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The correlation between DNA adducts and chromosomal aberrations in the target organ of benzidine exposed, partially-hepatectomized mice

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An experimental system was developed to test the association between benzidine–DNA adduct levels and chromosome aberrations in the target organ, the liver, of mice. A 2/3 partial hepatectomy was performed (0 h), then the animals were treated with benzidine (0, 7.8, 19.5, 38.2 or 97.8 mg/kg, i.p.) and an agar-coated 50 mg 5-bromodeoxyuridine tablet was implanted subcutaneously (58 h). Colcemid was given at 4 mg/kg i.p. (70 h), and the animals were sacrificed 2 h later. The liver from each animal was divided, with portions allocated for cytogenetics and DNA adduct analysis. DNA adducts were analyzed with the ^{32}P-postlabeling technique. DNA adduct and chromosomal aberration data were available on a total of 43 animals. Benzidine was shown to be a potent clastogen in liver, the target organ, as opposed to its reported weak activity in the bone marrow. A linear dose response was demonstrated for benzidine–DNA adducts found in the liver. The correlation between adduct levels and aberrations in individual animals was 0.43 ($P < 0.05$). However, most of the residual variance was due to four outlying cases. When these cases were removed from the data set and the analysis repeated, the linear correlation coefficient increased to 0.74. When the data were analyzed by dose groups, the correlation was 0.91. These data support the hypothesis that carcinogen–DNA adducts are responsible for the induction of chromosomal aberrations, and perhaps other genotoxic events, including neoplasia.

Introduction

A central tenet of the somatic mutation theory as it pertains to chemical carcinogenesis is that DNA adduct formation induces heritable genetic damage such as mutation and/or chromosomal aberrations (1). Mutation and aberrations, in turn, under appropriate circumstances, can allow a cell to escape the growth control of the organism and become a neoplasm (2–4).

An association between carcinogen–DNA adduct levels and *in vitro* genotoxicity has been described. Arce *et al.* (5) and Beland *et al.* (6) gave proof of a linear relationship between aromatic amine DNA adduct levels and frameshift mutagenesis in *Salmonella typhimurium*. Similar relationships were shown between DNA adduct levels and *in vitro* cell transformation (7,8), mutation at the hypoxanthine guanosine phosphoribosyl transferase gene in cultured Chinese hamster ovary cells (9) and with mutation and chromosome damage in *Drosophila melanogaster* (10–12). These data suggest that carcinogen–DNA adducts are directly mutagenic, a conclusion corroborated by structural analysis of DNA models containing adducts (13–15).

The focus of this work was the development of a system to test the association between carcinogen–DNA adduct levels and chromosome aberrations in the intact animal. Chromosome aberration analysis is an established method of testing genotoxic effects of exposure in humans and animals exposed to radiation or chemical carcinogens (16,17). Specific chromosome aberrations have been associated with specific tumors and with the activation of certain oncogenes (3,18,19).

Benzidine, an industrial chemical used in the production of dyestuffs and certain laboratory reagents (20), is a liver carcinogen in mice, rats and hamsters, but a bladder carcinogen in dogs and humans (21). The reason for this species-specific organotropism is apparently the relative ability of the species to acetylate aromatic amines. Species which rapidly acetylate aromatic amines (rodents) develop hepatic tumors, while species which are relatively slow acetylators develop bladder tumors following benzidine exposure (22).

Martin and Ekers (23) monitored the binding of radiolabelled benzidine to hepatic DNA, RNA and protein. DNA binding peaked 24 h following an i.p. dose of 32 mg/kg, but detectable levels were seen as early as 3 h following administration. A single DNA adduct was detected in the liver following treatment with benzidine (23–25). This adduct co-chromatographed with *N*-(deoxyguanosin-8-yl)-*N'*-acetylbenzidine which was synthesized *in vitro*. The chemical identity of the adduct was confirmed by mass spectrometry (24,26).

Evidence of *in vivo* benzidine clastogenicity has been equivocal. Cihak *et al.* (27) enumerated micronuclei in the peripheral blood of mice given 100, 200 or 300 mg/kg i.p. and reported a statistically significant response only at the high dose (which was ~1.5 times the LD50), but no consistent dose–response relationship was seen. Similar results were reported in preliminary studies of mouse bone marrow cytogenetics (28,29). These findings would seem to indicate that benzidine is not, or is at best a weak animal clastogen.

