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Induction of an adaptive response in *Drosophila* imaginal disc cells exposed *in vivo* to low doses of alkylating agents

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The adaptive response of *Drosophila* larvae to three alkylating agents (ethyl methanesulfonate, methyl methanesulfonate and *N*-nitroso-*N*-ethylurea) has been studied in the wing spot test. The experimental procedure included a 24 h pretreatment of 2-day-old larvae with two different adaptive doses followed by a challenge dose applied until the end of development. The genotoxic effects were analysed in trans-heterozygous larvae for the third chromosome recessive markers multiple wing hairs (*mwh*) and flare (*flr*^{3}). Genetic changes induced in somatic cells of the wing imaginal discs led to loss of heterozygosity, expressed as mutant clones of the genetic markers used. From our results it appears that the adaptive doses clearly reduce the frequency of mutant clones induced by the challenge dose. As far as we know, this is the first time that the existence of an adaptive response to alkylating agents after *Drosophila* larval treatment has been reported using the wing spot assay.

Introduction

Exposure to low doses of DNA damaging agents can induce resistance to later moderate or relatively high genotoxic exposures. This phenomenon, known as an adaptive response, has already been demonstrated in organisms as diverse as bacteria, plants and animals (Samson and Cairns, 1977; Olivieri *et al.*, 1984; Rieger *et al.*, 1985; Mahmood and Vasudev, 1992; Wolff, 1992). The widespread expression of this phenomenon suggests that the adaptive response is an evolutionarily well conserved cellular defence mechanism.

Although most of the experimental studies on the adaptive response have been carried out by detecting radio-resistance, this response has also been observed after exposure to different chemical agents (Samson and Schwartz, 1983), where cross-adaptation occurred (Wolff *et al.*, 1988). This cross-resistance indicated that in addition to the first phenomenon reported (Samson and Cairns, 1977), where the response was caused by induction of an alkyltransferase that removes alkylated bases (Karran *et al.*, 1979), another repair mechanism must underlie this effect (Wolff, 1996).

The phenomenon of adaptive response has also been found in *Drosophila*. Thus, by analysing the induction of dominant lethal mutations after irradiation with X-rays, an adaptive response was observed in oocytes. This effect was detected when both repair-proficient and repair-deficient strains (*mus 302*^{D1} and *mei 41*^{D5}) were used (Fritz-Niggli and Schäppi-Büchi, 1991). These results indicate that the mechanism responsible for the adaptive response is not related to the main excision and post-replication repair mechanisms.

Since somatic mutation plays an important role in initiation of the oncogenic process (Rigaud and Moustacchi, 1996), the protective process of the adaptive response might also contribute to a reduction in cancer incidence. Although it is not clear at present whether adaptation lowers cancer rates in humans, there are some data supporting this hypothesis. Thus, pre-irradiation in Swiss mice seems to cancel induction of thymus lymphoma (Bhattarcharjee, 1996).

In *Drosophila* the somatic mutation and recombination tests (SMART) have been shown to be very useful in the study of induced genetic damage in somatic cells (Würgler and Vogel, 1986). Here we report results obtained in the wing spot assay after larval treatment with three alkylating agents, being evidence of the existence of an *in vivo* adaptive response in somatic cells.

Materials and methods

Chemicals

The mutagens used in this study were ethyl methanesulfonate (EMS) (CAS no. 62-50-0), methyl methanesulfonate (MMS) (CAS no. 66-27-3) and *N*-nitroso-*N*-ethylurea (ENU) (CAS no. 759-73-9), supplied by Sigma Chemical Co. (St Louis, MO). Just before treatment these compounds were dissolved in distilled water to the different concentrations used.

Strains

Two *Drosophila melanogaster* strains were used: the flare strain with genetic constitution *flr*^{3}/*In*(*3LR*)*TM3*, *Bd*^{S} and the multiple wing hairs strain with genetic constitution *y*; *mwh jv* (both kindly provided by Prof. F.E.Würgler, University of Zürich, Switzerland). More detailed information on the genetics and descriptions can be found in Lindsley and Zimm (1992).

Wing spot test

The wing spot test is based on loss of heterozygosity in somatic cells of larvae (Graf *et al.*, 1984). The trans-heterozygous larvae were obtained by parental crosses between *flr*^{3}/*TM3*, *Bd*^{S} virgin females and *mwh* males. Eggs were collected during 8 h periods in cultured bottles containing standard medium. The resulting larvae were fed with the different test chemicals in plastic vials containing 4.5 g of *Drosophila* instant medium (Carolina Biological Supply Co., Burlington, NC) rehydrated with 9 ml of the respective test solutions. All surviving flies were collected and stored in 70% ethanol. For the observation of mutant spots in the *mwh*/*flr*^{3} trans-heterozygous flies, the wings were removed and mounted in Faure's solution on microscope slides and both surfaces were inspected at 400× magnification for the presence of *mwh* and *flr* spots. Mutant clones were classified into three types: (i) small single spots consisting of 1 or 2 *mwh* cells; (ii) large single spots consisting of three or more cells; (iii) twin spots consisting of adjacent *mwh* and *flr* cells. We used this classification because it is biologically meaningful (Frei and Würgler, 1988).

