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Genotoxic evaluation of ten carcinogens in the *Drosophila melanogaster* wing spot test

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Abstract. To provide further background data on the wing spot somatic mutation and recombination assay, 10 selected carcinogens (acetamide, acrylamide, benzo(a)pyrene, cyclophosphamide, diethylstilbestrol, 4-nitroquinoline N-oxide, propyleneimine, safrole, thiourea, and o-toluidine) were tested in this assay. 72-h-old third-instar larvae, trans-heterozygous for 2 recessive wing cell markers: *multiple wing hairs* (*mwh*) and *flare*^{3} (*flr*^{3}) were fed with 3 concentrations of each carcinogen during the rest of their development until pupation, and the genotoxic effects were measured as significant increases in the appearance of visible mutant hair clones on the adult wing blade. Our results show that 6 of the carcinogens tested produce significant increases in wing spot frequency, at least at one of the concentrations assayed. Benzo(a)pyrene, diethylstilbestrol, safrole and thiourea were the compounds that did not increase the incidence of mutant clones.
Key words. Carcinogens; genotoxicity testing; *Drosophila melanogaster*; somatic mutation.

The *Drosophila* wing spot test is a fast one-generation somatic mutation and recombination assay (SMART) detecting the induction of genetic damage in the wing tissue. This assay seems to be effective in checking various types of genetic endpoints (gene mutations, deletions and mitotic recombination), and in detecting different types of genotoxic chemicals[1]. It is based on the treatment, in larvae, of wing primordial cells contianing specific genetic markers. Later, the genetic alterations induced can be detected on the wings of the adult flies. The alterations will appear as clones of mutant cells expressing the phenotype regulated by the specific genetic marker. The wing spot test and other *Drosophila* SMART assays appear to be useful tools in the genotoxic evaluation of chemicals with *Drosophila*. These tests have been proposed as a useful complement to the sex-linked recessive lethal test (SLRLT), which detects germinal mutation, since although the SLRLT is up to now the best-validated *Drosophila* assay, the somatic assays present many advantages, being less time-consuming and allowing detection of a broad range of genetic alterations[2,3]. To validate the different somatic mutation and recombination tests it is necessary to collect information on the response of such assays to a wide spectrum of compounds of different chemical families and with different biological properties.

In this report we provide information about the response of 10 carcinogenic compounds in the wing spot test, to increase the database file and to contribute to the validation of this assay.

Materials and methods

Strains. 2 *Drosophila* strains were used: the *multiple wing hairs* strain with genetic constitution: *y*; *mwh jv* and the *flare* strain with genetic constitution: *flr*^{3}/*In*(3*LR*)*TM*3, *Ser*. Both strains were obtained by courtesy of Prof. F. E. Würgler (Zürich).

Chemicals. Acetamide (CAS 60-35-5), acrylamide (CAS 79-06-1), benzo(a)pyrene (CAS 50-32-8), cyclophosphamide (CAS 6055-19-2), diethylstilbestrol (CAS 56-53-1), 4-nitroquinoline N-oxide (CAS 56-57-5), propyleneimine (CAS 75-55-8), safrole (CAS 94-59-7), thiourea (CAS 62-56-6) and o-toluidine (CAS 95-53-4) were obtained from Sigma Chemical Co., St. Louis, Missouri (USA). The compounds were dissolved in distilled water or in Tween-80 (1%) plus ethanol (5%).

Experimental procedures. Eggs from the cross between *mwh* virgin females and *flr*^{3} males were collected during 8-hour periods, and 3 days after emergence the larvae were placed in plastic vials (50 larvae/vial) containing 2.5 ml Drosophila Instant Medium (Carolina Biological Supply Co., Burlington, North Carolina, USA) prepared with 2.5 ml of solutions of different concentrations of the carcinogens tested. For the negative controls, instant medium was prepared with distilled water or with Tween-80 plus ethanol, only. The larvae were fed on this medium for the rest of their development until pupation. Flies of the trans-heterozygous (*mwh* + / + *flr*^{3}) genotype were selected and stored in 70% ethanol. Afterwards, wings were removed and mounted in Faure's solution on slides. The wings were scored at 400 × magnification for the presence of clones of cells showing malformed wing-hairs. Such somatic spots appeared as single spots, showing either the multiple wing hairs (*mwh*) or the flare (*flr*^{3}) phenotype, and twin spots showing adjacent *mwh* and *flr*^{3} areas. 3 separate endpoints were recorded: 1) small single spots (1–2 cells); 2) large single spots (>2 cells); and 3) twin spots.

Statistical analysis. The statistical significance of the results obtained was calculated by the use of the 2-alternative-hypotheses method[4].