On the other hand, benzidine is a potent clastogen *in vitro* when an appropriate metabolic activation system is employed (30–32). Petzold and Swenberg (33) provided a likely explanation for the anomalous results of *in vivo* and *in vitro* tests of benzidine clastogenicity. They noted that benzidine produced an increase in alkaline labile sites in DNA from the livers, but not the lungs or bladders of treated mice. These findings, together with the known species-specific organotropism of benzidine carcinogenicity and a complex metabolic activation mechanism which includes both acetylation and oxidation (34), suggested that clastogenic activity might not be expected in the bone marrow,

*Abbreviations: bicine, *N,N*-bis-(2-hydroxyethyl)-glycine; 5BrdU, 5-bromodeoxyuridine; DMSO, dimethylsulfoxide; SDS, sodium dodecylsulfate; PEI, polyethyleneimine–cellulose; RAL, relative adduct labeling; <RAL>, relative adduct labeling carrier free conditions; IF, intensification factor.

a non-target organ, but that benzidine may be a clastogen in the liver, the target organ.

In order to test this hypothesis it was decided to assay benzidine for clastogenicity in the target organ, the liver. Cytogenetic analysis can be performed on hepatocytes because of the regenerative capabilities of the liver. Hepatocytes are usually in the G_0 stage of the cell cycle, but surgical or chemical partial hepatectomy can induce many of the remaining hepatocytes to proliferate (35).

Materials and methods

Materials

The following reagents were obtained from Sigma Chemical Co., St. Louis, MO: benzidine, urea, dithiothreitol, calf thymus DNA, ethylenediamine tetraacetic acid, micrococcal endonuclease (Grade VI, 100 U/mg), potato apyrase (Grade I, 2 U/mg), spermidine, sodium pyruvate (Type II), sodium adenosine diphosphate, ribonuclease (RNase) T1 (Grade IV, 400 000 U/ml), RNase A (Type IIIa, 75 U/mg), *N,N*-bis-(2-hydroxyethyl) glycine (bicine*), 5-bromodeoxyuridine (5BrdU), collagenase (Type IV, 360 U/mg), dimethylsulfoxide (DMSO) and Hoechst 3325T stain. Boehringer-Mannheim Biochemicals, Indianapolis, IN supplied: calf spleen endonuclease (phosphodiesterase) (2 U/mg), proteinase K (20 U/mg), glycerol-3-phosphate dehydrogenase (rabbit muscle, 170 U/mg), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 100 U/mg), triose phosphate isomerase, rabbit muscle (5000 U/mg), 3-phosphoglycerate kinase (yeast, 450 U/mg) lactate dehydrogenase (rabbit muscle 500 U/mg), L-glycerol-3-phosphate and beta-nicotinamide adenine dinucleotide (NAD) (Grade I, 100%). Fisher Scientific, Houston, TX supplied tris(hydroxymethyl)aminomethane–HCl (Tris–HCl), lithium chloride, ammonium formate, sodium dodecylsulfate (SDS), ammonium sulfate, phenol, chloroform, isoamyl alcohol and colchicine. Aldrich Chemical Co., Milwaukee, WI was the source of formic acid. Polyethyleneimine–cellullose (PEI) thin layer plastic-backed sheets were manufactured by Merck and purchased from Alltech Inc., Waukeegan, IL. Sheets were predeveloped with distilled water before use and stored at 2°C. PL Biochemicals (Milwaukee, WI) was the supplier for deoxyguanosine-3′-phosphate deoxycytidine-3′-phosphate, deoxyadenosine-3′-phosphate, thymidine-3′-phosphate and T4 polynucleotide kinase (11.5 U/μl). [^{14}C]Ink and carrier free [^{32}P]H_3PO_4 were obtained from ICN Biochemicals, Irvine, CA.

Animal care

Male ICR strain outbred mice were obtained from Harlan Sprague Dawley, Houston, TX. Animals weighed between 20 and 24 g when received. Animals were housed in the laboratory's animal care facility for a minimum of one week to allow acclimatization. Animals were allowed food and water *ad libitum*. The temperature, humidity and day-night cycles of the animal room were controlled. Animals were assigned randomly to treatment groups. Care was taken to perform surgery and other treatments at the same time of day for all animals to minimize variation due to circadian rhythms.

The temporal sequence of events for the study is given in Figure 1.

