Influence of the Liver on the Profile of Circulating Antigens Recognized by Antiserum against Hepatoma Membrane Glycoproteins

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ABSTRACT

Rat hepatoma tissue culture cells have retained only a few liver-specific membrane functions. To find out whether transformation of these cells led to the appearance of tumor-specific membrane glycoprotein structures, which might also be present in released form in the circulation of tumor-bearing animals, we compared immunologically the sera and ascites fluids of normal and of hepatoma-bearing rats. For structural identification, we used polyclonal antibodies against the membrane glycoproteins of the hepatoma cells. Crossed immunoelectrophoresis of the body fluids resulted in the separation of several antigens, one of which appears to be tumor specific. Immunoelectrophoretic analyses of the glycoproteins from the membranes of liver and hepatoma cells revealed many shared antigens; in one instance, we could assign the membrane associated form to major antigen detected in the serum. Characterization of the molecular size and charge of two selected antigens by two-dimensional gel electrophoresis indicated that the hepatoma cell products were not completely sialylated. In the case of the circulating, hepatoma-specific antigen, this property appears to result in hepatic clearance of the more basic (''asialo'') portion of the glycoprotein species via the receptor-mediated uptake of galactose-terminated glycoproteins. The result was the accumulation of the acidic forms of the antigen in the serum and ascites fluid of tumor-bearing rats.

INTRODUCTION

When a cell undergoes transformation to a malignant state, a multitude of cellular, subcellular, and molecular properties are subject to change (18). Alteration of the cellular phenotype is often coupled with the appearance of specific markers, such as proteins, glycoconjugates, enzymatic activities, and metabolites, etc. These markers can be found intracellularly, on the plasma membrane, or secreted into the surrounding milieu (7). The release of specific and recognizable products by the tumor tissue into the circulating system of the organism would provide a useful tool to monitor the presence and the growth of a particular tumor (19). Currently, efforts are being made to establish a catalog of such markers and to determine their general diagnostic usefulness. There are numerous reports on the occurrence and characterization of tumor-specific marker molecules in transformed cells in tissue culture. Although these findings seldom allow generalization because they are restricted to unique systems, studies on tissue culture cells nevertheless provide useful information about the biogenesis and metabolic behavior of such tumor products.

In analyzing the turnover properties of the plasma membrane in rat HTC cells, we noticed that a minor but specific set of surface-exposed glycoproteins were turned over not by lysosomal degradation but instead by extracellular proteolytic fragmentation and the subsequent release of specific glycopeptides into the culture fluid (4). The question arises as to whether a similar mechanism exists in vivo, when HTC cells are growing i.p. as solid tumors, and whether hepatoma-specific glycopeptides can be detected in the circulation of tumor-bearing rats. To test this hypothesis, we prepared antibodies against the nonfractionated mixture of glycoproteins purified from the membrane fraction of hepatoma cells in tissue culture. In this report, we used these immunoglobulins to screen the ascites fluid and serum of HTC cell tumour-bearing rats for major antigenic markers. We found that both the liver and the hepatoma released antigens into the serum of HTC cell-bearing rats, the levels of which in the body fluids were changed during progressive tumor growth.

MATERIALS AND METHODS

Cells and Hepatomas. A cloned cell line of rat HTC cells (Morris hepatoma 7289C) (14, 16) was grown in monolayer culture in Eagle's minimal essential medium containing 50 mM N-tris(hydroxymethyl)-methylglycine, 1 mM CaCl2, and 10% fetal calf serum and under an atmosphere of 95% air-5% CO2. For growing solid tumors, freshly trypsinized HTC cells in 2 ml of phosphate-buffered saline (140 mM NaCl-10 mM KCl-10 mM sodium phosphate, pH 7.4) were injected i.p. into male Buffalo rats (300 to 400 g). The tumors present in the abdominal cavity were excised and freed of host tissues. Their wet weights were measured to nearest g values. These determinations are less accurate in cases of large tumor burdens because not all tumor could be recovered. Bleeding of the animals was performed by heart puncture.

