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Chromosomal aberrations in mouse lymphocytes exposed in vitro and in vivo to benzidine and 5 related aromatic amines

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Summary

Mouse lymphocytes were exposed in vitro for 2 h or in vivo for 24 h to benzidine and related aromatic amines to test for chromosome aberrations (CA) and mitotic indices. Uninduced mouse S9 was used to activate the amines for the in vitro tests to be consistent with the in vivo tests. Contrary to a previous report, no difference could be established in the genotoxicity of benzidine following activation with uninduced S9 compared to induced S9. There were concentration related increases in CA for benzidine and all the amines in vitro except for 4,4′-diaminostilbene which exhibited the greatest cellular toxicity towards cultured lymphocytes. Benzidine and its derivatives showed significant increases in CA in vivo compared to its negative control. The CA values for 4-aminostilbene were significantly higher than the other amines in both in vivo and in vitro studies. These genotoxicity results for 4-aminostilbene are consistent with our previous report of the pronounced CA effects in murine bone-marrow cells but would not be predicted from Salmonella mutagenicity tests.

Benzidine, because of its bifunctionality and in the case of dyes because of its contribution of extending conjugated unsaturation, was an important intermediate in the polymer and dye industries. However, the restrictions on its continued manufacture and use since its recognition as a human carcinogen (Zavon et al., 1973; Haley, 1975) has led to the utilization of benzidine analogs. Therefore, we have been interested in developing structure–genotoxicity relationships for aromatic amines related to benzidine to aid in making risk assessments of such analogs (Messerly et al., 1987; Sinsheimer et al., 1992; You et al., 1993). In these studies, it was indicated using the Ames Salmonella test that mutagenicity in vitro was promoted by increases in the electron-withdrawing ability of substituents on 4-amino-biphenyl, 4-aminostilbene and 3,3′-disubstituted benzidine derivatives. However, the results with bacteria were not predictive of genotoxicity in vivo, as followed by chromosomal aberrations (CA) in murine bone-marrow cells following intraperitoneal (i.p.) administration of these aro-

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matic amines. The most genotoxic compounds in vivo were the conjugated amines without substituents in the *para'* position. For example, 4-aminostilbene had exceptionally high CA values in vivo which would not have been predicted from the in vitro mutagenicity data.

It is the purpose of the present investigation to extend such studies of the genotoxicity of benzidine and its derivatives by using the same mammalian end-point in vitro and in vivo. In this manner it should be possible to avoid comparing in vitro bacterial to in vivo mammalian results in establishing relative genotoxicity in our series of aromatic amines.

The testing for chromosomal effects in the blood lymphocytes of humans following occupational chemical exposure is well established (Lambert et al., 1982; Galloway et al., 1986). Methods have also been developed to follow chromosomal damage in peripheral blood lymphocytes of rodents following exposure to chemicals in vivo (Kligerman et al., 1982; Rithdech et al., 1987). Cultured human blood lymphocytes after in vitro exposure to chemicals have also been examined for chromosomal damage in the absence of S9 activation (Norppa et al., 1981; Sasiadek et al., 1991) and in the presence of S9 activation (Asquith et al., 1985).

However, we have not been able to find direct comparisons in lymphocytes of chromosomal damage following in vitro versus in vivo exposure to chemicals in laboratory animals. We have previously made that type of comparison for a series of direct-acting epoxide mutagens (Das et al., 1993). In the present study, we needed to extend this approach to a series of aromatic amines that require metabolic activation prior to exhibiting genotoxicity in vitro. A useful starting point for this study is the work of Asquith et al. (1985) where liver S9 fractions were used with the promutagen benzidine to study CA effects in cultured lymphocytes. Thus, in the present investigation, in addition to benzidine, 4-aminobiphenyl, 4-amino-4'-nitrobiphenyl, 4-aminostilbene, 4,4'-diaminostilbene and 4-amino-4'-nitrostilbene were examined for CA in mouse lymphocyte cultures following direct in vitro exposure of the amines to the cultures as well as in lymphocyte cultures prepared from mice subsequent to the i.p. administration of these amines. The relative genotoxicity of these amines in vitro compared to that in vivo is of primary interest.

