Alteration in the Distribution of Basic Soluble Rat Liver Proteins during Azo Dye Carcinogenesis

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SUMMARY

Antibodies against rat liver \( h \) proteins and against a similar charge class of azoproteins have been labeled with fluorescein isothiocyanate (FITC), and the labeled antisera have been used as histoserosiological stains on unfixed frozen sections of rat liver. Fixation of antibody was defined by fluorescence microscopy and was used to determine the intracellular distribution of the appropriate antigens. FITC-labeled anti-\( h \) protein stained the cytoplasm of both normal and 3'-methyl-4-dimethylaminoazobenzene (3'MeDAB)-treated rat livers uniformly but did not stain 3'MeDAB-induced rat hepatomas. On the other hand, fixation of FITC-labeled antiazoprotein by normal rat liver was concentrated mainly at the periphery of the cell, in the perinuclear region ("membrane" fluorescence) and, to a lesser extent, in the cell cytoplasm. Pretreatment of sections of normal rat liver with unlabeled anti-\( h \) protein followed by FITC-labeled antiazoprotein inhibited only the cytoplasmic fluorescence, whereas pretreatment with unlabeled antiazoprotein inhibited both cytoplasmic and "membrane" fluorescence. In sections of liver from 3'MeDAB-treated rats, FITC-labeled antiazoprotein stained only the cytoplasm. This cytoplasmic fluorescence was inhibited by pretreating the sections with unlabeled antiazoprotein but not with unlabeled anti-\( h \) protein. The findings have demonstrated that, following administration of 3'MeDAB to rats, there is an alteration in the intracellular distribution of certain liver proteins from a particularly high concentration in the region of the nuclear and plasma membranes to a more uniform dispersal throughout the cell cytoplasm.

INTRODUCTION

Carcinogen binding to protein of target organs is thought to be of causal significance in carcinogenesis (16). Studies in this field have been directed toward establishing binding sites in the carcinogen molecule (16) and toward characterizing the nature of the proteins involved (19). Some morphological and biochemical studies have been made with the aim of determining the intracellular spatial distribution of the dye-binding proteins. It has been shown that bound dye is associated first with the microsomal fraction and later with the cell sap (4, 9, 10). Baldwin et al. (2) induced an antiserum in rabbits against a conjugate of an azo dye derivative and bovine serum albumin and localized bound azo dye in the cytoplasm of liver cells. Tanigaki et al. (25) induced antibodies in rabbits against 2-azofluorenyl-hemocyanin conjugates which they rendered specific to the 2-azofluorenyl group. Using this antibody, they demonstrated that bound 2-acetylaminofluorene derivatives were distributed through the liver cytoplasm and were also associated with the nuclear and plasma membranes. However, while these studies have provided information concerning the distribution of protein-bound carcinogens in the cell, they did not provide any information regarding the origin and components of the electrophoretic class of azoproteins present in the cell sap following the administration of \( \alpha \)-azo dyes. This class may well contain other proteins which are not associated with carcinogen (15), and either of these types of protein may have originally been associated with organelles. This study was conducted with the aim of providing information as to the intracellular origin of the components of the charge class of azoproteins.

In previous studies, with the use of agarose gel microelectrophoresis of liver cell sap from rats given i.p. injections of 3'MeDAB-\( ^3 \text{H} \) (14), approximately 80% of the azoprotein was recovered from 2 slow-moving bands which were equated with the group of \( h \) proteins described by Sorof and Cohen (20). The fraction of cell sap contained in these slow-moving bands was temporarily increased shortly after the administration of carcinogen, but in the resulting hepatomas this fraction was absent or grossly diminished in quantity (14). These findings confirmed earlier studies of soluble proteins during carcinogenesis by some other carcinogens (1, 20—22).