Radioactive Labeling. HTC cells were metabolically labeled for 24 hr with L-[3H]glucose (22 Ci/mmol; Amersham/Searle, Arlington Heights, III.); in a concentration of 15 μCi/ml of culture medium or with [35S]methionine (1100 Ci/mmol, New England Nuclear, Boston, Mass.) in a concentration of 50 μCi/ml. For recovering labeled and secreted proteins, the media were removed from the cells, centrifuged for 5 min at 1000 x g and for 60 min at 200,000 x g, and dialyzed against 50 mM NH4HCO3. After freeze-drying, the medium proteins were redissolved in phosphate-buffered saline in one-tenth of the original volume. To obtain higher concentrations of medium proteins (see Fig. 7, A and B), the cells were labeled initially in medium containing 2% fetal calf serum. For labeling hepatoma tissue, the technique described earlier was used (3). To label soluble proteins (serum and ascites fluid), we treated 200-μl aliquots of the protein solution with [35S]methionine as outlined by Hunter and Greenwood (10). To remove the nonreacted radiolotope, the proteins were chromatographed on Sephadex G-25 in phosphate-buffered saline. Membrane glycoproteins were labeled with [35S] and chloramine-T while still bound to concanavalin A-Sepharose. After reaction, the lectin column was washed free of...
radioactive iodide. Then, the labeled glycoproteins were eluted with α-methylmannoside as outlined below.

**Isolation of Membrane Glycoproteins.** To obtain a membrane glycoprotein fraction suitable for immunological and electrophoretic analysis, we used a technique modified slightly from that described earlier (5). All buffers and solutions contained 0.5 mM phenylmethylsulfonyl fluoride, and each step was carried out at 0–4°C. HTC cells were removed by scraping from the support and were incubated prior to homogenization for 15 min in 5 mM Tris-HCl, pH 8.0, at a concentration of 5 × 10^5 cells/ml to allow them to swell. Livers were perfused with phosphate-buffered saline. Then the cells and liver tissue (1 g in 10 ml solution) were disrupted in a Teflon-glass homogenizer in 0.25 M sucrose-5 mM Tris-HCl, pH 8.0. The homogenates were cleared of cell fragments, nuclei, and mitochondria by centrifugation for 10 min at 2000 x g followed by 10 min at 15,000 x g. The supernatant fractions were centrifuged for 60 min at 200,000 x g. To remove secretory proteins, the resulting membrane pellets were ultrasonicated for 3 sec in 50 mM Tris-HCl, pH 7.4, and the membrane fragments were recovered by ultracentrifugation. This washing procedure was repeated once. The glycoproteins in the final membrane fractions were extracted by sonication in 1% sodium deoxycholate in 10 mM Tris-HCl, pH 8.0. After centrifugation of the extract for 1 hr at 200,000 x g, the supernatant solutions containing not more than 25 mg of protein were applied to a 300-μl column of concanavalin A-Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.). The nonbound material was eluted with 20 μl of deoxycholate-Tris buffer, followed with 10 μl of phospho-buffered saline containing 1% Nonidet P-40. The bound glycoproteins were eluted after incubation of the column with 0.5 M α-methylmannoside in phosphate-buffered saline for 1% Nonidet P-40 18 hr. The total volume collected was kept to a minimum (400 to 500 μl) to avoid dilution. Aliquots from the final eluates could be used for electrophoretic analyses without any further treatment.

**Affinity Chromatography on Hepatic Receptor for Galactose-terminating Glycoproteins.** The receptor for galactose-terminating glycoproteins was purified from acetone powder of rat liver as described earlier (9). Two mg of the receptor protein were covalently coupled to 4 ml of Sepharose CL-4B, using the method of March et al. (13). The activity of the immobilized receptor protein was verified by specific binding of 125I-labeled human asialoorosomucoid. For affinity chromatography, the column containing 0.5 ml of receptor beads was washed with 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, and 5 mM CaCl2. The glycoprotein sample in the above buffer was applied and recycled 5 times through the column for efficient binding. Then, the column was washed free of unbound material with 30 ml of buffer. The bound glycoproteins were eluted in 50 mM Tris-HCl, pH 7.4, containing 5 mM EDTA, 0.5 M β-lactose, and 1% Nonidet P-40.

**Antiserum.** As described previously (3), a goat was immunized with a preparation of nonfractionated glycoproteins purified from the membrane fraction of HTC cells by chromatography twice on concanavalin A-Sepharose. Concanavalin A was chosen because, unlike other lectins, it recognizes most when not all glycoprotein species present in HTC cell plasma membranes (4–6). The antiserum used in this study was derived from the final bleeding of the animal performed after the sixth antigen injection (6).

