Only one protein product has been described in these species, but different forms of GT have been described in organs and various tumour tissues which differ in their carbohydrate composition and structure [2–4]. ALP is coded for by four gene loci in humans and by two genes in rats [5]. Tumour-specific isoenzymes, such as the group of placenta-like ALP, have been described [5] and a recent study comparing normal rat liver ALP and hepatoma ALP demonstrated significant differences in their carbohydrate chains [6], including high amounts of fucosylated high mannose chains in the tumour isoform.

Although tumour-derived isoforms of GT and ALP have different glycan structures when compared with their normal counterparts, there is also a considerable microheterogeneity among each isoform, which will affect the diagnostic utility of the tumour isoforms. An experimental model is required for the study of how such variants can be detected, how they are released into the circulation and what factors affect their clearance from the circulation. To be detectable in serum, the

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Received 17 Nov. 1997; revised 10 Mar. 1998; accepted 11 Mar. 1998.

tumour-specific variants must be cleared at a low rate. The elimination of GT and ALP from the circulation has been shown to occur via the hepatic galactose (or asialoglycoprotein) receptor [7–9]. Variants with reduced sialic acid content possess a higher affinity for the receptor, making such forms difficult to detect [7]. The elimination rates of GT and ALP isolated from human liver were relatively low, due to the high content of sialic acid on both enzymes.

We used an experimental liver metastases model in the rat to study the mechanisms behind serum GT and ALP changes during tumour growth. Two colon carcinoma cell lines metastatic to liver were used. Both cell lines possess significant GT and ALP activity. A search for specific tumour variants of these enzymes in serum was performed after comparing the enzymes in tumour tissue with the enzymes in liver. We also investigated factors that could affect the release of GT and ALP from liver and tumour tissue into the circulation, as well as factors affecting their elimination from the circulation. A better understanding of the determinants of serum enzyme increases may result in a better clinical interpretation of these parameters during growth of liver metastases.

MATERIALS AND METHODS

Tumour cells

Two tumour cell lines, CC531 and DHD/K12 (DHD cells) [10, 11] derived from primary colon carcinomas after chemical carcinogenesis in rats (WagRij and BD IX rats, respectively) were used. The CC531 cells were kindly donated by Dr C. Thomas (University of Groningen, The Netherlands) and the DHD cells by Dr M.S. Martin (Faculty of Medicine, Dijon, France). Both cell lines were grown in RPMI 1640 medium with 10% fetal calf serum and antibiotics, using an atmosphere of 100% humidity and 5% CO_2. Tumour cell suspensions for *in vivo* injections were obtained from nearly confluent cell layers by trypsin ethylenediamine tetraacetic acid (EDTA) treatment at room temperature. The cells were washed once with RPMI and resuspended in medium at a concentration of 2×10^6 cells/ml or 2×10^7 cells/ml.

Animal experiments

Male WagRij and BD IX rats (200–250 g) were obtained from Charles River (WIGA GmbH, Sulzseld, Germany). They were maintained in regularised conditions in the Animal Department at the Institute, and fed standard chow *ad libitum*. All experiments were approved by the National Council for Animal Research. The animals were anaesthetised by subcutaneous injections of a 1:1 mixture of Hypnorm (fluarisol plus fentanyl, obtained from Jannsen Pharmaceutica, Belgium) and Dormicum (obtained from Roche, Basel, Switzerland). Temgesic (Burprenorfin, Reckitt & Colman, U.K.) was used postoperatively as a painkiller.

Liver metastases were initiated by injecting tumour cells (1×10^6 CC531 cells or 1×10^7 DHD cells) suspended in 0.5 ml RPMI medium into the spleen of the respective syngeneic rats. Sham operated controls were injected with 0.5 ml RPMI medium. The higher number of DHD tumour cells and performing a splenectomy ensured a satisfactory incidence of liver metastases in the BD IX rats [12, 13]. A total of 20 WagRij rats and seven BD IX rats were included in the experiments, but one animal in each of the two groups died postoperatively due to bleeding. The CC531 rats were examined daily after 2 weeks and the BD IX rats after 6 weeks for signs of tumour growth. The animals were terminated as soon as signs of distress were evident. Blood samples were obtained by heart puncture or from a tail vein. Serum was isolated after coagulation and centrifugation (15 min at 3,000***g***) and kept frozen (− 20°C) prior to analysis.