In an immunoelectrophoretic study (15), antibodies against normal rat liver \( h \) proteins reacted specifically with the slow-moving bands of liver cell sap from either normal or 3'MeDAB-fed rats but did not react with any component of 3'MeDAB-induced rat hepatoma cell sap. However, only 26.6% of the azocarcinogen present in the slow-moving bands was recovered from the precipitin arc. While it was not possible at the time to account for the failure of 73.4% of azoprotein to react with this antibody, a suggestion was made that perhaps only 26.6% of this charge class of azoprotein was antigenically similar to the \( h \) proteins of normal rat liver and that the major portion of azoprotein present in this charge class of cell sap might have been derived from breakdown of membrane-bound or insoluble dye-bound proteins as suggested by Sorof (19).
addition, other proteins which were not involved in carcinogen binding could also have become dislodged from organelles or membranes as a result of the toxicity of the administered compound and might have migrated with the azoproteins.

In an effort to explain why the major portion of azoproteins did not react with normal anti-h protein, and to try to determine the possible intracellular origin of the proteins in this charge class following azo dye administration, antibodies were prepared against normal h proteins and against a similar charge class of azoproteins from a rat given 3'MeDAB by i.p. injection. These antibodies were then labeled with FITC and used as histoserological stains on frozen sections of livers from normal rats and from other rats which received 3'MeDAB either in the diet or by i.p. injection. This procedure was adopted as there is evidence that the dye-binding proteins have different characteristics following either a single large dose of 3'MeDAB or the inclusion of 3'MeDAB in the diet for several days (24). By these means, it has been possible to demonstrate that a protein component present in a high concentration in the region of the nuclear and plasma membranes in normal rat liver (membrane-associated protein) appears in the cytoplasm following administration of the azocarcinogen. This protein component is either absent from or grossly diminished in hepatomas which subsequently develop.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats, random bred from departmental stock, were used for the preparation of h and azoprotein antigens and for preparation of frozen tissue sections for histological studies.

For the preparation of h and azoprotein antigens, cell sap was isolated from the livers of 4 adult rats. One of these received no treatment, one was given an injection i.p. of corn oil (1 ml/100 g body weight), and a further 2 were given i.p. injections of 2% (w/v) 3'MeDAB dissolved in corn oil (1 ml/100 g body weight, equivalent to 20 mg of 3'MeDAB/100 g body weight). These rats were killed by exsanguination under light ether anesthesia after 24 hr.

Frozen tissue sections of various organs for histoserological studies were prepared from an additional 13 rats. Of these rats, 5 were fed a control diet ad libitum for 10 days and a further 3 rats likewise received a 3'MeDAB-containing diet for a similar period. Two other rats were given i.p. injections of 2% (w/v) 3'MeDAB dissolved in corn oil (1 ml/100 g body weight) and were killed 24 hr later. In addition, 3 rats bearing 3'MeDAB-induced hepatomas were used. These rats had been fed a 3'MeDAB-containing diet ad libitum for 20 weeks.

For antibody production, a total of 10 female Swiss mice were used. These were random bred from departmental stock and were divided into 2 groups of 5.

**Diets.** All animals received a standard pellet diet ad libitum (I.R.M. pellets, Victorian Wheatgrowers Co-operative, Melbourne, Australia), except where otherwise indicated. The control diet and 3'MeDAB-containing diet comprised crushed pellets to which were added 30 ml maize oil per kg of diet and 30 ml maize oil containing 2% (w/v) 3'MeDAB/kg, respectively. Both rats and mice were allowed tap water ad libitum.

**Reagents.** 3'MeDAB (m.p. 119°) was synthesized as described by Giese et al. (5). Freund's incomplete adjuvant and Freund's complete adjuvant were obtained from the Baltimore Biological Laboratory, Baltimore, Md., and FITC was obtained from British Drug Houses, Poole, England. The corn oil used was a local proprietary brand (Sherman Edible Oil Co. Pty. Ltd., Melbourne, Australia). All solutions were prepared with metal-distilled water passed through an Elgastat deionizer.

**Preparation of Rat Liver Cell Sap.** Liver cell sap was prepared from rats which had received I.R.M. pellets, i.p. corn oil, or i.p. 3'MeDAB dissolved in corn oil by homogenizing samples of liver in equal volumes of ice-cold 0.08 M sodium phosphate buffer, pH 7.8, containing 0.075 M NaCl as described previously (15). All procedures were performed at approximately 4°, and the samples of cell sap were stored at −20°.