Partial hepatectomies and cytogenetics

Partial hepatectomies were performed essentially as described by Brooks and Mead (36). Mice were ether-anesthetized and the median and left caudal liver lobes were excised. An estimated 55% of the liver mass is removed by this procedure (36). Fourteen hours prior to sacrifice (58 h post surgery) the animals were anesthetized and an agar-coated, 50 mg 5BrdU tablet was implanted subcutaneously by removing a staple suture (37). The staple was replaced and the animal was then given an i.p. dose of benzidine and/or DMSO vehicle at 0.037 ml/10 g body weight. Two hours prior to sacrifice (70 h post-surgery), an i.p. injection of colcemid (0.01 mg/g of a 0.4 mg/ml saline solution) was administered.

Animals were killed by cervical dislocation 72 h following surgery. Livers were rapidly excised, then minced coarsely. Liver pieces were allocated to either a watchglass (~1/3 the total amount) for cytogenetic analysis or to a 1.5-ml cryotube which was then immediately placed in a beaker of ethanol and dry ice for later adduct analysis. These tissues were stored at −80°C until analysis. The liver portion for cytogenetic analysis was then finely minced and incubated for 10 min in 7 ml of McCoy's media to which 100 U/ml of collagenase was added. The tubes were centrifuged at 1500 r.p.m. for 5 min, the media poured off and replenished with 10 ml of McCoy's media with 10% plasma. The cell button was then treated with 0.075 M KCl hypotonic solution and then with 3:1 methanol:acetic acid fixative.

Slides were prepared by washing with acetic acid, then several drops of the mixed cell pellet were dropped onto the slides from a height of 6 inches. The method for staining the chromosome preparations was essentially that of McFee *et al.* (38). In brief, the cells were stained with Hoechst 3325T fluorescent stain (6.25 μg/ml), washed with citrate buffer, exposed to ultraviolet radiation, then counterstained with 5–7% Giemsa and air dried.

TIME [hours]	
0	Partial Hepatectomy.
58	a. IP dose of benzidine in DMSO (0, 7.8, 19.5, 38.2, or 97.8 mg/kg in 3.7μl/g body weight volume).
	b subcutaneous implantation of a 50mg 5-bromodeoxyuridine tablet.
70	IP injection of colcemid (0.04mg/10g body weight in 0.1 mg/10g saline).
72	Animal killed by cervical dislocation; liver was coarsely chopped and 2/3 of the pieces frozen for adduct analysis; 1/3 used to prepare cytogenetics slides.

Fig. 1. Sequence of events for chromosome aberration–DNA adduct study. Temporal sequence of events for the treatment of animals and isolation of tissues involved in this study. See text for details of each procedure.

A primary concern of the study design was to score only those cells in their first metaphase following benzidine exposure (39). Benzidine and 5BrdU were administered concomitantly. Both agents are rapidly absorbed and reach maximal tissue concentrations within 2 h (23,37). However, benzidine must be activated metabolically before a species capable of binding DNA is produced. To produce a similar time lag of absorption of 5BrdU, the 5BrdU tablets were coated with agar as described by King *et al.* (37). These workers observed that peak blood levels of 5BrdU occurred 5–7 h following implantation of coated tablets and remained steady for 5–7 h thereafter, whereas the peak blood levels with uncoated tablets were seen within 2–3 h and decined rapidly.

Slides were scored by a single reader using a Zeiss microscope. Only cells readily identifiable as being in their first mitotic division in the presence of 5BrdU were scored. No cells were counted unless all 40 chromosomes were present and spreading was sufficient so that each chromatid could be discerned. Data from any animal was entered into analysis only if 50 metaphase spreads could be read and counted according to the above criteria.

^{32}P-Postlabeling assay of benzidine DNA adducts

The methods used to quantify DNA adduct formation were essentially as described in Gupta *et al.* (40), Randerath *et al.* (41,42), Reddy *et al.* (43,44) and Schurdak and Randerath (45), with modifications as noted. Adduct levels were determined routinely using carrier-free labeling conditions where [^{32}P]ATP is the rate-limiting reagent (42). Randerath *et al.* (42) have shown that adducts are often labeled in preference to normal nucleotides by polynucleotide kinase under these conditions. The degree of intensification under carrier free conditions varies depending upon the nature of the adduct and the conditions employed. The intensification will then vary with each adduct formed by a particular carcinogen. In order to determine the degree of intensification, separate experiments were performed using both carrier free and conditions where excess cold ATP was added (standard conditions). Under the latter conditions nucleotides are limiting and are labeled to completion. The methods for this experiment for this experiment were described earlier (42). A stratified block sampling method (by dose) was employed to minimize potential run-to-run variations in the assay.