**Crossed Immunoelectrophoresis.** The separation of antigenic glycoproteins was carried out by crossed immunoelectrophoresis as described previously (3, 17). The sample volumes ranged between 15 and 30 μl. The separation in the first dimension (in the figures from left to right) occurred at 5 V/cm for 4 hr and that in the second dimension occurred at 2 V/cm for 22 hr. When serum, ascites fluid, or culture medium was analyzed, the second dimension contained 7.5% antisera, and when membrane glycoproteins were separated (generally 30 μg), it contained 2% antisera. To identify antigens on crossed immunoelectrophoresis plates, we used in situ adsorption of antibodies during second-dimension electrophoresis (Figs. 7 and 8). For this, we separated the first-dimension gel strip from the antiserum-containing gel of the second dimension by a 6-mm-wide lane of 1% agarose containing either 10% normal rat serum or 10% ascites fluid of a tumor-bearing rat. The precipitin lines were visualized by staining of the fixed and dried gels with Coomassie Brilliant Blue. For quantitation of antigen concentrations, the area under the precipitin lines were determined. Because purified antigens are not available for standardization of the measurements to w/w terms, we expressed the concentration in arbitrary units (see Chart 1). Zero values represent nondetectable levels, and values of 0.1 to 0.2 represent the lowest amounts which can be measured by the method used. Fluorographic detection of titrated antigens were achieved by impregnation of the agarose gels with Enhance (New England Nuclear, Boston, Mass.). To recover radioactive antigens from crossed immunoelectrophoresis plates for further electrophoretic analysis, the precipitin lines of interest were localized and cut out according to the autoradiographic image of the dried but not fixed gels. The agarose gel was melted off the plastic backing by boiling for 5 min in 400 μl of 25 mM Tris-HCl, pH 8.8, containing 1% sodium dodecylsulfate and 5% 2-mercaptoethanol. The liquified gel was either applied directly to polyacrylamide gels in sodium dodecyl sulfate or poured into glass tubes. After solidification in the tubes, the proteins were electrophoretically eluted into a dialysis bag. Following dialysis against 50 mM NH4HCO3, the samples were freeze-dried.

**Polycrylamide Gel Electrophoresis.** One-dimensional electrophoresis of proteins was carried out on 10% polyacrylamide gels in 0.1% sodium dodecyl sulfate (12). Two-dimensional gel separation was performed according to the method of Ames and Nikaido (1). The second dimension consisted of a uniform concentration of 10% acrylamide. The following molecular weight markers were used: β-galactosidase (M, 116 x 103), phosphorylase (M, 93 x 103), albumin (M, 68 x 103), ovalbumin (M, 43 x 103), and chymotrypsinogen (M, 25 x 103). For fluorography of the gels, the method of Bonner and Laskey (8) was followed.

**RESULTS**

Goat antiserum against membrane glycoproteins of HTC cells recognized one major antigenic component in the serum of a normal rat when analyzed by crossed immunoelectrophoresis (Fig. 1A). This component will be referred to as NSA. Similarly, when the serum of a rat with a large HTC cell-derived tumor was analyzed, an additional major antigen with a higher chart. 1. Concentrations of antigens in rat sera in relation to tumor size. Aliquots of 15 μl of serum from control and tumor-bearing rats were separated under constant conditions by crossed immunoelectrophoresis (see Fig. 2). The areas under the precipitin lines of the NSA and the TAA were determined (1 sq cm = 1 antigen unit). The obtained values are shown in relation to the weights of the tumors present in the animals at the time of the bleeding. The growth periods ranged from 6 to 17 days for tumors below 5 g, from 9 to 21 days for tumors between 5 and 15 g, and from 13 to 24 days for tumors above 15 g.