**Preparation of h Proteins and Azoproteins.** The method of preparative electrophoresis has been described in detail previously (15). Azoproteins and h proteins were separated, extracted, and concentrated from the liver cell sap of 3'MeDAB-treated and normal rats, respectively. All procedures were performed at 4°.

**Preparation of Antibodies and Conjugation with FITC.** Antibodies were induced in mice by giving them i.p. injections of antigen, Freund's complete adjuvant, Freund's incomplete adjuvant, and phosphate buffer as described previously (15). The ascites thus produced was tapped on Day 52, and the γ-globulin fractions were isolated from portions of the ascitic fluid aspirates according to the method of Pillemer and Hutchinson (18). Fibrin was removed subsequent to the addition of acetate buffer by centrifuging and discarding the precipitates. The deposits of γ-globulin obtained on the addition of methanol reagent were suspended in 0.9% NaCl solution (20 ml) and carbonate-bicarbonate buffer, pH 9.0, was added until the suspensions cleared (approximately 3 to 4 ml). Finally, acetone (2 ml) and FITC (25 mg) were added to the solutions and allowed to react with continuous stirring for 18 hr. Free fluorescein derivatives were removed by dialysis for 24 hr against repeated changes of 0.08 M phosphate buffer, pH 7.8, containing 0.075 M NaCl. The final conjugates were am pupilied in aliquots of 2 ml and stored in the dark at −20°.

Further portions of ascitic fluid were used for the preparation of unconjugated γ-globulin fractions. These were suspended in 0.9% NaCl solution and carbonate-bicarbonate buffer, pH 9.0, was added as before. The unconjugated γ-globulin fractions were then stored at −20° and were later thawed and used as unlabeled antibody solution for pretreatment of frozen sections prior to staining with FITC-labeled antibody. All procedures were performed at approximately 4°.

**Preparation of Unfixed Frozen Sections.** Fresh slices of rat liver and other organs, not more than 4 mm thick, were snap-frozen by dropping in a tube of precooled isopentane kept at −70° in a Dewar flask containing a mixture of solid CO₂ and ethanol. From these blocks, unfixed frozen sections, 4 to 5 μm, were cut at −20° with a rotary microtome as described previously (12). Sections were picked up on ethanol-moistened slides, also at −20°, and allowed to dry at 4°. The sections were again immersed in cold ethanol at −20°
for a further 20 min to remove free azo dye and then dried overnight at 4°C.

Staining of Tissue Sections, Microscopy, and Photography. The dried tissue sections were covered with a few drops of FITC-labeled antibody solution for 20 min at room temperature and then washed gently for 30 min in 3 changes of 0.08 M phosphate buffer, pH 7.3. In experiments involving blocking of the staining reaction, the sections were covered with a few drops of unlabeled antibody for 20 min at room temperature and washed for 30 min in 3 changes of 0.08 M phosphate buffer, pH 7.3. Immediately following this treatment, the sections were stained with the appropriate FITC-labeled antibody for 20 min and washed as above.

Leitz fluorescence equipment was used for fluorescence microscopy. This consisted of an Ortholux microscope with an Orthomat fully automatic microscope camera and an HBO 200-watt mercury vapor lamp as light source. A 5-mm BG 12 emission filter with a wavelength of 400 nm was placed between the light source and condenser and a K530 suppression filter below the ocular lens. For photography, Tri-X film (Kodak, Melbourne, Australia) was used. Appropriate areas were selected and photographed under UV light; then the sections were fixed in 10% formol-0.9% NaCl solution and stained with hematoxylin and eosin, and the same areas were rephotographed under visible light for comparison with the photographs made with UV light (Figs. 3 and 4).

RESULTS

The direct fluorescent antibody technique has been used to determine localization of antibody to cellular components of rat liver. Observations were made on unfixed frozen liver sections from normal rats, from rats fed 3'MeDAB for 10 days, and from rats given i.p. injections of 3'MeDAB 24 hr previously. A positive staining reaction was indicated by a bright green fluorescence in UV light. The staining pattern of liver from rats fed 3'MeDAB was similar to that of rats given i.p. injections of 3'MeDAB; hence the results are recorded collectively as those of 3'MeDAB-treated rat liver. The results are summarized in Table 1.

