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Immunological Detection and Quantitation of DNA Adducts Formed by 4-Aminoazobenzene Species *in vivo*

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Antibodies to 3-methoxy-4-aminoazobenzene (3-MeO-AAB) and 2-methoxy-4-aminoazobenzene (2-MeO-AAB) DNA adducts were raised in rabbits against *in vitro*-adducted DNA samples. The enzyme-linked immunosorbent assay (ELISA) was used to determine the sensitivity and specificity of these antibodies. They proved highly specific for the modified DNA used as the immunogen, but cross-reacted with each other. Moreover, they showed cross reactivity with DNA modified by 4-(*o*-tolylazo)-*o*-toluidine, but not by other carcinogens, such as 4-aminobiphenyl or 4-nitroquinoline 1-oxide. The 50% inhibition level of antibody binding in the competitive ELISA was at 10–20 fmol of modified base per assay (equivalent to 1–2 adducts per 10^6 bases). Immunohistochemical staining indicated that these antibodies bind specifically to nuclear components of the liver in rats given either 3-MeO-AAB or 2-MeO-AAB at the dose of 50 mg/kg body weight.

Key words: Anti-3-MeO-AAB-DNA-antibody — Anti-2-MeO-AAB-DNA-antibody — Immunoassay — Immunohistochemistry

Over several years, we have focused our attention on elucidation of the relationships between structure and carcinogenicity of aminoazo dyes, especially methoxy-substituted 4-aminoazobenzene derivatives.[1-3] Clarification of the observed variation in genotoxic activity between 3-methoxy-4-aminoazobenzene (3-MeO-AAB)[6] and 2-methoxy-4-aminoazobenzene (2-MeO-AAB), which differ only in the position of the methoxy substituent on the same benzene ring, has been our aim. The former is a potent hepatocarcinogen in the rat, while latter is a noncarcinogen and nonmutagen.[4-6] For elucidation of the mechanisms of carcinogenesis, an understanding of carcinogen adduct formation, structure, persistence and repair remains fundamental.

Recently, reliable methods have become available for the determination of DNA adducts in cells and tissues[7] through the ^{32}P-postlabeling[8-10] and immunoassay[11,12] approaches. Using the ^{32}P-postlabeling assay, we determined adduct levels in liver of rats treated with 3-MeO-AAB or 2-MeO-AAB, and found quantitative and qualitative differences regarding adduct formation between the 2 chemicals. The carcinogen 3-MeO-AAB generated more than 20-fold higher amounts of DNA adducts than did 2-MeO-AAB. Furthermore five adducts, one major (70%) and 4 minor, were detected in DNA of 3-MeO-AAB-treated rats, whereas only one adduct was apparent in DNA of 2-MeO-AAB-treated rats.[1]

Previously, we raised polyclonal antibodies specific for DNA modified by 4-nitroquinoline 1-oxide (4NQO) and 3,2′-dimethyl-4-aminobiphenyl (DMAB) in order to be able to monitor adduct formation by immunoassay and immunohistochemical staining.[13-17] Specific detection of 4NQO- or DMAB-DNA adducts was thus possible at both molecular and cellular levels.

The immunohistochemical approach in particularly useful for investigation of adduct formation and removal in different cell types in tissues, in relation to sensitivity to tumor induction. It also allows application of non-radiolabeled chemicals.

The present report concerns (i) the production and characterization of antibodies that specifically recognize 3-MeO-AAB- or 2-MeO-AAB-adducts in DNA, (ii) the development of convenient immunoassays for quantitative determination of the extent of aminoazo-dye adduct formation in DNA and (iii) immunohistochemical detection of such adducts in target tissues.