Enzyme elimination measurements were performed as previously described [8]. Partially purified enzyme samples were injected into a tail vein. Volumes of 200 µl blood were collected frequently during the following 30 min from the tail tip. After coagulation and centrifugation (15 min at 3,000***g***), serum was collected for enzyme analysis, which was performed within 2–3 h. All data on serum enzyme activities were expressed as a percentage of the activity in blood sampled 2 min after enzyme injections, after subtracting the basal enzyme activity level in a serum sample collected prior to sample injections. Clearance studies with inhibitors of glycoprotein receptors were performed by injections of 5.0 mg of asialofetuin or a mixture of 1.5 mmol mannose and 5.0 mg invertase prior to the injection of enzyme samples.

Enzyme measurements

Enzyme activity was measured at 37°C according to the recommendations of IFCC [14, 15], using commercial reagent kits from Boehringer Mannheim, Germany. The measurements were performed in Cobas Fara centrifugal analyser (Hoffman-LaRoche, Basel, Switzerland).

Comparisons of median values for groups of enzyme activities were performed using Wilcoxon's *t*-test, and differences with *P* values, less than 0.05 were considered significant.

Enzyme preparations

GT was partially purified from liver metastases and unaffected liver tissue using modifications of previously described methods [8, 16]. Briefly, the enzyme was solubilised with deoxycholate (1% final concentration) after homogenisation. Polyethyleneglycol 6000 was added to 4% final concentration, discarding the precipitate by centrifugation. The GT solution was treated with papain and the enzyme was eluted through a phenyl Sepharose column in the presence of 0.15 M NaCl. A final purification was obtained using Concanavalin A Sepharose and gel filtration. The specific activity of the final tumour tissue preparation was 20–50 U/mg and 0.5 U/mg for the rat liver preparation which possess a much lower GT content. GT in pooled serum from rats with activities higher than 15 U/l was concentrated to approximately 450 U/l using the same procedure. ALP was partially purified after solubilisation of tissues with 1% Triton X100 in 0.1 M Tris–HCl (pH 6), followed by butanol treatment [9]. Preparations with a specific activity of 20–200 U/mg were obtained after further purification using phenyl Sepharose, Concanavalin A Sepharose chromatography and gel filtration.

Agarose gel electrophoresis

For the separation and comparison of both GT and ALP isoforms, electrophoresis using Beckman Paragon SPE agarose gels was performed as recommended by the manufacturer (Beckman Instruments, Brea, California, U.S.A.). Sample volumes of 3–5 µl with GT activities of 300–900 U/l and ALP activities of 4–800 U/l were applied to the gels. The zymograms were developed in 10 ml of the GT assay mixture with 50 mg naphthylethylenediamine and 10 mg $NaNO_2$ or in 10 ml diethanolamine buffer (pH 9.8) with the NBT/BCIP substrates for ALP (50 mg/ml nitroblue tetrazolium and 25 mg/ml 5-bromo-4-chloro-3-indolylphosphate).

RESULTS

Liver metastases were detected in all 19 WagRij rats injected with CC531 cells, and 3 weeks after the injections the total liver mass was increased by 30–60%. Significant elevations in serum GT activity (3–30-fold) were observed in all animals after 3 weeks (Figure 1). Serum ALP activity was increased in 11 of 19 animals when compared with the control group, but their median values were not significantly different ($P = 0.075$). The animals with high tumour burden also showed highly elevated serum activities, but no significant correlations were found between enzyme activities and tumour mass. A total of seven metastatic animals had jaundice after 3 weeks, with bilirubin values > 8 μM (Figure 1) and the remaining animals showed low bilirubin values (< 3 μM). There were no significant differences in GT and ALP activities between the jaundice and the non-jaundice animals ($P > 0.05$). In the control rats, no changes in serum GT and ALP activities or bilirubin were detected. The activities of both enzymes were higher in tumour tissue than in unaffected liver tissue (Table 1). The low basal level of serum GT (< 3 U/l) is clearly related to the low GT level in rat liver.

The growth of DHD metastases in BD IX rats was much slower than the growth of CC531 metastases in WagRij rats. These animals were terminated after 10–12 weeks, at which time the tumour mass was comparable with that in WagRij rats after 3 weeks. However, six of seven animals showed no rise in serum GT (Figure 1) and the one that did presented a tumour mass much larger than the other six. This animal also showed a highly increased ALP activity (780 U/l).

