Microfluorometric Determination of DNA Adducts in Immunofluorescent-stained Liver Tissue from Rats Fed 2-Acetylaminofluorene

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ABSTRACT

The intensities of immunofluorescence in nuclei stained by an antibody specific for the DNA adduct N-deoxyguanosin(8-yl)aminofluorene (dG-8-AF), were quantified by microfluorometry in frozen liver sections from male Fischer rats fed 2-acetylaminofluorene (AAF). Results of previous studies demonstrated that dG-8-AF is the predominant adduct (80–100%) formed in livers of rats fed AAF continuously, and that nuclei of hepatocytes and bile duct epithelial cells in rats fed AAF exhibit an adduct-specific immunofluorescence. In the present investigation, nuclear staining for dG-8-AF was quantified by microfluorometry in liver sections from male Fischer rats fed 0.02% AAF continuously for 2, 4, 8, 12, 16, 20, and 28 days. Microfluorometric determinations of the intensities of nuclear immunofluorescence within periportal, midzonal, and centrilobular hepatocytes and bile duct epithelial cells revealed that levels of the dG-8-AF adduct increased in these cells during AAF feeding, reaching a plateau by 12 days. However, significant differences were detected in dG-8-AF levels within cells of each lobular area. Nuclei of periportal hepatocytes exhibited the most intense immunofluorescence, nuclei of centrilobular hepatocytes and bile duct epithelial cells emitted the least intense fluorescence, and nuclei of midzonal hepatocytes exhibited an intermediate fluorescence intensity. Quantitation of whole-liver levels of the dG-8-AF adduct by RIA, after extraction of DNA, also revealed that adduct accumulation reached a plateau by 12 days of AAF feeding. Thus, similar profiles of adduct accumulation were obtained by microfluorometric analysis of immunofluorescence staining within frozen liver sections, and by RIA analysis of DNA extracted from whole livers. The perportal concentration of DNA adducts in livers of rats continuously fed a carcinogenic dose of AAF may be an important early event in AAF-induced liver tumorigenesis.

INTRODUCTION

Immunohistochemical techniques have achieved morphological localization of carcinogen-DNA adducts formed within a complex tissue, such as the liver, following in vivo carcinogen exposure (1–3). This technology allows visualization of the extent and persistence of carcinogen-induced DNA damage within specific cell populations of target tissues and organs. Thus, it may well become a powerful tool with which to investigate carcinogen-DNA interactions in vivo. In previous immunohistochemical studies (3), we employed an antiserum specific for the deacetylated C-8-substituted adduct of AAF with dG-8-AF to localize this adduct within nuclei of hepatocytes and bile duct epithelial cells in frozen liver sections obtained from male Fischer rats fed 0.02% AAF for 5 or 28 days. The results showed a distinctly nonuniform pattern of adduct distribution, with a predominant localization peripherally, intermediate localization midzonaingly, and sparse localization centrilobularly. In addition, the same adduct distribution was found to persist during feeding of a control diet subsequent to AAF feeding (3), even though the overall adduct concentration was diminishing (4). One disadvantage of conventional immunofluorescence is an inherent lack of quantitative potential. The recent development of microfluorometry (5) allows semiquantitative measurement of fluorescence intensity to be made on a cell-by-cell basis. In the present study microfluorometry was employed to quantitate the predominating deacetylated C-8 adduct in different regions of the liver lobule during 1 month of continuous AAF feeding. The microfluorometric data obtained for accumulation of dG-8-AF within nuclei of periportal, midzonal, and centrilobular hepatocytes and bile duct epithelial cells were compared to quantitation of this adduct in whole liver DNA by RIA.

MATERIALS AND METHODS

Animals, Diets, and Tissue Sections. Young adult male Fischer rats, weighing 180–200 g, were maintained at Microbiological Associates Inc., (Bethesda, MD) and given food and water ad libitum. Animals on the control diet received purified, semisynthetic basal Bio-Mix no. 0007 (Bio-Serv, Inc., Frenchtown, NJ). AAF (m.p., 192–196°C; Aldrich Chemical Co., Milwaukee, WI) was mixed into the diet at a concentration of 0.02% (w/w). Two AAF-fed rats and one rat fed the control diet were sacrificed at 2, 4, 8, 12, 16, 20, and 28 days of feeding. Tissue from the median liver lobe of each animal was placed so that a piece of control liver was between pieces of livers from AAF-fed rats, covered with optimum cutting temperature compound (Miles, Naperville, IL) and frozen. From each tissue block, one hematoxylin and eosin-stained slide and several 6-μm frozen sections were prepared (American Histo Labs, Gaithersburg, MD). The remaining liver was immediately frozen and later used for DNA preparation on CsCl buoyant density gradients (6) and RIA quantitation of adducts (4).