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[6] Abbreviations: 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; 2-MeO-AAB, 2-methoxy-4-aminoazobenzene; OAT, 4-(*o*-tolylazo)-*o*-toluidine; 4NQO, 4-nitroquinoline 1-oxide; 4ABP, 4-aminobiphenyl; DMAB, 3,2′-dimethyl-4-aminobiphenyl; Glu-P-1, 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2, 2-amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole; AAF, 2-acetylaminofluorene; ELISA, enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

Chemicals 2-MeO-AAB and 3-MeO-AAB, and their N-hydroxy compounds were prepared in our laboratory.[6, 18] Seryl-AMP was prepared according to the method of Berg.[19] Complete and incomplete Freund's adjuvants were purchased from Difco Lab., Detroit MI. Goat anti-rabbit IgG-peroxidase conjugate was obtained from Tago Inc., Burlingame, CA. Biotin-labeled goat anti-rabbit IgG antibodies and avidin-biotin-peroxidase complex (ABC) were purchased from Vector Laboratories Inc., Burlingame, CA. Single-stranded DNA cellulose was obtained from Wako Pure Chemicals Inc., Osaka.

Preparation of modified DNA Commercial native calf thymus DNA (0.5 mg/ml) in 20 m*M* Na-cacodylate (pH 7.5) was incubated with 0.25 m*M* N-hydroxy-2-methoxy-4-aminoazobenzene (N-OH-2-MeO-AAB) or N-hydroxy-3-methoxy-4-aminoazobenzene (N-OH-3-MeO-AAB), 2 m*M* seryl-AMP and 1 m*M* DTT for 30 min at 37°C. In this reaction, DNA became modified by N^4-O-seryloxyaminoazobenzene derivatives generated from the N-OH compound and seryl-AMP *in situ*.[1-3, 20] Nonreacted chemicals were then removed by extraction with ethyl acetate and diethyl ether. The modified DNA was dialyzed against 0.15 *M* NaCl in 20 m*M* Na-phosphate buffer (pH 7.0) to ensure complete removal of chemicals. The amounts of adducts in DNA were estimated by spectrophotometric analysis as described previously.[21] Briefly, modified DNA samples (0.5–1.0 mg) were dissolved in 2 *N* HCl-75% formic acid and kept for 2 h at room temperature. The amounts of aminoazo dye were calculated from the molecular extinction values of 66,000 at 502 nm for 2-MeO-AAB and 57,000 at 545 nm for 3-MeO-AAB adducts. The adduct levels for the two DNA used as immunogens were 4.3 adducts/10^3 nucleotides for 2-MeO-AAB and 3.13 adducts/10^3 nucleotides for 3-MeO-AAB. N-hydroxy-4-(*o*-tolylazo)-*o*-toluidine (OAT)-modified DNA was also prepared as above and the extent of adduct formation was measured by spectrophotometry (molecular extinction value of 26,000 at 378 nm). Other carcinogen-modified DNA samples were prepared as described previously.[22, 23] These modified DNAs contained 1–5 adducts/10^3 nucleotides.

Preparation of antibodies The antibodies against 2-MeO-AAB- or 3-MeO-AAB-modified DNA were produced by using the published procedure.[13, 15] Rabbits were immunized with 0.5 mg aliquots of each modified DNA-methylated BSA mixture (1 ml) first in Freund's complete adjuvant (1.0 ml) into the footpads and then with incomplete adjuvant intramuscularly for 4 weeks at weekly intervals. A booster injection without adjuvant was performed 4 weeks after the last injection.

IgG fractions were isolated by DEAE-Sepharose CL-6B chromatography of the 40% saturated ammonium sulfate-precipitated antisera fraction. The pooled IgG fraction was dialyzed against 20 m*M* Tris-HCl (pH 7.2) containing 1 m*M* EDTA and 0.1 *M* NaCl, and passed through affinity columns bearing single-stranded DNA cellulose to remove nonspecifically binding materials.

Enzyme immunoassay ELISA was performed as described.[3] Polystyrene U-bottomed microtiter plates (Immunoplate type II, Nunc, Inc. Naperville, IL) were coated with 20 ng of control unmodified DNA or 2-MeO-AAB- or 3-MeO-AAB-modified DNA containing 1–2 adducts per 10^3 nucleotides in 50 μl of PBS and dried overnight.[13, 15] After blocking with 0.02% gelatin, sequential incubations with dilutions of IgG and goat anti-rabbit IgG-peroxidase conjugate were performed to colorimetrically detect specific antibody binding. For competitive ELISA, the inhibition of antigen-antibody binding was determined after mixing serial dilutions of the modified or unmodified DNA (0–50 μg/ml) with purified IgG (2–4 μg/ml) overnight at 4°C prior to antibody assay. The amount of inhibitor required for 50% inhibition of antibody binding to immobilized antigen (IA_{50}) was taken as a measure of the sensitivity of the competitive ELISA.[13, 15]

