Antigenic Changes in Nonhistone Proteins during Azo Dye Hepatocarcinogenesis

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ABSTRACT

We have investigated the appearance of specific nonhistone proteins during azo dye-induced hepatocarcinogenesis in the rat. Groups of animals fed azo dye-containing diet were sacrificed at approximately 3-week intervals, portions of their livers were examined histologically, and the remaining material was fractionated into chromatin and cytoplasmic fractions. Livers of the azo dye-fed animals exhibited histological changes that have been classically attributed to the course and development of cancer; by 28 to 30 weeks of treatment, nearly all animals had developed hepatomas. Heterogeneous rabbit antisera were prepared to dehistonized chromatin from several azo dye-induced hepatomas. These antisera were then used to assess various chromatin for the appearance of antigens specific for neoplasia during induced carcinogenesis using immunodetection of antigens separated electrophoretically and transferred to nitrocellulose. Changes in the immunoreactivity of liver chromosomal proteins during carcinogen treatment were evident after 3 weeks, and the antigenic profiles of various chromatin samples gradually assumed the characteristics of the hepatoma. The transformation was accompanied by qualitative changes in chromosomal protein antigens, and although these antigenic species were not directly quantitated, noticeable enrichment of tumor-specific species occurred with treatment time. Immunotransfer assays of cytoplasmic fractions indicated most antigens to be specific for chromatin. Normal tissue chromatin exhibited minimal immunoreactivity, and slightly more antigenic homology was noted with regenerating liver and most transplantable tumor chromatin. Interestingly, the transplantable tumor Walker 256 carcinosarcoma was highly enriched in antigens recognized by antisera to azo dye hepatoma dehistonized chromatin. These studies establish a definite chronological correlation between the chemical induction of cancer and sequential changes in the immunological specificity of nonhistone protein antigens.

INTRODUCTION

Azo dye hepatocarcinogenesis presents a useful model system for assessment of the morphological and physiological changes occurring during neoplastic transformation (15, 33). According to current views, most if not all induced neoplasia is a multistage process in which the carcinogenic stimulus provokes the appearance of preneoplastic hyperplastic cells which in the ensuing progression may be converted to malignant invasive ones (2, 3, 16, 24, 31, 32). Since neoplastic cells manifest a variety of morphological and biochemical phenotypes different from their normal cell counterparts, substantial reprogramming of the cellular genome must occur during neoplastic transformation. Because the nonhistone proteins have been implicated in various aspects of differential gene expression, considerable attention has focused on the identification and characterization of nonhistone proteins specific for neoplasia due to potential diagnostic and therapeutic applications (13, 22, 48). However, in addition to the elucidation of the specificity and function of nonhistones related to neoplasia, it is important to determine the temporal appearance of these species during induced carcinogenesis. These latter studies are essential to define the role (if any) for nonhistones as mediators of the course and progression of neoplasia and may eventually facilitate the identification of cells committed to cancer.

In this report, we have immunologically examined chromosomal proteins during experimental hepatocarcinogenesis in rats maintained on a diet containing azo dye. Striking changes were observed in the kinds and quantities of nonhistone protein antigens observed during hepatocarcinogenesis as revealed by immunological identification of antigens separated electrophoretically and immobilized on nitrocellulose sheets. These results provide additional support for a role of nonhistone proteins in neoplastic transformation and suggest that a number of these antigenic species may be useful markers for neoplastic progression.

MATERIALS AND METHODS

Treatment of Animals, Histology, and Sources of Tissue. Male Fischer 344 rats (150 g) were fed a diet consisting of 0.06% N,N-dimethyl-p-tolylazoaniline (azo dye; Eastman Kodak Co., Rochester, N. Y.) and 10% corn oil (Mazola; CPC International, Englewood Cliffs, N. J.), mixed with normal laboratory rat chow (Wayne Laboratory Chow; Allied Mills, Inc., Chicago, Ill.). Control-fed animals obtained normal laboratory chow with 10% corn oil only. Unless indicated otherwise, animals were maintained on control or azo dye-containing diet until sacrifice and autopsy. The first hepatoma growing outside of the liver was recorded after 22 weeks of azo dye feeding.

