Antigens Common to Rat Hepatomas Induced with 2-Acetylaminofluorene

Shinzo Isojima, Yasuo Yagi, and David Pressman

Department of Biochemistry Research, Roswell Park Memorial Institute, Buffalo, New York 14203

SUMMARY

Autochthonous rat hepatomas induced by 2-acetylaminofluorene were found to have antigenic components in common which were not detected in normal rat liver. The hepatoma antigens were also detected in cultured cells established from the hepatoma. Antigenic components present in normal liver were also found.

Sections from 6 hepatoma nodules, 2 each from individual rats, were examined by the indirect fluorescent antibody technic with 6 rabbit antisera prepared against microsomes of each nodule. Each of the antisera gave positive results with essentially all the hepatoma nodules after exhaustive absorption with normal liver. Two antisera against normal rat liver gave no reaction with hepatoma after such treatment.

INTRODUCTION

In our previous study with fluorescent antibody technic (5), 2 antisera prepared against individual nodules of 2-acetylaminofluorene-induced rat hepatoma were shown to react with the hepatoma used for immunization even after exhaustive absorption with normal liver. These antisera did not react with hepatomas from other rats. Individual antisera against 9 other hepatomas failed to react with even the homologous hepatoma after absorption with normal liver. We have now studied this system further using an improved technic of higher sensitivity and using a number of antisera prepared against individual hepatoma nodules. Even after exhaustive absorption with normal liver, each of the antisera reacted to some degree with all of the hepatoma nodules tested, indicating that some antigens which are lacking in normal liver, or present only in undetectable amounts, are commonly present in all the hepatoma nodules.

A comparison of the intensity of the reaction between various antisera and hepatoma nodules, including those used for immunization, indicated that several antigenic components are present in common in the tumors, but in different concentrations in each of the tumors. These antigens were also detected in hepatoma cells grown in continuous cell culture.

In addition, the presence of some normal liver antigens in hepatoma cells was also shown by the fact that specific antisera against normal liver were able to react with hepatoma.

MATERIALS AND METHODS

Rat Hepatoma. Hepatomas were induced in groups of Fisher male rats by feeding 2-acetylaminofluorene as described previously (4). Rats which had more than 2 large well-segregated hepatoma nodules were chosen. Rats were numbered, and 2 large hepatoma nodules selected from each rat were designated as nodules A and B. From each nodule, a small portion was frozen with dry ice-acetone and used later for preparing sections. The remaining portion was used for preparing the microsome fractions as described previously (6). Livers from 3 normal rats were treated in a similar manner.

Antisera to Rat Hepatoma and Normal Liver. Microsome fractions from thoroughly perfused hepatoma nodules and normal livers were used for immunizing rabbits. Two rabbits were used for each microsome preparation. Fifty mg of the microsome fraction (wet) in 1 ml of borate-buffered saline (BBS) was emulsified with 1 ml of complete Freund's adjuvant and injected intradermally and intramuscularly into the footpads and backs of rabbits. Four and 6 weeks after the first injection, rabbits were again injected in the same way. Blood samples taken 2 and 4 weeks after the third injection were assayed for antibody activity after absorption with rat plasma by immunodiffusion against a 0.4% deoxycholate extract of the microsome fraction of pooled hepatomas. Based on this information, antiserum from the rabbit with the higher titer in each group was pooled and used. Antisera prepared against 3 pairs of hepatoma nodules, each pair derived from a single rat, and 2 normal livers were chosen for the study. Antisera were designated as anti-hepatoma IA, IB, IIA, IIB, IIIA, or IIIB and anti-normal liver A or B, respectively. The γ-globulin fraction of each serum was obtained by precipitation at one-third saturation of ammonium sulfate.

Purification of Antibodies. All antisera to microsome fractions of rat hepatoma and normal liver contained fairly large amounts of antibodies reactive to plasma proteins. To remove anti-rat plasma antibodies, rat plasma conjugated with the insoluble polymer of bovine γ-globulin was used for absorption. Polymers of bovine γ-globulin were prepared as described previously (9, 11). A crude plasma protein fraction of rat plasma...
Rat Hepatoma Antigens