Immunohistochemistry. Anti-G-8-AF, elicited in rabbits at Microbiological Associates, Inc., has been described previously (3). This antisera is specific for dG-8-AF and aminofluorene-DNA, with a 100-fold lower specificity for dG-8-AAF. It does not recognize AAF, dG, or unmodified DNA. Mouse monoclonal anticytokeratin PKK1 (Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, NIH, Bethesda, MD) was mixed into the diet at a concentration of 0.02% (w/w). Two AAF-fed rats and one rat fed the control diet were sacrificed at 2, 4, 8, 12, 16, 20, and 28 days of feeding. Tissue from the median liver lobe of each animal was placed so that a piece of control liver was between pieces of livers from AAF-fed rats, covered with optimum cutting temperature compound (Miles, Naperville, IL) and frozen. From each tissue block, one hematoxylin and eosin-stained slide and several 6-μm frozen sections were prepared (American Histo Labs, Gaithersburg, MD). The remaining liver was immediately frozen and later used for DNA preparation on CsCl buoyant density gradients (6) and RIA quantitation of adducts (4).

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2 Norwegian Cancer Society Research Fellow (Landsforeningen mot Kreft). To whom requests for reprints should be addressed, at National Cancer Institute, NIH, Laboratory of Cellular Carcinogenesis and Tumor Promotion, Building 37, Room 3B25, Bethesda, MD 20892.

3 The abbreviations used are: AAF, 2-acetylaminofluorene; RIA, radioimmunoassay; dG-8-AF, N-deoxyguanosin(8-yl)aminofluorene; dG-8-AAF, N-deoxyguanosin(8-yl)acetylaminofluorene; MC, 3-methylcholanthrene; FITC, fluorescein isothiocyanate; G-8-AF, N-guanosin(8-yl)aminofluorene.
Microscopy and Photomicrography. Stained slides were examined with a Nikon Labophot microscope containing an epifluorescence attachment (HBO 100-W lamphouse). A B2 filter block (dichroic mirror, 510 nm; excitation filter, 450–490 nm; barrier filter, 520 nm) was used to visualize FITC, while a G filter block (dichroic mirror, 580 nm; excitation filter, 510–560 nm; barrier filter, 590 nm) was used for Texas Red. ASA 400 daylight film was used for photography, with a 10-s exposure time for FITC staining and a 5-s exposure time for Texas Red staining.

For counting fluorescent nuclei an eye-piece reticle was aligned on the fluorescence-stained tissue slides defining an area 80 µm wide between a portal triad and a central vein. All of the fluorescent nuclei within eight to 11 of such areas were counted. To determine total nuclei, a frozen section from a control rat was stained with ethidium bromide, visualized with the G filter block and the nuclei counted in 11 reticel-defined areas.

Microfluorometry. Paired immunofluorescence slides were examined with a modified Leitz Orthoplan microscope photometer system as described previously (5). For microfluorometric analyses of FITC staining, a K2 filter cube (excitation, 470–490 nm; KRP 510 beam-splitting mirror; LP 515 suppression filter) was used, and fluorescent light emitted from circular areas (6 µm in diameter) within nuclei was transmitted through a Leitz microfluorometric attachment to a Schoeffel GM 100 monochromator. Fluorescence emission at 525 nm was detected by a 9658 A photomultiplier tube, amplified by a Schoeffel M 460 photometer and fed into a Digimetry MK4 data coupler (Computer Inquiry Services, Inc.) which interfaces the photometer with an HP-85F minicomputer. Emitted fluorescence is expressed as one absor-bit unit (7). The photographs shown in Fig. 1 are double exposed, illustrating as a function of the duration of AAF feeding, were assessed morphologically by counting the number of stained nuclei in uniform areas of liver 80 µm wide, extending from a bile duct to a central vein. All of the fluorescent nuclei were counted in eight of 11 of such areas, and the data are shown as mean ± SE in Table 1. The total nuclei, in 11 similar areas of liver from a rat fed control diet were counted after nuclear staining with ethidium bromide. This allowed the data for fluorescent nuclei to be expressed as a percentage of total nuclei (Table 1). The results shown in Table 1 comprise values from two frozen sections, each from a different rat, and demonstrate an overall adduct accumulation profile similar to that obtained by microfluorometry.