For animals on azo dye-containing diet, groups of 3 or 4 animals were killed at approximately 3-week intervals. Hepatomas from these animals were processed separately from what was recognizably liver from visual inspection. As expected, these chemically transformed livers contained a variety of abnormal cell types from hyperplastic nodules and distorted hepatic architecture in the early weeks of feeding to true in situ carcinoma in the later weeks of feeding. In this report, azo dye liver and azo dye hepatoma merely refer to the separation of tissue at autopsy time. From either azo dye livers or hepatomas, random portions of the tissue were fixed in Bouin's fixative, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histological analysis.

Normal tissues were obtained from healthy donors, immediately
washed in ice-cold phosphate-buffered saline (0.01 M sodium phosphate: 0.14 M sodium chloride, pH 7.2) and processed as described below. Fetal rat livers were obtained from 18-day fetuses. For regenerating rat liver, mature male rats were partially hepatectomized under light ether anesthesia (21), and regenerating livers were then excised and used 24 or 48 hr after surgery. Novikoff ascites hepatoma was maintained by weekly transplantations in male Sprague-Dawley rats. The sources of the other cell lines used in this study were: 13762 MAT-B rat breast adenocarcinoma solid-tumor and 13762 MAT-B tissue culture line and Walker 256 solid-tumor carcinosarcoma and ascites line, all kindly supplied by Dr. A. Bogden, Mason Research Institute, Worcester, Mass. These lines were passed 2 to 3 times into healthy 150-g Fischer (MAT-B) or Sprague-Dawley (Walker 256) female hosts before use. MAT-B, adapted for tissue culture, was grown in McCoy’s Medium 5A supplemented with 5% FBS$^3$ and 5% CS. Adult rat liver tissue culture cells ARL16-T2 (38, 39) were generously supplied by Drs. G. Williams and T. Shimada from Naylor Dana Institute, Valhalla, N. Y. This line was grown and maintained in William’s medium E (Flow Laboratories, Inc., McLean, Va.), supplemented with 10% FBS. Novikoff hepatoma tissue culture line (strain N1S1-67; see Ref. 30) was kindly donated by Dr. P. Plagemann, Department of Microbiology, University of Minnesota, Minneapolis, Minn. These cells were passed in Joklik’s modified minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.), containing 5% FBS and 5% CS. Samples of Fischer 344 rat sarcoma (35) were a gift of Dr. Murray Brennan, National Cancer Institute, and AS-30D hepatoma carcinoma cells (43) were contributed by Dr. E. Walborg, University of Texas, Science Park, Worchester, Mass. These lines were passed 2 to 3 times into healthy 150-g Fischer (MAT-B) or Sprague-Dawley (Walker 256) female hosts before use. MAT-B, adapted for tissue culture, was grown in McCoy’s Medium 5A supplemented with 5% FBS$^3$ and 5% CS. Adult rat liver tissue culture cells ARL16-T2 (38, 39) were generously supplied by Dr. David Bloch, University of Texas, Austin, Texas.

Isolation of Nuclei, Chromatin, and Cytosol. All the following procedures were performed on ice at 0–4°C. Azo dye livers or hepatomas were disrupted by intermediate-speed homogenization with a Polytron or VirTis homogenizer in 0.25 M sucrose:50 mM Tris-HCl (pH 7.5):25 mM KCl:5 mM MgCl$_2$ (1:4, buffer:tissue, v/v). Homogenates were filtered through 8 layers of prewetted cheesecloth, centrifuged (800 $\times$ g, 10 min), and resuspended in 10 volumes of 10 mM Tris-HCl:0.25 M sucrose:0.1 mM MgCl$_2$ (pH 7.5), and homogenized using a tight-fitting Teflon:glass homogenizer. The crude nuclei were collected by low-speed centrifugation, homogenized in 2.2 M sucrose:10 mM Tris-HCl:5 mM MgCl$_2$ (pH 7.5), and purified by ultracentrifugation (100,000 $\times$ g, 60 min). Nuclei from transplantable tumors and tissue culture lines were obtained using methods described previously (41). Nuclei from normal tissues were purified using the method of Blobel and Poter (4).