Results and discussion

Tables 1 and 2 show the data obtained in the *Drosophila* wing spot test for the 10 carcinogens assayed. Table 1 presents the results obtained with the 5 water-soluble carcinogens, together with their respective controls, and table 2 presents the results for the water-insoluble carcinogens. The validation of genotoxicity assays for the screening of carcinogenic compounds requires sets of experimental data from which the predictivity values can be assessed. Therefore we report here results on the sensitivity of the wing spot assay to 10 well known carcinogens. A description of the results obtained by us, compound by compound, compared with those obtained in other genotoxicity assays using *Drosophila*, is as follows:

Acetamide only induced a significant increase in the frequency of both small and large single wing spots at the highest concentration tested (50 mM). In *Drosophila*, acetamide has been reported to be negative in the SLRLT[5], although a weak mutagenic response was obtained in the *zeste-white* eye somatic mutation test[6,7].

Acrylamide also induced a small but significant increase in the frequency of wing spots. This induction affects the frequency of small and large single spots. This response agrees with the positive results previously reported by other authors using the same assay[8,9]. In the *zeste-white* somatic mutation eye system, acrylamide also showed a weak mutagenic response[7]. On the other hand, when the ability of this chemical to induce mutagenicity in the germinal cell line was tested, both negative and positive results were obtained[8,9].

Cyclophosphamide was found to be a strong inducer of single wing spots, although no increase in the frequency of twin spots was detected. A clear positive response has been reported previously in the same assay[10,11], as well as in the *zeste-white* system[7,12], and in the somatic white-ivory reversion test[13,14]. Nevertheless, in the *white/white-coral* assay, cyclophosphamide exerted only marginal genotoxic activity[15]. In the SLRLT this compound was scored as positive[16].

Propyleneimine acts as a strong mutagen in the wing spot test, inducing significant increases in the 3 genetic endpoints recorded. This behaviour agrees with the results previously reported by us in the *zeste-white* system[7] and in the *white-ivory* reversion test[17]. To the best of our knowledge these are the only reports on the genotoxicity of propyleneimine in *Drosophila*.

Table 1. Wing spot test data for the 5 water-soluble carcinogens

Compound conc. (mM)	Number of wings	Small single spots (1–2 cells) (m = 2.0)			Large single spots (>2 cells) (m = 5.0)			Twin spots (m = 5.0)			Total spots (m = 2.0)		
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.
Acetamide													
0.0	48	7	0.15		1	0.02		3	0.06		11	0.23	
20.0	36	7	0.19	i	2	0.06	i	0	0.00	−	9	0.25	i
30.0	48	11	0.23	i	1	0.02	i	0	0.00	−	12	0.25	i
50.0	84	36	0.43	+	16	0.19	+	3	0.04	−	55	0.65	+
Acrylamide													
0.0	48	7	0.15		1	0.02		3	0.06		11	0.23	
1.0	70	19	0.27	i	10	0.14	+	3	0.04	−	32	0.46	+
1.5	64	16	0.25	i	7	0.11	i	2	0.03	−	25	0.39	i
2.0	68	23	0.34	+	8	0.12	i	3	0.04	−	34	0.50	+
Cyclophosphamide													
0.0	52	17	0.33		3	0.06		2	0.04		22	0.42	
0.5	48	13	0.27	−	9	0.19	i	3	0.06	i	25	0.52	i
1.0	46	25	0.54	i	7	0.15	i	1	0.02	i	33	0.72	+
5.0	42	108	2.57	+	24	0.57	+	2	0.05	i	134	3.19	+
Propyleneimine													
0.0	40	5	0.12		1	0.02		0	0.00		6	0.15	
0.1	42	8	0.19	i	1	0.02	i	0	0.00	i	9	0.21	i
2.0	48	26	0.54	+	36	0.75	+	20	0.42	+	82	1.71	+
8.0	40	65	1.62	+	53	1.32	+	19	0.47	+	137	3.42	+
Thiourea													
0.0	48	11	0.23		1	0.02		0	0.00		12	0.25	
0.1	48	16	0.33	i	0	0.00	i	0	0.00	i	16	0.33	i
0.5	56	15	0.27	i	3	0.05	i	1	0.02	i	19	0.34	i
1.0	92	27	0.29	i	2	0.02	i	2	0.02	i	31	0.34	i

Fr., frequency; D., statistical diagnosis; m., multiplication factor; +, positive, −, negative, i, inconclusive (at the 5% level, based on the 2-alternative-hypotheses method).

