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Detection of mitotic recombination and sex chromosome loss induced by adriamycin, chlorambucil, demecolcine, paclitaxel and vinblastine in somatic cells of *Drosophila melanogaster in vivo*

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The conventional *w*/*w*$^+$ eye assay in *Drosophila* has been used for the last 10 years for genotoxic evaluation of a broad number of chemicals with different mechanisms of action. Although chemicals that induce genotoxic effects by mechanisms other than covalent binding to DNA are difficult to detect. The aim of this study was the parallel detection of both mitotic recombination and X chromosome loss induced by five chemical compounds used worldwide as antineoplastic drugs using the *w*/*w*$^+$ somatic assay in *Drosophila melanogaster*. The compounds tested were the intercalating agent adriamycin (AD), the alkylating compound chlorambucil (CAB) and the spindle poisons demecolcine (DEM), paclitaxel (taxol, TX) and vinblastine (VBL). We used a cross between heterozygous females with a rod-X and a ring-X chromosome mated with *ywf* males. All four genotypes in the next generation are heterozygous or hemizygous for the *w*$^+$ reporter gene and were inspected for the occurrence of *white* clones in their compound eyes. We found differences in the induction of mitotic recombination when compared with chromosome loss. Genotoxic profiles obtained for the antineoplastic drugs studied indicate direct and indirect effects. While AD seems to be clastogenic due to its induction of X chromosome loss in XrX females; DEM, CAB and TX produced both structural chromosome aberrations through clastogenic activities and mitotic recombination through DNA interactions; the cytotoxic VBL induced rX loss only in XrY and intra-chromosomal recombination (XY) males, probably due to sister strand recombination, generating a *w*$^+$*w*$^+$ duplication and a *w*$^-$ deletion, forward mutations or small deletions at the *white* locus.

Introduction

Antineoplastic drugs are responsible for the survival of cancer patients around the world, however, like many other cancer therapeutics they may themselves cause mutation and secondary malignancies. Cancer induction is therefore a toxic consequence predicted by short-term tests of genotoxicty and should be weighed against the potential therapeutic benefits of several antitumor drugs (Anderson and Berger, 1994). It is therefore essential that effective anticancer drugs should be tested not only for their cytotoxic potential but also for their ability to disturb genomic integrity, in order to render a deeper understanding of the potential risks related to their clinical use (Tiburi *et al*., 2002).

Adriamycin (AD), an anthracycline antibiotic, is a topoisomerase-interactive agent that may induce mutations through simple DNA intercalation or via generation of oxygen free radicals (Anderson and Berger, 1994). The compound is strongly mutagenic against frameshift-sensitive strain TA98 in the *Salmonella* microsome test (Brookes, 1990) and positive in the conventional *w*/*w*$^+$ bioassay in *Drosophila* (Vogel and Nivard, 1993). Chlorambucil (CAB), a nitrogen mustard derivative used in the treatment of cancer, is a broad spectrum mutagen. CAB can both crosslink DNA in the absence of metabolism (McLean *et al*., 1980) and monoalkylate DNA following metabolic removal of one of its chloroethyl groups (Adams *et al*., 1996). CAB produces micronuclei in both spleen and bone marrow cells of rats, while structural chromosome aberrations were shown to be an end-point less sensitive to damage (Moore *et al*., 1995). CAB induces ^{32}P-labeled DNA adducts in calf thymus DNA used as a target for the direct detection of adducts (Yourtee *et al*., 1992) and also induces inter-chromosomal mitotic recombination in the *w*/*w*$^+$ assay in *Drosophila* (Vogel and Nivard, 1993). Spindle poisons may inhibit the polymerization of tubulin into microtubules and block the cell cycle during mitosis in the transition from prometaphase to metaphase and thus might be expected to induce numerical chromosome aberrations (aneuploidy). Demecolcine (DEM) exerts a selectively destructive action on rodent (basal cell) carcinomas, solar keratoses, Bowen's disease and keratoacanthomas, while sparing surrounding normal tissues (Belisario, 1965). The vinca alkaloids have been used in cancer therapy for more than 30 years (Zhou and Rahmani, 1992). Among them, vinblastine (VBL) is used in the treatment of breast and lung cancer (Maral *et al*., 1984). VBL was classed as a weakly active genotoxin in the *w*/*w*$^+$ assay and positive in the *mwh*/*flr* somatic test in *Drosophila* (Tiburi *et al*., 2002). Paclitaxel (taxol) is perhaps one of the most successful drugs used in the treatment of a variety of cancers, having been shown to be non-genotoxic in the *Drosophila* wing somatic mutation and recombination test (SMART) (Cunha *et al*., 2001). The spindle poison taxol exerts its action through stabilization of microtubules by promoting assembly, rather than disassembly, leading to the formation of non-functional microtubule bundles (Kumar, 1981; Ringel and Horwitz, 1991).