H. Baumann, unpublished observation.
Because in Fig. 1 the serum of a rat with a tumor in a terminal stage was used, we followed the change in antigen pattern by analyzing sera and ascites fluids of rats at intermediate stages of tumor growth (Fig. 2). In many but not all rats bearing a HTC cell tumor larger than 5 g, an accumulation of ascites fluid free of tumor cells can be observed. The data in Fig. 2 are a representative selection of antigen analysis of over 40 individual tumor-bearing rats. The tumor burden at the time of bleeding was determined for some of these rats. In Chart 1, we have summarized the concentrations of the antigens present in the serum in relation to the measured tumor weight. Although a rather large variation in tumor growth rates and in absolute concentrations of NSA and TAA was noted, major trends in the pattern development, as illustrated in Figs. 1 and 2 and Chart 1, were regularly observed: (a) the concentration of the NSA declined during tumor growth and was always lower in ascites fluid than serum; and (b) the accumulation of TAA in serum, and to some extent in ascites fluid, occurred at a relatively late stage of tumor growth. Conversely to NSA results, the concentration of the TAA was in almost every case higher in the ascites fluid than in serum.

Because both the NSA and the TAA represent antigens of HTC cells, we analyzed the culture media of metabolically labeled HTC cells to determine whether these antigens are

**Fig. 1.** Antigenic proteins in normal rat serum and serum of a tumor-bearing rat. Fifteen-μl aliquots of serum of a normal rat (A) and of a rat with a 20-day-old HTC cell tumor (23 g) (B) were separated by crossed immunoelectrophoresis. In C, the samples of A and B were coelectrophoresed. The Coomassie blue patterns are reproduced.

electrophoretic mobility appeared (termed TAA), while the NSA was drastically reduced in amount (Fig. 1B). In the normal serum, there is a minor precipitation line discernible which exhibits the same electrophoretic mobility in the first dimension as the TAA. To show whether these are antigenically identical, the 2 sera were combined and analyzed (Fig. 1C). The minor antigenic component could still be recognized at its original position, hence not incorporated in the TAA peak, which suggests that the 2 molecules do not share common antigenic determinants. The same analysis also shows clearly that NSA and TAA do not have any antigenic properties in common. A crossed immunoelectrophoretic analysis of plasma instead of serum yielded the same qualitative and quantitative pattern as in Fig. 1 (data not shown), indicating no preferential loss of antigens during serum preparation.

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Similarly synthesized and released by the cells in tissue culture. As illustrated in Fig. 3A, one major and several minor antigens labeled with [35S]methionine are recognized by the antiserum. The incorporation of [3H]fucose verifies that the major antigen is a glycoprotein (Fig. 3B). In both separations, the antigen peak showed an asymmetrical form with an extended leading edge, indicating heterogeneity in electrophoretic mobility. To identify the relationship of this major antigen in the tissue culture medium with the NSA and the TAA, coelectrophoretic analyses were performed (Fig. 4). As is apparent from Fig. 4B, the major medium antigen and the TAA share common antigenic determinants, resulting in a continuous precipitation line. Although differing in the overall electrophoretic mobility, the antigens from the 2 sources overlap in the region of the leading side of the medium antigen peak and trailing end of the ascites TAA. The combination of medium and normal serum under conditions used in Fig. 4A failed, however, to pinpoint a recognizable antigen synthesized and released by HTC cells and related to the NSA [see below (Fig. 7)].

To determine whether the tumors synthesize and secrete the TAA and if so in what form compared to that of HTC cells, we labeled tumor explants in tissue culture with [35S]methionine. The released material indeed contained an antigen with migration properties identical to those of the HTC cell antigen (Fig. 3C). Moreover, a mixing experiment with ascites fluid yielded the same positive identification on crossed immunoelectrophoresis plates as in Fig. 4B (data not shown). The immunoelectrophoretic analyses show in addition that tumor explants synthesize and secrete the TAA in amounts comparable to HTC cells.

To find whether liver cells synthesize an antigen related to the TAA, we prepared liver explants and primary hepatocytes

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Fig. 3. Antigens synthesized and released by HTC cells and hepatoma explants in tissue culture. HTC cells were labeled for 24 hr with [35S]methionine (A) or with [3H]fucose (B). Immediately after excision, tumor explants were labeled for 24 hr with [35S]methionine (C). The cell-free media were concentrated 10-fold, and aliquots of 20 μl (A, C) and 15 μl (B) were analyzed. The autoradiographs in A and C and the fluorograph in B were exposed for 3 weeks.