Treatment of rats with 2-MeO-AAB or 3-MeO-AAB: isolation of DNA Male F344 rats (Charles River Japan; 8 weeks old) were given a single i.p. injection of 2-MeO-AAB or 3-MeO-AAB dissolved in corn oil at 50 mg/kg body weight. Control animals received the solvent alone. After 20 h, the rats were killed and the livers were removed and rapidly frozen at −80°C. DNA samples were isolated from the tissues as described[15] and heated in PBS for 5 min at 90°C, followed by rapidly cooling on ice, and used for the competitive ELISA.

Immunohistochemical staining Slices of excised liver were fixed in cold acetone or 10% buffered formalin[16] and routinely processed for embedding. Paraffin sections (4 μm) were placed on poly-L-lysine-coated slide glass (sections of 2-MeO-AAB- and 3-MeO-AAB-treated livers were placed on the same slide for comparison). They were deparaffinized with 3 xylene changes and hydrated in a graded series of ethanol. After washes in 0.01 *M* phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20, PBS and 2×saline sodium citrate (SSC), sections were treated with RNase A (100 μg/ml) at 37°C for 2 h, and 2.5 *N* HCl at 37°C for 20 min. After being washed with PBS several times, they were treated with 0.02% actinase in distilled water at 37°C for 3 min, and incubated with 100 μl of 5% normal goat serum at 20°C for 20 min and then with 100 μl of anti-2-MeO-AAB- or 3-MeO-AAB-DNA antibodies at 1:50–250 dilution (20–100 μg IgG/ml) in PBS containing 1% BSA and 0.05% NaN_3 for 60 min at 37°C. After treatment with 0.3% H_2O_2 for 30 min at 37°C, binding of the antibody was visualized by the avidin biotin peroxidase

complex (ABC) method (37°C for 30 min) with 0.05% diaminobenzidine (for 10 min). The sections were also counterstained weakly with hematoxylin, before dehydration through graded ethanol and xylene, and mounted.

To confirm the specificity of immunohitochemical staining, antibody was replaced with diluted normal rabbit sera.

RESULTS

Immunoassay Immunization of rabbits with 2-MeO-AAB- or 3-MeO-AAB-modified DNA resulted in the development of antigen-specific antibodies at 4–8 weeks after the first immunization. These antibodies were characterized by direct or competitive ELISA as previously established in our laboratory.[13, 15] Antibody titers were determined by direct ELISA, the amount of antibody giving of 1.0 absorbance (at 492 nm) after 15 min incubation with substrate being found to be 2–5 ng IgG per assay. No cross reactivity could be observed with unmodified calf thymus single or double stranded DNA (Fig. 1). The capacity of antibody to specifically distinguish aminoazo-dye modified base adducts was confirmed by competitive ELISA using 2-MeO-AAB- or 3-MeO-AAB-DNA as the immobilized antigen.

Inhibition of antigen-antibody binding could be observed with increasing concentration of DNA modified with both aminoazo-dyes, DNA samples contained 8.7 adducts per 10^3 nucleotide (29.0 nmol/mg) for 2-MeO-AAB-DNA and 7.1 adducts 10^3 nucleotides (23.7 nmol/mg) for 3-MeO-AAB-DNA, and inhibition became apparent with as little as 3 ng of inhibitor. Anti-2-MeO-

Table I. Competitive Inhibition of Antibody Binding to 2-MeO-AAB-DNA and 3-MeO-AAB-DNA by Various Compounds