Somatic cells are an indispensable target for the study of loss of heterozygosity (LOH) produced by several mechanisms, including large deletions, mitotic recombination and chromosome loss (Lasko *et al*., 1991). LOH by mitotic recombination, an important cancer-prone mechanism, can be detected in *Drosophila* by several systems, such as the *multiple wing*/*flare wing* spot system (Graf *et al*., 1994) and the *white*/*white*$^+$ (*w*/*w*$^+$) eye mosaic test (Vogel and Zijilstra, 1987). The basic principle of the *w*/*w*$^+$ assay is the detection of phenotypically visible light spots in the red eyes of adult females, resulting

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Table I. Number of eyes, distribution of spots (frequency of spots), average clone size, clones per 10^4 cells and statistical diagnoses induced in the eyes of flies after acute (6 h) treatment with five antineoplastic drugs

Genotype[a]	Conc. (mM)	No. of eyes	No. of spots scored (frequency of spots/100 eyes)					Average clone size	Clones per 10^4	Statistical diagnoses[b] (m = 2)
			Clone size (ommatidia affected)				Total			
			1 + 2	3 + 4	5–8	>8				
Adriamycin (AD)										
F XX (+)	Control	386	22 (5.7)	1 (0.3)	2 (0.5)	4 (1.0)	29 (7.5)	4.14	7.78	
F XX (+)	0.05	374	28 (7.5)	5 (1.3)	3 (0.8)	2 (0.5)	38 (10.1)	3.37	8.56	–
F XX (+)	1	428	26 (6.1)	6 (1.4)	6 (1.4)	3 (0.7)	41 (9.6)	4.05	9.7	–
F XX (+)	3	404	25 (6.2)	5 (1.2)	2 (0.5)	0	32 (7.9)	2.69	5.33	–
F XrX (yf)	Control	358	26 (7.3)	5 (1.4)	2 (0.6)	2 (0.6)	35 (9.9)	3.08	7.53	
F XrX (yf)	0.05	318	57 (17.9)	4 (1.2)	3 (0.9)	0	64 (20.0)	2.33	11.73	+
F XrX (yf)	1	328	71 (21.6)	8 (2.4)	2 (0.6)	0	81 (24.6)	2.33	14.92	+
F XrX (yf)	3	270	72 (26.7)	13 (4.8)	21 (5.9)	5 (1.8)	106 (39.2)	3.57	31.4	+
M XY (+)	Control	300	17 (5.7)	0	0	0	17 (5.7)	2	2.83	
M XY (+)	0.05	402	29 (7.2)	5 (1.2)	1 (0.2)	0	35 (8.6)	2.26	4.92	–
M XY (+)	1	372	27 (7.3)	2 (0.5)	0	0	29 (7.8)	2.38	4.64	–
M XY (+)	3	348	25 (7.2)	3 (0.8)	2 (0.6)	1 (0.3)	31 (8.9)	3.37	7.5	–
M XrY (yf)	Control	266	27 (10.1)	0	0	0	27 (10.1)	1.93	4.9	
M XrY (yf)	0.05	334	48 (14.4)	3 (0.9)	1 (0.3)	0	52 (15.6)	2.15	8.37	–
M XrY (yf)	1	280	40 (14.3)	5 (1.8)	0	0	45 (16.1)	2.27	9.12	–
M XrY (yf)	3	358	27 (7.5)	13 (3.6)	3 (0.8)	0	43 (11.9)	2.87	8.62	–
Chlorambucil (CAB)										
F XX (+)	Control	252	15 (5.9)	6 (2.4)	2 (0.8)	0	23 (9.1)	2.71	6.45	
F XX (+)	0.5	500	42 (8.4)	8 (1.6)	5 (1.0)	6 (1.2)	61 (12.2)	3.77	11.5	–
F XX (+)	1	504	84 (16.7)	10 (1.9)	6 (1.2)	3 (0.6)	103 (20.4)	3	15.33	+
F XX (+)	3	502	186 (37.0)	24 (4.8)	8 (1.6)	2 (0.4)	220 (43.8)	2.44	26.73	++
F XrX (yf)	Control	168	20 (11.9)	5 (3.0)	1 (0.6)	3 (1.8)	29 (17.3)	3.41	14.71	
F XrX (yf)	0.5	502	180 (35.9)	15 (3.0)	5 (1.0)	2 (0.4)	202 (40.3)	2.48	23.71	++
F XrX (yf)	1	500	168 (33.6)	26 (5.2)	2 (0.4)	5 (1.0)	201 (40.2)	4.39	44.12	++
F XrX (yf)	3	502	175 (34.9)	13 (2.6)	5 (1.0)	7 (1.4)	200 (39.9)	2.82	28.09	++
M XY (+)	Control	252	18 (7.1)	2 (0.8)	0	0	20 (7.9)	2.2	2.18	
M XY (+)	0.5	380	38 (10.0)	1 (0.3)	1 (0.3)	0	40 (10.6)	2.2	5.79	–
