The Reaction of Specific Antibody with 2-Acetylaminofluorene Fixed in Liver Cells

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SUMMARY

Rabbit antibody specific to the 2-azofluorenyl group (anti-2-AzF antibody) was used to investigate the behavior of cellular components capable of binding 2-acetylaminofluorene (2-AAF) in rat livers during the chemical carcinogenesis.

The fixation of the anti-2-AzF antibody in vitro by liver sections of rats fed or injected with fluorenyl derivatives was quantitatively measured by the radioiodine-labeled antibody technic. The anti-2-AzF antibody was fixed to a higher extent at earlier stages of 2-AAF feeding than at later stages. No definite fixation of the antibody was observed 2 weeks after completing the feeding schedule of 16 weeks. The antibodies fixed after and before the beginning of 2-AAF feeding were injected with 2-AAF and assayed, the antibody reacted more weakly with hepatomatous nodules than with preneoplastic or normal liver, indicating the presence of carcinogen-binding components at the preneoplastic stage. The concentration of these components in hepatomatous nodules was much lower.

Cellular distribution of the carcinogen in the livers of these rats was determined by fluorescent antibody technic. The results clearly demonstrated that carcinogen-binding components of hepatic cells are present at a particularly high concentration in the cell boundary and the perinuclear zone and that these components are partially deleted in cells of hyperplastic nodules which appear at the later stage of the 2-AAF feeding, and almost completely deleted in hepatomatous cells.

INTRODUCTION

Our previous studies (9, 10) showed that surface and endoplasmic reticulum of hepatic cells of rats possess some cellular components which bind 2-AF or 2-AAF in vivo and that some of these components are apparently not found in livers of rats fed with 2-AAF diet for long periods or in liver tumors induced by 2-AAF feeding. This was demonstrated by the use of a purified rabbit antibody specific to the 2-azofluorenyl group (anti-2-AzF antibody). Localization of radioiodine-labeled anti-2-AzF antibodies in vivo (10) and radioimmunoelectrophoresis (9) were used for the detection and identification of carcinogen-binding components.

The present study was undertaken to determine the cellular distribution of the carcinogen-binding components by a direct reaction of anti-2-AzF antibody with frozen sections of liver from rats treated with fluorenyl derivatives, using the radioiodine labeled antibody technic and the fluorescent antibody technic.

The results indicated that the carcinogen-binding components of hepatic cells are present at a particularly high concentration in the perinuclear zone and the cell boundary and an observable loss of these components is found during 2-AAF feeding in hyperplastic nodules.

MATERIALS AND METHODS

Carcinogens. 2-Aminofluorene was purchased from Distillation Products Industries, Rochester, N. Y., and 2-acetylaminofluorene from Mann Research Laboratories Inc., New York, N. Y.; 2,7-diaminofluorene was obtained from Chemicals Procurement Laboratories, Inc., College Point, N. Y., and 2-amino-7-azofluorene from Bios Laboratories, Inc., New York, N. Y.

Antiserum against 2-Azofluorenyl Group. The methods for preparing antibody against 2-azofluorenyl-hemocyanin conjugates were described previously (10).

Purification of Anti-2-AzF Antibody. Rabbit antibodies against 2-azofluorenylhemocyanin were purified by the method described previously (10) with some modification in the elution procedure. An immunoabsorbent specific to 2-azofluorenyl group (insoluble polymer of rabbit serum albumin to which diazotized azofluorene had been coupled) was mixed with the antiserum. The anti-2-AzF antibody adsorbed on the immunoabsorbent was eluted by two treatments with 1 M propionic acid in an ice bath under stirring. The supernatants were separated by centrifugation, neutralized with 1 N NaOH, and dialyzed against pH 8.0, borate buffer saline.6 Elution with glycine hydrochloric acid

6 A stock of borate buffer, pH 8.0, was prepared by dissolving 21.2 gm of H3BO3 and 16.0 gm of NaCl in water, adding 45.3 ml of 1 N NaOH, and diluting to 2 liters with water. Buffered saline was prepared by mixing 1 volume of stock borate buffer with 9 volumes of 0.8% NaCl.
buffer, pH 2.4, used in previous studies resulted in only 30% elution of the protein adsorbed on the immunoadsorbent, while that with 1 M propionic acid produced usually more than 70% elution.

