Carcinogen-binding Antigens in Rat Liver Microsomes

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Summary

Microsomal fractions were prepared from livers of normal rats and rats treated with 2-aminofluorene or 2-acetylaminofluorene as well as from hepatomas induced by feeding 2-acetylaminofluorene. The microsomes were solubilized with deoxycholate and examined by immunoelectrophoresis using rabbit antiserum against normal liver microsomes which had been absorbed with rat plasma and kidney microsomes.

At least 9 precipitate arcs were detected with preparations from livers of normal rats. Similar patterns were obtained with preparations from livers of rats which had been injected with 2-aminofluorene or 2-acetylaminofluorene 72 hr earlier. In the latter case, at least 2-3 arcs were shown to fix anti-2-azofluorene-\(^{125}\)I antibody, indicating that some microsomal components combined with the injected carcinogen.

Some of the liver microsomal antigens including one of the carcinogen-binding components were not detected in preparations from livers of rats which had been fed 2-acetylaminofluorene for 16 weeks and removed from the carcinogen diet for 1, 2, or 4 weeks.

Similar but more extensive changes in antigen composition were observed with preparations from hepatomas. Some antigens, including the carcinogen-binding components, were not observed. In contrast, some antigens not clearly detectable in normal liver preparations were observed in hepatoma preparations.

The role of the binding of carcinogen to the microsomal components and the role of these components themselves in carcinogenesis remain to be seen.

Introduction

Since the studies by Miller et al. (11, 14), it has been known that azo dye carcinogens are bound to proteins in the liver of rats during the process of chemical carcinogenesis. Sorof et al. (14) pointed out that the aminoazo dyes appeared to combine with specific liver proteins which migrated relatively slowly upon electrophoresis. Baldwin (2) reported that at least 1 abnormal antigen containing bound carcinogen could be detected in the cell sap of liver from rats fed with 4-dimethylaminoazobenzene (DMAB). This was shown by immunodiffusion test with rabbit antiserum containing antibody directed to DMAB. Recently, Green and Ghose (6) demonstrated that rabbit antibodies against liver of normal rats after proper absorptions were fixed in livers of normal rats when injected i.v., but not in livers of 3'-methyl-4-dimethylaminoazobenzene (3'-DMAB)-treated rats. On the other hand, antibodies against liver of 3'-DMAB-treated rats when injected did not localize in livers of normal rats, but did do so in livers of treated rats. They suggested that 3'-DMAB was bound to particular liver components and modified their immunologic specificities.

In our previous study (10), rabbit antisera directed specifically to the 2-azofluorenyl group (2-AzF) were prepared by immunization of rabbits with 2-AzF-protein conjugate. We showed that the purified anti-2-AzF antibody, when injected, localized in livers of rats pretreated with 2-aminofluorene (2-AF) or 2-acetylaminofluorene (2-AAF). This indicated that 2-AF or 2-AAF was bound to some cellular components of liver in a form capable of reacting with anti-2-AzF antibody in vivo.

In the present study, direct evidence has been found that some of the liver microsomal components participate in combination with 2-AF or 2-AAF. This was demonstrated on radioimmunoautoradiography by the specific fixation of radiolabeled anti-2-AzF antibody on the precipitate arcs which were formed between antiliver microsome serum and microsomal antigens prepared from livers of rats treated with 2-AF or 2-AAF. Some of these components are apparently not formed in livers of rats fed 2-AAF for long periods of time or in tumors induced by feeding 2-AAF.

Materials and Methods

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PREPARATION OF MICROSOMAL FRACTION. The microsomal fractions were prepared from livers, hepatomas, and kidneys according to the method described by Isojima et al. (8). The microsomes were solubilized with sodium deoxycholate (DOC) in the cold (1). These particulates were suspended in 0.2 M glycylglycine buffer, pH 8.06 (100 mg of wet weight/ml), and mixed with 4% DOC to give a final concentration of 0.4%. After 20 min, the mixture was centrifuged at 105,000 \( \times g \) for 120 min. The upper layer of supernatant was taken and dialyzed against 10-liter portions of borate buffer, pH 8, for 2 days in the cold. The microsomal fractions and the DOC-solubilized materials were stored in frozen state, unless used immediately.

ANTISERUM AGAINST THE MICROSOMAL FRACTION OF NORMAL RAT LIVER. Three rabbits were injected intradermally in the footpads twice at 2-week intervals with 100 mg (wet weight) of the microsomal fraction in 1 ml of borate buffer, pH 8, emulsified with 1 ml of complete Freund’s adjuvant. After 3 weeks’ rest, a booster injection with 100 mg of the microsomal fraction in 1 ml of borate buffer, pH 8, was given i.p. Rabbits were bled 10 days after the last injection. Antiserum was used after absorption with normal rat plasma (0.2 ml/ml of antiserum) and with the microsomal fraction from the kidney of normal rats.