Fig. 4. Comparison of the antigens in HTC cell medium with those in serum and ascites fluid. Ten-μl aliquots of 10-fold concentrated medium of HTC cells labeled with [3H]fucose (similar to Fig. 3B) were combined with 10 μl of normal rat serum (A) or 10 μl of ascites fluid of a tumor-bearing rat (see Fig. 1B) (B). Each combination was separated on 2 identical crossed immunoelectrophoresis plates. One plate of each set was stained with Coomassie blue, and the other plate was processed for fluorography (exposed for 3 weeks).
and labeled them similarly with methionine (Fig. 3). In no case were we able to detect any immunoprecipitation related to the TAA of HTC cells or of ascites fluid (data not shown). Even the use of a 50-fold concentration of culture media of primary hepatocytes for analysis was negative.

If the tumor is the sole source of the TAA in the animal, as suggested by the above findings, we must explain why the molecular form of the TAA of HTC cells (a) does not predominate in vivo, and (b) what the biochemical difference between the TAA of hepatoma cells in tissue and that in the circulation of the tumor-bearing rat is. To learn more about physicochemical properties of the antigen, the medium of HTC cells labeled with \[^{35}S\]methionine and the ascites proteins labeled chemically with \[^{125}I\] were separated by immunoelectrophoresis. The labeled proteins in TAA precipitation lines were isolated from the gel plates and analyzed on 2-dimensional polyacrylamide gels (Fig. 5). The TAA present in the tissue culture medium appeared as a charge heterogeneous band \([M, 37,000; pI 5 to 5.6 \text{ (Fig. 5B)}]\). The predominant portion of the antigen was found to be on the basic side of that charge range. Of the total proteins synthesized and released into the medium by HTC cells (Fig. 5A), the TAA represents a rather minor component. The TAA of the ascites fluid appeared to belong to the same protein band; its major part, however, was localized on the acidic end at pI 5 or slightly less (Fig. 5C). The apparent molecular weight of the ascites TAA was consequently slightly larger \((M, 38,000)\). Because we have already demonstrated in Fig. 3 that TAA is a glycoprotein, the charge heterogeneity of this molecule most probably is due to differences in content of sialic acid residues on an identical polypeptide backbone (5).

The in vivo form of TAA contains on the average more sialic acid residues (about 4, if each charge interval is counted as one residue) (5), which explains also its higher molecular weight and higher electrophoretic mobility on the immunoelectrophoretic plate relative to the form released in tissue culture (Fig. 4B). To evaluate further this possibility, the \[^{125}I\]-labeled ascites proteins were treated with neuraminidase and then immunoelectrophoretically analyzed (Fig. 6). The removal of sialic acid residues gave the TAA a slower electrophoretic mobility (Fig. 6/) and lower apparent molecular weight which were now identical to the TAA present in the medium of HTC.

**Fig. 5.** Two-dimensional gel electrophoresis of the tumor-associated antigen isolated from tissue culture medium and ascites fluid. HTC cells were labeled with \[^{35}S\]methionine for 24 hr. The medium was concentrated 10-fold, and an aliquot of 30 \(\mu\)l was separated by crossed immunoelectrophoresis, as in Fig. 3A. Ascites proteins (see Fig. 2C) were labeled with \[^{125}I\]. An aliquot \((6\mu\)l) of the labeled proteins, containing \(5 \times 10^5\) cpm, was combined with \(10\mu\)l nonlabeled ascites fluid and was immunoelectrophoretically analyzed. The labeled antigens were recovered from the precipitin lines and separated on 2-dimensional polyacrylamide gels. The fluorographs were exposed for 3 weeks. A, total \(^{35}S\)-methionine-labeled medium proteins of HTC cells \((3 \times 10^6\text{ cpm})\); B, the TAA of HTC cell medium; C, the TAA of ascites fluid.