M XY (+)	1	504	59 (11.7)	11 (2.2)	3 (0.6)	2 (0.4)	75 (14.9)	2.35	25.49	–
M XY (+)	3	502	61 (12.1)	4 (0.8)	1 (0.2)	1 (0.2)	67 (13.3)	2.39	7.97	–
M XrY (yf)	Control	220	27 (12.3)	2 (0.9)	1 (0.4)	1 (0.4)	31 (14.0)	2.55	11.23	
M XrY (yf)	0.5	500	71 (14.2)	4 (0.8)	0	2 (0.4)	77 (15.4)	2.49	9.59	–
M XrY (yf)	1	484	182 (37.6)	25 (5.2)	1 (0.2)	2 (0.4)	210 (43.4)	2.37	8.93	+
M XrY (yf)	3	502	183 (36.4)	19 (3.8)	4 (0.8)	6 (1.2)	212 (42.2)	2.54	26.82	+
Demecolcine (DEM)										
F XX (+)	Control	502	37 (7.4)	11 (2.2)	6 (1.2)	3 (0.6)	57 (11.4)	3.61	10.25	
F XX (+)	0.025	500	76 (15.2)	36 (7.2)	5 (1.0)	0	117 (23.4)	2.5	14.6	+
F XX (+)	0.05	500	93 (18.6)	30 (6.0)	11 (2.2)	9 (1.8)	143 (28.6)	4.57	32.7	++
F XX (+)	0.075	500	269 (53.8)	103 (20.6)	79 (15.8)	10 (2.0)	461 (92.2)	3.35	77.3	+++
F XrX (yf)	Control	418	38 (9.1)	11 (2.6)	2 (0.5)	3 (0.7)	54 (12.9)	2.82	9.14	
F XrX (yf)	0.025	500	73 (14.6)	29 (5.8)	3 (0.6)	3 (0.6)	108 (21.6)	3.49	18.85	+
F XrX (yf)	0.05	500	194 (38.8)	41 (8.2)	11 (2.2)	11 (2.2)	257 (51.4)	3.6	46.2	+++
F XrX (yf)	0.075	500	207 (41.4)	74 (14.8)	26 (5.2)	14 (2.8)	321 (64.2)	3.37	54.05	+++
M XY (+)	Control	502	45 (9.0)	4 (0.8)	9 (1.8)	0	58 (11.6)	2.31	6.72	
M XY (+)	0.025	500	94 (18.8)	42 (8.4)	9 (1.8)	0	145 (29.0)	2.58	18.7	+
M XY (+)	0.05	500	92 (18.4)	14 (2.8)	12 (2.4)	0	118 (23.6)	2.65	15.65	+
M XY (+)	0.075	500	223 (44.6)	102 (20.4)	113 (22.6)	14 (2.8)	452 (90.4)	4.11	92.9	+++
M XrY (yf)	Control	426	48 (11.3)	4 (0.9)	2 (0.5)	1 (0.2)	55 (12.9)	2.49	8.07	
M XrY (yf)	0.025	500	99 (19.8)	34 (6.8)	16 (3.2)	5 (1.0)	154 (30.8)	4.45	34.3	+
M XrY (yf)	0.05	500	149 (29.8)	50 (10.0)	14 (2.8)	1 (0.2)	214 (42.8)	2.71	29.05	+++
M XrY (yf)	0.075	500	193 (38.6)	92 (18.4)	95 (19.0)	14 (2.8)	394 (78.8)	4.29	84.6	+++
Paclitaxel (TX)										
F XX (+)	Control	972	64 (6.6)	13 (1.3)	7 (0.7)	12 (1.2)	96 (9.8)	4.71	11.63	
F XX (+)	0.01	806	56 (6.9)	20 (2.5)	10 (1.2)	15 (1.9)	101 (12.5)	2.71	16.26	–
F XX (+)	0.1	320	117 (36.6)	7 (2.2)	1 (0.3)	1 (0.3)	126 (39.4)	4.91	31.97	++
F XX (+)	1	250	77 (30.8)	26 (10.4)	12 (4.8)	3 (1.2)	118 (47.2)	3.19	37.64	++
F XrX (yf)	Control	606	77 (12.7)	9 (1.5)	3 (0.5)	1 (0.2)	90 (14.9)	3.9	11.26	
F XrX (yf)	0.01	794	152 (19.1)	29 (3.6)	3 (0.4)	5 (0.6)	189 (23.7)	2.78	16.54	++
F XrX (yf)	0.1	334	81 (24.2)	8 (2.4)	2 (0.6)	2 (0.6)	93 (27.8)	2.46	17.12	+
F XrX (yf)	1	296	149 (50.3)	22 (7.4)	4 (1.3)	2 (0.7)	177 (59.7)	2.75	41.11	+++
M XY (+)	Control	620	41 (6.6)	2 (0.3)	2 (0.3)	2 (0.3)	47 (7.5)	2.74	5.19	
M XY (+)	0.01	832	60 (7.2)	13 (1.6)	1 (0.1)	3 (0.4)	77 (9.3)	5.08	7.33	+
M XY (+)	0.1	330	151 (45.8)	20 (6.1)	6 (1.8)	4 (1.2)	181 (54.9)	2.61	35.79	+++
M XY (+)	1	108	45 (41.7)	16 (14.8)	1 (0.9)	4 (3.7)	66 (61.1)	3.3	50.42	+++
M XrY (yf)	Control	448	33 (7.4)	6 (1.3)	4 (0.9)	0	43 (9.6)	2.56	6.14	
M XrY (yf)	0.01	622	68 (10.9)	13 (2.1)	6 (1.0)	3 (0.5)	90 (14.5)	3.03	10.96	+
M XrY (yf)	0.1	344	154 (44.8)	34 (9.9)	16 (4.6)	5 (1.4)	209 (60.7)	2.97	45.11	+++
M XrY (yf)	1	176	84 (47.7)	26 (14.8)	5 (2.8)	2 (1.1)	117 (66.4)	2.69	44.7	+++