Competitor		Amount of competitor (fmol) causing 50% inhibition	
		Antibodies	
		2-MeO-AAB-DNA	3-MeO-AAB-DNA
2-MeO-AAB-DNA			
native		14	400
denatured[a]	pH 7.0	17	58
	0.02 *N* NaOH	16	58
3-MeO-AAB-DNA			
native		130	60
denatured[a]	pH 7.0	23	13
	0.02 *N* NaOH	23	12
OAT-DNA			
native		90	850
denatured[a]	0.02 *N* NaOH	5	40
4NQO-dDNA[b]		$>10^{5c}$	$>10^5$
4ABP-dDNA		$>10^5$	$>10^5$
DMAB-dDNA		$>10^5$	$>10^5$
Glu-P-1-dDNA		$>10^5$	$>10^5$
Glu-P-2-dDNA		$>10^5$	$>10^5$
AAF-dDNA		$>10^5$	$>10^5$

Microtiter plates were coated with 20 ng of 2-MeO-AAB-DNA (15.6 nmol/mg DNA) or 3-MeO-AAB-DNA (11.1 nmol/mg DNA), and 20 ng of IgG was applied per well.
a) Modified DNA was heated in PBS or 0.02 *N* NaOH at 90°C for 5 min.
b) DNAs were denatured in PBS at 90°C for 5 min.
c) No inhibition was detected at the highest concentration tested.

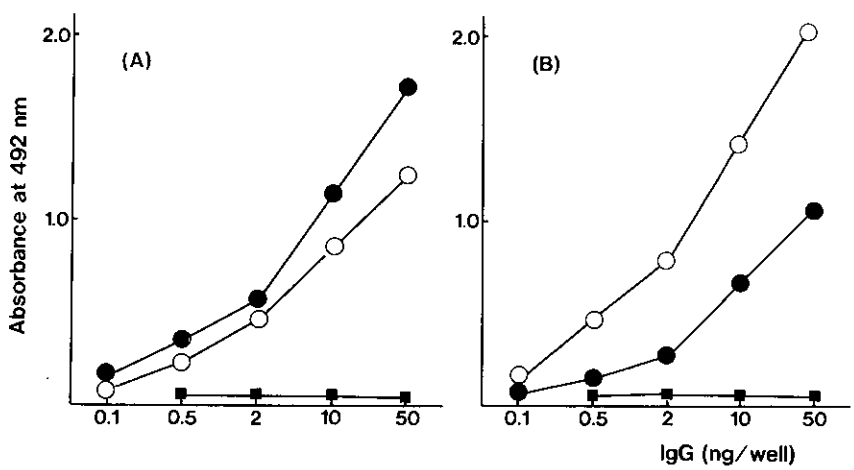

Fig. 1. ELISA titers of 2-MeO-AAB-DNA (A) and 3-MeO-AAB-DNA (B) specific antibodies. Wells of microtiter plates were coated with 20 ng of single-stranded 2-MeO-AAB-DNA (●), 3-MeO-AAB-DNA (○) and unmodified DNA (■). Antibody binding was determined by ELISA as described in "Materials and Methods." The absorbance values were obtained after a 15 min incubation with substrates.

AAB-DNA antibody binding was 50% inhibited by 140 fmol of 2-MeO-AAB-DNA adducts and 130 fmol of 3-MeO-AAB adducts, while anti-3-MeO-AAB-DNA antibody binding to 3-MeO-AAB-DNA was 50% inhibited by 400 fmol of 2-MeO-AAB-DNA adducts and 60 fmol of 3-MeO-AAB-DNA adducts in double-stranded DNA.

Denaturation of DNA samples by heating at 90°C for 5 min in PBS or 0.02 *N* NaOH resulted in about 5- to 10-fold increase in sensitivity (Table I). Neither anti-2-MeO-AAB-DNA antibody nor anti-3-MeO-AAB antibody cross-reacted with control calf thymus DNA at the highest concentration tested (15 μg/well). Several other aromatic amine-modified DNAs were also tested, but only OAT-modified DNA showed significant cross reactivity, with almost the same sensitivity as the 2-MeO-AAB- or 3-MeO-AAB-DNA adducts. No cross reactivity was seen with a high concentration (15 μg DNA/well) of DNAs modified with 4NQO, DMAB, 4-aminobiphenyl (4ABP), 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-amino-dipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) or 2-acetylaminofluorene (AAF). Nuclease P_1 digestion of modified DNA to the constituent deoxynucleotides resulted in loss of antigenicity. These antibodies did not react with adducted nucleotides. Treatment with acid to release adducts caused complete loss of reactivity.