DNA isolation

DNA was isolated by a solvent extraction procedure (46). DNA was resolubilized using 1.5 mM NaCl, 0.1 mM sodium citrate (1/100 SSC). DNA concentration was estimated spectrophotometrically taking 20 A_{260} units per milligram of DNA. DNA recoveries averaged 2.75 mg/g liver tissue. Hydrolysis of the DNA to 3′-phosphonucleotides is described in the legend to Figure 2.

ATP synthesis

The method used to synthesize [^{32}P]ATP was essentially that of Johnson and Walseth (47) except that the final concentration of the isotope is 100 mCi/ml.

Carrier-free labeling

Carrier-free labeling was performed as described by Reddy *et al.* (43) and Randerath *et al.* (41,42). Two micrograms of 3′-phosphonucleotides were incubated with 200 μCi of [^{32}P]ATP; the concentration of nucleotides was ~300 times the ATP concentration.

Standard labeling conditions

The standard (ATP excess) labeling conditions were as described by Gupta *et al.* (40). Each sample was incubated with 250 μCi of [^{32}P]ATP, the excess of which was destroyed by incubation with apyrase following labeling. The chromatographic conditions used in all assays are described in the legends to Figures 2 and 3. The magnet-mediated transfer technique was employed (48).

Calculation of DNA adduct levels

Count rates of normal nucleotides and adduct spots were obtained and the relative

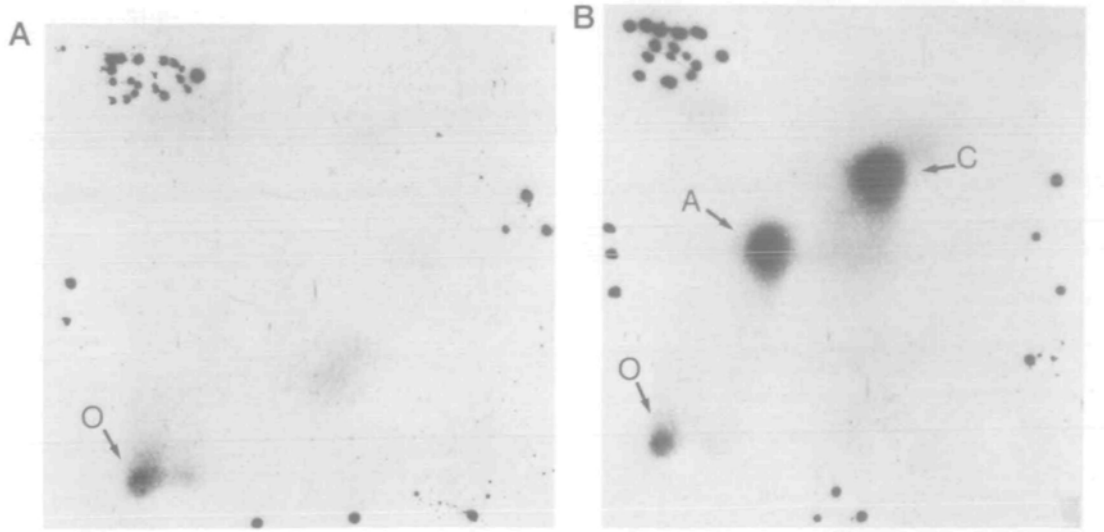

Fig. 2. Autoradiograms of chromatograms from control mouse liver DNA (**panel a**), or the DNA from a benzidine-treated mouse (**panel b**). DNA (2 μg) was isolated as described in Materials and methods, then hydrolyzed by incubation for 3 h with 2.5 μg spleen phosphodiesterase and 0.25 U micrococcal endonuclease in 3.5 mM $CaCl_2$ and 6.7 mM sodium succinate, pH 6.0 (total volume 10 μl). Samples were ^{32}P-postlabeled under adduct intensification conditions as described (42), then each sample was spotted at an individual origin along a PEI sheet. The chromatogram was developed overnight in 1.1 M LiCl, washed and dried and the origins of each sample excised and contact-transferred to an equivalent origin on a separate 10 cm^2 PEI sheet (48). The sheet was pre-developed to the origin with water then developed in 7.65 M urea, 3.2 M lithium formate, pH 3.5. The sheets were washed and dried then rotated 90° clockwise, pre-developed as above, then developed in 8.0 M urea, 0.76 M LiCl, 0.48 M Tris–HCl, pH 8.0. Sheets were again washed and dried, then developed in the same direction with 1.7 M sodium phosphate onto a 1.5 cm Whatman #1 wick. The wicks were discarded and the sheets dried. The sheets were marked with ^{14}C-containing ink, then placed in Kodak cassettes with Kodak X-omatic regular intensifying screens overnight (14–16 h) at −80°C. The origin (O) and adducts A and C are indicated. Inked markings for alignment with the chromatograms are evident.