Antisera and Immunoassays. Chromatin from 3 hepatomas (collected from animals 26 to 30 weeks on azo dye diet) was dehistonized in 5 M urea:2.0 M NaCl:phosphate buffer (pH 6.0) as outlined by Spelsberg et al. (45). Dehistonized chromatin was rehydrated in 2 mM Tris-HCl:0.1 mM phenylmethylsulfonyl fluoride (pH 7.5), and 300-$\mu$g samples (as DNA) were mixed with Freund’s adjuvant (1:1, v/v) and used to immunize New Zealand White rabbits (10). Blood was obtained 7 days after a 50-$\mu$g i.v. booster injection. Sera were heat inactivated at 56°C for 0.5 hr and stored at -20°C.

The PAP staining method of Sternberger (49) was used to identify immunoreactive antigens after transfer of proteins separated by SDS-PAGE to nitrocellulose sheets (type HA, 0.45 $\mu$m; Millipore Corp., Bedford, Mass.) as described previously (18).

For immunoabsorption, isolated chromatin was used as an absorbent in the ratio of 1 mg of chromatin (as DNA) to 1.0 ml of 1:10 diluted antiserum in phosphate-buffered saline. Antisera were absorbed at least 3 times as described previously (42).

SDS-PAGE and Immunodetection of Antigens with PAP. Electrophoresis supplies and high-molecular-weight standards were purchased from Bio-Rad Laboratories, Richmond, Calif. Hydrated chromatin were treated (1 hr, 2°C) with 50 $\mu$g of DNase I (Worthington Biochemical Corp., Freehold, N. J.) per ml in 10 mM Tris-HCl (pH 7.5):1 mM MgCl$_2$;0.1 mM phenylmethylsulfonyl fluoride (1 mg/ml as DNA). Samples were then made 2% sodium dodecyl sulfate:10% glycerol:5% 2-mercaptoethanol:0.0625 mM Tris-HCl (pH 6.8), boiled for 5 min, and electrophoresed under conditions described by Laemmli (25) using a 3% stacking gel and a 7.5% resolving gel. Other cellular fractions were dissolved (1 mg protein/ml) directly in electrophoresis sample buffer. Proteins were visualized by Coomassie Brilliant Blue staining (14).

Proteins separated with SDS-PAGE were electrophoretically transferred to nitrocellulose as described by Towbin et al. (51). Antigens were visualized using the PAP procedure (49). The molecular weights of the immunoreactive bands were calculated from the mobility of high-molecular weight standards electrophoretically transferred to nitrocellulose sheets and stained with amido black (40).

RESULTS

Histological Changes during Azo Dye Feeding. Rats maintained on azo dye diet developed classical liver histological changes in accordance with the earlier results of others (15, 33) (Fig. 1). Early changes included damage to portal areas, localized hyperplasia of bile duct cells, and enlarged atypical nuclei. These changes were followed by a trend toward nodularity (Fig. 1A), increased bile duct proliferation, and very serious distortion of hepatic architecture. The adenosis of bile ducts was extreme by the 19th week and included moderate nuclear atypia at this time. Definite neoplastic foci were evident by the 23rd week on azo dye diet, and both adenocarcinoma (Fig. 1B) and trabecular patterns (Fig. 1C) of hepatoma were seen. Metastases to the lung were observed in several cases in the 26th week and were of a trabecular- or solid-pattern histology (Fig. 1D).

Immunological Changes in Nonhistone Proteins during Hepatocarcinogenesis. In these experiments, dehistonized chromatin (see “Materials and Methods”) was prepared from 3 azo dye hepatomas which were collected from animals after 26 and 27 weeks on azo dye diet. Dehistonized chromatin was then injected into rabbits to prepared heterogenous antisera. Using immunodetection on nitrocellulose, we then assayed various chromatins prepared from the livers or hepatomas of animals after varying periods of time on azo dye diet (Fig. 2). Both qualitative and quantitative changes in the liver nonhistone protein antigens between azo dye-fed animals and those of control-fed animals were evident. Some of the new or enriched antigens which appeared during the treatment time are summarized in Table 1.