Competitive ELISA frequently involves calibration with highly modified DNA diluted with various amounts of unmodified DNA, but our previous report on anti-4NQO-DNA-adduct antibodies[13] and two other recent reports[24, 25] suggest that the use of such highly modified DNA as a standard competitor in ELISA leads to erroneous results (in most cases an underestimation) when determining adduct levels in biological samples with a low level of modification. To establish an optimal assay for biological samples, we prepared modified DNA *in vitro* by treatment with various concentrations of N-OH compound and seryl-AMP. The amount of aminoazo dye covalently bound with DNA was measured spectrophotometrically, adduct formation being found to increase dose-dependently (data not shown). After heat denatur-

Table II. The Extent of DNA Adduct Formation by N-Hydroxy Derivatives of 2-MeO-AAB and 3-MeO-AAB in Calf Thymus DNA *in vitro* Measured by Competitive ELISA

Aminoazo-dye	No. of DNA adducts/10^4 nucleotides[a]				
	Aminoazo-dye concentration (μM)				
	1	5	50	100	250[b]
N-OH-2-MeO-AAB	0.036	0.081	1.30	2.6	7.1
N-OH-3-MeO-AAB	0.043	0.11	1.35	2.2	4.7

a) Values are averages of 3 different determinations.
b) Amounts of aminoazo dye in DNA were measured spectrophotometrically and these modified DNAs were used as standards.