**Fig. 6.** Influence of neuraminidase on electrophoretic mobility of the ascites TAA. I. \(^{125}I\)-Labeled ascites proteins (see Fig. 5), \(7 \times 10^5\text{ cpm}\) in \(15\mu\)l, were combined with \(25\mu\)l nonlabeled ascites fluid (see Fig. 2C). To one half of the mixture (A), 0.005 unit of neuraminidase (Vibrio cholerae; Calbiochem-Behring Corp., La Jolla, Calif.) was added and incubated for 3 hr at 37°. The other half of the mixture, without neuraminidase (A), was treated identically. Then, the 2 samples were analyzed by crossed immunoelectrophoresis. The autoradiographs after 4 days of exposure are shown. II. The TAA precipitin lines in I were cut out and separated on one-dimensional 10% polyacrylamide gels. 1, diluted aliquot of the \(^{125}I\)-labeled ascites fluid mixture used in I (70,000 cpm); 2, control-treated TAA of IA; 3, TAA treated with neuraminidase of IB; 4, TAA of \[^{35}S\]methionine-labeled HTC cell medium; 5, 10 \(\mu\)l \[^{35}S\]-methionine-labeled medium (30,000 cpm). Lanes, fluorographic pictures after different exposure times (1, 6 hr; 2 to 4, 2 weeks; and 5, 3 days).
cells. The analysis of this desialylated form of the TAA on 2-dimensional polyacrylamide gel revealed that the major portion of the radioactivity migrated to the basic end of the charge range indicated in Fig. 5C and that the overall charge heterogeneity was slightly less than that of the antigen produced by HTC cells (data not shown).

It appears that in the tumor-bearing animal only the most acidic members of the charge heterogenous TAA molecules are accumulated. The question is what happened to the major portion of the TAA with the more basic charge. On the basis of the result of the neuraminidase experiment of Fig. 6, it can be assumed that those basic members represent undersialylated molecules with, most probably, exposed galactose residues (11). Once in the circulation, these "asialo" TAA are possible substrate for hepatic clearance through the receptor-mediated uptake of galactose-terminating serum glycoproteins (2). Examination of this hypothesis by in vivo clarity studies could not be performed due to the lack of sufficient radiolabeled and purified TAA. We therefore decided to test the interaction of the asialoglycoprotein receptor with the TAA in vitro. For this purpose, we isolated from rat liver the receptor for galactose-terminating glycoproteins by affinity chromatography (9). The receptor proteins were immobilized by covalent linkage to Sepharose beads. With the use of these receptor beads for affinity chromatography of culture medium of [35S]methionine-labeled HTC cells, we were able to demonstrate that TAA was indeed recognized by the hepatic receptor (Fig. 7C). To support the conclusion that terminal galactose residues in the TAA were actually recognized, we carried out a similar chromatography with immobilized ricin instead of receptor. Essentially, the same result as in Fig. 7C was obtained (data not shown).

The antisera used in this study is directed against membrane glycoprotein of HTC cells. Thus, the question arises: do we find antigens related to the TAA and the NSA associated with the membrane fraction of HTC and liver cells? To detect such components in the total membrane glycoprotein fractions containing antigenic determinants of the NSA and TAA, we used the identification technique based on in situ depletion of the specific antibodies by providing a vast antigen excess. In such cases, the proteins with antigenic determinants common to the additionally added excess antigen can be recognized by the fact that they will no longer form a precipitin line in the same region of the second dimension of the crossed immunoelectrophoresis. Unfortunately, a comparison of the patterns from HTC cell glycoproteins in Fig. 7/1 did not allow any positive assignment of an antigenic membrane band to the TAA. Because any manipulation of the conditions for crossed immunoelectrophoresis used in Fig. 7/1 would have adverse effects on separation and resolution, we did not pursue further our identification by this technique. Mixing experiments, as done in Fig. 4, failed also in pinpointing of a membrane-associated antigen corresponding to the TAA.

Surprisingly, by the same experiments of Fig. 7/1, we found a distinct band cross-reacting with the NSA (arrow in Fig. 7/1). We achieved a definitive identification by coelectrophoresis of membrane glycoproteins and normal rat serum, resulting in a common peak (data not shown). The question now is whether HTC cells release low amounts of NSA which are below the level of detection by the methods used. Therefore, we analyzed higher concentrated culture medium and detected among the minor bands one which shared antigenic determinants with the NSA (arrows in Fig. 7I, A and B). By virtue of its presence in normal rat serum, the NSA may have a corresponding antigen in the liver. We, therefore, isolated from the liver the membrane glycoproteins in the same manner as those from HTC cells and subjected them to crossed immunoelectrophoresis (Fig. 8I). One major band proved to be cross-reacting with the NSA (arrow in Fig. 8/A). This membrane form of the NSA had a slightly slower migration in the first dimension than did the serum form. Moreover, the antigen of the liver membranes formed a symmetrical peak (Fig. 8/A), whereas of HTC cells always exhibited a pronounced trailing end (see Fig. 7/A). When we analyzed the different membrane and released forms of the NSA from liver and HTC cells by gel electrophoresis (Fig. 81), we noted a larger molecular weight of released forms (M, 63,000 for the antigen in rat serum, and M, 61,000 for that in culture medium) than for the membrane-associated form (M, 58,000 for both liver and HTC cells). Whether the difference in size lies in the polypeptide or in the carbohydrate structure has not yet been established.