The anti-2-AzF antibody thus purified showed a rather high nonspecific reactivity with tissue sections as measured by a paired label technic (see below). One of the preparations was analyzed in the ultracentrifuge and showed a major component of 6.8 and two minor components. It showed a single precipitate arc of IgG on immunoelectrophoresis when tested against a goat antirabbit globulin serum. Reduction of this nonspecific reactivity was accomplished by chromatography on a DEAE-cellulose column according to Sober et al. (15). The fraction obtained by elution with pH 6.3, 0.0175 M phosphate buffer (IgG) was used.

Radioiodination. Purified anti-2-AzF antibody was labeled with 125I using chloramine T [Greenwood et al. (6)]. Unreacted iodine was removed by dialysis against borate-buffered saline, pH 8.0, or by column chromatography on Sephadex G-25.

Similarly, normal serum IgG prepared by Sober's method (15) was labeled with 125I. Both preparations had 0.7 atom iodine per molecule IgG protein and 0.4-0.5 mc of radioiodine/mg protein.

Animals. Male rats of the inbred Fisher Strain (Charles River Farms) were used throughout. Feeding of 2-AAF was carried out as described previously (3).

Preparation of Test Specimens. Rats were killed by perfusion through the abdominal aorta with borate-buffered saline following anesthesia with Nembutal. The organs were excised and quickly frozen. Frozen sections were cut at a 4-micon setting in a cryostat and attached to cover slips (25 x 18 mm) for the radioiodinated antibody technic or to ordinary microscope slides for the fluorescent antibody technic.

Radioiodine-labeled Antibody Technic. 125I-labeled anti-2-AzF antibody (500 μg) in 5 ml of borate-buffered saline containing 10% fetal calf serum was absorbed with 500 mg (wet weight) of rat-liver sediment for 45 min at room temperature. The supernatant separated by high-speed centrifugation was mixed with 125I-labeled normal serum IgG, approximately 500 μg in 5 ml of the above diluent (paired mixture). Tissue specimens, fixed with acetone for 10 min at room temperature, were washed with phosphate-buffered saline (pH 7.2, 0.01 M phosphate) and covered with a few drops of the absorbed anti-2-AzF antibody solution. They were centrifuged and the absorbed anti-2-AzF antibody solution, and incubated with repeated agitation for 60 min in a cold room. They were washed with phosphate-buffered saline and stained with the horse anti-rabbit-globulin-fluorescein conjugate for 30 min in the same way as above. Increase of autofluorescence during the staining procedures seemed to be reduced by the use of low temperature.

RESULTS AND DISCUSSION

Radioiodine-labeled Antibody Studies. The fixation of carcinogen in livers of rats injected with various fluorine derivatives was studied by the paired label antibody technic where the uptake of anti-2-AzF antibody on liver sections was determined. A fine suspension (10 mg/ml) of 2-AF, 2-AAF, 2-A-7-AAF, and 2,7-DAF was prepared by dissolving the solid with ethanol and adding 20 volumes of saline. Fifty mg of each derivative were injected i.p. into normal rats. Seventy-two hr later, rats were perfused and liver sections were treated with a mixture of 125I-labeled anti-2-AzF antibody and 131I-labeled normal rabbit globulin to determine the specific uptake of anti-2-AzF antibody. All the rats injected with 2-AF, 2-AAF, and 2-A-7-AAF survived, but two of the three rats injected with 2,7-DAF died before the test. Livers of rats injected with saline containing 5% ethanol were used as controls. Values in Table 1 indicate the uptake quotient of 125I-labeled anti-2-AzF antibody by each specimen in duplicate determinations.