Table 2. Wing spot test data for the 5 water-insoluble carcinogens

Compound conc. (mM)	Number of wings	Small single spots (1–2 cells) (m = 2.0)			Large single spots (>2 cells) (m = 5.0)			Twin spots (m = 5.0)			Total spots (m = 2.0)		
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.
Benzo(α)pyrene													
0.0	48	10	0.21		1	0.02		1	0.02		12	0.25	
5.0	48	11	0.23	i	1	0.02	i	1	0.02	i	13	0.27	i
10.0	48	14	0.29	i	0	0.00	i	1	0.02	i	15	0.31	i
15.0	52	11	0.21	i	3	0.06	i	1	0.02	i	15	0.29	i
Diethylstilbestrol													
0.0	48	9	0.19		4	0.08		2	0.04		15	0.31	
5.0	60	14	0.23	i	3	0.05	–	0	0.00	–	17	0.28	–
10.0	44	13	0.30	i	2	0.05	–	0	0.00	–	15	0.34	i
20.0	50	11	0.22	i	0	0.00	–	0	0.00	–	11	0.22	–
4-Nitroquinoline N-oxide													
0.0	36	11	0.31		1	0.03		0	0.00		12	0.33	
2.0	48	19	0.40	i	35	0.73	+	10	0.21	+	64	1.33	+
3.0	46	23	0.50	i	46	1.00	+	2	0.04	i	71	1.54	+
8.0	40	33	0.82	+	53	1.32	+	25	0.62	+	111	2.77	+
Safrole													
0.0	48	9	0.19		4	0.08		2	0.04		15	0.31	
0.1	48	12	0.25	i	5	0.10	i	0	0.00	–	17	0.35	i
0.5	96	20	0.21	i	4	0.04	–	0	0.00	–	24	0.25	–
1.0	72	14	0.19	i	1	0.01	–	4	0.06	i	19	0.26	–
o-Toluidine													
0.0	48	12	0.25		1	0.02		0	0.00		13	0.27	
1.0	44	11	0.25	i	0	0.00	–	2	0.05	i	13	0.30	i
2.0	48	15	0.31	i	0	0.00	–	0	0.00	i	15	0.31	i
5.0	42	18	0.43	i	4	0.09	i	1	0.02	i	23	0.55	+

Fr., frequency; D., statistical diagnosis; m., multiplication factor; +, positive, –, negative, i, inconclusive (at the 5% level, based on the 2-alternative-hypotheses method).

Thiourea was negative with respect to the induction of different types of spots in the wing assay. The only available data on the mutagenicity of thiourea in other somatic assays using *Drosophila* indicates that this compound induces a weak response in the *zeste-white* system[7], although it was effective in the induction of SLRL mutations[18].

Benzo(a)pyrene was unable to increase significantly the frequency of wing spots in our study. However, this compound has been reported to be effective in the same assay[2,19,20] and in the *zeste-white* system[7,21,22]. Positive results were also found when benzo(a)pyrene was tested for the induction of SLRL mutations[16]. It must be pointed out that for this polycyclic hydrocarbon the mutagenic response depends on the metabolic activity of the strain used[20].

Diethylstilbestrol does not induce significant increases in the frequency of wing spots. This result agrees with those previously reported in the same test[2,15,23], and with those found in a *Drosophila UZ*-strain carrying the excision-repair gene *mei-9* (ref. 24). In a previous study[7] we found that diethylstilbestrol induced a weak positive response in the *zeste-white* test, although only at the highest concentration tested (20 mM). In the SLRLT, this chemical produced inconclusive results when adult males were treated[2,25], while a positive response was found after larval feeding experiments[26].

4-Nitroquinoline N-oxide was found to be strongly[1] genotoxic, inducing both single and twin spots. These data agree with other positive results in the same test[11], and with those reported by us in the *zeste-white* and in the *white-ivory* systems[7,17]. 4-Nitroquinoline has also been reported to be a powerful mutagen in the SLRLT[27].

Safrole was unable to induce significant increases in the frequency of wing spots. Our results are in contrast with the positive response found by using the *flare*-strain instead of the *flare*[3]-strain[2,15,23]. In the *zeste-white* system, negative results with a *UZ*-strain deficient in excision-repair were reported[24], but a positive response was found by our group[7]. In the SLRLT safrole was negative[28,29].

o-Toluidine induces a significant increase in the frequency of total spots only at the highest concentration tested (5 mM). In the same assay, both positive and marginal results were reported after acute and chronic treatments[15,23]. In the *zeste-white* assay we reported positive results[7], while o-toluidine was negative using a *UZ*-strain carrying the excision-repair-deficient gene *mei-9*[24]. In the SLRLT this chemical was found to be negative[29].

From the analysis of the information summarized above, our results indicate that 6 out of the carcinogens studied were shown to be genotoxic, inducing significant

increases in the frequency of wing spots. Among the 4 carcinogens which led to negative results in our study, one, the polycyclic hydrocarbon benzo(a)pyrene is known to give negative or positive results depending on the *Drosophila* strain used[30], since this compound requires metabolic activation. Thus, Frölich and Würgler[20] obtained a clear positive response in the wing spot test by using a strain with high biometabolic activation. The other 3 carcinogens usually given negative responses in most of the assays testing for genotoxicity. Thus, the negative results found by us are in agreement with the expectations.

From the comparison of our results with those reported in the literature on germ cell assays in *Drosophila*, it appears that, at least for some compounds, the somatic cells of *Drosophila* are more sensitive than the germ cells. Thus the wing spot test was able to demonstrate the genotoxicity of three compounds that give negative results in the SLRLT, acetamide, acrylamide and o-toluidine, though benzo(a)pyrene, which gives positive SLRLT results gave negative results in our experiments. The ability of the wing spot test to detect genotoxic carcinogens, and even some non-genotoxic ones, confirms previous data[31] and supports the conclusion that this *Drosophila* somatic mutation and recombination assay is useful, and is sensitive enough for mutagenicity/carcinogenicty testing.

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