precipitated at 50% saturation of ammonium sulfate was con-
jugated with insoluble polymer of bovine γ-globulin (BGG-
polymer) by biiodiazotized benzidine (BDDB). This fraction was
used since it contains albumin but not in the overwhelming propor-
tion as is the case with whole plasma. Five hundred mg of BGG-polymer in 8 ml of ice-cold phosphate buffer (pH 7.5, 0.2 M) were mixed rapidly with 7.5 ml of BBD prepared by
titrating 0.02 M benzidine in 0.1 N HCl with 0.04 M NaNO3 in
ice. After 1 min, 13.2 ml of the rat plasma fraction (18.8 mg
protein/ml) were added and the pH was adjusted to 7.5. The
mixture was stirred in ice for 2 hr, then kept in the cold room
overnight. It was centrifuged at 2,000 rpm for 10 min, and the
precipitated polymer was washed repeatedly with physiologic saline, cold glycine-HCl buffer (pH 2.5), and borate buffer (pH
8.0) to remove any soluble material. The washed polymer was
used as immunoadsorbent for absorbing anti-rat plasma anti-
body.

In the absorption procedure, 100 mg (wt) of washed rat
plasma-BGG-polymer were shaken with γ-globulin fractions of
anti-hepatoma or anti-normal liver antisera (350—500 mg pro-
tein) for 120 min at 4°C, then centrifuged at 2,000 rpm for 10
min. The supernatant was examined for anti-plasma antibody
titer and the same absorption procedures were repeated until
the anti-plasma antibody titer was almost completely removed. The
anti-plasma antibody titers were examined by the tanned he-
magglutination test. Formalized sheep red cells were treated with
tannic acid (1:40,000) and then sensitized with Fisher rat
plasma which had been diluted to a concentration of 50 μg
protein per ml (10).

After removal of anti-plasma antibodies, IgG fractions were
isolated from the supernatants by passage through diethyl-
aminoethylcellulose (DEAE) columns previously equilibrated
with 0.0175 M phosphate buffer (pH 6.4).

The IgG fractions were then treated with rat tissue or hepa-
toma. Sediments from 4 ml of 20% normal rat tissue homogen-
ate in phosphate buffer (pH 7.0) was washed repeatedly by
centrifugation at 16,000 rpm for 30 min until the supernatant
was clear. Tissue sediments were mixed with 2.0 ml of IgG
from each antiserum. The mixture was shaken at room tem-
perature for 30 min and then centrifuged at 16,000 rpm for 30
min. The supernatant was examined for its reaction with sec-
tions of the tissue used for absorption, and the absorption was
repeated until no reaction was observed.

Fluorescein-Conjugate of Goat Anti-Rabbit IgG Antibody.

Goat antibody against rabbit IgG was specifically purified with
BGG-polymer conjugated with rabbit IgG as described previ-
ously (9, 11). The anti-rabbit-IgG antibody adsorbed on the
immunoadsorbent dissociated with glycinem-HCl buffer (pH
2.3). The fluorescein-conjugate was prepared according to
Wood et al. (12) with slight modifications as follows. Purified
goat anti-rabbit IgG antibody (47.2 mg in 4 ml 0.1 M carbon-
ate buffer, pH 9.5) was reacted with 1.2 mg of fluorescein
isothiocyanate (25.4 μg/mg of protein) for 20 hr at 4°C in the
dark and then passed through a Sephadex G-25 column (1.6 x
15 cm), which had been equilibrated with 0.01 M phosphate
buffer (pH 7.5), to remove unconjugated fluorescein deriva-
tives. The fluorescein-conjugated globulin was applied on a
DEAE cellulose column (1.6 x 12 cm) which had been equili-
brated with 0.01 M phosphate buffer (pH 7.5). The conjugated
globulin eluted with 0.1 M NaCl in the same phosphate buffer
was used for staining at a concentration of 0.8 mg/ml. The
mole ratio of fluorescein to protein in the conjugate was 1.6.

The fluorescein-conjugate was absorbed twice with rat liver
sediments prior to use for staining.

Cultured Hepatoma Cells. The hepatoma cells induced in
Fisher rats by feeding with 2-acetylaminofluorene were con-
verted to an established cell line by Dr. George E. Moore of
this Institute. These cultured hepatoma cells were collected by
gentle centrifugation and washed 6 times with phosphate-
buffered saline (pH 7.5). For assay, smears were made from
the sedimented cells. The smears were dried completely, fixed
in 95% ethanol for 30 min at room temperature, and then
rinsed briefly in phosphate buffer before application of rabbit
antibody globulin.