There was a general progressive increase in the amount of both total nonhistone proteins (Fig. 2A) and immunoreactive antigens (Fig. 2B) until about 28 weeks of treatment. Most new

$^3$ The abbreviations used are: FBS, fetal bovine serum; CS, calf serum; PAP, peroxidase:antiperoxidase; SDS-PAGE, sodium dodecyl sulfate:polyacrylamide gel electrophoresis.
antigens were present in chromatin samples from 28-week azo dye liver (Fig. 2B, compare Lanes 13 to 16 with Lane 9); however, noticeable decreases in major antigens and nonhistone protein species were seen thereafter in 30- to 32-week azo dye livers (Fig. 2, Lanes 10 to 12). Since animals were selected randomly at autopsy time, these differences could represent a partial resistance to transformation in animals surviving for longer treatment times. It is interesting that chromatin samples from animals treated with carcinogen for only 3 weeks showed noticeable increases in antigens (Fig. 2B, Lane 2), but the Coomassie-stained gel of this preparation (Fig. 2A, Lane 2) revealed a loss or reduction of several nonhistone bands that were present in normal liver chromatin (especially high-molecular-weight species). This may be the consequence of the toxic effect of the azo dye on normal liver cells resulting in increased necrosis and enzymatic degradation.

If one compares the electrophoretic mobilities of bands in normal liver chromatin with those of azo dye liver, many polypeptides of similar mobilities are common throughout the various chromatin. This finding may suggest that many of the antigens specific to the hepatomas have normal liver analogs; perhaps proteins in an unmodified nonimmunoreactive state. A few antigens, most notably a pair of proteins with molecular weights of about 68,000 and 70,000 and a prominent antigen of high-molecular-weight (M, 200,000), are common in all the chromatins examined. This latter protein, however, becomes visibly enriched in azo dye-fed animals.

Azo dye:protein complexes may have been present in the initial immunogen such that antibodies could have been raised to the azo dye or metabolites. If extensive formation of azo dye:protein complexes occurred during the continuous carcinogen feeding, then all proteins covalently linked to azo dye would appear immunoreactive. To test this possibility, one group of animals was removed from azo dye diet after 21 weeks of treatment and fed control chow for an additional 7 weeks. When the chromatins from these livers or hepatomas were assayed (Fig. 2, Lanes 9 and 15, respectively), all antigens characteristic of the other continuously treated hepatomas were present. Assuming sufficient protein turnover during this 7-week interval, we concluded that antibodies to carcinogen:protein complexes contributed little if any to the observed immunospecificity. Moreover, this conclusion was further supported by the finding that a majority of the azo dye hepatoma antigens was found in the transplantable Walker 256 carcinosarcoma tumor (see below).

A pair of low-molecular-weight antigens (about M, 18,000 and 20,000, appearing in about 19 weeks; Fig. 2, Lanes 7 and 8), were suspected to be H1 histones since they electrophoresed close or directly in front of H1 histones, depending upon the bisacrylamide cross-linker concentration in the resolving gel. However, these proteins could not be extracted from chromatin with either 0.63 M NaCl or 5% perchloric acid, i.e., conditions which nearly entirely solubilized the H1 histones from intact chromatin (23, 29). Hence, these antigens are not likely to be H1 histones.

The presence of antigens immunoreactive with antisera to azo dye hepatoma dehistonized chromatin in other cellular fractions was also examined (Fig. 3). Some antigens, in particular the prominent high-molecular-weight protein(s) (200,000), the M, 45,000 to 55,000 triad, and some weak antigens of various molecular weights, were found in cytosol preparations. However, most antigens observed in chromatin were not present in the cytosol. Analysis of antigens present in mitochondrial (10,000 x g) or microsomal (100,000 x g) pellets of azo dye liver or hepatoma homogenates was also performed. These fractions showed about the same immunoreactive species exhibited by the corresponding cytosol fractions (data not shown).