Specificity of the Staining Reactions. Primary or autofluorescence in tissues was excluded by examining unstained sections in UV light. Some of these showed a bright yellow linear fluorescence emanating from the elastic laminae of blood vessels. In all slides examined, emphasis was placed on the characteristic green fluorescence of fluorescein. Repeated observations showed that washing the sections for 30 min after staining removed all background and nonimmune staining but not the immunologically specific staining.

Since the addition of 3'MeDAB to the protein molecule would confer new antigenic determinants not present in the parent protein, antibodies against azoproteins would be expected to cross-react with normal target proteins, with azoprotein conjugates and with free azo dye. In these experiments, cross-reaction with free azo dye has been excluded since treatment for 20 min with ethanol would remove any free 3'MeDAB from the liver sections. Furthermore, the specificity of these staining reactions was established by the demonstration of inhibition of part or all of the fluorescence after pretreatment with the appropriate unlabeled antibody (Table 1).

Staining with FITC-labeled Mouse Anti-Rat Liver h Protein. The cytoplasm of normal rat liver parenchymal cells showed a strong affinity for the labeled antibody and fluoresced brightly in UV light. The fluorescence was uniformly distributed throughout the cytoplasm and was limited centrally by the nuclear membrane and peripherally by the cytoplasmic membrane. Neither nuclear nor intercellular material showed a positive staining reaction. The appearances were similar to those of 3'MeDAB-treated rat liver stained with FITC-labeled antiazoprotein (Fig. 1). Pretreatment of sections of normal rat liver with either unlabeled anti-h protein or unlabeled azoprotein blocked the antigenic receptor sites and, on subsequent staining with FITC-labeled anti-h protein, the cytoplasm showed no affinity for the labeled conjugate and failed to fluoresce in UV light.

In sections prepared from 3'MeDAB-treated liver and stained with FITC-labeled anti-h protein, the cytoplasmic fluorescence showed a distribution similar to that of normal rat liver. A similar inhibition of fluorescence was also observed when these sections were pretreated with either of the unlabeled antibody preparations.

Staining with FITC-labeled Mouse Anti-Rat Liver

Table 1

<table>
<thead>
<tr>
<th>Pretreatment (unlabeled antibody)</th>
<th>Stain (FITC-labeled antibody)</th>
<th>Normal liver</th>
<th>3'MeDAB-treated liver</th>
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*a h, mouse anti-rat liver h protein; +, bright green fluorescence; -, no fluorescence; Azo, mouse anti-rat liver azoprotein.*
Azoproteins. The cell cytoplasm of normal rat liver again showed a uniform bright green fluorescence but, in addition, there was a linear concentration of fluorescing material in the region of the nuclear and plasma membranes ("membrane" fluorescence, Fig. 3). Hematoxylin-eosin staining of these sections subsequent to the fluorescent studies demonstrated that the linear fluorescence was in the region of the cell periphery and around the nucleus (Fig. 4). Pretreatment of sections of normal rat liver with unlabeled antiazoprotein inhibited both cytoplasmic and "membrane" fluorescence. However, when the sections were pretreated with unlabeled anti-\( h \) protein and then stained with FITC-labeled antiazoprotein, only the cytoplasmic fluorescence was inhibited, whereas the linear "membrane" fluorescence persisted and remained at approximately the same intensity (Fig. 5).

Repeated observations on sections prepared from 3'MeDAB-treated rat liver that were stained with FITC-labeled antiazoprotein showed no linear concentration of fluorescing material around cell membranes but only a uniformly distributed, bright green fluorescence throughout the cytoplasm (Fig. 1). Pretreatment of these sections with unlabeled antiazoprotein inhibited all the fluorescence; but when the sections were pretreated with unlabeled anti-\( h \) protein, the cell cytoplasm retained most of its affinity for the FITC-labeled antiazoprotein and fluoresced brightly in UV light (Fig. 2).