Fluorescent Antibody Staining Procedure. Hepatoma nod-
ules and normal rat livers, while frozen, were sectioned in 5-μ
thicknesses. Sections were fixed in cold acetone for 15 min
and then rinsed in phosphate buffer (pH 7.5) for 15 min. They
were then treated with one drop of rabbit antiserum globulin,
kept for 60 min in a moist chamber, and then washed in
phosphate buffer for 15 min. Subsequently the section was
treated with 1 drop of fluorescein-conjugated goat anti-
rabbit γ-globulin antibody, kept in a moist chamber for an-
other 60 min, and then washed with the buffer for 15 min.
Sections were observed with an ultraviolet microscope.

RESULTS

The titers of the anti-rat plasma antibody in the rabbit globu-
lin preparations from anti-hepatoma and anti-normal-liver
sera before and after absorption with the rat plasma-BGG-
polymer adsorbent are shown in Table 1. All had fairly high
titers of anti-rat plasma antibody originally, but after treat-
ment with the immunoadsorbent conjugated with rat plasma,
the anti-plasma antibody titer was reduced by a factor of 102
or 103. None of the globulin preparations used agglutinated
sensitized erythrocytes at a concentration of 125 μg/ml or
lower.

The IgG of the anti-hepatoma antisera which had been freed
of anti-plasma antibodies reacted strongly with normal liver, as
demonstrated by subsequent staining with the fluorescein-
conjugate of goat anti-rabbit IgG antibody. After 4 successive
absorptions with normal liver sediments, the reactivity for nor-
mal liver disappeared, but the reactivity for hepatoma re-
ained. Although normal rabbit IgG showed a weak reactivity
for normal liver and hepatoma, this was completely removed
after 2 absorptions with normal liver sediments. The results of
staining of various hepatoma nodules or normal livers with
these absorbed preparations of anti-hepatoma antisera are
shown in Table 2.

There was some difference in the reactivity pattern of vari-
ous anti-hepatoma antisera. The reactivity of a particular anti-
serum was not always strongest with the nodule against which
the antiserum had been prepared. Only in the case of antiser-
um IIA was the reaction with the homologous nodule strong-
est. In addition, the two nodules from an individual rat be-
haved quite differently against various antisera. For example,
nodule IIA reacted more strongly than nodule IIB with anti-
sera IA, IB, IIA, and IIB, while both nodules reacted equally with antiserum IIA and nodule IIA reacted more weakly than nodule IIB with antiserum IIB. None of the anti-hepatoma serum reacted with any of the normal livers after absorption with liver.

The staining was not uniform within the section of hepatoma. Some portions stained more brightly than the others, indicating uneven distribution of hepatoma antigens. Histologically, both cytoplasm and cell membranes of hepatoma cells were stained, although some antisera stained both cytoplasm and cell membranes equally (Fig. 1) while another antiserum (anti-IIB) stained cell membranes more strongly than cytoplasm (Fig. 2). Staining was not generally observed in connective tissues. However, staining was observed in some fibrous structures within the connective tissue. One nodule, IIA, was particularly reactive with all the antisera.

To test the specificity of the staining, three anti-hepatoma preparations, anti-IA, anti-IIA, and anti-IIB, were absorbed 3 times with pooled sediments from other hepatomas and tested for the ability to react with normal livers and all the hepatomas. No staining was observed, showing that the pooled hepatomas were capable of removing all the detectable antibodies from anti-hepatoma antisera.

The cultured cells which were established from the 2-acetylaminofluorene-induced rat hepatoma reacted with all the anti-hepatoma preparations, especially with anti-IA, IIA, IIB, and IIIB, after complete absorption with normal liver. This means that the established hepatoma cell lines have retained antigens associated with the hepatoma.

The anti-normal liver antisera, after absorption with normal liver sediments, did not react with normal livers nor with any of the hepatoma nodules. Absorption with rat kidney sediments was not as effective. After 3 absorptions the sera still reacted with normal livers and the hepatomas even though they no longer reacted with kidney. The staining of normal liver sections was uniform and in the cytoplasm (Fig. 3). However, the staining of the hepatoma was not uniform. Although the staining was still in the cytoplasm, patches of cells or rows of adjacent cells in dendritic patterns showed much brighter fluorescence than the others (Fig. 4).

**DISCUSSION**

The present results indicate that rat hepatoma induced by 2-acetylaminofluorene contain some antigens which can be detected by antisera against hepatoma microsomes after exhaustive absorption with normal liver. Whether these antigens represent antigens which are **totally** absent in normal liver cannot be determined by this type of study since a great increase of some normal liver components would also give similar results. However, antisera prepared similarly against normal liver microsomes failed to react with hepatoma sections, indicating that such components, if any, must be present in such a small amount in normal liver as not to produce detectable amounts of antibodies upon injection into rabbits.