Table I. *Continued*

Genotype[a]	Conc. (mM)	No. of eyes	No. of spots scored (frequency of spots/100 eyes)					Average clone size	Clones per 10^4	Statistical diagnoses[b] (m = 2)
			Clone size (ommatidia affected)				Total			
			1 + 2	3 + 4	5–8	>8				
Vinblastine (VBL)										
F XX (+)	Control	268	16 (6.0)	7 (2.6)	5 (1.9)	4 (1.5)	32 (12.0)	3.58	10.35	
F XX (+)	0.025	280	22 (7.8)	9 (3.2)	3 (1.1)	7 (2.5)	41 (14.6)	5.55	20.62	–
F XX (+)	0.05[c]	178	18 (10.1)	10 (5.6)	2 (1.1)	1 (0.6)	31 (17.4)	3.42	14.89	–
F XrX (yf)	Control	212	40 (18.9)	10 (4.7)	3 (1.4)	3 (1.4)	56 (26.4)	2.98	19.33	
F XrX (yf)	0.025	366	101 (27.6)	21 (5.7)	10 (2.7)	2 (0.5)	134 (36.5)	2.98	27.48	–
F XrX (yf)	0.05[c]	142	35 (24.6)	7 (4.9)	5 (3.5)	2 (1.4)	49 (34.4)	3.9	33.64	–
M XY (+)	Control	272	10 (3.7)	0	0	1 (0.4)	11 (4.1)	1.8	1.65	
M XY (+)	0.025	290	24 (8.3)	5 (1.7)	3 (1.0)	0	32 (11.0)	3.64	10.35	+++
M XY (+)	0.05[c]	160	24 (15.0)	8 (5.0)	4 (2.5)	0	36 (22.5)	2.31	12.99	+++
M XrY (yf)	Control	160	14 (8.7)	4 (2.5)	0	1 (0.6)	19 (11.8)	2.41	6.4	
M XrY (yf)	0.025	256	38 (14.8)	14 (5.5)	3 (1.2)	2 (0.8)	57 (22.3)	3.29	18.63	+
M XrY (yf)	0.05[c]	178	40 (22.5)	7 (3.9)	7 (3.9)	3 (1.7)	57 (32.0)	3.79	30.34	++

[a]F, female; M, male.
[b]Statistical diagnosis according to Frei and Würgler (1988): +, positive; –, negative; ++, $P < 0.01$; +++, $P < 0.001$; m, multiplication factor for the assesment of negative results; χ^2 test for proportions.
[c]Cytotoxic.

from LOH and expression of the reporter gene *white* in female genotypes heterozygous for this marker (Vogel and Nivard, 1993). The use of multiple inverted X chromosomes, which in XX cells suppress recombination between the two X chromosomes, identified homologous (inter-chromosomal) mitotic recombination as the predominant cause generating loss of the w^+ reporter gene (Vogel and Szakmary, 1990). Recently, a novel method for the parallel monitoring of homologous mitotic recombination (inter-chromosomal), intra-chromosomal recombination and structural chromosome aberrations has been developed (Vogel and Nivard, 1999). The assay is based on the generation of four regular distinct genotypes, which can be recognized by their phenotypes. This study focused on the ability of several antineoplastic drugs to induce mitotic recombination and X sex chromosome loss in the *in vivo white/white*$^+$ eye somatic assay in *Drosophila melanogaster*.

Materials and methods

Chemical compounds and concentrations tested

AD (CAS no. 23214-92-8), CAB (CAS no. 305-03-3), DEM (CAS no. 477-30-5), taxol (TX) (CAS no. 33069-62-4) and vinblastine sulfate (VBL) (CAS no. 143-67-9) were purchased from Sigma (St Louis, MO). The chemicals were dissolved in a mixture of 1% Tween-80 and 3% ethanol immediately prior to use. The final concentration of this solvent mix was 4%. The higher exposure dose was determined as the LD_{50} and two additional lower doses were tested.

Somatic assay

Wild-type (Hague-79) females were mated with rX, R(1)2; *y* (*yellow*) *f* (*forked*)/y^+ Y males. Virgin F_1 females heterozygous for a ring-X and a rod-X chromosome were mated with *ywf* males (for a description of the genetic markers see Lindsley and Zimm, 1992), generating in the F_2 four regular classes: XX, $y^+w^+f^+$/*ywf* female (wild-type phenotype); XY, $y^+w^+f^+$/Y male (wild-type); XrX, *yw*$^+$*f*/*ywf* female (*yellow*, *forked*); XrY, *yw*$^+$*f*/Y male (*yellow*, *forked*). All four regular genotypes are heterozygous or hemizygous for the w^+ reporter gene and thus express the red wild-type color in their eyes. Loss of the w^+ reporter gene in *w*/w^+ (XX or XrX) or in w^+ (XY or XrY) pre-ommatidia cells during larval development leads to *white* clones which become visible in the adult fly (Vogel and Nivard, 1999). Besides these four regular F_2 classes, five additional genotypes are expected from double crossovers in ring/rod heterozygotes and from rX loss. The frequency of these exceptional flies in the present study in unexposed cultures was 0.016 (60 flies among a total progeny of 3690 flies). According to Vogel and Nivard (1999) these exceptional progeny have to be excluded from the scoring procedure.

Visible light spots in the red compound eyes of the four regular phenotypes are produced by several mechanisms. (i) LOH in XX females by homologous inter-chromosomal recombination between the two rod-X chromosomes. (ii) In all three genotypes carrying the w^+ allele several genetic end-points can be produced: unequal sister strand recombination generating a w^+w^+ duplication and a w^- deletion (intra-chromosomal recombination), forward mutations and small deletions at the *white* locus. Thus LOH predominantly monitors homologous recombination in XX females while it represents rX loss due to breakage events in XrX females (Vogel and Nivard, 1999).