The technic clearly detected the anti-2-AzF antibody specifically fixed on the specimens, as indicated by its high uptake quotient on liver sections of rats treated with 2-AAF or 2-AAF.
This indicates that livers of these rats contained carcinogens or their metabolites reactive toward anti-2-AzF antibody. The \(^{125}\)I-labeled anti-2-AzF antibody reacted most strongly with livers of rats treated with 2-AAF, and somewhat more weakly with those of rats treated with 2-AF. However, it did not react with those of rats treated either with 2-A-7-AAF or with 2,7-DAF. The negative results in the latter specimens do not exclude the possible presence of these compounds or their metabolites in the livers since the anti-2-AzF antibody was previously found to react only poorly with 2,7-disubstituted fluorene compounds (10).

These results are in accord with our previous results on the localization in vivo of \(^{125}\)I-labeled anti-2-AzF antibody in livers of rats injected with various fluorene derivatives (10).

As shown in Table 2, the uptake quotient of the anti-2-AzF antibody for rats on 2-AAF diet for 2 to 4 weeks was larger than that for rats on the diet for 8 to 16 weeks: The uptake quotient of the latter ranged from 2.4 to 3.7 while those of the former ranged from 3.7 to 5.0. This difference may reflect the accumulation of carcinogen at earlier stage of feeding as observed by Miller et al. (12) on DMAB-carcinogens. The livers of rats on normal diet for at least 2 weeks after completing the 16-week feeding schedule did not fix the anti-2-AzF antibody at high enough level to give a definite reaction. This indicates a rapid decrease of carcinogen during the time the carcinogen-fed rats were on normal diet.

In order to see whether there is a decrease of carcinogen-binding components in liver of carcinogen-fed rats while on normal diet, rats fed with normal diet for 2, 4, 8, and 16 weeks after the complete course of 2-AAF feeding and rats bearing liver tumors were injected i.p. with 50 mg of 2-AAF. Seventy-two hr later, the livers were assayed by the paired label technic. The results are in Table 3 along with reference to the morphologic changes observed.

Carcinogen-induced hepatomatous nodules gave definitely lower uptake quotients than livers of normal rats. Hyperplastic, cirrhotic livers of carcinogen-fed rats did not show definite differences from normal livers, although somewhat low uptake was obtained in a few rats. This indicates that carcinogen-binding components are still present in premalignant livers, but they are considerably reduced in hepatomatous liver.

Predominant histologic changes in these livers were formation of hyperplastic nodules associated with necrobiosis and proliferation of connective tissues. The degrees of the hyperplasia varied from specimen to specimen. Some livers contained disseminated marked hyperplastic nodules and some only a few weakly hyperplastic nodules. The changes seemed severe just after discontinuing 2-AAF feeding and to reduce during feeding with normal diet. However, it was difficult to correlate these morphologic changes to the uptake quotient values of anti-2-AzF antibody.

Fluorescent Antibody Studies. The location of carcinogen bound to cellular components of liver was determined in rats fed with 2-AAF diet by the indirect fluorescent antibody technic. Liver sections of rats fed with 2-AAF diet for 4, 8, and 16 weeks and of rats fed with normal diet for 2, 16, and 32 weeks after feeding with 2-AAF diet for 16 weeks were treated with anti-2-AzF antibody and subsequently stained with horse anti-rabbit-globulin-fluorescein conjugate.