Treatments

The experimental procedure used to detect an adaptive response was as follows. Two-day-old larvae were placed into vials containing medium rehydrated with the adaptive dose of the compounds to be tested. Twenty-four hours later, larvae were floated off with tap water, rinsed and placed in new vials with new medium prepared with the challenge dose. Distilled water was used instead of the chemicals for the control experiments. In addition, treatments lasting for 24 h (with the adaptive doses) and from 72 h until pupation (with the challenge doses) were also carried out. All experiments were performed at 25 ± 1°C and at a relative humidity of ~60%. Strict

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Table I. Wing spot test data obtained after EMS treatment

Compound conc. (mM)	Small single spots (1–2 cells) (m = 2)			Large single spots (>2 cells) (m = 5)			Twin spots (m = 5)			Total *mwh* (m = 2)			Total spots (m = 2)			Frequency of clone formation per 10^5 cells
	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Control water (48 h)	13	0.16		2	0.02		2	0.02		15	0.19		17	0.21		0.77
EMS																
0.005	11	0.14	–	3	0.04	i	1	0.01	–	12	0.15	–	15	0.19	–	0.61
0.02	18	0.22	i	3	0.04	i	0	0.00	–	21	0.26	i	21	0.26	i	1.08
Control water (72 h)	18	0.22		2	0.02		1	0.01		21	0.26		22	0.27		1.08
EMS																
0.5	38	0.47	+	13	0.16	+	5	0.06	i	50	0.62	+	56	0.70	+	2.56
Control water (48 +72 h)	19	0.24		2	0.02		0	0.00		21	0.26		21	0.26		1.08
EMS																
0.005 + 0.5	17	0.21	–	5	0.06	i	5	0.06	+	21	0.26	i	27	0.34	i	1.08
0.02 + 0.5	19	0.24	i	5	0.06	i	2	0.02	i	24	0.30	i	26	0.32	i	1.23

A total of 80 wings were examined for each concentration. No., number of spots; Fr., frequency (no./80); D., statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; i, inconclusive; m, multiplication factor; probability levels: $\alpha = \beta = 0.05$.

Table II. Wing spot test data obtained after ENU treatment

Compound conc. (mM)	Small single spots (1–2 cells) (m = 2)			Large single spots (>2 cells) (m = 5)			Twin spots (m = 5)			Total *mwh* (m = 2)			Total spots (m = 2)			Frequency of clone formation per 10^5 cells
	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Control water (48 h)	13	0.16		2	0.02		2	0.02		15	0.19		17	0.21		0.77
ENU																
0.0002	10	0.12	–	3	0.04	i	0	0.00	–	12	0.15	–	13	0.16	–	0.61
0.001	17	0.21	i	2	0.02	i	0	0.00	–	19	0.24	i	19	0.24	i	0.97
Control water (72 h)	18	0.22		2	0.02		1	0.01		21	0.26		22	0.28		1.08
ENU																
0.01	29	0.36	i	10	0.12	+	4	0.05	i	38	0.47	+	43	0.54	+	1.95
Control water (48 +72 h)	19	0.24		2	0.02		0	0.00		21	0.26		21	0.26		1.08
ENU																
0.0002 + 0.01	13	0.16	–	2	0.02	i	3	0.04	i	15	0.19	–	18	0.22	–	0.77
0.001 + 0.01	20	0.25	i	1	0.01	i	1	0.01	i	21	0.26	i	22	0.28	i	1.08

A total of 80 wings were examined for each concentration.
No., number of spots; Fr., frequency (no./80); D., statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; i, inconclusive; m, multiplication factor; probability levels: $\alpha = \beta = 0.05$.

control of temperature is important as it influences the speed of larval development and, thus, the number and size of mutant clones.

Statistical analysis

For evaluation of the induced genotoxic effects, the frequencies of spots per wing obtained in the chemically treated series were compared with the concurrent negative control. The data obtained were computed for statistical significance using a multiple decision procedure based on two alternative hypotheses (Frei and Würgler, 1988). For the statistical calculations, the conditional binomial test according to Kastenbaum and Bowman (1970) was used with 5% significance levels. The frequency of clone formation was calculated, without size correction, by dividing the number of *mwh* clones obtained per wing by 24 400, which is approximately the number of cells examined in one wing (Alonso Moraga and Graf, 1989).

Results and discussion

Tables I–III show induction of mutant spots observed after treatment with two adaptive doses and, 24 h later, with the challenge dose. In addition, the results obtained with the two types of single treatment are also shown. The different treatments were conducted in parallel, together with the concurrent negative water controls. Since there were no significant differences between the negative control values, the spontaneous frequency of total spots for the pooled data was determined, giving a value of 0.25. This frequency is within the usual range of variability previously reported in the literature (Tripathy *et al.*, 1990; Torres *et al.*, 1998).