Test protocol and data analysis

All crosses were set up as mass cultures, with 50 pairs of flies/bottle. Chemicals were administered by acute treatment. Eggs from crosses were collected for 6 h in culture bottles containing a solid agar base (5% w/v agar in water) covered with a 5 mm layer of live baker's yeast supplemented with sucrose. Three days later, larvae were collected by washing them out with an aqueous solution of 20% sucrose and seeded in bottles (500 larvae/bottle) containing 50 ml of standard medium and 2 ml of the solvent mixture with the test solution. Larvae were fed on the above medium for 6 h, then transferred to fresh medium until the end of development. Newly hatched females were scored 1–5 days later. Adult females were heterozygous for *white* and were inspected for the occurrence of *white* in their compound eyes. More details about the test procedure are given in Vogel and Nivard (1993). Spots separated from each other by at least four non-mutated ommatidia were counted as independent clones. Proliferation of pre-ommatidia cells in the imaginal discs of developing larvae increases the number of target cells from ~20 at the end of first instar, to 100–150 cells in second instar, reaching a final number of 780–800 pre-ommatidia cells at the end of third instar (Becker, 1976). Thus, primordial cells of the adult compound eye divide continuously throughout the larval period. Mosaic white clones induced in early larvae will be large but less frequent, whereas those produced later will be successively smaller and more frequent, as the number of potential target cells increases with larval age. The size of the white clone and their distribution among size classes therefore provides information on the time of induction of a LOH event (Vogel and Nivard, 1999).

For each experiment a concurrent control was run, in which larvae were treated with the solvent mixture alone. For evaluation of the genotoxic effects recorded, the frequency of mosaic eyes of the treated series was compared to its concurrent negative control series. These statistical comparisons were done using the χ^2 test for proportions. Statistical analyses were done exclusively for the total number of spots recovered. To test the alternative hypothesis (H_A) the parameter m = 2 (multiplication factor) was used due to the relatively high spontaneous incidence of total spots (Frei and Würgler, 1988).

Results

Detection of mitotic recombination and X chromosome loss induced by the five antineoplastic drugs was assayed in two acute and independent experiments. The data from each

experiment were compared and analyzed before being pooled for statistical testing by Fisher's exact test ($P < 0.05$) (Statistica Program version 6.0). Table I shows the results with the five compounds in this novel somatic *w/w*$^+$ assay in *D.melanogaster*.

The effects induced by all five antineoplastic drugs were mainly related to increases in the frequencies of small spots (1–2 ommatidia affected). Large spots (>2 ommatidia affected) were by far less frequent; the ratio between small and large spots varied between 0.94 and 19.0. Size classes are related to the number of cell divisions that had occurred between the time of clone induction in the imaginal larval cells and the beginning of eye differentiation. In acute treatments the number of cell divisions before pupation was between 2 and 3. Average clone size as a measure of genotoxicty was between 2.15 and 4.91 in all genotypes and concentrations tested (Table I).

The total number of spots per 100 eyes induced by AD were not significantly above solvent control levels for XX females and XY and XrY males. In contrast, for XrX females the results obtained were positive at all concentrations tested (Figure 1).

Spot frequencies obtained after exposure to CAB are similar for XX and XrX female genotypes and XrY males, thus homologous mitotic recombination and late rX loss, but clearly not intra-chromosomal events, dominate the activity of this alkylating agent. Thus mutations at the *white* locus do not measurably contribute to the observed activities; otherwise spot frequencies in XY males should have been higher (Figure 2).

DEM induced LOH by homologous inter-chromosomal recombination in XX females, indicating efficient induction of DNA damage. It also induced intra-chromosomal recombination, as can be seen from the significant spot induction in XY males. In addition, DEM induced rX loss due to chromosome breakage events in both XrX females and XrY males. The genetic profile of DEM appeared to be more recombinagenic than clastogenic (Figure 3).

TX induced structural chromosome aberrations more efficiently in both XrY males and XrX females through its clastogenic activity. It also induced inter-chromosomal recombination as well as mitotic intra-chromosomal recombination. For both end-points males seemed to be more affected than females (Figure 4).

VBL induced frequent irregularities of the compound eye, reduced survival of the four types of larvae and several malformations at the highest LD_{50} concentration assayed. It appeared to be clearly positive for intra-chromosomal recombination as well as for the induction of rX loss in males, while at all concentrations tested it was negative for the XX and XrX female genotypes (Figure 5).

Since large spots are rare events, and in order to make the differences between the activity patterns of the antineoplastic drugs more visible, several size classes were grouped together into three larger groups: group 1 (all spots >8 ommatidia), group 2 (spots between 3 and 8 ommatidia) and group 3 (1–2 ommatidia affected). An average of all dose groups was obtained before they were pooled, then corrected for spontan-

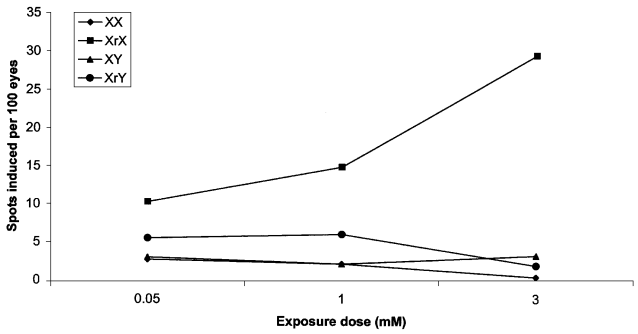

Fig. 1. Frequencies of total white spots induced by AD.