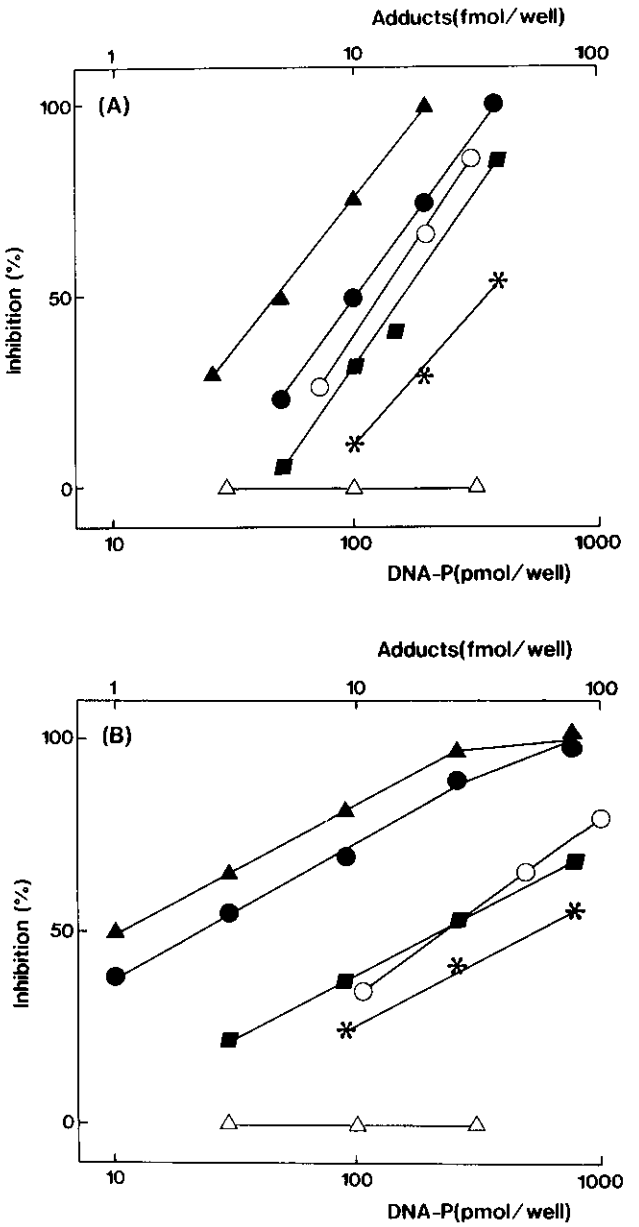

Fig. 2. Inhibition of antigen-antibody binding by inhibitors with various modification levels prepared by mixing various concentrations of N-OH compound and seryl-AMP. The inhibitory activity of each of the samples was compared with those of standard amounts of inhibitors (○). Anti-2-MeO-AAB-DNA antibody (A) and anti-3-MeO-AAB-DNA antibody (B). Concentrations of N-OH compound used in the binding reaction were 100 μM (▲), 50 μM (●), 5 μM (■) and 1 μM (*). Without seryl-AMP (△) (see Table II).

ation, adducts in DNA were determined by competitive ELISA. As shown Table II, a dose-dependent production of DNA adducts was evident with both 2-MeO-AAB and 3-MeO-AAB on competitive ELISA. Treatment with 1 μM N-OH compound is calculated to yield 2–5 adducts per 10^6 nucleotides (Fig. 2, Table II).

Quantitation of adducts in DNA from the livers of rats exposed to aminoazo dyes and from non-treated rats was performed by competitive ELISA. DNA samples were heated in PBS at 90°C for the time indicated in Fig. 3 and the immunological reactivity was increased. The number of adducts in DNA from livers exposed to 3-MeO-AAB was found to be 1–2 per 10^6 nucleotides. Values for adducts in DNA from 2-MeO-AAB-exposed rats were below the limit of detection, i.e., less than 5 per 10^7 nucleotides.

Immunohistochemical staining Our previous investigations of the immunohistochemical procedures indicated that the fixation steps are very critical.[14, 16] In the present case we investigated tissue fixation with 10% buffered formalin for 18 h and with cold acetone for 2 weeks at 4°C. The fixation with cold acetone was found to be the better procedure (Fig. 4 A and B) for the anti-3-MeO-AAB antibody. Although fixation with buffered formalin was better for the anti-2-MeO-AAB antibodies, the staining was still only faint (data not shown).

Under the selected experimental conditions, antibodies to 3-MeO-AAB-DNA adducts and 2-MeO-AAB-DNA adducts did not bind in detectable amounts to livers from unexposed rats (Fig. 4C). Control rabbit sera also did

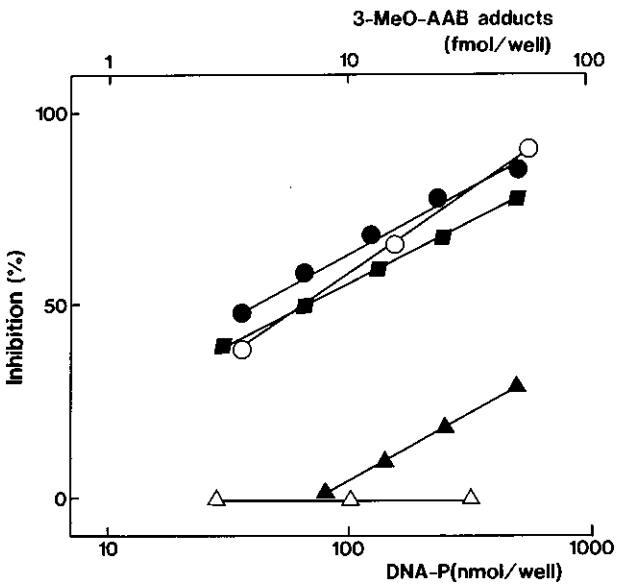

Fig. 3. Analysis of 3-MeO-AAB adducts in DNA from the liver isolated from rats 18 h after i.p. administration of 3-MeO-AAB (50 mg/kg body weight) by competitive ELISA. DNA was heated at 90°C for 0 min (▲), 3 min (■) 5 min and 10 min (●). DNA from untreated rats was heated at 90°C for 10 min (△). 3-MeO-AAB DNA containing 0.7 adduct per 10^3 nucleotides was used as the standard competitor (○).