Antisera prepared against several individual hepatomas showed varying degrees of reaction with the original and other individual hepatomas and with hepatoma cells in tissue culture. Differences were found in that an individual serum would

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### Table 1

<table>
<thead>
<tr>
<th>Antiserum globulin</th>
<th>Anti-plasma antibody titer</th>
<th>Before treatment</th>
<th>After treatment</th>
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</thead>
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<tr>
<td>Anti-hepatoma</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Anti-IA</td>
<td>256</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Anti-IB</td>
<td>1024</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anti-IIA</td>
<td>2048</td>
<td>&lt;2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-IIB</td>
<td>2048</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Anti-IIIA</td>
<td>2048</td>
<td>2</td>
<td></td>
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<tr>
<td>Anti-IIIB</td>
<td>1024</td>
<td>&lt;2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-normal liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-NL-A</td>
<td>256</td>
<td>&lt;1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Anti-NL-B</td>
<td>256</td>
<td>&lt;1&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Anti-rat plasma antibody titer of anti-hepatoma and anti-normal liver globulins before and after treatment with adsorbent conjugated with rat plasma. 

<sup>a</sup>Serial two-fold dilutions of each globulin solution (1 mg/ml) were tested for agglutination of sheep erythrocytes sensitized with rat plasma. Figures indicate the highest dilution which gave positive results.

<sup>b</sup>Not tested at higher concentrations.

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### Table 2

<table>
<thead>
<tr>
<th>Globulin used</th>
<th>Globulin concentration (mg/ml)</th>
<th>Tissue sections stained</th>
<th>Normal liver&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cultured hepatoma cells</th>
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</thead>
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<tr>
<td>Anti-hepatoma</td>
<td></td>
<td>IA IB IIA IIB IIIA IIIIB</td>
<td>NL-A NL-B NL-C</td>
<td></td>
</tr>
<tr>
<td>Anti-IA</td>
<td>35.1</td>
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<td>+</td>
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<tr>
<td>Anti-IB</td>
<td>10.5</td>
<td>+ + + + + +</td>
<td>- - - - - +</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IIA</td>
<td>5.9</td>
<td>+ + + + + +</td>
<td>- - - - - +</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IIB</td>
<td>10.1</td>
<td>+ + + + + +</td>
<td>- - - - - +</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IIIA</td>
<td>11.4</td>
<td>+ + + + + +</td>
<td>- - - - - +</td>
<td>+</td>
</tr>
<tr>
<td>Anti-IIIB</td>
<td>8.5</td>
<td>+ + + + + +</td>
<td>- - - - - +</td>
<td>+</td>
</tr>
<tr>
<td>Anti-normal liver</td>
<td></td>
<td>IA IB IIA IIB IIIA IIIIB</td>
<td>NL-A NL-B NL-C</td>
<td></td>
</tr>
<tr>
<td>Anti-NL-A</td>
<td>7.2</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>-</td>
</tr>
<tr>
<td>Anti-NL-B</td>
<td>13.2</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>-</td>
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<tr>
<td>Normal globulin</td>
<td>9.0</td>
<td>- - - - - -</td>
<td>- - - - - -</td>
<td>-</td>
</tr>
</tbody>
</table>

Reactions of anti-hepatoma and anti-normal liver globulins with sections of hepatoma nodules and normal liver after exhaustive absorption with normal liver sediments. Sections were reacted with IgG of rabbit antisera against individual hepatoma nodules or normal livers, washed, and then reacted with fluorescein-conjugates of specifically purified goat antibody against rabbit IgG.

<sup>a</sup>Concentration of IgG of rabbit antiserum used for reaction with tissue sections before absorption with normal liver sediment.

<sup>b</sup>NL-A, NL-B, and NL-C are the livers from 3 normal rats. Anti-NL-A and Anti-NL-B antisera were prepared against the corresponding livers.
react with one hepatoma, not necessarily the hepatoma against which it was prepared, more strongly than with other hepatomas. The pattern of reactivity of various anti-hepatoma sera with individual nodules was quite variable. Nodule IIA reacted more strongly than IIB with four antisera, anti-IA, anti-IB, anti-IIA, and anti-IIB, while another antisera, anti-IIIB, gave completely opposite results (Table 2).