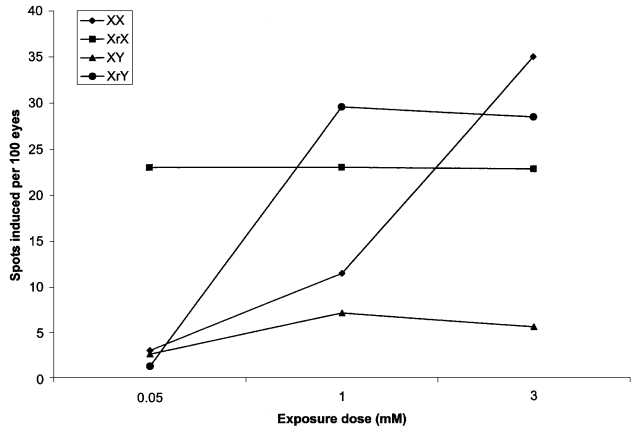

Fig. 2. Frequencies of total white spots induced by CAB.

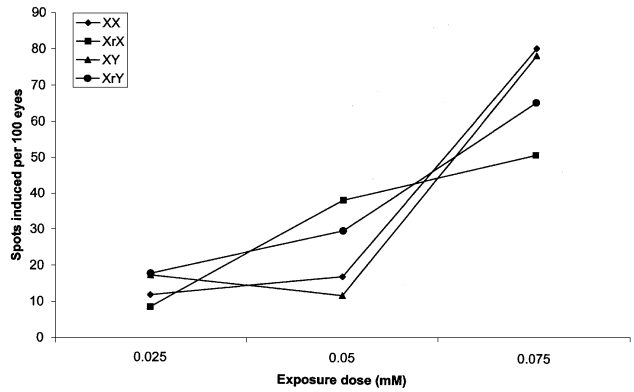

Fig. 3. Frequencies of total white spots induced by DEM.

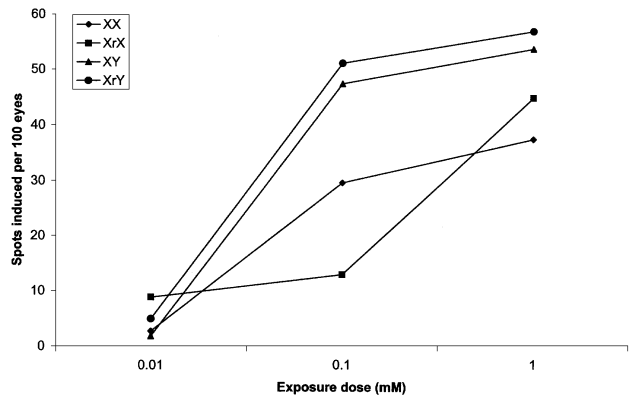

Fig. 4. Frequencies of total white spots induced by TX.

eous events in parallel controls. The results of these analyses showed a similar general response for both female and male genotypes (Figure 6). The genetic end-point induced by AD was only significant for rX chromosome loss in females. DEM, CAB and TX were very efficient in the induction of both rX loss due to breakage events and homologous inter-chromosomal mitotic recombination producing LOH in females. In addition, VBL was shown to be active only in male genotypes, producing a positive response for structural chromosome aberrations (XrY) and intra-chromosomal recombination, mutations and small deletions at the *white* locus (XY).

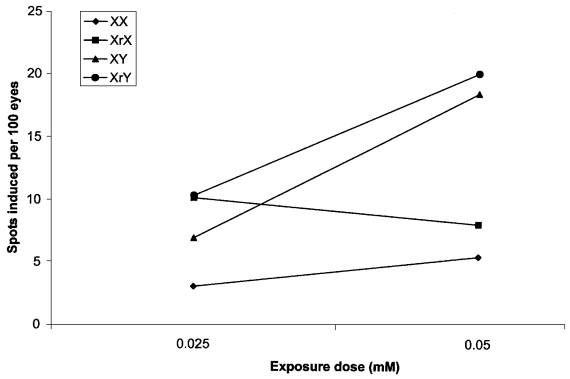

Fig. 5. Frequencies of total white spots induced by VBL.

Discussion

The conventional w/w^+ assay seems to be a good predictor of the genotoxicity of compounds with different mechanisms of interaction with DNA, while positive spindle poisons with an aneuploidogenic effect have typically shown weak effects (Vogel and Nivard, 1993). This behavior was found with the micronucleus antagonist chloral hydrate (Zordan *et al*., 1994), VBL, vincristine and vinorelbine, which induce significant increases only in the frequency of single (1–2 cells) spots; large spots were not produced (Tiburi *et al*., 2002). Chemicals which are able to induce genotoxic effects by mechanisms other than covalent binding to DNA are of special interest. Thus, genotoxicity tests able to detect drugs that cause genetic damage by interaction with other cellular targets, such as enzymes and microtubules, are particularly interesting because they play a significant role in DNA replication or in segregation of chromosomes during cell division. The w/w^+ assay for parallel monitoring of both mitotic recombination and clastogenicity in somatic cells *in vivo* in *Drosophila* seems to separate the two processes from each other (Vogel and Nivard, 1999).