Microfluorometry. Microfluorometric determinations of immunofluorescence staining intensity yield data in the form of relative fluorescence units, and allow meaningful comparisons to be made between or among cells and/or tissue regions. For these experiments, each tissue block contained livers from two rats fed the AAF diet and from one rat fed the control diet. One block was made for each time point taken. The microfluorometric values obtained from nuclei in specific regions of livers from rats fed control diet were subtracted from values obtained in corresponding regions of livers from AAF-fed rats. Measurements were taken from within circular, nuclear areas of 22 to 24 hepatocytes in periportal, midzonal, and centrifibular areas, and bile duct epithelial cells from each of two rats. Fig. 24 shows microfluorometric values for hepatocytes in the three regions of the liver lobule at times during 1 month of AAF feeding. The dG-8-AF adduct in these regions accumulated, during the first 12 days of continuous feeding, and the adducts were maintained at a constant level for the next week, with a decrease seen at 28 days. Marked differences were detected in the quantities of adduct found in each region, even though the overall adduct accumulation profiles were similar. At the plateau level (12–20 days of AAF feeding), the fluorescence intensity was 5-fold higher in the periportal hepatocytes as compared to the centrifibular hepatocytes. In addition, the plateau level in midzonal hepatocytes was about 25% less intense than that in periportal hepatocytes. The profile for dG-8-AF immunofluorescence in bile duct cells is shown in Fig. 2B. In these cells a plateau is less apparent, since adduct accumulation increased between 2 and 20 days of AAF feeding. Immunofluorescence intensity emitted from bile duct epithelial cells was much lower than that emitted from periportal hepatocytes, and was similar to that determined for centrifibular hepatocytes.

Radioimmunoassay. In order to compare the relative levels of dG-8-AF present within different liver cells with that in the liver as a whole, DNA was extracted from liver homogenates, and adducts were quantitated by RIA. This assay measured total C-8 adduct, in contrast to the immunohistochemistry which utilized an antiserum specific only for dG-8-AF. The results were expected to be comparable, however, since the dG-8-AF comprises 80% of the total at early times of AAF feeding and 95–100% by 2 weeks and thereafter (11). The results of RIA determinations are shown in Fig. 3. There is a parallel between profiles obtained by the two methods, since the plateau from day 12 is again clearly apparent. Even the decrease in adducts evidenced at day 28 was demonstrated by both techniques. The decrease at day 28 is probably caused by unique
Fig. 1. Immunofluorescence of dG-8-AF adducts in frozen liver sections from male Fischer rats fed either control diet (A) or 0.02% AAF diet for 2, 4, 8, 12, 20, and 28 days (B–G, respectively). Paired staining has been performed with anti-G-8-AF and anticytokeratin, as described in "Materials and Methods" (original magnification, × 55). P, bile duct areas. H, a portal area with a bile duct, in liver from a rat fed the AAF diet for 28 days (original magnification, × 220).
Table 1 Accumulation of fluorescent nuclei during chronic AAF feeding

The number of nuclei positive for dG-8-AF immunofluorescence were counted in eight to 11 areas of liver frozen sections. Uniform areas (80 μm wide) were chosen between a portal triad and central vein. Values are mean ± SE for two frozen sections, each from the median lobe of a different rat. Average total nuclei in 11 representative areas were determined after ethidium bromide staining of all nuclei.

<table>
<thead>
<tr>
<th>Days of AAF feeding</th>
<th>Fluorescent nuclei</th>
<th>Total nuclei</th>
<th>% Total nuclei fluorescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8.3 ± 0.9</td>
<td>44.5 ± 1.6</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>24.4 ± 1.4</td>
<td>55</td>
<td>55</td>
</tr>
<tr>
<td>8</td>
<td>31.8 ± 1.4</td>
<td>71</td>
<td>85</td>
</tr>
<tr>
<td>12</td>
<td>38.0 ± 1.6</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>16</td>
<td>33.6 ± 1.1</td>
<td>75</td>
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<tr>
<td>20</td>
<td>36.5 ± 1.2</td>
<td>82</td>
<td>82</td>
</tr>
<tr>
<td>28</td>
<td>30.2 ± 1.6</td>
<td>68</td>
<td>68</td>
</tr>
</tbody>
</table>

Fig. 2. Microfluorometric analyses of dG-8-AF immunofluorescence (as in Fig. 1) in nuclei of periportal (C), midzonal (O), and centrilobular (A) hepatocytes (A) and bile duct cells (B) of male Fischer rats fed 0.02% AAF for 2, 4, 8, 12, 16, 20, and 28 days (abscissa). Ordinate values, relative fluorescent units ±SE for 40 to 44 nuclei in each cell type from two animals. Fluorescence values from corresponding cells in livers of rats fed the control diet have been subtracted.

Fig. 3. RIA quantitation of dG-8-AF and dG-8-AAF adducts in whole livers of male Fischer rats fed 0.02% AAF in the diet for 2, 4, 8, 12, 16, 20, and 28 days (abscissa). Adduct values (ordinate) are mean ± range for two rats at all time points except 28 days, which is a single value for one rat. Livers analyzed were from the same animals from which microfluorometric data shown in Figs. 1 and 2 were obtained.