**Table 1**

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<tr>
<th>Time of azo dye treatment (wk)</th>
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<tr>
<td>3</td>
<td>120,000</td>
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<td>115,000</td>
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<td>6-13</td>
<td>120,000-200,000 (heterogeneous)</td>
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<td>64,000 (2)</td>
<td>110,000</td>
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<td>16-19</td>
<td>36,000</td>
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<td>20,000</td>
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<td>63,000-94,000 (heterogeneous)</td>
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**Distribution of Azo Dye Nonhistone Antigens in Other Tissues and Tumorigenic Cell Lines.** Several other transplantable tumors and normal or proliferating tissues were tested for the presence of antigens immunoreactive with antisera to dehistonized chromatin from azo dye tumors. In Fig. 4, several normal tissues, regenerating liver, and various tumorigenic cell lines were assayed by the immunotransfer technique. It is apparent that all of the chromatins assayed only Walker 256 carcinosarcoma contained a complement of nonhistone protein antigens comparable to that of the azo dye hepatoma. This result was highly reproducible, and both the solid Walker tumor (Fig. 4, Lane 16) and its ascites subline (data not shown) gave identical results. Normal tissues such as breast and kidney contained antigenic species similar to those of normal liver (Fig. 4, Lanes 1, 4, and 5), with a noticeable increase of a high-molecular-weight immunoreactive band at about M, 160,000 in breast chromatin. The 24- and 48-hr regenerating liver chromatins contained many of the antigenic bands characteristic of the azo dye hepatoma but in a considerably reduced quantity (Fig. 4, Lanes 2 and 3, respectively). It is noteworthy that the transplantable Novikoff ascites hepatoma (Lane 11) exhibited antigens very similar to those seen in regenerating liver (Lanes 2 and 3) and the azo dye hepatoma (Lane 6). However, not all of these antigens were consistently seen in the other transplantable ascites hepatoma, AS-30D (compare Lanes 10 and 11). The transplantable breast solid-tumor 13762 MAT-B adenocarcinoma also exhibited more antigens than its normal breast tissue counterpart (compare Lanes 13 and 4, respectively). It should be emphasized that the transplantable tumorigenic cell lines assayed significant differences in antigenic proteins were consistently seen when homologous cell lines were passed as in vitro-grown solid tumors or ascites suspensions or, alternatively, as in vitro-grown tissue-cultured cells (compare Fig. 4, Lanes 12 and 13 or Lanes 8 and 11). These latter observations may suggest that some of these antigens are preferentially expressed in particular cell types which may predominate depending on the growth conditions.

**Immunosorption Experiments.** The immunospecificity of antisera to azo dye hepatoma dehistonized chromatin was tested further with immunoabsorption experiments (Fig. 5). Antisera were absorbed at least 3 times with chromatins pre-
pared from normal liver, Novikoff ascites hepatoma, azo dye hepatoma, or fetal liver. By far, the most effective immunoadsorbent was azo dye tumor chromatin (Fig. 5C). Of the other chromatin tested, Novikoff hepatoma removed some of the activity (Fig. 5D), while normal liver or fetal liver (Fig. 5, E and F, respectively) left most of the immunological staining intact. In other studies, 24- and 48-hr regenerating liver chromatinas, as well as Walker tumor chromatin, were used to immunoadsorb these antisera. After absorption with regenerating liver chromatin, an antigenic pattern similar to that seen for antiserum-absorbed Novikoff hepatoma chromatin was observed, in accordance with the similar kinds and quantites of antigens that each of these chromatinas contained (see Fig. 4). Walker 256 chromatin was the most effective nonazo dye chromatin absorbent, and it nearly removed the immunoreactivity of nearly all the major antigens. However, Walker tumor chromatin was not as potent an absorbent as azo dye hepatoma chromatin (data not shown), especially for high-molecular-weight species. These results indicate a general specificity of these antisera for chromosomal proteins of azo dye hepatoma chromatin and that many but not all of these antigens are also present in Walker tumor chromatin.

**DISCUSSION**

From the classical models of skin and liver cancer, it is generally accepted that induced carcinogenesis is a stepwise multistage process involving transformation and cellular selection and eventually culminating in cancer (2, 3, 16, 24, 27, 31, 32). Hepatocarcinogenesis is accompanied by profound changes in cellular biochemical activity as compared to normal liver (16, 24, 32), which are likely to be influenced in part by different chromosomal nonhistone proteins whose putative role(s) as gene regulators have been investigated for some time (13, 22, 48).