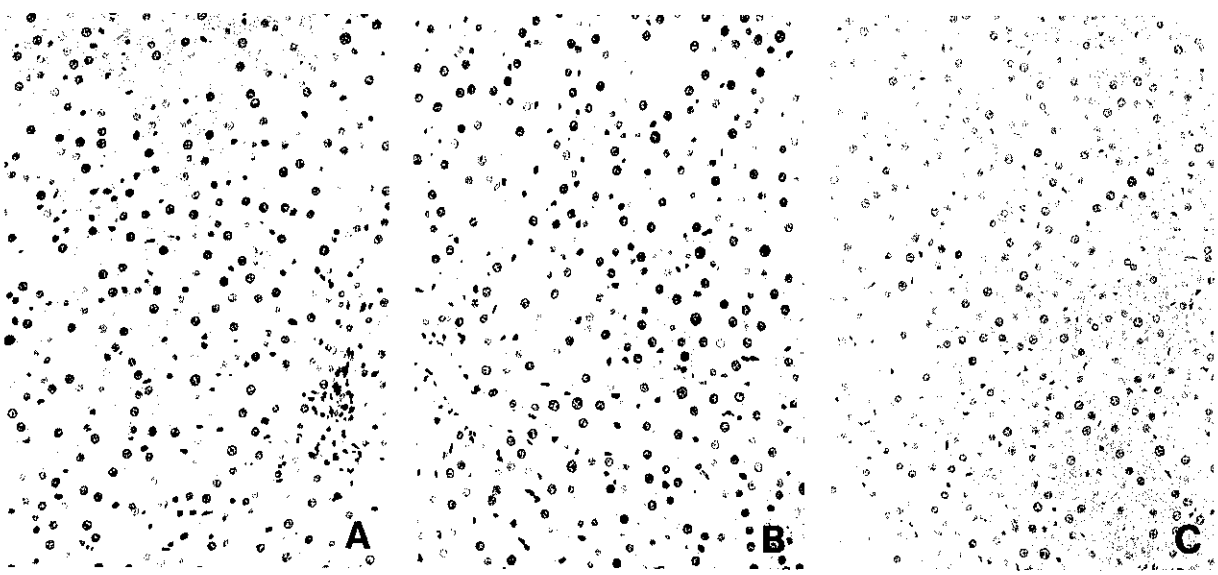

Fig. 4. Immunostaining of acetone-fixed liver sections from rats given an i.p. dose of 50 mg/kg body weight 3-MeO-AAB (A), 2-MeO-AAB (B) or corn oil only (C). Anti-3-MeO-AAB antibody (80 μg/ml) was used (original magnification ×200).

not react with liver of rats given aminoazo dyes (data not shown). In contrast, antibodies to 3-MeO-AAB-DNA adducts reacted with the liver exposed to either 2-MeO-AAB or 3-MeO-AAB. Immunohistochemical localization of aminoazo-dye adducts showed that the distribution over the liver lobule was rather homogeneous. Staining was observed in the nuclei of hepatocytes and also Kupffer cells. Weak cytoplasmic staining was present. With anti-2-MeO-AAB-DNA adduct antibody, weak staining was observed at 50 and 100 dilutions of the antibodies but not at 250 dilutions in the nuclei of rat livers exposed to 3-MeO-AAB or 2-MeO-AAB. These results were consistent with the ELISA findings, where cross-reaction was apparent.

DISCUSSION

Previous studies have indicated the usefulness of carcinogen DNA adduct-specific antibodies as tools to probe the relevance of DNA adducts to carcinogenesis *in vivo*.[13-17] In the present investigation, polyclonal antibodies against 2-MeO-AAB-DNA adducts and 3-MeO-AAB-DNA adducts were generated, and competitive ELISA revealed very high affinities of these antibodies for the modified DNAs used as immunogens. Cross reactivity with each other was, however, observed. Moreover, they cross-reacted with structurally related adducts in OAT-modified DNA, but not with DNA modified by other carcinogens such as 4NQO, 4ABP, DMAB, Glu-P-1, Glu-P-2 and AAF. Thus, the epitopes of these antibodies might be present in the structure surrounding the 4-aminoazobenzene base adducts or some location with close structural similarity to these adducts.