adduct labeling (RAL) values were determined from the standard labeling conditio assays as follows:

$$RAL = \frac{\text{c.p.m. adduct}}{\text{c.p.m. normal nucleotides}}$$

Reddy *et al.* (43) saw that labeling under standard conditions was quantitative because recoveries of nucleotides were not affected by substantial increases in nuclease concentration, or digestion time, ATP excess, or polynucleotide kinase concentration or by labeling incubation time. Relative adduct labeling under carrier free conditions (<RAL>) are equivalent to RAL values when divided by an intensification factor (IF) where:

$$IF = \frac{<RAL>}{RAL}$$

As noted above, the RAL values reported herein are calculated from <RAL> values. The RAL values when multiplied by 1.0×10^7 represent the number of adducts in 10^7 normal nucleotides, and one adduct per 10^7 normal nucleotides is equivalent to 0.3 pmol adducts per milligram DNA (42).

Statistical analysis

Demographic, cytogenetic, and DNA adduct data were compiled using Dbase II software (Ashton-Tate, Inc.). Variables were analyzed using the statistical software program Systat (Systat, Inc., Evanston, IL). The particular statistical analysis used is noted for each case in Results.

Results

Partial hepatectomies were performed on a total of 77 animals in the course of this work. Sixty-nine animals (89.6%) survived the operation. Animal weight and age were analyzed and there was no statistically significant difference between the mean values of different dose groups for these parameters.

Table I displays the summary results of chromosome aberrations caused by benzidine in partially-hepatectomized mice. No chromosome type aberrations were seen, as is usual with DNA synthesis-dependent clastogenic agents. Gaps, which did not strictly increase with increasing dose, were not included in these analyses. There was a clear, dose-related increase in both total aberration rate and in the percentage of cells which had aberrations. Statistically significant differences were also seen when

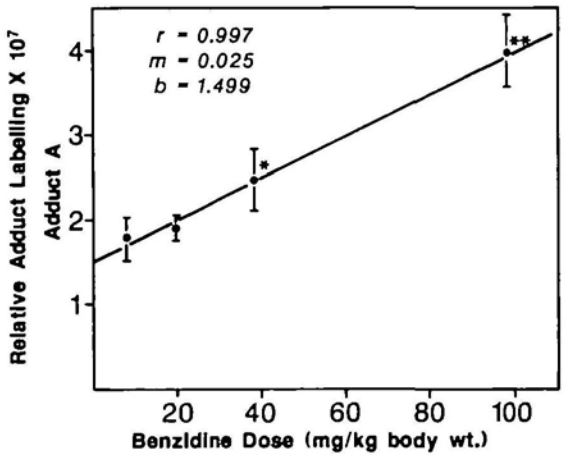

Fig. 3. Net relative adduct labeling times 10^7 for adduct A by benzidine dose. Samples were prepared and postlabeled as in Materials and methods. The benzidine dose, numbers of animals in each group and the group averages are given in Table II. The c.p.m. in the adduct spots were reduced by the c.p.m. in an equivalent area from a control animal chromatogram run at the same time. The mean values for each group are plotted along with the standard error of the means (error bars). The single asterisk indicates a significant ($P < 0.05$) difference between the indicated group and the lowest dosed group, a double asterisk indicates a difference significant at the 0.01 level.

Table I. Chromatid aberrations in male mice exposed to benzidine

Dose (mg/kg)	No. of animals	Total aberrations per 100 cells (standard deviation)	Percent aberrant cells (standard deviation)
0 (DMSO)	9	1.11 (1.05)	1.33 (1.41)
7.8	10	5.40 (2.50)[a]	5.0 (2.54)[a]
19.5	9	7.33 (3.00)[a]	7.33 (3.00)[a]
38.2	7	12.86 (7.29)[a]	10.57 (4.58)[a]
97.7	10	15.60 (7.23)[a]	11.80 (4.05)[a]

[a]Indicates $P < 0.001$ Mann-Whitney U statistic

Table II. Intensification factors for adducts A and C[a]

	Mean[b]	SEM[c]
Adduct A	3.64	0.29
Adduct C	18.70	1.78

[a]Hepatic DNA samples were obtained from non-hepatectomized mice 36 or 48 h after a dose of 97.8 mg/kg benzidine i.p. Concentration of nucleoside-3'-phosphates for carrier-free conditions was 495 nM and the concentration of carrier-free ATP was 1.65 nM.
[b]Values listed are the means of two or three replicates of DNA from two animals.
[c]SEM = standard error of the mean.