Staining of Rat Hepatoma Tissues. Sections prepared from the examples of 3'MeDAB-induced rat hepatomas all failed to stain with either FITC-labeled antibody preparation. The cells of the adjacent nonneoplastic liver stained uniformly but showed no linear concentration of fluorescing material over membranes (Fig. 6). These rats were still receiving the 3'MeDAB-containing diet at the time of sacrifice.

Staining of Other Rat Tissues. Tissues routinely examined were heart, lung, kidney, and spleen. Sections prepared from these organs and stained with either of the FITC-labeled antibodies showed no affinity for these conjugates and failed to fluoresce in UV light.

DISCUSSION

In vivo interactions between carcinogen and target proteins result in the presence of carcinogen-protein conjugates in the cell sap (19). In rat liver, the azocarcinogen present in these conjugates is contained mainly in a small electrophoretic class of basic proteins which has a similar mobility to the \( h \) proteins (14, 21, 22), but there is no evidence to indicate that normal \( h \) proteins are the initial target of the azo dyes. A suggestion has been advanced by Sorof (19) that the target proteins, although soluble when isolated, might initially be membrane or organelle bound. In support of his concept, he cited the work of Lotlikar et al. (11), who showed that covalent binding of carcinogen resulted in cleavage of peptide bonds. Carcinogen administration could also result in additional changes in the secondary and tertiary structure of proteins, and such alterations might be expected to facilitate the release of proteins from organelles and might modify their electrophoretic mobility. In the present investigation, a comparison of normal \( h \) proteins with a similar charge class of azoprotein has demonstrated differences in their antigenic composition. The results obtained have been correlated with those from a previous immunoelectrophoretic study (15) and have shown that a membrane-associated protein in normal rat liver is found in the cell sap following administration of azocarcinogen.

The present demonstration that both FITC-labeled anti-\( h \) and antiazoproteins stained liver sections from both normal and 3'MeDAB-treated rats confirms the previous observation made on immunoelectrophoresis that a portion of the slow-moving azoproteins cross-reacted with mouse anti rat liver \( h \) protein (15). The finding that only normal rat liver showed "membrane" fluorescence when stained with FITC-labeled antiazoprotein implies that the soluble basic protein fraction in livers from rats given azocarcinogen contains a protein component which is associated with the cell membrane in normal rat liver. Furthermore, when sections of liver from rats given 3'MeDAB were blocked with unlabeled antiazoprotein and subsequently stained with FITC-labeled anti \( h \) protein, no staining occurred, indicating that the azoprotein contains most of the \( h \) antigens. On the other hand, the persistence of cytoplasmic fluorescence when sections from the same rat livers were blocked with unlabeled anti-\( h \) protein and then stained with FITC-labeled antiazoprotein indicated that the basic azoproteins contain additional protein components as well as \( h \) proteins. These additional proteins, with different antigenic determinants from those of the \( h \) proteins, probably include a major portion of the slow-moving azoproteins which failed to cross-react with mouse anti-rat liver \( h \) protein in the previous immunoelectrophoretic study (15). They could also account for the increase in the level of slow-moving basic protein found in the livers of rats following 10 to 14 days of administration of certain hepatocarcinogens (23). This increase is found at approximately the same time as maximum carcinogen binding to protein (23). This evidence, considered together with the results of the present experiments, offers support to the notion that a significant proportion of the basic protein in livers of rats administered 3'MeDAB is antigenically different from an equivalent charge class of \( h \) protein.

It is likely that the changes observed in the staining reaction between normal rat liver and liver from 3'MeDAB-treated rats represents changes in the distribution of membrane-associated proteins. These observations suggest that membrane-associated proteins are released into the cell sap in response to administration of 3'MeDAB, and support the suggestion of Sorof (19) that some proteins migrating with the azoproteins were initially associated with organelles. However, it is not possible at present to state whether the appearance of these proteins in the cell sap is a result of their interaction with azocarcinogen or of some other effect of the carcinogen on the organelle. Evidence for the latter effect has been provided by Friedrich-Freksa et al. (3) who demonstrated a high concentration of a microsomal antigen in liver cell sap of rats fed azo dye. This protein did not contain bound carcinogen.