Immunoassays normally require the physicochemical characterization of an adduct before detection and quantitation in biological samples can be attempted. Unfortunately, the chemical structures of the aminoazo-dye adducts have not yet been clarified. Our previous findings suggested that mainly guanine-substituted adducts are produced in DNA after reaction with these chemicals.[21] A ^{32}P-postlabeling analysis revealed the major component of both 2-MeO-AAB and 3-MeO-AAB adducts to be C-8 deoxyguanosine substituents.[1] After administration of the hepatocarcinogenic aminoazo dye N-methyl-4-aminoazobenzene (MAB), the major carcinogen-DNA adducts formed in the liver were identified as N-(deoxyguanosin-8-yl)-MAB (C8-dG-MAB), 3-(deoxyguanosin-N^2-yl)-MAB(N^2-dG-MAB) and 3-(deoxyadenosin-N^6-yl)-MAB (N^6-dA-MAB).[26,27] Only C8-dG-MAB and N^2-dG-MAB were detected *in vivo* following a single i.p. dose of MAB.[28] The synthetic C8-dG-MAB and also C8-dG-AAB were both reported to be stable in 0.1 *N* NaOH upon heating at 60°C, yielding only deribosylated products.[29] Since the antigenicity of our aminoazo-dye adducts was not changed by heat treatment at neutral or alkaline pH, the epitopes of these antibodies might be mainly C8-dG-AAB and N^2-dG-AAB in DNA. The latter is also stable under alkaline conditions.[29]

The present studies using competitive ELISA indicated 50% inhibition of antibody binding by 10–20 fmol of adducts in a maximum of 5 μg of DNA per well for denatured modified DNA. We could thus detect a level of 0.5–1 adduct per 10^6 nucleotides or 3 fmol of adducts in 1 μg of DNA. This allowed calculation of the numbers of aminoazo-dye adducts in DNA isolated from 3-MeO-AAB-exposed rats to be between 1–2 adducts per 10^6 nucleotides. Values for 2-MeO-AAB-exposed rats were, in contrast, below the detection limit of 0.5 adduct per 10^6 nucleotides. These values are in agreement with those based on our previous ^{32}P-postlabeling analysis,[1] where the maximum values for total DNA adducts formed by 3-MeO-AAB and 2-MeO-AAB were found to be 3.6 and 0.16 per 10^7 nucleotides, respectively. The significance of the finding that the carcinogen 3-MeO-AAB generated more than 20-fold greater amounts of adducts in rat liver than the non-carcinogen appears clear.

The reason why the immunohistochemical studies revealed positive staining in the nuclei of rat livers exposed to both aminoazo dyes is not known. No positive correlation between nuclear staining intensity and the number of adducts determined by immunoassay or ^{32}P-postlabeling analysis was found. In our previous experiments concerned with immunostaining of 4NQO-DNA adducts in tissues using anti-4NQO-DNA antibodies, the lower limit of detection for staining was 4–5 adducts per 10^6 nucleotides, i.e., about 10^4 adducts per cell.[14] This is in the same general range as the detection limit of 5×10^4 molecules per cell reported for O^6-ethyldeoxyguanosine in liver sections of rats exposed to dimethylnitroamine[30] and of 1×10^5 molecules of 2-acetylaminofluorene-dG adducts per liver cell (5–15 adducts/10^6 bases).[31,32] From the immunoassay results, the immunological activities of the anti-aminoazo-dye-DNA antibodies were similar to that of anti-4NQO-DNA antibody or anti-DMAB-DNA antibody, while far higher concentrations of IgG (80 μg/ml) were used for immunostaining of aminoazo-dye adducts than in the cases of anti-4NQO-DNA antibody or anti-DMAB-DNA antibody (1–2 μg/ml). Even with these large amounts of IgG, no positive staining was observed in control livers.

Aminoazo-dye adducts formed in the liver after single i.p. administration amount to less than 10^4 per cell, but could be detected immunohistochemically. Thus, although we could not obtain a clear positive correlation between the nuclear staining intensity and the number of adducts calculated from immunoassay or ^{32}P-postlabeling analysis, the immunohistochemical technique should prove useful for further investigations.

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