At 4 weeks after initiation of the 2-AAF diet, the specific
TABLE 3
Fixation of Radiolabeled Anti-2-AzF° Antibody in Vitro on Livers of Rats Injected with 2-AAF after Previous Feeding with 2-AAF°

<table>
<thead>
<tr>
<th>Period (weeks) of normal feeding before injection</th>
<th>Rat No.</th>
<th>Uptake quotient of anti-2-AzF° antibody</th>
<th>Histologic changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>3.9 3.7</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.7 3.6</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.2 2.9</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2.8 2.6</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.3 1.2</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.8 2.7</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>2.6 2.5</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.3 1.9</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.5 3.0</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>3.1 2.9</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.4 3.4</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.9 2.9</td>
<td>Cirrhotic nodules</td>
</tr>
<tr>
<td>16 or more</td>
<td>1</td>
<td>1.8 1.5</td>
<td>Mainly hepatomatous</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.4 1.2</td>
<td>Mainly hepatomatous</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.7 0.7</td>
<td>Mainly hepatomatous</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.6 0.5</td>
<td>Mainly hepatomatous</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.6 2.5</td>
<td>Partially hepatomatous</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.6 2.2</td>
<td>Partially cholangiomatous</td>
</tr>
<tr>
<td>Normal rats</td>
<td>1</td>
<td>3.8 3.6</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>Normal</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.0 3.5</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* 2-AzF, 2-azofluorene; 2-AAF, 2-acetylaminofluorene.  
*° Rats were fed 2-AAF diet for 16 weeks and were on normal diet for the indicated period before injection of 2-AAF.  
* Values for duplicate consecutive sections.  
* From a different part of the liver.

Fluorescence was diffusely distributed through liver lobules, but seemed brighter at the perportal area than at central vein area (Fig. 1). The staining was observed in cytoplasm and no definite nuclear staining was obtained, except a few fluorescent granules which may be due to nucleolus staining. Prominent staining was seen at cell boundary and perinuclear zone. The livers at this stage of feeding were practically normal, although at the perportal area there were a number of hepatic cells containing vacuoles.

The livers of rats fed with 2-AAF diet for 8 and 16 weeks showed staining patterns distinctly different from those obtained with rats at earlier stage of feeding (Fig. 3). The observed staining was not even. Some areas stained brightly and some areas stained poorly. In the borders of these areas, the intensity of staining varied from cell to cell. Some areas of poorly stained cells still showed bright staining of cell boundary, while some completely lost such staining. The cells composing poorly staining areas were usually larger than normal hepatic cells in both nuclear size and cytoplasmic volume. These areas were, in some places, demarcated by fine connective tissues from adjacent normal-appearing areas, but usually not associated with definite border. Brightly stained cells were intimately intermingled with poorly stained cells. The livers of normal rats used as controls and rats on normal diet for 2, 16, and 32 weeks following the 16-week 2-AAF feeding schedule did not show any definite fluorescence.

The livers of rats fed with 2-AAF for long periods were usually larger than normal livers and macroscopically cirrhotic. At 16 to 32 weeks on normal diet following 16 weeks of 2-AAF feeding, livers of some rats showed multiple nodules. Microscopically, the cirrhotic livers contained hyperplastic areas of various size, which consisted of large hepatic cells with cytoplasm which stained faintly with hematoxylin and eosin stain. These morphologic changes in the livers of rats fed with 2-AAF were described along with hyperplastic changes in other epithelial organs by Wilson et al. (19) and Cox et al. (2). The areas which stained poorly by the fluorescent technic were these hyperplastic areas of hepatic cells.

Clearer results were obtained by injecting 2-AAF into rats on normal diet after the 16-week 2-AAF feeding schedule.

Rats on normal diet for 2, 4, 8, and 16 weeks after a 16-week 2-AAF feeding schedule were injected i.p. with 50 mg of 2-AAF. Seventy-two hr later, the liver sections were cut and stained in the same way as above. Normal rats injected with 50 mg of 2-AAF and of 2,7-DAF were also stained.