These results indicate that the anti-hepatoma antibodies remaining after exhaustive absorption with normal liver are heterogeneous, and they are directed against several antigenic components which are present in different concentrations in the individual hepatomas. The variable pattern of reactions is not due to the difference in antigenic composition of individual rats, since two nodules from the same rat behaved quite differently against various antisera. The multiple nature of these hepatoma antigens and the corresponding antibodies was also reflected in the difference of the staining pattern among these antisera. Staining was mostly limited to the cell membranes with antiserum IIB (Fig. 2), while staining of both cytoplasm and cell membranes was observed with other antisera (Fig. 1).

No antigens specific to a particular nodule were apparent in this study although there may well have been some (5). There was insufficient hepatoma sediment available from any one of the hepatomas to permit specific absorption with the pertinent heterologous tumor. However, absorption with sediments from pooled hepatomas other than those used for the immunization was carried out with three of the anti-hepatoma preparations, and it was found that they lost their reactivity to all the hepatoma nodules.

It is of particular interest that all the anti-hepatoma preparations reacted with hepatoma cells in established tissue culture even after exhaustive absorption with normal liver. Apparently, these hepatoma antigens are still produced in hepatoma cells in culture.

Retention of normal liver antigens in hepatomas was shown by the reactivity of anti-normal liver preparations after absorption with kidney sediments. Although all the hepatoma nodules reacted, the observed staining was not uniform in the sections, indicating uneven distribution of these normal liver antigens in hepatoma nodules. Apparent disappearance of some normal liver components and a great increase of some normal liver components was shown previously in our study on immunoelectrophoretic analyses of hepatoma microsome extracts with anti-normal liver microsome antibodies (7). The change of antigenic components accompanying chemical induction of tumors has been reported by many investigators (for references see 1, 2, 3, 8). Whether the components not found in normal liver are related to embryonal liver components remains to be seen.

A major technical feature in the experiments carried out here was that the goat antibody reagent used for preparing fluorescein-conjugate was specifically purified so that practically all of the fluorescein-conjugate would react specifically with rabbit globulin fixed on the sections. This reduced considerably the background fluorescence due to non-specific fixation of conjugates of non-antibody globulins and resulted in a clearer contrast between the specific staining due to rabbit antibody fixed on the tissue and non-specific background staining. Thus, the sensitivity of the method was considerably increased.

Moreover, the rabbit globulin used was a chromatographically purified IgG. This avoids the nonspecific background due to the β-globulins which are present in crude globulin preparations. In addition, all anti-plasma antibodies had been carefully removed using a specific adsorbent. In our previous study, anti-plasma antibodies were neutralized by addition of excess rat plasma to the antisera. By removal of the anti-plasma antibodies with a solid adsorbent, the possibility of false positive results due to adherence of soluble complexes of rat plasma proteins and anti-plasma antibodies could be completely eliminated. Such soluble complexes containing rabbit antibody globulin may adhere to tissue sections nonspecifically or may adhere specifically to the remaining rat plasma in sections through unsaturated antibody sites of the complex.

ACKNOWLEDGMENTS

We are grateful to Dr. G. E. Moore of this Institute for establishing a cell line from 2-acetylaminofluorene-induced rat hepatoma and supplying the cultured cells. We thank Mr. Jakob Planinsek, Mr. Joseph Berneycke, and Mr. Arthur Trott for technical assistance.

REFERENCES


JANUARY 1969
Fig. 1. Section of hepatoma IA treated with the globulin fraction of anti-IA antiserum (absorbed with normal liver) and then stained with fluorescein-conjugated goat anti-rabbit globulin antibody. Note the staining of cytoplasm and cell membranes.

Fig. 2. Section of hepatoma IIA treated with the globulin fraction of anti-IIB antiserum (absorbed with normal liver) and then stained with fluorescein-conjugated goat anti-rabbit globulin antibody. Note the preferential staining of the cell membranes.

Fig. 3. Section of normal liver, NL-B, treated with the globulin fraction of anti-NL-B antiserum (absorbed with normal kidney) and then stained with fluorescein-conjugated goat anti-rabbit globulin antibody. Note the uniform staining.

Fig. 4. Section of hepatoma IB treated with the globulin fraction of anti-NL-B antiserum (absorbed with normal kidney) and then stained with fluorescein-conjugated goat anti-rabbit globulin antibody. Note the discontinuous pattern of staining.
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