In this paper we report genotoxic damage induced by several chemotherapeutic agents which act on different targets, as well as topoisomerase (AD), spindle poisons (DEM, TX and VBL) and DNA (CAB). The events that cause visible light spots in the four regular F_1 phenotypes are: (i) LOH in XX females by homologous inter-chromosomal recombination between the

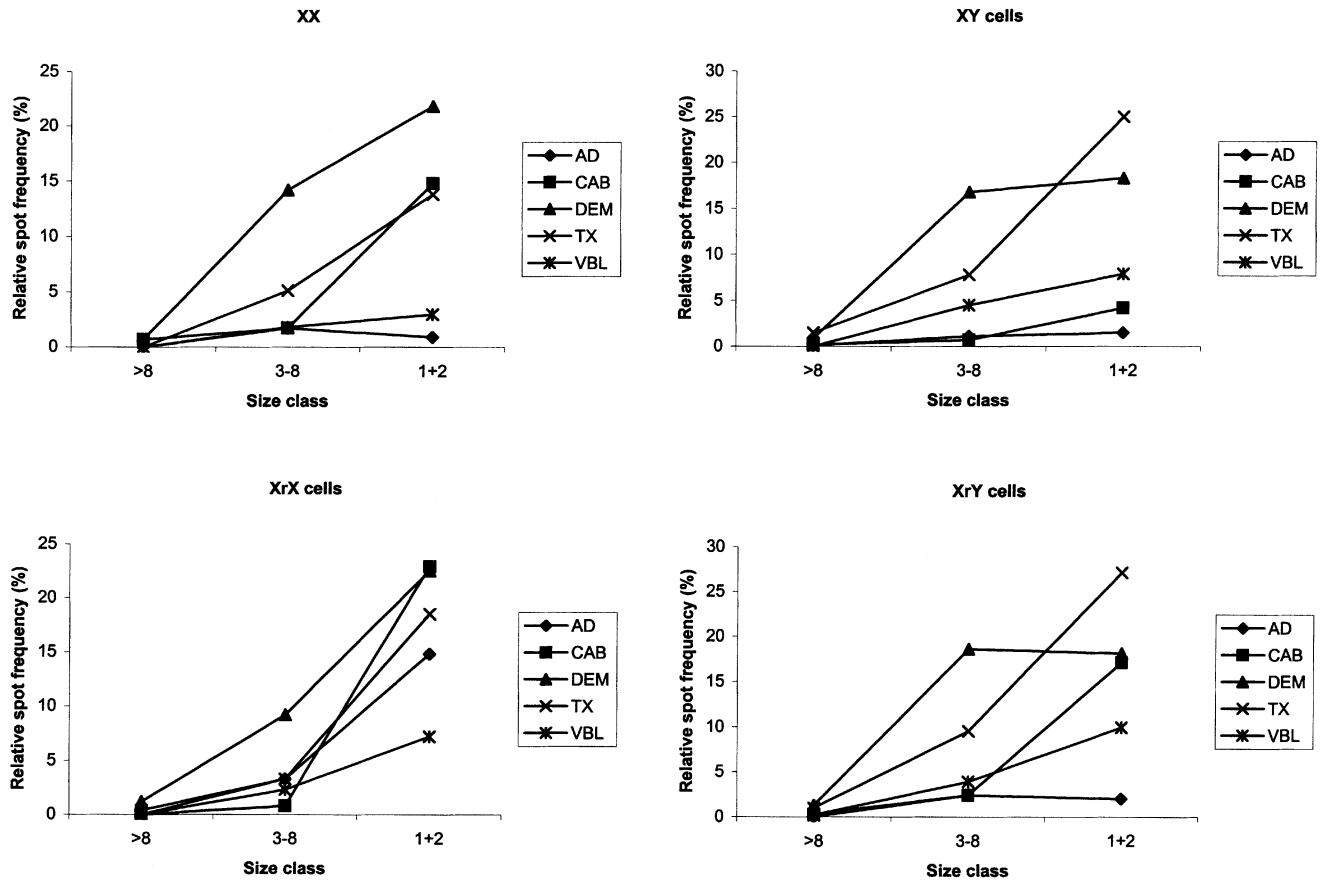

Fig. 6. Distribution of white spots induced per chemical in XX, XrX, XY and XrY cells (several size classes are grouped together).

two rod-X chromosomes; (ii) unequal sister strand recombination generating a w^+w^+ duplication and a w^- deletion (intra-chromosomal recombination), forward mutations or small deletions at the *white* locus in all three genotypes carrying the w^+ allele on their rod-X chromosome; (iii) structural chromosome aberrations and sister-chromatid exchanges in cells carrying a ring-shaped chromosome, resulting in its loss. Whole ring-X chromosome loss as a result of clastogenicity is cell lethal when generated at or shortly after the blastoderm stage, however, if rX loss is induced in preommatidia cells of third instar larvae, viable clones visible as small white patches in the red compound eye are generated (Vogel and Nivard, 1999). In somatic cells, ring chromosome loss may result either from chromosome breakage or from sister-chromatid exchanges (Ashburner, 1989). In XrX cells mitotic recombination between rX and the rod-X in heterochromatin, loss of the dicentric element and death of the rX/0 cell (Becker, 1976) might produce a light spot. Another cause of rX loss could be non-disjunction, resulting in aneuploidy. With the antineoplastic drugs analyzed a relatively low clone induction, generally no more than a 2- to 4-fold increase in the number of mosaic spots, was found in this study. In addition, with the three spindle poisons tested clone induction was always associated with cytotoxicity: reduced survival and frequent irregularities of the structure of the compound eye. This effect was also observed with five spindle poisons in the conventional w/w^+ assay (Vogel and Nivard, 1993).

The direct and indirect genotoxic potential of antineoplastic drugs, evaluated by means of LOH of the reporter *white* gene, could be predominantly due to whole X chromosome loss (AD, CAB, TX and VBL) as well as through mitotic inter-chromosomal recombination and intra-chromosomal recombination (CAB, DEM, TX and VBL).