The livers of normal rats injected with 2,7-DAF did not give any definite positive staining. The staining pattern of normal rats injected with 2-AAF was exactly the same as that of rats fed with 2-AAF for 4 weeks (Fig. 2). The hepatic cells stained uniformly and produced brighter fluorescence in the cell boundary and perinuclear zones. When rats on normal diet after 16-weeks of feeding with 2-AAF were injected with 2-AAF, they gave staining patterns similar to those of rats fed with 2-AAF for more than 8 weeks. Hyperplastic nodules showed some weak staining, but the intensity was much lower than the bright staining of adjacent normal-appearing areas (Fig. 4). Hepatomatous nodules present in some of the rats showed very little staining although some cells still retained weak cell boundary and perinuclear staining (Fig. 6). Usually, atrophic hepatic cells surrounding hyperplastic nodules or hepatomatous nodules showed brighter fluorescence than normal-appearing cells.

Even in the midst of hepatomatous nodules, hepatic cells, which were either apparently normal or atrophic, stained brightly (Fig. 5). This finding eliminates the possibility that the poor staining of hepatomatous nodules could be due to the limited access of injected 2-AAF to the area.

Baldwin (1) and Green et al. (5) reported the presence of abnormal carcinogen-bound components in rat livers at the early stage of DMAB or 3'-DMAB carcinogenesis, which do not react with antisera prepared against normal liver. Bound carcinogen in such components would have escaped detection by the radioimmunoelectrophoresis test we used previously (9). It depended...
on the binding of radioiodine-labeled anti-2-AzF antibodies to liver components precipitated by antinormal liver微some antibodies. However, the present results were obtained by a direct reaction of anti-2-AzF antibodies with liver sections, so that all the carcinogen bound to cellular components in insoluble form would be expected to participate in the reaction.

Whether cellular components capable of binding carcinogens are actually deleted in hyperplastic or hepatomatous cells is something open to question. The lack of reaction with anti-2-AzF antibodies is an indication that there is no AAF fixed on these cells. This can be due to the absence of the components capable of binding the carcinogen or, alternatively, to the absence of an enzyme system necessary for metabolizing the carcinogen to a stage where it can couple to the cell components as described by Miller et al. (13, 14), or to both effects. However, our previous study of radioimmunoelectrophoresis (9) along with the present stage where it can couple to the cell components as described by binding the carcinogen or, alternatively, to the absence of an antibody is an indication that there is no AAF fixed on these cells.

ACKNOWLEDGMENTS

We thank Mrs. H. Kantor, Messrs. J. Bernecky, J. Planinsek, and A. Trott for their technical assistance.

REFERENCES


FIGS. 1-6. Sections were stained with anti-2-AzF antibodies and subsequently with fluorescent horse anti-rabbit globulins. Anti-2-AzF, anti-2-azo-fluorene; 2-AAF, 2-acetylaminofluorene.

Fig. 1. Liver of rat fed 2-AAF diet for 4 weeks. Bright fluorescence is evenly seen in hepatic cells. Note predominant staining of cell boundary and perinuclear zone.

Fig. 2. Liver of normal rat injected with 2-AAF. The staining pattern is the same as that of rats fed 2-AAF diet for 4 weeks.

Fig. 3. Liver of rats fed 2-AAF diet for 16 weeks. Hepatic cells in the upper left part shows brighter cell boundary and cytoplasmic staining than those in the lower right part. Hyperplastic changes are not distinct.

Fig. 4. Liver of rat injected with 2-AAF after 2-AAF feeding (16 weeks). Note reduced staining in hyperplastic cells. The poorly stained cells have a larger nuclear size and cytoplasmic volume than the brightly stained cells.

Fig. 5. Liver of hepatoma-bearing rats injected with 2-AAF. Hepatic cells, either apparently normal or atrophic, are stained brightly in the midst of hepatomatous cells, which are poorly stained.

Fig. 6. The same liver as the above. A portion of a typical hepatomatous nodule. Hepatomatous cells are poorly stained but still retain weak cytoplasmic staining. Some of the cells show cell boundary staining.
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