Tumour-specific enzyme variants were detected in the serum of WagRij rats using agarose gel electrophoresis. GT isolated from tumour tissue showed a different electrophoretic mobility when compared with the enzyme from unaffected liver, but identical to the enzyme isolated from pooled serum from rats with liver metastases (Figure 2a). The liver GT isoform was not detectable in this serum preparation. GT isolated from DHD metastatic tissue migrated more slowly than the CC531 enzyme, caused by the absence of sialic acid on the DHD enzyme (Figure 2b). ALP isolated from tumour tissue showed a different electrophoretic mobility compared with liver ALP (Figure 3a), and its mobility differed also from those of intestinal and placental ALP. In all serum samples from rats with metastases, the presence of tumour ALP was clearly detected (Figure 3b) together with the liver ALP (Figure 3b). The amount of tumour ALP in the serum was apparently equal to or even higher than the liver ALP band, although increased liver ALP was also detected.

Table 1. Enzyme activities in CC531 metastatic tumour tissue and unaffected liver of WagRij rats. Activities were measured in homogenised and solubilised tissue, and related to total protein content. Results are mean ± standard deviation from five different animals

	GT (U/g)	ALP (U/g)
Unaffected liver	2.4 ± 0.4	10.1 ± 4.1
Tumour	69.9 ± 20.4	104.9 ± 48.3

GT, γ-glutamyltransferase; ALP, alkaline phosphatase.

The elimination rate of GT and ALP from the rat circulation was measured after intravenous (i.v.) injections of purified enzyme preparations into rats without tumours. The clearance of GT isolated from CC531 tumours (Figure 4a) was more rapid than the clearance of ALP from both CC531 tumours (Figure 4b) and normal liver (Figure 4b). To test whether liver metastases reduced the uptake of circulating enzymes, the clearance of GT isolated from CC531 tumours was measured after injection into animals with metastases (2.5 weeks after tumour cell injection). No significant change in elimination rate was observed when compared with that in

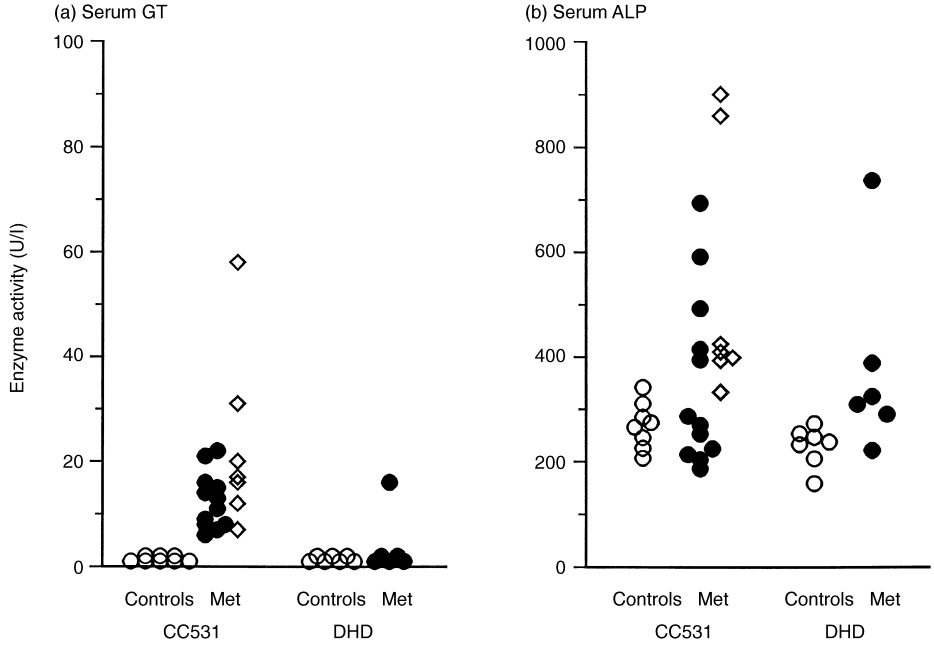

Figure 1. Serum γ-glutamyltransferase (GT) (a) and alkaline phosphatase (ALP) (b) activities in control and metastasised rats. The enzyme activities were measured in serum of controls or metastasised rats (Met), 3 weeks after injections of 1×10^6 CC531 cells in WagRij rats, and 10–12 weeks after injections of 1×10^7 DHD cells in BD IX rats, respectively. The metastasised WagRij rats indicated by ◇ had jaundice.

untreated animals (Figure 4a). As shown in Figure 5(a) the clearance of CC531 GT was inhibited by both asialofetuin and by the invertase/mannose mixture. The DHD GT enzyme was cleared very rapidly, and the elimination of this enzyme was inhibited by invertase/mannose, but not by asialofetuin (Figure 5b).