The 2-dimensional gel pattern of the membrane-associated NSA (Fig. 9) revealed that the antigen is itself quite charge...
Fig. 8. Antigenic glycoproteins in the membrane of the rat liver. I. The glyco-
proteins were isolated from the membrane fractions of the liver as described in
"Materials and Methods." Twenty-five-μl aliquots of the final fraction, containing
30 μg of protein, were separated by crossed immunoelectrophoresis identical to
that used for HTC cells (Fig. 7II, A and B). A, without in situ; adsorption B, with
in situ adsorption of immunoglobulins against the NSA by inclusion of a lane
containing 10% normal rat serum. The patterns stained with Coomassie blue are
shown. II. To determine the apparent molecular weight of the NSA in serum,
medium, and membranes, membrane glycoproteins of the liver and the normal
rat serum proteins were labeled with 125I as described in "Materials and Meth
ods." Those of HTC cell membranes and medium were labeled with [35S]methi-
onine. The radiolabeled material was immunoelectrophoretically separated as in
Figs. 1A, 7II and II, and Bl. The radioactive immunoprecipitin lines corresponding
to the NSA were further separated by one-dimensional electrophoresis on 10%
polyacrylamide gel. Lane 1, total 125I-labeled glycoproteins of rat liver mem-
branes (20,000 cpm); Lane 2, NSA in the rat liver membrane glycoprotein
fraction; Lane 3, NSA in the serum; Lane 4, NSA in the medium of HTC cells;
Lane 5, the NSA in the membrane glycoprotein fraction of HTC cells; and Lane 6,
total [35S]methionine-labeled glycoproteins of HTC cell membranes (30,000
cpm). The fluorograph was exposed for 1 week.

Fig. 9. Identification of the NSA on the 2-dimensional gel pattern of membrane
 glycoproteins. From the experiment in Fig. 8II, the following samples were
separated by 2-dimensional polyacrylamide gel electrophoresis: A, [35S]methio-
nine-labeled glycoproteins of HTC cell membranes (100,000 cpm); B, 125I-labeled
glycoproteins of liver membranes (150,000 cpm); C, 125I-labeled NSA in the liver
membrane and isolated by crossed immunoelectrophoresis. The fluorographs
were exposed for 3 weeks. Brackets, position of the NSA.

DISCUSSION

The experiments reported here were designed to determine
whether antibodies raised against membrane glycoproteins of
hepatoma cells in tissue culture are a useful tool to compare
membrane properties of hepatoma and liver and to detect
tumor markers in vivo. Tissue culture cells are most attractive
as a source of antigens because they provide sufficient material
for immunization, which does not contain any contamination
from other cell types. With HTC cells, the antiserum proved to
be successful not only to monitor antigenic glycoproteins in the
circulation of normal and hepatoma-bearing rats but also to
reveal some additional interesting features: (a) that liver can
modify the pattern of hepatoma-derived antigens; (b) that quan-
titatively minor membrane glycoproteins were major antigens;
(c) that membrane glycoproteins from liver and hepatoma share
many antigenic determinants with differences in biochemical
properties; and (d) that an antigenic glycoprotein released by
the hepatoma cells does not necessarily have a recognizable
form associated with the membrane fraction.

Hepatoma cells retain always to a certain degree liver-spe-
cific properties (14). It is therefore to be expected that liver
contains proteins cross-reacting with antibodies raised against
hepatoma products. However, previous studies by metabolic
or cell surface labeling have shown that the 2 rat hepatoma
cell lines, HTC and H-35, have each a specific composition of
membrane glycoproteins without any recognizable common
forms (4). Furthermore, both compositions differ completely
from that of liver cells. How different the patterns can be is
apparent in Fig. 9, A and B. In anticipation that these differ-
ences might be recognized in a xenogenic immunization, we
considered membrane glycoproteins as a potential target for
Antigenic Glycoproteins Released by Hepatoma Cells

comparative analyses using immunochemical detection methods. The remarkable differences in the antigenic glycoprotein patterns between HTC cells and liver are illustrated in Figs. 7IIA and 8IIA. Because the difference of the antigenic glycoprotein lies mainly in the electrophoretic mobilities in the first dimension, we will have to determine the biochemical basis for this phenomenon. In the only case analyzed here, i.e., the membrane-associated NSA, we suspect, on the basis of the result of 2-dimensional gel separation (Fig. 9, A and B), that the difference in mobility on crossed immunoelectrophoresis (Figs. 7II and 8II) is probably due to cell-specific variation in sialylation of that glycoprotein species.