ANTISERUM AGAINST THE 2-azofluorenyl GROUP (2-AzF). The methods for preparation of 2-azofluorenyl-hemocyanin conjugate as immunizing antigen, immunization of rabbits, and specific purification of anti-2-AzF antibody were described previously (10).

LABELING OF PROTEIN WITH RADIOACTIVE IODINE. Purified anti-2-AzF antibody was labeled with \(^{125}\)I, and \( \gamma \)-globulin prepared from normal rabbit serum by Sober’s method (13) was labeled with \(^{125}\)I as described previously (10).

IMMUNOELECTROPHORESIS AND RADIOIMMUNOELECTROPHORESIS. Electrophoresis of a test material was carried out in an agar layer, 1.5–2 mm thick, on a microscope slide (1.5% Difco Noble agar in Veronal buffer, pH 8.2, \( \Gamma / 2.05 \)). The microsomal fraction solubilized with DOC (10 mg protein/ml) was applied in 2 portions of 4–5 \( \mu l \) to the starting well. A 30-volt potential was applied across the end of the slide (7 cm) for 60 min. at room temperature. After electrophoresis, a rabbit antiserum against normal liver microsomes was used to produce precipitate arcs by diffusion from the center channels. The channels were filled 3 times with the antiserum during the diffusion for 48 hr. For radioimmunoelectrophoresis, the antiliver microsome serum mixed with anti-2-AzF-\(^{125}\)I antibody (1 \( \mu l/cm \) of antiserum) or normal \( \gamma \)-globulin-\(^{125}\)I (0.2 \( \mu l/cm \) of antiserum) was applied once to the channel and then the antimicrosome serum alone was applied twice. Slides were rinsed with several changes of borate buffer, pH 8, for 48 hr. Staining with amido black and radioautography were carried out as described previously (10).

Results and Discussion

The microsome fractions were prepared from livers of normal rats and rats injected with 2-AF or 2-AAF or fed 2-AAF. These fractions were solubilized in 0.4% deoxycholate. Immunoelectrophoresis was carried out using the solubilized materials and rabbit antiserum against the liver microsomes of normal rats. The antisera had been absorbed with rat plasma and kidney microsomes.

The results are shown in the left half of Fig. 1 (Stained Slide). An immunoelectrophoretic pattern obtained with a preparation from normal rats (Fig. 1a, left) is presented as a drawing to show arcs of 9 readily discernible components (a1–d1). The microsomal preparation of rat liver has been shown to contain liver-specific antigens (5, 7). Vogt (15, 16) has shown that these specific antigens are located exclusively in the membranous fraction prepared from microsomes and that they are probably lipoproteins.

The treatment of the microsomal preparation with deoxycholate apparently solubilizes lipoproteins of the membranes without great effect on the immunologic properties (1, 3).

Similar immunoelectrophoretic patterns were obtained with the preparations from livers of rats which had received an i.p. injection of 10 mg of 2-AF or 2-AAF 72 hr previously (Fig. 1–b, c, left).

In order to test the possibility that some of these liver microsomal antigens are the components which bind administered carcinogen in vivo, anti-2-AzF-\(^{125}\)I antibody was used to identify the precipitate arcs containing complexes of carcinogen and liver components. As reported previously (10), anti-2-AzF antibody used here did not form precipitates directly with 2-AzF-protein on agar diffusion test. As seen by the radioautographs in Fig. 1, b and c (right), radiolabeled anti-2-AzF antibody was fixed at least on 3 of the components (a2, b2, b3) in preparations from rats injected or fed 2-AAF and on 2 of the components (b2, b3) in those from rats injected with 2-AF. The lack of antibody fixation on the a2 are in the latter might be due to a difference of reactivity of this protein with 2-AF and 2-AAF or their metabolites. This requires further study. The arcs around the starting well (region of c1–c3) also seemed to fix radiolabeled antibody, but the results were not clear because of residual radioactivity not easily removed from this region. No radioactive arc was observed when normal \( \gamma \)-globulin-\(^{125}\)I was used instead of anti-2-AzF-\(^{125}\)I antibody. Also, the precipitate arcs obtained with the preparation from normal rats did not fix anti-2-AzF-\(^{125}\)I antibody (Fig. 1 a10) or normal \( \gamma \)-globulin-\(^{125}\)I. Thus, the results indicate that at least 2–3 of the liver micro-

*This was prepared by dissolving 0.2 mole of glycylglycine in water, adding 1 N NaOH to bring pH to 8.0 and diluting to 1 liter with water.

†This was prepared by dissolving 21.2 gm of \( \text{H}_3\text{BO}_3 \) and 16.0 gm of NaCl in water, adding 45.3 ml of 1 N NaOH, and diluting 2 liters with water.