In accordance with these concepts, numerous examples exist in the literature of nonhistone proteins apparently specific for cancer cells (22, 48). In the liver, an increase in protein:DNA ratio, qualitative changes, and quantitative differences in nonhistones have been noted for spontaneous primary as well as transplantable hepatomas (1, 7, 34, 36, 37, 47, 55). Some controversy exists, however, as to the definite presence of unique nonhistone species in transplantable hepatomas. For example, Reek and Morris (34) detected no differences in liver and hepatoma nonhistone protein composition while Rodriguez et al. (37) noted more dramatic qualitative and quantitative variations. A possible explanation here is that the latter authors examined density gradient enriched chromosomal protein fractions. The important point, however, is that all of these studies have compared SDS-PAGE profiles of normal liver chromosomal proteins with those of hepatomas and only differences in electrophoretic mobility were subsequently detected. These assessments were probably overly simplified since subtle alterations in nonhistones and the heterogeneity of polypeptide bands of similar molecular weight could not be ascertained. For these reasons, studies incorporating sensitive 2-dimensional gels have been more revealing of the differences in nonhistones of hepatomas and normal liver. Busch and Busch (6), Davis et al. (11), and Yeoman et al. (56) have purified a number of tumor-specific or oncofetal nonhistone proteins following the examination of these proteins on 2-dimensional gels.

Although there are relatively few data concerning changes in the composition of liver nonhistone proteins during chemically induced hepatocarcinogenesis, it has been known for some time that variations in the protein:DNA ratio of liver chromatin occurs during this process (46). More recent studies have better defined compositional differences during hepatocarcinogenesis, and although quantitative variations in nonhistone proteins were easily observed, overt qualitative changes in the nonhistone complement of carcinogen-treated liver and normal liver have not been reported (19, 20, 26, 52).

Because of their selectivity and specificity, antibodies would be expected to provide a more sensitive assessment of the changes occurring in liver nuclear proteins during neoplastic progression. Indeed, our earlier studies indicated not only differences in the antigenicity of nonhistone proteins in transplantable hepatomas and normal liver but also progressive alterations in the immunological specificity of nonhistone protein:DNA complexes concomitant with azo dye-induced neoplasia (8, 9, 18, 22, 53, 54). Using immunotransfer methods and antisera to azo dye hepatoma dehistonized chromatin, we now have demonstrated immunological changes to be occurring in the kinds and quantities of nonhistone proteins during hepatocarcinogenesis. It should be emphasized, however, that comparison of the polypeptide electrophoretic profiles of normal liver chromatin and azo dye-transformed chromatin shows many homologies of polypeptides with similar if not identical mobilities. Thus, with only SDS-PAGE as a criterion, most neoplastic changes in nuclear proteins appear merely quantitative. Our data indicate that more extensive changes, detectable immunologically, may be occurring.

The diversity of nuclear antigens induced by azo dye treatment suggests not only the appearance of new possibly unique nuclear proteins but that other nuclear regulatory processes may be contributing to the observed specificity. It is known that posttranslational modification of nuclear proteins occurs in neoplastic cells (48), and we consider it a distinct possibility that extensive modification of nonhistones may confer additional immunological specificity to nuclear antigens during neoplastic progression. If a common antigenic modification such as glycosylation or poly(adenosine diphosphate) ribosylation occurs on a number of proteins, then all modified polypeptide bands on the nitrocellulose immunoassay would appear as distinct antigens. With regards to poly(adenosine diphosphate-ribose), we have assayed nuclear antigens before and after treatment with snake venom phosphodiesterase or Tris-base using conditions shown to cleave poly(adenosine diphosphate-ribose) from protein (28) or at the pyrophosphate linkage (17) and seen no difference in antigenicity. Although these experiments do not unequivocally establish the presence or absence of this modification on azo dye hepatoma antigens, they do indicate this modification to contribute little to the antigenicity. Furthermore, our experiments establishing the low-molecular-weight antigens (about M, 18,000 and 20,000) to be distinct from the nonantigenic H1 histones also support these conclusions. H1 histones are the major poly(adenosine diphosphate-ribose)-modified proteins (50).