Materials and methods

Animals

Male C57BL/6J mice (2–4 months old; 25–30 g) were obtained from Jackson Laboratory (Bar Harbor, ME) and were provided rodent lab chow (Purina) and water ad libitum. They were kept 5 per cage and housed 4–5 days prior to the start of the experiment. Ambient temperature of the animal room was controlled at 72–74°F with relative humidity of 25–50% and a 12-h photoperiod (light cycle 0600–1800 h). Bedding consisted of sterilized hardwood chips (Bed-O'-cobs, Anderson Industrial Products Division, Maumee, OH).

Chemicals

Cyclophosphamide monohydrate (6055-19-2) (CP) and N-acetylaminofluorene (53-96-3) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO, 99.9%) and 4-aminobiphenyl (92-67-1) were obtained from Aldrich Chemical CO. (Milwaukee, WI). Benzidine (4,4'-diaminobiphenyl) (92-87-5) was purchased from Harleco (Philadelphia, PA). 4-Amino-4'-nitrobiphenyl (1211-40-1) as well as 4-amino-4'-nitrostilbene (7297-52-1), 4-aminostilbene (4309-66-4) and 4,4'-diaminostilbene (7314-06-9) were synthesized as described earlier (Sinsheimer et al., 1992).

Lymphocyte assays following in vivo exposure

Amines (25 mg/kg body weight) dissolved in DMSO (2 ml/kg) were injected i.p. into mice. Because of cell toxicity, 4-aminostilbene was also tested at 10 mg/kg. Negative control mice received only DMSO (2 ml/kg) and positive control mice were injected with CP (25 mg/kg in DMSO). 3 mice were used for each test compound and for the controls with 2 culture tubes prepared from each animal. After 24 h, mice were anesthetized with Metafane (methoxyflurane; Pitman-Moore, Washington, NJ) and blood was drawn by cardiac puncture. Culture tubes were prepared just before inoculation with blood (0.2 ml) following the method of Davisson

and Akeson (1987). Each culture tube contained 0.95 ml of supplemented medium, 0.15 ml fetal bovine serum (Gibco, Gaithersburg, MD), 0.1 ml of lipopolysaccharide (50 μg/ml; Sigma) and 0.15 ml of purified phytohemagglutinin (60 μg/ml; Burroughs Wellcome, Greenville, NC). Supplemented medium was prepared with 100 ml of medium (RPMI 1640; Gibco), 1.2 ml of 200 mM L-glutamine (Gibco) and 1 ml of a mixture of penicillin (100 IU/ml) and streptomycin (100 μg/ml) (Irvine Scientific, Santa Ana, CA).

After inoculation with blood, the tubes were incubated for 42 h in a humidified 5% CO_2 atmosphere at 37°C. Colcemid (0.3 μg, Sigma) was added 2–3 h before hypotonic treatment with KCl (0.075 M for 20 min). Then the cells were fixed in acetic acid:methanol (1:3). Samples of cell suspensions were added to precleaned slides, air-dried and chromosomes stained with diluted Giemsa (1:20) and evaluated by a single observer. A total of 100 well-spread metaphase figures (50 cells per tube) with 40 ± 2 chromosomes per animal were scored according to the criteria of Carrano and Natarajan (1988). Chromosome and chromatid aberrations were scored separately and the total percentage of abnormal cells was expressed for statistical analysis (Sharief et al., 1986). Gaps were recorded but not included in the total CA frequency. Mitotic indices (MI) were estimated from 1000 cells/animal and expressed as percentages. Test chemicals and their controls were run under the same conditions at the same time. Results for the amines were compared to those of their controls using Student's t test.

Lymphocyte assays following in vitro exposure

Blood was drawn from untreated male mice and added to culture tubes as described above. S9 was prepared from the livers of 5 male mice (C57BL/6J) and the S9 mix made according to the method of Maron and Ames (1983). For benzidine, the activation treatment was both with Aroclor 1254-induced S9 and uninduced S9 mix. All the other amines were treated only with uninduced S9. After 21 h, the original medium was replaced with fresh serum-free medium containing 10% of the mouse S9 mixture. Test amines in DMSO (0.01 ml/ml medium) were added at the concentrations indicated in Table 1. After exposure for 2 h with the chemicals in the presence of S9, the cultures were washed 3 times with fresh medium and incubated again for 20 h under the same conditions as used for the cell cultures obtained following in vivo administration of amines. Culture tubes (2 from each of 3 animals) were prepared for each concentration of the amines and for the controls. The negative control was DMSO (0.01 ml/ml medium) and the positive control was *N*-acetylaminofluorene (100 μg/ml). Scoring and analysis of CA results were as described above for the in vivo exposure experiments. Results at each concentration were compared to those of their negative control using Dunnett's test.