‡The protein concentration of DOC-solubilized fraction was about 10 mg/ml on the assumption that 1 mg protein/ml gives an optical density of 1.50 at 280 nm with a 1-cm light path. When the protein concentration was less than 10 mg/ml, the solution was concentrated by pervaporation in the cold to give a concentration of over 10 mg/ml. The ratio of optical density at 280 nm to that at 260 nm was about 1.0.

†Absorption was carried out by 2 treatments with 20 mg (wet weight) of the microsomal fraction of kidney and 1 treatment with 0.2 ml (2 mg protein) of DOC-solubilized microsomal fraction of kidney per ml of antiserum.

\[ {\text{2-AzF-125I}} \]

In a few cases anti-2-AzF-\(^{125}\)I antibody was fixed on 1 of the precipitate arcs (b3) obtained with preparation of liver from untreated rats (Fig. 1a). However, it was very faint as compared with the fixation on the arcs from 2-AF- or 2-AAF-treated rats. We believe that this fixation was due to a nonspecific globulin binding.
organ-specific antigens were not produced in tumors (9, 12, 18). This is in agreement with other previous observations that certain or greatly decreased in hepatomas. One of the slowest migrating components (a1 and a2) which were seen in the latter, were not detected in the former. A decrease in the amounts of the fastest (d1) and some of the faster (arcs in c1–c3 region) migrating components was also observed. This may indicate that the cells lost the ability, either totally or partially, to produce these antigens during the process of carcinogenesis, or that their antigenicity was modified by the carcinogen treatment to be unreactive with the antibodies against normal liver microsomes. However, the 2 slower migrating components (b2 and b3) were shown to fix the radiolabeled anti-2-AzF antibody on radioautographs (Fig. 1—d–f), indicating that these antigens when complexed with 2-AAF could still react with antibodies against normal liver microsomes, although the radioactivity on the arcs was much weaker than that on the arcs obtained by the preparations from rats injected with 2-AAF.

Microsomal fractions solubilized with deoxycholate were also prepared from hepatomas induced with 2-AAF. The rats had been removed from the carcinogen diet at least 10 months prior to the experiments. The immunoelectrophoretic patterns are shown in Fig. 2. Each of the 1st 5 preparations (H1–H5) was from a single nodule of hepatoma from different rats. The last 2 preparations (H6, H7) were from pools of hepatomas from several rats. N was a preparation from normal rat liver. The hepatomas varied with respect to the number of normal liver antigens. Several antigens (corresponding to a1, a2, c1, c2, c3, and d1 in the drawing on the top of Fig. 1) were absent or greatly decreased in hepatomas. Radiolabeled anti-2-AzF antibody was weakened than that on the arcs obtained by the preparations from rats injected with 2-AAF. Some normal liver antigens in hepatomas varied with respect to the number of normal liver components which were shown to be absent in hepatoma microsomes in the present study, are on the cell surface of normal liver cells and are responsible for the fixation of carcinogen and anticanine antibodies in liver of pretreated rats. Microsomal fractions were shown to contain components of cell membranes (17) which presumably play an important role in the fixation of antibodies in vivo. Green and Ghose (6) reported that liver-specific antigens were present on cell membranes and were modified by their combination with 3'-DMAB.

The role of the binding of carcinogen to microsomal components and the role of these components themselves in carcinogenesis remain to be seen.

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References

Carcinogen-binding Antigens


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**FIG. 1.** Immunoelectrophoretic and radioimmunoelectrophoretic patterns of the deoxycholate-solubilized microsomal fractions from livers of normal rats and rats treated with 2-AF or 2-AAF. A sample, 0.1 mg in 10 μl, was applied in 2 portions and submitted to electrophoresis in agar layer on a microscope slide. After electrophoresis, a mixture of an antiserum against normal liver microsome and 2-AzF-3H antibody, was allowed to diffuse for 48 hr from the channel. The slide was washed, dried and stained with amidoblack (left), and then a radioautograph was taken by placing it in contact with X-ray film (right). The top figure is a sketch of stained slide a. In a few cases, a very faint radioactive line was observed corresponding to the precipitate arc b3 of normal rats as shown by the broken line. a, sample from liver of normal rats; b, c, samples from liver of rats 72 hr after injection with 2-AAF (b) or 2-AF (c); d–f, samples from liver of rats fed 2-AAF for 16 weeks and removed from 2-AAF diet for 1 week (d), 2 weeks (e), or 4 weeks (f).

**FIG. 2.** Immunoelectrophoretic patterns of the deoxycholate-solubilized microsomal fractions from hepatomas induced by feeding 2-AAF. Immunoelectrophoresis was carried out as described in legend of Fig. 1. Hr–Hs, Samples from single nodules of individual rats; Hr, Hs, samples from pooled nodules of hepatoma from several rats; N, sample from liver in normal rats.

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