DISCUSSION

The present experimental model on liver metastases is based on the injection of a large bolus of colon carcinoma cells into the spleen of syngeneic rats [12, 17–19]. In all WagRij rats injected with CC531 cells, a significant tumour mass was detected after 3 weeks, with a concomitant elevation in serum GT and ALP. As such elevations may parallel the frequent rise in serum enzymes in patients with liver metastases, this rat model appears useful for the investigation of the mechanisms behind the increases in serum enzyme activities and in the search for tumour-specific variants.

The increases in serum GT were detectable due to the very low and constant level of normal rat serum GT (< 3 U/l). In fact, slightly elevated values (2–6 U/l) were also measured in several animals after 2 weeks (data not shown). The increased serum GT was further identified as the tumour enzyme by agarose gel electrophoresis. Thus, the standard assay for the measurement of GT [15] apparently quantifies the tumour enzyme. The increased serum ALP observed in the third week was caused by ALP released from tumour tissue as well as from liver. The quantification of the two ALP forms using electrophoresis was difficult, due to the presence of several isoforms with similar mobilities (liver, bone and tumour). However, the amount of tumour ALP was detectable in significant amounts in all sera tested and in fact in larger amounts than liver ALP in several rats.

The GT and ALP isoforms may be released as a result of necrosis, inflammation, as well as cholestasis due to the influence of the growth of the metastases [17–20]. Cholestasis has been reported in rats with liver metastases at a terminal stage [17] and also during rapid tumour growth following selective elimination of Kuppfer cells [19]. The occurrence of increased bilirubin in seven of 19 animals is

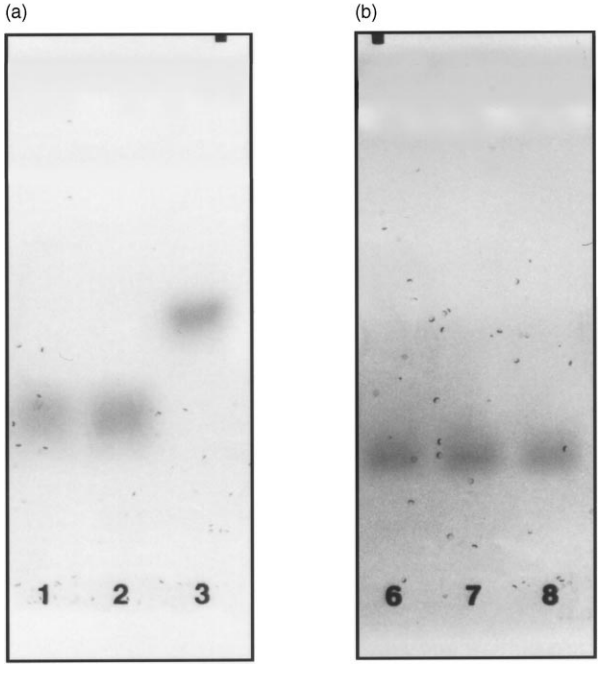

Figure 2. Agarose gel electrophoresis of γ-glutamyltransferase (GT) isoforms. (a) shows the mobilities of partially purified GT from CC531 tumour tissue (lane 1), pooled serum from rats with CC531 liver metastases (lane 2), and from unaffected liver (lane 3). (b) shows the mobilities of partially purified GT from DHD tumour tissue (lane 6), and neuraminidase treated enzyme preparations from CC531 and DHD tumour tissues (lanes 7 and 8).