Table III. Net relative adduct labeling in the livers of male mice exposed to benzidine

Dose (mg/kg)	N	Relative adduct labeling ($\times 10^7$)		
		Adduct A	Adduct C	Total adducts
7.8	10	1.77 (0.28)	0.29 (0.05)	2.06 (0.32)
19.5	11	1.89 (0.14)	0.39 (0.08)	2.27 (0.19)
38.2	10	2.48 (1.12)	0.42 (0.13)	2.90 (0.48)
97.8	10	3.97 (1.34)	0.86 (0.16)	4.84 (0.56)

Values in parentheses are the standard errors of the means. *N* denotes the number of animals with at least duplicate, independent determinations made for each animal.

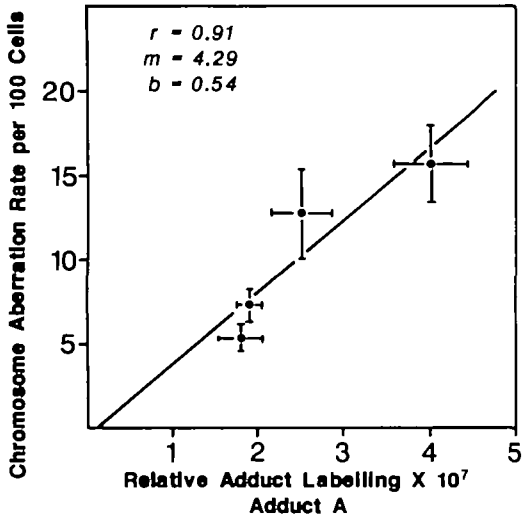

Fig. 4. The correlation between chromosomal aberration rate and net relative adduct labeling for adduct A. Samples were grouped according to benzidine dose. The number of animals is given in Table II. The values plotted are the means for each group with the standard error of the mean for each measurement (error bars).

the low dose was compared to the two highest doses. Chromatid breaks accounted for the vast majority of damage detected, as only one isochromatid break and chromatid exchange were scored. These data demonstrate clearly a clastogenic effect for benizidine *in vivo*.

Figure 2 depicts typical autoradiograms from the postlabeling experiments. Figure 2a is an autoradiogram from a control animal. Figure 2b is an autoradiogram from a treated animal. Two adduct spots are labeled. Adduct A was generally the predominant spot in these analyses. Adduct C occurred in all the treated animals, but levels were more variable. Results of experimentation to determine the intensification of adducts A and C under carrier free conditions are given in Table II. The relative

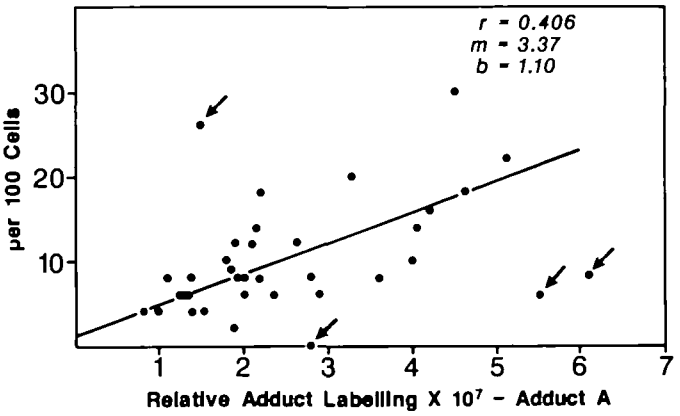

Fig. 5. The correlation between chromosome aberration rate and net relative adduct labeling for adduct A for individual mice. Arrows indicate data points which contribute heavily to the residual variance.

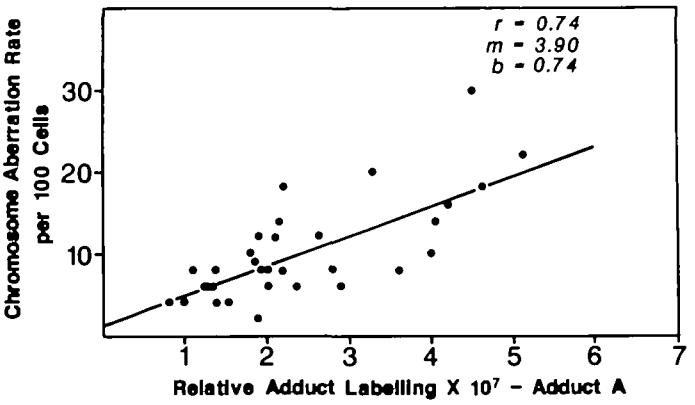

Fig. 6. The correlation between chromosome aberration rate and net relative adduct labeling for adduct A with four outliers removed.

adduct labeling from experiments performed under carrier free conditions (<RAL>) were divided by these intensification factors to estimate the true relative adduct labeling as reported here. The average RAL values (multiplied by 1.0×10^7) are given in Table III for each group of animals. As with the chromosome data, there was a clear dose-response relationship. This relationship is plotted in Figure 3 for adduct A. The data were linear over the dose range tested. The dose responses for adduct C and total adducts are similar.