DISCUSSION

In this investigation, patterns of dG-8-AF adducts localized within hepatocytes in the liver lobule and bile duct epithelial cells during 4 weeks of continuous AAF feeding were determined by microfluorometry and compared to quantitation of dG-8-AAF and dG-8-AF in the whole liver by RIA. This confirms a previous morphological study which showed predominantly periportal localization of dG-8-AF at 5 and 28 days of AAF feeding, and during time on control diet (3). In these experiments, semiquantitative microfluorometric determinations of immunofluorescent nuclei demonstrated adduct accumulation during the initial 12 days of continuous AAF feeding, followed by a plateau phase at later times. The adduct accumulation profiles were similar in hepatocytes of the three lobular areas as well as in bile duct epithelial cells, but the adduct concentrations were highest in periportal areas, somewhat less in midzonal areas and lowest in centrilobular areas and bile duct cells.

RIA determinations of adduct levels in whole liver revealed an adduct accumulation profile similar to those obtained by microfluorometry, even to the point of showing a decrease in adducts at 28 days of AAF feeding. There is some discrepancy, however, between the results generated by the two techniques at the earliest time points (2 and 4 days), since the RIA values were not as high, relative to the plateau, as the microfluorometric values. The overall liver adduct concentration, as determined by RIA, includes both the adduct concentration of the separate nuclei, also measured by microfluorometry, and the total number of adduct-positive nuclei. Microfluorometry does not yield data representative of the whole liver, since adduct-negative nuclei are not recognized by this staining technique. This is illustrated in Table 1, which shows that at the early time points only a minority of the hepatocytes were fluorescent, and that the fraction of fluorescent cells was indeed increasing as adducts accumulated during AAF feeding. It must also be appreciated that the two techniques have different sensitivity ranges, since adduct levels below 30 fmol/μg DNA will not show detectable fluorescence, while the RIA is 10-fold more sensitive.

At all time points, the microfluorometric data showed a distinct intralobular gradient, with periportal hepatocytes exhibiting the most intense fluorescence. Immunofluorescence emitted from nuclei of midzonal hepatocytes was consistently 25% less intense, while staining in centrilobular hepatocytes
and bile duct epithelial cells was about 80% less intense than that in periportal hepatocytes. These differences were constant at all times of feeding and were reflected in the magnitude of the plateau phase determined within each lobular area. Since hepatocytes in each of the lobular regions showed the plateau phenomenon, saturation of the nuclear antigens could not have occurred in any area except the periportal area. In addition, even if the metabolic enzymes were limiting in the periportal areas, this limitation would probably not have caused the plateaus in the other hepatocytes since the induced enzyme distribution is relatively uniform (12, 13, 14). In bile duct epithelial cells the low levels of adducts formed may reflect the fact that these cells metabolize AAF less-efficiently than hepatocytes (3, 15, 16).

Localization of some enzymes involved in the metabolic activation of AAF by hepatocytes has been determined in activated and nonactivated rat liver, while localization of others is unknown. The MC-inducible cytochrome P450, responsible for initial oxidation of AAF (17), is present in 2–3-fold higher concentrations in livers of rats pretreated with MC, and is present in activated periportal, midzonal, and centrlobular hepatocytes at similar concentrations (12). The NADPH-cytochrome C reductase is present in all lobular areas of normal liver, but at significantly higher concentrations in midzonal and centrlobular hepatocytes (13). The enzyme is not induced significantly in any area by pretreatment with MC (13). In our experiments, at most of the times studied, the rat livers could be considered to be activated because of the continuous AAF feeding. The fact that immunofluorescence with the anti-G-8-AF antiserum shows adduct predominance in the periportal areas suggests that something other than the cytochrome P450 enzymes may be determining adduct localization. The two enzymes responsible for metabolism of N-hydroxy-2-acetylaminofluorene to nucleic acid-bound products have not yet been visualized within the liver lobule. The sulfotransferase, which appears to carry out sulfate conjugation primarily in the periportal areas (18), is responsible for formation of acetylated AAF-DNA adducts (19), which have been measured by RIA in this study but not by microfluorometry. The deacetylated adducts are primarily the product of N,O-acetyltase activity (20, 21) which has not been localized, although an antiserum has been elicited and characterized (22). If these, like the other enzymes, are present in all lobular areas, it will be necessary to seek an alternative explanation. One possibility is that the periportal areas may simply have the highest consistent supply of drug. That is, since the afferent blood supply enters through the portal triad, the adduct gradient may, in fact, be reflective of a drug concentration gradient across the liver lobule.

In summary, microfluorometry has been employed to quantitate G-8-AF adducts localized within cells in lobular areas of livers from rats fed AAF. During 4 weeks of AAF feeding, adducts accumulated predominately in the periportal areas. Combined with studies demonstrating localization of AAF-metabolizing enzymes and foci of altered enzyme phenotypes, these investigations should yield new insights into carcinogen-DNA interactions in a complex target tissue occurring as a result of carcinogen exposure.

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