We currently have experiments in progress to further classify and eventually identify antigens on the basis of physicochemical parameters such as isoelectric point, DNA-binding ability,
and distribution in various fractions of chromatin after disruption with urea and salt. In this regard, it is noteworthy that our preliminary results indicate most of the azo dye hepatoma antigens to be firmly associated with chromatin since they are not removed from bulk chromatin with 0.35 to 0.6 M NaCl. Whether indeed these results imply a functional association with chromatin or are a reflection of antigen insolubility requires further examination. The especially prominent antigens with molecular weights of about 35,000, 45,000, and 55,000 may indicate the presence of insoluble structural proteins in these preparations. It is noteworthy that distinct quantitative changes in polypeptides of this mobility during the early weeks of chemical hepatocarcinogenesis have also been reported by Tsanev and Hadjiolov (52) and Martinez-Sales et al. (26). The former authors also noted the appearance of new nonhistone species in chemically induced primary liver neoplasms with molecular weights of 60,000, 90,000, and 120,000 which may correspond to some of the antigens shown in our studies.

In our analysis of transplantable tumors and normal or proliferating tissues, only Walker carcinosarcoma contained a complement of antigenic nonhistone proteins comparable to azo dye hepatomas. While the significance of this finding requires further investigation, it might reflect the poorly differentiated state of this tumor or its cellular heterogeneity (12). Conceivably, this tumor may approximate the situation of the primary azo dye hepatoma. Other transplantable tumors and tumorogenic cell lines showed weak activity although in general more than that of normal tissues. Since these transplantable tumors have been carried for many generations, it is possible that they may have acquired properties different from those apparent in primary neoplasm. There were small but noticeable increases in the antigenicity of several proteins in regenerating liver chromatin and a few high-molecular-weight proteins in fetal liver chromatin. These data indicate that some of these antigens may be associated with rapid proliferation.

It is significant that during azo dye hepatocarcinogenesis there was a temporal appearance of numerous antigens until the immunogenicity of nonhistone proteins characteristic of the hepatoma was achieved. Thus, in accordance with our previous studies (9), the carcinogenic process can be related to sequential changes in the immunological specificity of nonhistone antigens. We are uncertain presently what types of relationships (if any) exist among the various antigens and whether the appearance of some may be a prerequisite for others. Because numerous cell types were observed in azo dye hepatomas and they were the source of the dehistonized chromatin immunogen, it was not possible to equate different cell phenotypes and morphologies with the presence or absence of specific antigens. These studies must await monoclonal antisera and immunohistochemical experiments. The chronological appearance of antigens with carcinogen treatment, however, establishes a precedence for the correlation of these nuclear antigens with preneoplasia and the course and development of cancer.