These experiments were designed to determine if, on an individual basis, chromosome aberration rate could be predicted from DNA adduct levels. Hepatic DNA adduct levels and chromosome aberration rate were determined individually for each animal. The treatment regimen was designed so that cytogenetic analysis was performed on cells undergoing their first metaphase after being exposed to and metabolizing the benzidine dose. Because of the low mitotic index of the hepatocytes (six to nine metaphases per thousand cells), the DNA for adduct analysis was obtained mainly from cells in either the G_0, G_1, S or G_2 stage of the cell cycle before their first mitosis in the presence of benzidine. The data presented here show that the dose reponses of both endpoints are similar. When the dose group average aberration rate was plotted as a function of the dose group average adduct rate for adduct A (Figure 4), an approximate linear relationship was seen ($r = 0.91$, $P < 0.05$). A similar relationship was found for adduct C, however, the correlation coefficient was reduced ($r = 0.85$). The same data were plotted

for each animal in Figure 5. These data approximately fit a linear model ($r = 0.41$, $P \leq 0.05$), although there was considerable scatter of individual points along the line. Four points (indicated by arrows in Figure 5) contributed heavily to the variability of the data. The effect of these outliers on the total covariance was estimated by removing them from the data set and re-analyzing the remaining data. When the four outliers identified in Figure 5 were removed from the original data set, the correlation coefficient improved from 0.41 to 0.74 ($P < 0.001$) (Figure 6). There was no acceptable experimental reason for removing the four outlying cases other than to determine their effect on the underlying linearity of the data. The reasons why such outlying points may have occurred as a function of random variability will be discussed below.

Discussion

Benzidine and the aromatic amines in general are important occupational and environmental carcinogens (20,21,49). They have been implicated in the induction of bladder tumors in dyeworkers (49,50) and forensic technicians (51). Leatherworkers may also be at increased risk (20,52). Benzidine is a species-specific organotropic carcinogen. Our data indicate that benzidine–DNA adducts are correlated with chromosome aberrations induced in the target organ of exposed animals, while others have shown that the aberrations have been associated with oncogene activation and certain neoplastic diseases (3,18,19). Thus, there is a possibility that the induction of chromosome aberrations is a mechanism of benzidine carcinogenicity.

Although many classes of clastogenic compounds have been detected using standard *in vivo* bone marrow assays (53), target organ specificity ought to be an important consideration when screening potentially genotoxic agents. Our findings with benzidine underscore the importance of viewing negative cytogenetic reports on chemicals with caution, especially if there is an indication that the agent has a target organ-specific effect.

^{32}P-Postlabeling is a sensitive technique for the detection of benzidine–DNA adducts. For adduct C, one adduct was detected on average in ~4.8 million normal nucleotides at the 7.8 mg/kg dose. At this dose the c.p.m. in the adduct spots was greater than three times background, on average. The DNA adduct levels reported by Martin and Ekers (23) and Martin *et al.* (24) are very high relative to the levels reported in this study. Martin and Ekers reported detection of one adduct in 26 000 normal nucleotides at a dose of 32 mg/kg benzidine i.p. in the rat. At a similar dose in mice, we saw one adduct in 3.5 million normal nucleotides. Martin and Ekers measured adducts in non-hepatectomized animals 24 h following dosing, while 14 h was the harvest time in this study. Our preliminary results (G.Talaska and V.M.Sadagopa Ramanujam, in preparation) indicate that partial hepatectomy, or the difference in harvest times would not cause such a significant difference. A species difference might also contribute, but probably not to the magnitude seen. Martin and Ekers (23) and Martin *et al.* (24,25) resolved but a single adduct after i.p. injection of benzidine at 32 mg/kg. These authors estimated a detection limit of one adduct per 100 000 nucleotides. It seems likely that adducts present at the levels detected in the current study would not have been detected by Martin and Ekers. It also seems very likely that the adduct Martin *et al.* (24) identified as *N*-(deoxyguanosin-8-yl)-*N'*-acetylbenzidine is adduct A of our studies. Adduct A was the predominant benzidine adduct. Adduct C levels were generally an order of magnitude less than adduct A, so it is not surprising that a second adduct (possibly adduct C) has not been detected by other investigators using less sensitive methods.