When HTC cells were injected i.p. in a rat, the cells grew within 3 weeks to large solid tumors attached to the peritoneum. Soon after that stage, the animal will normally die. During the development of the hepatomas, we detected in the serum and ascites fluid, with the aid of the antiserum against HTC cell membrane glycoproteins, the appearance of a new antigen. Because we failed to detect by crossed immunoelectrophoresis a corresponding antigen in the normal serum or in the medium of metabolically labeled liver explants and primary hepatocytes, we consider this antigen as tumor specific. We cannot, however, exclude the possibility that the liver actually produces this protein but in amounts below the detection limit of the techniques used here. Nevertheless, we found that the tumor explants synthesize and release the same antigens in comparable quantities as do the hepatoma cells in tissue culture (Fig. 3). We assume that this property measured in vitro reflects the behavior of the tumor in vivo. Therefore, we had to explain why in serum and ascites fluid a more acidic form of the TAA prevailed than was found in the in vitro culture medium (Fig. 5).

We excluded the possibility of an enzyme mechanism present in the ascites fluid that was responsible for further sialylation of the released TAA, because incubation of ascites fluid with radiolabeled HTC cell medium did not cause any change in electrophoretic mobility of the labeled antigen (data not shown). The most probable explanation was the removal of the more basic components of that antigen species by hepatic clearance through uptake of galactose-terminating glycoproteins. This, however, is possible only when the antigen carries carbohydrate structures common for most secretory glycoproteins but lacking only sialic acid residues attached to penultimate galactose residues (11). The in vitro binding experiment with immobilized hepatic receptor for galactose-terminating glycoprotein and with immobilized ricin strongly supported the expected structural feature of the TAA, which favors the hypothesis of hepatic elimination.

The antigen present in normal rat serum was found to be a major antigen in the membrane fraction of both the liver and HTC cells (Figs. 7II and 8II). This antigen differs not only antigenically (Fig. 1C) but also physicochemically from the TAA (Figs. 6II and 8II). Furthermore, a preliminary mapping of fragments generated by partial digestion of radiolabeled NSA and TAA with Staphylococcus aureus protease did not show any common peptide structures (results not presented). The analysis of the culture medium of HTC cells revealed that a minute amount of the NSA, as compared to that of the TAA, is released. It is conceivable that this is the reason that little or no NSA was found in the ascites fluid of tumor-bearing rats (Fig. 2). However, there is also a possibility that the NSA released by the hepatoma is cleared by the liver as well. As we have demonstrated in Fig. 8II, the NSA released by HTC cells differs from that of the serum by a molecular weight of 2000. If this smaller size is due to a lower amount of sialic acid residues, then the same phenomenon described above for the TAA can be applied. Future experiments should give definitive answers.

The circulating NSA decreases in concentration during progressive tumor growth (Fig. 2; Chart 1). This could be the result either of a reduced release by the liver (and other organs) or of an increased removal somewhere in the organism. Thus far, neither possibility can be supported with experimental data. The electrophoretic comparison of the NSA from the serum and from the glycoprotein fraction of the liver membranes showed a 5000-molecular weight difference (Fig. 8II). Because the released form is larger in molecular weight than that associated with the membrane, the former cannot be considered to be a proteolytic fragment of a surface glycoprotein. An unproven possibility could be that the membrane-associated NSA represent a precursor form of a secretory glycoprotein, which did not yet completely undergo secondary glycosylation (15).

We believe that immunoglobulins against hepatoma tissue culture cells are very useful to determine the similarity and differences between hepatomas and liver. The immunological approach combined with biochemical analysis offers the opportunity to understand the structural basis leading to the hepatoma phenotype. Furthermore, we can learn more about the occurrence and fate of potential tumor markers in vivo by these approaches.

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REFERENCES

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