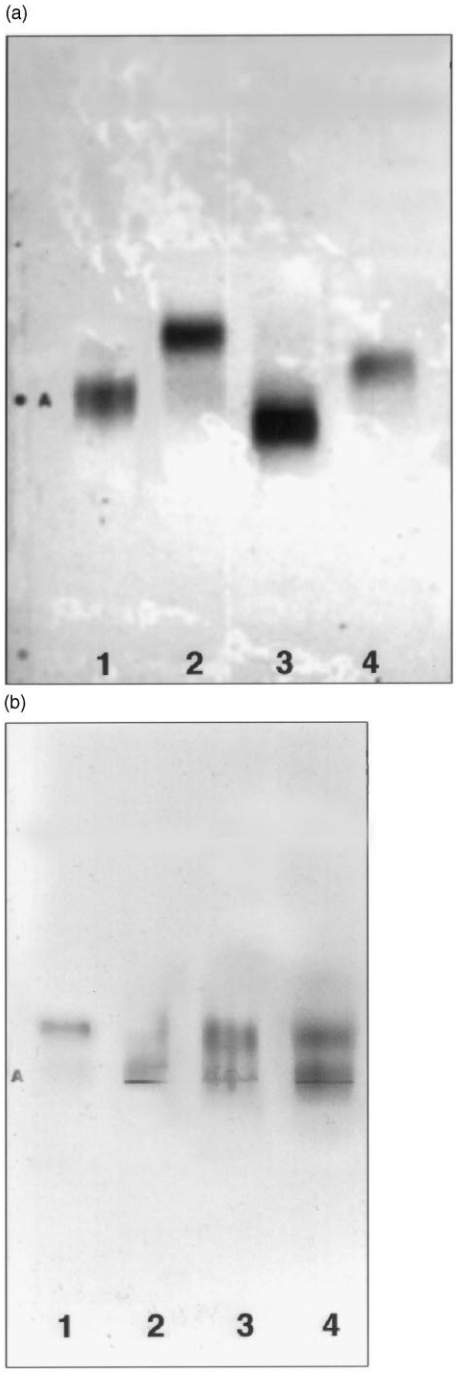

Figure 3. Agarose gel electrophoresis of alkaline phosphatase (ALP) isoforms. (a) shows partially purified ALP from CC531 liver metastases (lane 1), unaffected liver (lane 2), large intestine (lane 3), and placenta (lane 4). (b) shows ALP from serum of a control rat (lane 1), and from rats with liver metastases (lanes 2–4).

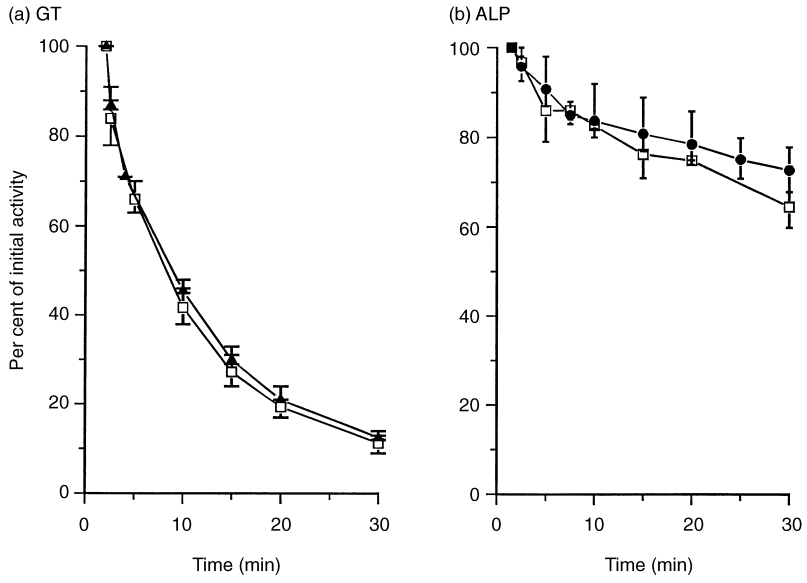

Figure 4. Elimination rate of γ-glutamyltransferase (GT) (a) and alkaline phosphatase (ALP) (b) from rat circulation. Partially purified enzyme preparations were injected intravenously into rats. GT and ALP activities were measured in blood sampled 30 min after injection, and expressed as a percentage of the initial activity measured 2 min after injection and after subtracting the rat's basal (endogenous) activity. Each curve is the mean of three to four experiments and the bars indicate the range. (a) shows the elimination rate of GT from CC531 tumour after injection into untreated rats (-□-) and in rats with liver metastases (-▲-). (b) shows the elimination rates of ALP from CC531 tumour (-□-) and liver (-●-) after injection in untreated rats.