The indication from the dose-response of benzidine–DNA adducts is either that adduct levels are not zero in control animals or that slope of the dose–response curve is very steep at doses below 7.8 mg/kg. While the latter seems the most likely, the possibility needs to be addressed in further studies at low doses.

The chemical identity of the carcinogen–DNA adducts cannot be determined by postlabeling. The differences in adduct intensification indicate that there may be significant structural differences between adducts A and C. Work is currently in progress to identify the benzidine adducts by postlabeling DNA modified *in vitro*, a procedure yielding adducts which have been analyzed by mass spectroscopy.

Vogel (10) has shown that O^6- and N^2-guanine adducts of alkylating agents induce different genotoxic events in *Drosophila*. The former adduct was better correlated with mutations, while the latter was correlated with chromosome aberrations. In the present work, the linear correlation coefficient for adduct A and chromosomal aberration rate was greater than when adduct C was used as an independent variable. Stepwise addition of adduct C to the regression equation of adduct A did not increase the predictive value. Total adduct correlation with aberration rate was intermediate between those of adducts A and C. However, the difference in the correlation between levels of adducts A and C and chromosome aberrations was not statistically significant. It was therefore not possible to conclude that one adduct more than the other was better correlated with the chromosomal effect. It may be that large, bulky adducts differ from simple alkylated nucleotides in that they may be similarly potent in inducing genotoxicity. This possibility requires testing with a wider range of carcinogens.

The degree of the linear correlation seen in this study reflects the diverse response of individuals of an outbred strain to an insult, and the error inherent in attempting to correlate measurements, each of which is itself an estimate of a true effect. However, averaging the data by taking the mean values of the dose groups increases the association. As indicated in Figure 4 there is much dose group variation in both assays; on an individual basis this error can be quite large and extreme values can be expected when sampling a relatively large number of animals. It was possible to score only 50 metaphases per animal, and this is likely to be the greatest source of measurement error. Using the aberration rates of the control group, the number of metaphases that ought to be scored to maximize precision and optimize effort can be determined (54). According to the formula provided by Whorton (54), a minimum of 180 metaphases would have provided an optimum for this study. While our data indicate that such precision was not necessary to demonstrate an effect of benzidine treatment between dose groups, the loss of precision was probably critical when an accurate estimate of mean effect was required, as when the individual correlations were made. Each of the four outlying datum had extreme values of chromosome aberrations which may have been due to the sampling error inherent in scoring 50 metaphases. Scoring 180 metaphases per animal would not have been possible due to the low mitotic index in the regenerating liver.

This study presents the results of diverse assays concerning the *in vivo* effects of exposure to a model carcinogen. The importance of testing for chromosome damage in the target organ was demonstrated. In general, DNA adduct levels were shown to be sensitive indicators of early genetic effects and correlated with subsequent chromosomal damage. The 5BrdU labeling

allowed the temporal sequence of events to be determined. Our results indicate that at 14 h following exposure, only six to nine cells per thousand were undergoing cell division and that the majority of those were in the first division (data not shown). Accordingly DNA adduct analysis was performed predominantly on DNA from cells which did not yet divide in the presence of benzidine. We could be reasonably sure, therefore, that the majority of the DNA for adduct analysis was from cells which were in either the G_0, G_1, S or G_2 phase of the first cell cycle following exposure, and, all the cells scored for aberrations were in the first metaphase following exposure. This allowed the establishment of the proper temporal sequence of events in the relationship between adducts and aberrations. Similar studies should be conducted with other compounds to test the generality of these findings.

These data indicate that benzidine–DNA adducts are associated with chromosomal aberrations *in vivo*, corroborating and extending the findings of investigators who observed associations between *in vitro* mutagenesis and transformation and levels of particular carcinogen–DNA adducts (5–12). However, since each of the two benzidine adducts observed had an approximately equal correlation with aberrations, it is possible that various bulky carcinogen–DNA adducts are similarly genotoxic; quite unlike the situation with alkylating agents where genotoxicity often differs depending on the position of substitution (10). Thus, it appears at least for bulky carcinogens, that DNA adduct levels may be important predictive factors in genotoxicity, including neoplasia, as predicted by the somatic mutation model.

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