Neither of the low doses, meant to induce an adaptive response with the three alkylating agents, caused any increase in the frequency of mutant spots, indicating that the selected doses did not exert genotoxic effects. Nevertheless, the challenge doses significantly increased the frequency of total spots. When challenge doses were applied 24 h after the adaptive doses, no significant increases in the frequency of mutant clones were found, suggesting that there was an adaptive response.

In spite of extensive experimental efforts to explain the mechanisms underlying the adaptive response, they are largely not understood. Up to now, several hypotheses have been put forward to provide a comprehensive explanation. Thus, the induction of alterations in cell cycle progression has been proposed, causing cell cycle delay, probably by signal transduction mechanisms (Meyers *et al.*, 1992). Other studies found

Table III. Wing spot test data obtained after MMS treatment

Compound conc. (mM)	Small single spots (1–2 cells) (m = 2)			Large single spots (>2 cells) (m = 5)			Twin spots (m = 5)			Total *mwh* (m = 2)			Total spots (m = 2)			Frequency of clone formation per 10^5 cells
	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Control water (48 h)	13	0.16		2	0.02		2	0.02		15	0.19		17	0.21		0.77
MMS																
0.001	14	0.18	i	3	0.04	i	0	0.00	–	17	0.21	i	17	0.21	i	0.87
0.002	12	0.15	–	1	0.01	–	1	0.01	–	13	0.16	–	14	0.17	–	0.67
Control water (72 h)	18	0.22		2	0.02		1	0.01		21	0.26		22	0.28		1.08
MMS																
0.1	22	0.27	i	9	0.11	+	3	0.04	i	34	0.42	i	40	0.50	+	1.74
Control water (48 +72 h)	19	0.24		2	0.02		0	0.00		21	0.26		21	0.26		1.08
MMS																
0.001 + 0.1	12	0.15	–	2	0.02	i	0	0.00	i	14	0.18	–	14	0.17	–	0.72
0.002 + 0.1	17	0.21	–	3	0.04	i	0	0.00	i	20	0.25	–	20	0.25	–	1.02

A total of 80 wings were examined for each concentration.
No., number of spots; Fr., frequency (no./80); D., statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; i, inconclusive; m, multiplication factor; probability levels: $\alpha = \beta = 0.05$.

no changes in the rate of cell progression to mitosis after a challenge dose, as well as that cell stage sensitivity is not a factor in the adaptive response (Wolff, 1996; Oliveira *et al.*, 1997).

Other authors have proposed that the adaptive response could be the result of protective functions, such as an increase in the expression of superoxide dismutase (SOD), a key enzyme that protects cells from the deleterious effects of superoxide radicals (Laval, 1988). However, the use of embryonic fibroblasts from transgenic mice with overexpression of human Cu-Zn SOD did not prove this assumption (Wolff, 1996).

Induction of metallothionein has also been proposed as a mechanism protecting against oxidative damage (Sato and Bremmer, 1993) and against the toxicity of alkylating anticancer drugs and other electrophilic compounds (Lazo and Pitt, 1995).

In addition to the protective mechanisms mentioned above, DNA repair mechanisms have classically been considered responsible for the adaptive response. The existence of a repair mechanism has been strengthened by experiments in which an inhibitor of poly(ADP-ribose)polymerase, an enzyme implicated in DNA strand break rejoining, prevented the adaptive response (Wiencke *et al.*, 1986: Shadley and Wolff, 1987).

Although much of the work dealing with the adaptive response has been done *in vitro*, it was also observed when animals were exposed *in vivo*. Thus, Cai and Liu (1990) showed a clear adaptive response in both somatic (bone marrow) and germ cells (spermatocytes) of irradiated mice. Such a response has also been observed in humans, although with important individual variability (Sankaranarayanan *et al.*, 1989; Tedeschi *et al.*, 1995). This variability, also observed in mice (Wojcik *et al.*, 1992), indicates that the individual genetic constitution can be a confounding factor for the adaptive response.

This *in vivo* response has also been found in germ cells of *Drosophila*, where an adaptive response was obtained after ionizing radiation exposure for dominant lethals induced in oocytes (Fritz-Niggli and Schäppi-Büchi, 1991). In that study, repair-proficient and repair-deficient strains were used and the response did not seem to be affected by their repair abilities. This would indicate that the repair mechanism involved in the adaptive response is not related to the standard post-replication (*mei 41*^{D5}) and excision repair (*mus 302*^{D1}) mechanisms of *Drosophila*. In addition, Schäppi-Büchi (1994) has also shown that the adaptive response to X-ray treatment was very dependent on the genotype of the flies tested. It appeared that the genetic factor responsible for the adaptive response was located in a specific region of the X chromosome. Our positive findings in somatic cells of *Drosophila* add to the data indicating that an adaptive response in *Drosophila* does take place *in vivo*. Moreover, the many advantages of the wing spot test in the detection of genotoxicity could be very useful in further studies on the mechanisms underlying the adaptive response.

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