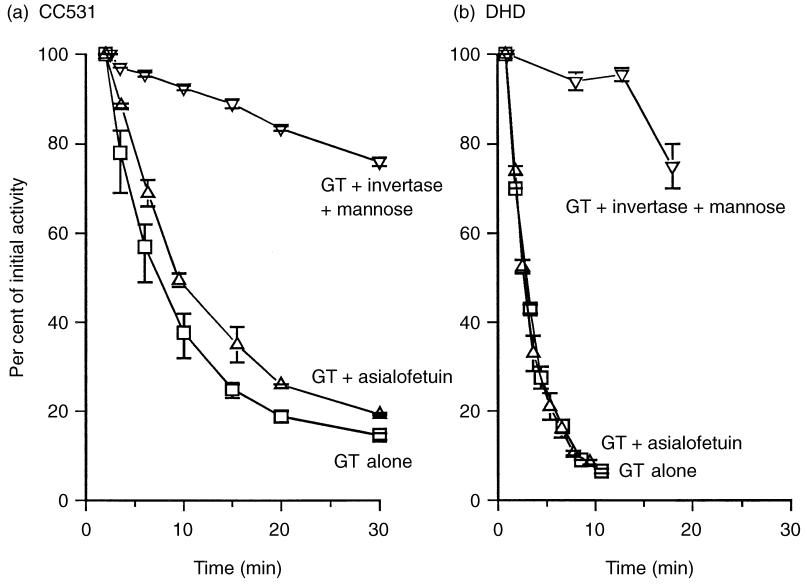

Figure 5. Elimination rates of γ-glutamyltransferase (GT) from rat circulation. Partially purified GT from CC531 tumour (a) and DHD tumour (b) were injected intravenously into rats. The GT activity in blood samples was measured and expressed as described in Figure 4. The curves indicate the elimination of GT alone (-□-), or after injection of 5.0 mg asialofetuin (-△-) or 5 mg invertase and 1.5 mmol mannose (-▽-). Each curve is the mean of three to four experiments and the bars indicate the range.

difficult to explain, but may be related to anatomical differences in the tumour cell deposition and subsequent growth of metastases, which in some cases would have grown close to and thereby blocked biliary ductules [12, 13, 17–19]. Both ALP and GT can be induced in liver during cholestasis [21–23], leading to increased enzyme release into the circulation. A local accumulation of bile salts may lead to solubilisation of plasma membranes [21, 22] of both hepatocytes and tumour cells, resulting in further release of GT and ALP into the circulation. We were unable to detect the liver GT in pooled serum, but this result needs confirmation, particularly in jaundice rats.

A slightly lower GT level was detected in the DHD tumour compared with the CC531 tumour and may partly explain the low level of serum GT in BD IX rats with liver metastases. Probably more important is the rapid clearance of the DHD GT from the circulation. The DHD enzyme showed a high affinity for the mannose receptor, as indicated by the significant inhibition of the clearance rate by the mannose/invertase mixture (B. Smedsrød, University of Tromsø, Norway). As this isoform also lacks sialic acids, the DHD GT may possess only high mannose chains. The competitive clearance studies on the CC531 GT indicated that this tumour variant was eliminated by both the asialoglycoprotein

receptor and the mannose receptor. This finding, together with the electrophoretic studies, suggest that CC531 GT possesses both complex, partly sialylated chains and high mannose chains. We have previously demonstrated two GT variants in human colon carcinomas and liver metastases with similar differences; one being sialylated and the other non-sialylated [24]. This indicates, as asialoforms of GT are cleared rapidly from the circulation [7, 8], that GT variants in several colon carcinomas and metastasis may be difficult to detect in serum, leading to low sensitivity as tumour markers. The elimination of ALP was significantly slower than for GT, and this finding, in combination with the high concentration of ALP and tumour, may well explain the relatively higher amounts of the tumour ALP detected in serum. The presence of several isoforms and the large heterogeneity of both enzymes in various rat and human tumours [2, 6], clearly show that it is necessary to confirm whether specific glycan changes in GT and ALP exist that are constant findings in specific tumour tissues [6]. A more detailed study of the two tumour-derived enzymes is now in progress and the possibility of making tumour-specific assays for GT and ALP is being investigated.

The present study has shown that tumour-specific variants of GT and ALP can be detected in serum during experimental liver metastases, and that the level of these variants depends on the amount of enzymes in the tumour and on the carbohydrate composition of the enzymes, which in turn determines their low elimination rate from the circulation. The CC531 tumour cells express sialylated GT and ALP forms which are detectable in serum due to their low elimination rates. The particular low clearance of tumour ALP and its high level in tumour tissue indicate that this isoform may be an interesting tumour marker in our model. However, to evaluate the diagnostic utility of tumour-derived forms of GT and ALP, more sensitive and specific assays must be developed.

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Acknowledgements—This study was supported by the Norwegian Cancer Society (grant no. A 97053/003) and the Aakre Foundation, Tromsø, Norway.