REFERENCES


Fig. 2. Identification of azo dye hepatoma antigens in various chromatins prepared from the livers or hepatomas of rats fed azo dye-containing diet. A, SDS-PAGE of various chromatins. Chromatins were prepared in sodium dodecyl sulfate sample buffer and electrophoresed as described in "Materials and Methods." Each lane contained 25 μg (as DNA) of the respective chromatin. Lane 1, normal (control-fed) liver; Lane 2, 3-week azo dye-treated liver; Lane 3, 6-week azo dye-treated liver; Lane 4, 9-week azo dye-treated liver; Lane 5, 13-week azo dye-treated liver; Lane 6, 16-week azo dye-treated liver; Lane 7, 19-week azo dye-treated liver; Lane 8, 27-week azo dye-treated liver; Lane 9, 26-week azo dye-treated liver; Lane 10, 30-week azo dye-treated liver; Lane 11, 31-week azo dye-treated liver; Lane 12, 32-week azo dye-treated liver; Lanes 13 to 16, azo dye hepatoma chromatins isolated after 23, 26, 28, and 30 weeks of treatment, respectively; Lane 17, high-molecular-weight standards (Bio-Rad) (myosin, M, 200,000; β-galactosidase, M, 116,500; phosphorylase β, M, 95,000; bovine serum albumin, M, 68,000; ovalbumin, M, 43,000). With the chromatins for Lanes 9 and 15, animals were removed from azo dye-containing diet after 21 weeks of feeding and fed control chow for an additional 7 weeks until autopsy and chromatin preparation. B, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated chromatins. Chromatins were prepared and electrophoresed as in A and transferred to nitrocellulose, and the immunoreactive antigens were localized by incubation with antisera to azo dye hepatoma dehistonized chromatin (1:200 dilution), followed by the PAP procedures as described in "Materials and Methods." Lanes are as indicated in A, with the omission of molecular weight standards (Lane 17).
Fig. 3. Identification of azo dye hepatoma antigens in various cytosols prepared from the livers or hepatomas of rats fed azo dye-containing diet. A, SDS-PAGE of various cytosols. Cytosols were prepared and electrophoresed as described in "Materials and Methods." Each lane contained 25 μg cytosol protein. Lane 1, normal (control-fed) liver; Lane 2, 3-week azo dye-treated liver; Lane 3, 6-week azo dye-treated liver; Lane 4, 9-week azo dye-treated liver; Lane 5, 13-week azo dye-treated liver; Lane 6, 16-week azo dye-treated liver; Lane 7, 19-week azo dye-treated liver; Lane 8, 27-week azo dye-treated liver; Lane 9, 28-week azo dye-treated liver; Lane 10, 32-week azo dye-treated liver; Lane 11, 23-week azo dye hepatoma; Lane 12, 26-week azo dye hepatoma; Lane 13, 28-week azo dye hepatoma; Lane 14, 30-week azo dye hepatoma; Lane 15, 31-week azo dye hepatoma; Lane 16, molecular weight standards (see Fig. 2). B, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated cytosols. Cytosols were electrophoresed as in A and transferred to nitrocellulose, and the immunoreactive antigens were localized by incubation with antiserum to azo dye hepatoma dehistonized chromatin (1:200 dilution), followed by the PAP procedure as described in "Materials and Methods." Lanes are as indicated in A, with the omission of molecular weight standards.
Fig. 4. Identification of azo dye hepatoma chromosomal antigens in chromatins from various sources. A, SDS-PAGE of various chromatins. Chromatins were prepared in sodium dodecyl sulfate sample buffer and electrophoresed as described in "Materials and Methods." Each lane contained 25 µg (as DNA) of chromatin. Lane 1, normal liver; Lane 2, 24-hr regenerating liver; Lane 3, 48-hr regenerating liver; Lane 4, rat breast; Lane 5, rat kidney; Lane 6, azo dye hepatoma (30 week); Lane 7, ARL-16T; Lane 8, Novikoff ascites hepatoma (tissue culture); Lane 9, N1S1-67; Lane 10, AS-30D; Lane 11, Novikoff ascites hepatoma; Lane 12, 13762 MAT-B (tissue culture); Lane 13, 13762 MAT-B (solid tumor); Lane 14, Fischer 344 rat sarcoma; Lane 15, mouse Ehrlich ascites; Lane 16, Walker 256 (solid tumor); Lane 17, molecular weight standards (see Fig. 2). B, localization of immunoreactive antigens on nitrocellulose containing SDS-PAGE-separated chromatins. Chromatins were prepared and electrophoresed as in A and transferred to nitrocellulose, and the immunoreactive antigens were localized by incubation with antiserum to azo dye hepatoma dehistonized chromatin (1:200 dilution), followed by the PAP procedure as described in "Materials and Methods." Lanes are as indicated in A, with the omission of molecular weight standards.
Fig. 5. Immunoabsorption of antiserum to azo dye hepatoma chromosomal antigens with various chromatins. A, Coomassie-stained gel of various chromatins. Lane 1, normal liver; Lane 2, Novikoff ascites hepatoma; Lane 3, azo dye hepatoma (30 week); Lane 4, azo dye treated liver (27 weeks); Lane 5, fetal liver; Lane 6, molecular weight standards (see Fig. 2). Each lane contained 25 μg (as DNA) of each chromatin. B to F, antiserum was absorbed 3 times with chromatin as described in "Materials and Methods." Absorbed antisera were then incubated (1:200 dilution) with nitrocellulose sheets containing SDS-PAGE-separated chromatins with the lanes identical to those shown for A. The immunoreactive species were then visualized with the PAP reaction. The absorbed antisera used to stain each sheet were: unabsorbed (B); absorbed with azo dye hepatoma (C); absorbed with Novikoff ascites hepatoma (D); absorbed with normal liver (E); and absorbed with fetal liver chromatins (F).
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