Some workers, using "organ-specific" and nonimmune fluorescein-labeled antisera, have demonstrated a similar loss of fluorescence staining to that shown in this report in rat hepatomas induced by azo dyes or acetylamino fluorene.
aim of identifying these proteins and their relevance to carcinogenesis. Further experiments are in progress with the membranes in normal rat liver appear in the cytoplasm following carcinogen administration. When tumors develop, reproducible observation that a cytostructural alteration of the present FITC-labeled antibody preparations with the differences in electrophoretic profile between the cell sap of liver and that of 3'MeDAB-induced hepatoma, kidney, heart, lung, or spleen was subjected to electrophoresis under similar conditions (Ref. 21; C. J. Louis and J. M. Blunck, unpublished data). It is therefore tempting to correlate the apparent organ specificity of the present FITC-labeled antibody preparations with the organ-specific antigen from normal, acetylaminofluorene-and azo dye-treated rats. They showed that, in each case, this microsomal organ-specific antigen did not cross-react with the principal carcinogen-protein conjugates, it did not migrate with the h or azoproteins on electrophoresis and it did not contain bound carcinogen. Therefore such a protein could not have been present in either of the h or azoprotein preparations used in this study and, as a result, loss of this organ-specific antigen could not have been responsible for the differential staining of normal and neoplastic tissue. However, although both FITC-labeled anti-h and antiazoproteins stained sections of rat liver, they did not stain sections from kidney, heart, lung, or spleen; in fact, they behaved as organ-specific stains. Also, while in rat liver cell sap 2 prominent bands migrated towards the cathode when electrophoresis was carried out at pH 8.6, very little protein migrated in this region when cell sap from 3'MeDAB-induced hepatoma, kidney, heart, lung, or spleen was subjected to electrophoresis under similar conditions (Ref. 14; C. J. Louis and J. M. Blunck, unpublished data). It is therefore tempting to correlate the apparent organ specificity of the present FITC-labeled antibody preparations with the differences in electrophoretic profile between the cell sap of liver and that of 3'MeDAB-induced hepatoma and the other rat organs.

The most significant finding of this study was the reproducible observation that a cytostructural alteration occurs in membrane-associated proteins in response to administration of azocarcinogen to rats. By use of the fluorescent antibody technique, it has been shown that proteins which are localized in the region of the cell membranes in normal rat liver appear in the cytoplasm following carcinogen administration. When tumors develop, these proteins are not present in detectable amounts in tumor tissue cytoplasm. Further experiments are in progress with the aim of identifying these proteins and their relevance to carcinogenesis.

REFERENCES


Fig. 1. Fluorescence photomicrograph of an unfixed frozen section of liver from a rat fed 3'MeDAB for 10 days and stained with FITC-labeled antiazoprotein, showing uniform fluorescence of the cytoplasm of the parenchymal cells. The nuclei do not fluoresce. X 560.

Fig. 2. Fluorescence photomicrograph of an unfixed frozen section of liver from a rat fed 3'MeDAB for 10 days treated first with unlabeled anti-β protein and then stained with FITC-labeled antiazoprotein. The cytoplasmic fluorescence is similar to that in Fig. 1. X 560.

Fig. 3. Fluorescence photomicrograph of an unfixed frozen section of normal rat liver stained with FITC-labeled antiazoprotein. In addition to the uniform cytoplasmic fluorescence, there is a linear concentration of fluorescence in the region of the cytoplasmic membrane (2 arrows on right) and in the region of the nuclear membrane (2 arrows on left). X 560.

Fig. 4. The same area as shown in Fig. 3, subsequently stained with hematoxylin and eosin for comparison. X 560.

Fig. 5. Fluorescence photomicrograph of an unfixed frozen section of normal rat liver first blocked with unlabeled anti-β protein and then stained with FITC-labeled antiazoprotein. The cytoplasmic fluorescence has been blocked but the “membrane” fluorescence has persisted. X 560.

Fig. 6. Fluorescence photomicrograph of an unfixed frozen section from the margin of a 3'MeDAB-induced rat hepatoma stained with FITC-antiazoprotein. The cytoplasm of the nonneoplastic liver cells (left) fluoresce brightly, whereas the hepatoma tissue (right) does not fluoresce. X 280.
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