Both chromosome breakage events and sister chromatid recombination remain as major rX loss generating mechanisms. If it occurs in the ring a dicentric ring is formed which will be eliminated leading to single *white* clones, presuming they are viable (Becker, 1975). The distribution of small spots among the size classes revealed strict correlations of spot size and larval age: the later the treatment the smaller the size of the *white* clone induced by the compound. Small spots must represent independent events because there are no more than two or three mitotic divisions left for the production of visible *white* patches in the context of red eyes. In order to avoid an underestimation of the total induced spot frequency, particularly at higher dose levels (data not shown, but calculated), the frequency of spots per eye, rather than the frequency of mosaic eyes, was determined (Vogel and Nivard, 1999).

Mitotic aneuploidy may contribute to tumorigenesis by facilitating loss of a chromosome involving tumor suppressor genes that harbor oncogenes (Pihan and Doxsey, 1999; Duesberg *et al*., 1999). Chemicals that can interact with the spindle apparatus or interfere with spindle function, preventing normal segregation of chromosomes or chromatids (Bourner *et al*., 1998), are proven carcinogens (Oshimura and Barret 1986; Dellarco *et al*., 1985).

Direct DNA intercalation induced by AD (Anderson and Berger, 1994) could be the mechanism underlying the genotoxic effect observed in XrX females. LOH due to homologous mitotic recombination was not observed, in contrast to the results obtained by Vogel and Nivard (1993) in the conventional w/w^+ assay.

CAB produced structural chromosome aberrations more consistently through chromosome breakage events than mitotic recombination through its DNA crosslinking activity (Moore *et al*., 1995; Yourtee *et al*., 1992). Thus CAB is more clastogenic than recombinogenic. The recombinogenic activity responsible for induced somatic cell recombination in *Drosophila* observed for CAB is in agreement with the results previously reported in the conventional w/w^+ assay (Vogel and Nivard, 1993).

The data obtained with DEM seem to indicate that this antitubulin compound induces mitotic intra- and inter-chromosomal recombination equally, as well as rX loss. The type of response for both genetic end-points was very similar between male and female genotypes (Figure 6). DEM has been shown to be active without requiring any cytochrome P450 isoform (Dvorak *et al*., 2000).

While TX was found to be non-genotoxic in the wing somatic assay in *Drosophila* in the standard cross as well as in the higher bioactivation cross (Cunha *et al*., 2001), in the bone marrow micronucleus test TX give a strong positive response. Some of the micronuclei induced by this antineoplastic drug are either large or of aberrant morphology (Tinwell and Ashby, 1994) and may be associated with metaphase spindle disturbances and consequently with an aneugenic action of this inhibitor of tubulin disassembly. In our study TX was found to be a potent inducer of both mitotic recombination as well as X chromosome loss (Figure 4), with a very similar dose–response relationship for male and female genotypes. It is well known that biotransformation mediated by cytochrome 450 enzymes leads to the generation of active metabolites, in particular, TX has been shown to undergo two pathways of metabolism; the major one leads to 6-hydroxylation catalyzed by CYP2C8 (Cresteil *et al*., 1994) and a minor human metabolite results from hydroxylation of the lateral chain by CYP3A (Royer *et al*., 1996). The constitutive overexpression of CYP6 subfamily genes in resistant *Drosophila* strains (Waters and Nix, 1988; Brun *et al*., 1996; Ffrench-Constant *et al*., 1999), which is similar to the CYP3 subfamily in humans (Aoyama *et al*., 1989), could be responsible for the LOH observed.

Spindle poisons such as VBL are non-DNA targeting mutagens and do not damage, intercalate in or form adducts with DNA directly, as evidenced by their non-mutagenicity in bacterial and most mammalian gene mutation assays (Dickins *et al*., 1985; Galloway and Ivett, 1986; Mortelmans *et al*., 1986). VBL induced somatic recombination in the wing somatic assay while the vinca analogs vincristine and vinorelbine were shown to induce mutagenic and recombinagenic events almost equally (Tiburi *et al*., 2002). In this study VBL did not produce inter-chromosomal recombination or X chromosome loss in females, although it was active for inter-chromosomal recombination and whole X chromosome loss in males. In the *white-ivory* test in *Drosophila* VBL induces non-disjunction (Clements *et al*., 1990).

Our data indicate that the genotoxicity of spindle poisons is due to their clastogenic effects, an activity that is expected for compounds that are potent inhibitors of mitotic cellular division. Microtubules play an important role in cell proliferation and inhibition of microtubule dynamics appears to be the mechanistic basis underlying the antitumor effects of most antimitotic compounds. Coupled with their chemical efficacy in cancer chemotherapy, spindle poisons seem to disturb the integrity of the genome, mainly inducing loss of whole chromosomes. The responsiveness of the antineoplastic drugs

studied by the eye *w*/*w*$^+$ method for parallel monitoring of mitotic recombination and clastogenicity could be a result of their effectiveness as aneuploidogens. The importance of aneugenic events in the expression of recessive deleterious genes and in the development of cancer is well documented (Oshimura and Barrett, 1986; Cimino *et al*., 1986; Tinwell and Ashby, 1994).

In summary, from the results obtained in this study it has been shown that the *in vivo w*/*w*$^+$ eye somatic assay for parallel monitoring of mitotic recombination and clastogenicity seems to be a good predictor of the mechanisms underlying genotoxic effects and thus for determination of the genotoxic profile induced by cancer therapeutic drugs.

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