Results and discussion

The in vitro assay results for benzidine following activation by S9 with and without induction by Aroclor 1254 are given in Table 1. Asquith et al. (1985), in their study of CA in human lymphocyte cultures, reported a greater response for activation with uninduced S9 than for induced S9. In the present study with mouse lymphocytes, a concentration–response relationship was found with both treatments but they were not significantly different. All subsequent in vitro comparisons of the amines to benzidine were performed with uninduced S9 to parallel our in vivo tests. The results are given in Table 1. Benzidine and its derivatives, except for 4,4′-diaminostilbene, produced a significant increase ($p < 0.01$) in the percentage of CA compared to their negative controls and concentration–response relationships could be established for most of these amines. However, toxicity at the higher concentrations is a factor in narrowing this relationship for 4-aminostilbene and the two nitro compounds. Toxicity became most evident when these compounds were tested at 400 μg/ml and there were insufficient metaphase cells for evaluation. 4-Aminostilbene exhibited this toxicity at 200 μg/ml and 4,4′-diaminostilbene even at 25 μg/ml. This latter compound was the only derivative for which CA and MI results could not be established. Toxicity is a limitation in ranking these amines by the in vitro lymphocyte assay in comparison to our previous study (Sinsheimer et al.,

TABLE 1

CHROMOSOMAL ABERRATIONS IN MOUSE LYMPHOCYTE IN VITRO CULTURES INDUCED BY BENZIDINE AND RELATED AMINES

Treatment Dose (μg/ml)	Gaps [a]	Aberrations/cell		Aberrant cells [b] (%) (mean ± S.D.) [d]	Slope [c] (r^2)	Mitotic indices (%) (mean ± S.D.) [d]
		Chromatid type	Chromosome type			
Induced S9						
Solvent control						
DMSO (0.01 ml/ml)	3.33	0.020	0.000	2.00 ± 1.00		2.16 ± 0.27
Benzidine						
100	4.00	0.030	0.013	4.33 ± 0.58 **	0.017	1.55 ± 0.26 **
200	6.67	0.047	0.010	5.67 ± 1.15	± 0.002	1.41 ± 0.35
400	9.00	0.050	0.047	9.00 ± 1.00	(0.91)	1.25 ± 0.45
Positive control *N*-acetylamino-fluorene	6.33	0.043	0.007	5.00 ± 0.00 **		1.50 ± 0.41 **
Uninduced S9						
Solvent control						
DMSO (0.01 ml/ml)	3.00	0.020	0.000	2.00 ± 1.00		2.23 ± 0.37
Benzidine						
100	3.33	0.026	0.013	4.00 ± 1.00 **	0.016	1.69 ± 0.50 **
200	6.33	0.047	0.017	6.00 ± 1.00	± 0.002	1.45 ± 0.21
400	7.67	0.056	0.027	8.33 ± 0.58	(0.89)	1.29 ± 0.28
4-Aminobiphenyl						
50	4.67	0.026	0.020	4.67 ± 0.58 **	0.039	1.75 ± 0.26 *
100	6.33	0.053	0.017	6.33 ± 1.15	± 0.004	1.54 ± 0.46 **
200	8.33	0.063	0.043	10.00 ± 1.00	(0.92)	1.17 ± 0.30
4-Amino-4′-nitrobiphenyl						
50	4.33	0.044	0.000	4.33 ± 0.58 **	0.024	1.57 ± 0.20 **
100	5.33	0.047	0.007	5.33 ± 0.58	± 0.004	1.48 ± 0.35
200	6.67	0.053	0.017	7.00 ± 1.00	(0.82)	1.27 ± 0.41
4-Aminostilbene						
25	7.00	0.053	0.023	7.67 ± 0.58 **	0.083	1.51 ± 0.25 **
50	9.33	0.077	0.030	10.33 ± 0.58	± 0.016	1.38 ± 0.22
100	12.33	0.080	0.033	11.00 ± 1.00	(0.72)	1.11 ± 0.31
200	cell toxicity					
4,4′-Diaminostilbene						
25	insufficent metaphase cells					0.82 ± 0.24 **
100	cell toxicity — no division					
4-Amino-4′-nitrostilbene						
50	5.33	0.043	0.010	5.33 ± 0.58 **	0.026	1.55 ± 0.26 **
100	7.33	0.060	0.000	6.00 ± 1.00	± 0.005	1.43 ± 0.14
200	7.00	0.077	0.000	7.67 ± 1.15	(0.74)	1.28 ± 0.22
Positive control *N*-Acetylamino-fluorene	4.33	0.040	0.013	5.33 ± 1.66 **		1.43 ± 0.17 **

[a] Total chromatid and chromosome gaps per 100 cells at each concentration were recorded but not included as aberrations.

[b] Cells with at least 1 aberration. Results are for 3 animals at each concentration (100 cells/animal from 2 culture tubes).

[c] Slope ± S.D. and correlation coefficent (r^2) for the aberrant cell concentration–response curve for each compound.

[d] Lowest dose for which the mean is significantly greater than its negative control using Dunnett's test is indicated by * $P < 0.05$ and ** $P < 0.01$.

1992) in which Salmonella strains were used and 4,4′-diaminostilbene was found to be weakly mutagenic.

CA frequencies and MI values for the lymphocytes of mice following i.p. administration of benzidine and the 5 related aromatic amines were observed at a single dose of 25 mg/kg for an initial comparison of their in vivo genotoxicity. This dose was based upon the previous dose–genotoxicity relationships found for benzidine in our in vivo CA bone-marrow cell study (Sinsheimer et al., 1992). The fixation times used for the lymphocyte cultures in this study are the same as those previously employed in our epoxides study (Das et al., 1993). DeBoer et al. (1977) found for murine lymphocytes that similar fixation times resulted in predominantly 1st division metaphase cells. For a more definitive in vivo genotoxicity comparison, an optimal fixation time should be established for these compounds and the comparison should be based upon a dose–response evaluation for each compound (Preston et al., 1987).

In the present in vivo CA assay, all the compounds with the exception of 4-aminostilbene had a significant increase in the percentage of abnormal cells at 25 mg/kg when compared to the negative control ($1.67 \pm 0.58\%$). 4-Aminostilbene could not be compared at this concentration because of cell toxicity but at 10 mg/kg it had a higher percentage of CA ($7.00 \pm 1.00\%$) than any of the other amines at 25 mg/kg (3.00 ± 1.00–$5.67 \pm 1.16\%$), where for these values 4,4′-diaminostilbene had the least and 4-amino-4′-nitrostilbene had the greatest genotoxicity. These results are in comparison to $11.00 \pm 1.00\%$ for CP, the positive control. The most frequent aberrations were chromatid breaks. Benzidine and all 5 aromatic amines produced a significant decrease in MI compared to the negative solvent control.

The results of the present studies on cultured mouse lymphocytes subsequent to either in vivo or in vitro exposure to the aromatic amines were compared to our previous in vivo genotoxicity studies of the same amines (Sinsheimer et al., 1992). In general, there is an increase in observable results for the in vivo lymphocyte assay compared to the in vivo bone-marrow cell examination for CA. That is, CA values for 25 mg/kg i.p. doses in the lymphocyte assay are about twice those of the bone-marrow cell results at this dose. These results for the non-dividing lymphocytes in comparison to rapidly dividing bone-marrow cells were unexpected.

The relative order of in vitro lymphocyte genotoxicity is 4-aminostilbene > 4-aminobiphenyl > 4-amino-4′-nitrostilbene = 4-amino-4′nitrobiphenyl > benzidine. Thus, 4-aminostilbene is the most genotoxic of the present series of amines when the same lymphocyte-CA end point was used in vitro and in vivo. This was not the case when Salmonella strains were used for the in vitro evaluation of these amines. Moreover, the present study is in agreement with the pronounced in vivo genotoxicity of 4-aminostilbene as measured by its CA effects to the bone-marrow cells of mice.

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