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Dominance of resistance to the alkylating agent 1,2:5,6-dianhydrogalactitol in P388 mouse lymphoma hybrid cells

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Summary. Cultured P388/S mouse lymphoma cells resistant to 5-bromodeoxyuridine (BUdR) and deficient in thymidine kinase (TK^-) were fused with P388/DAG cells resistant to 1,2:5,6-dianhydrogalactitol (DAG), an anticancer alkylating agent, and to 6-thioguanine (6-TG) and deficient in hypoxanthine phosphoribosyl-transferase ($HPRT^-$). Sensitivity to DAG in the hybrid line was very close to that in the P388/DAG line, which means that resistance to DAG was inherited in a quasi-dominant manner. Hybrid cells showed cross-resistance, similar to that of the DAG-resistant line, to two other hexitols, dibromodulcitol (DBD) and disuccinyldianhydrogalactitol (DisuDAG).

Introduction

Cytotoxic hexitol derivatives represent a group of sugar alcohol compounds with alkylating properties [21, 22] and antitumor effects against experimental tumors [24, 27, 30, 31, 33, 38] and human malignancies [5, 11, 12]. The most effective compounds of this group are dibromomannitol (DBM, myelobromol) [9], dibromodulcitol (DBD) [5, 11, 12, 37] and 1,2:5,6-dianhydrogalactitol (DAG) [1, 10, 11, 27]. Two new derivatives, diacetyldianhydrogalactitol (DiacDAG) [29] and disuccinyldianhydrogalactitol (DisuDAG) [14] are under clinical trial. Resistance and cross-resistance studies have been carried out on experimental tumors with hexitol derivatives [16, 17]. A subline of the P388 mouse lymphoma resistant to DAG was recently induced [3].

Intra- and interspecific hybrids of various cell lines have been widely used to reveal the molecular mechanisms underlying the acquisition of resistance to various drugs [13, 15, 18, 26, 35]. Cultured P388 lymphoma and its resistant sublines are frequently used for drug sensitivity and cross-resistance studies [4, 40, 42–44]. In the present work, sensitive P388/S cells were hybridized with DAG-resistant P388/DAG cells; the resultant hybrid cells were checked for sensitivity to DAG, DBD, and DisuDAG. It was shown that the resistance to DAG in the hybrids was of a quasi-dominant character. The DAG-resistant parent line and the hybrid line showed cross-resistance to DBD and DisuDAG.

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Materials and methods

Cell culture. P388 mouse lymphoma was obtained from I. Wodinsky (Arthur D. Little Inc., Cambridge, Mass., USA) and maintained in DBA/2 mice. The DAG-resistant subline was induced by giving increasing doses of DAG to mice bearing P388/S-sensitive tumor [3]. Both the sensitive and the DAG-resistant P388/DAG tumors were established in culture. Culture conditions were the same as those described previously [33]. Briefly, cells were grown in Fischer medium (GIBCO) containing 20% horse serum (Phylaxia, Hungary) in static suspension culture. Both lines were subcultured twice a week. Doubling times were 12 h and 16 h for P388/S and P388/DAG cells, respectively.

Cell fusion. Cell fusion was carried out according to the methods of Davidson and Gerald [8] and of Vaughan et al. [41], with a slight modification [34]. Enzyme-deficient sublines were produced by treatment of P388/S and P388/DAG cells with 100 μg/ml ethyl-methane sulfonate (EMS) for 24 h. The cells were then centrifuged and plated in semisolid agar medium containing 10 μg/ml BUdR for TK^- mutants and 5 μg/ml 6-TG for $HPRT^-$ mutants. Surviving clones from both cultures were isolated with finely drawn pipettes and grown into mass cultures in media containing the selective agents. TK^- and $HPRT^-$ cells (2×10^7 each) were mixed and pelleted, and 2 ml 50% polyethylene glycol (PEG 6000, FLUKA) solution was layered carefully over the pellet and mixed gently for 3 min. Thereafter, 1, 2, 4, 8, and 16 ml serum-free medium were added at 1 min intervals and the mixture was gently resuspended after each dilution. Following centrifugation, the cells were washed and spun down twice in complete medium, then resuspended in a medium containing 5×10^{-5} *M* hypoxanthine, 4×10^{-7} *M* aminopterin, and 8×10^{-5} *M* thymidine (HAT medium) plus 0.25% agar and distributed into plastic petri dishes (NUNC). The formation of fused bi- and polynuclear cells was checked with aceto-orcein. The dishes were kept in a CO_2 incubator (HERAEUS) for 8–10 days. Under these conditions parental TK^- and $HPRT^-$ cells were destroyed, because they were incapable of using preformed nucleic acid precursors and their de novo nucleic acid synthesis was blocked by aminopterin. The true hybrid cells could survive in the HAT medium. Some colonies were picked up and grown to mass cultures. TK-deficient, HPRT-deficient, and hybrid lines were per-

manently kept in selective medium to avoid revertants by back mutation in the deficient lines or segregation in the hybrid line.

Chromosome spreads. Cells were treated with 0.1 μg/ml colcemid (CIBA) for 2 h and hypotonized in 0.15% NaCl solution, and the air-dried preparations were stained with Giemsa solution.

Isolation of tetraploid P388/S cells. Diploid P388/S cells (5×10^4 cells/ml) were treated with colcemid (GIBCO) at doses of 0.03–0.12 μg/ml for 20 h, then spun down and resuspended in fresh medium. Cells were then plated in soft agar at 200 cells/50-mm-diameter dish. After 10 days, eight surviving clones were picked up with Pasteur pipettes and grown to mass cultures. One clone, which seemed to be cytologically tetraploid, was designated P388/TS and used for comparison with the tetraploid hybrid cells.

Dose-response studies. Sensitivity of the cells to the drugs was assessed with the colony-forming ability method [33]. Cells were treated with different doses of the drugs for 1 h (DAG) or continuously, i.e. the drugs (DBD, DisuDAG) were left in contact with the cells throughout the experiments. Nontreated and drug-treated cells were plated in plastic petri dishes (50 mm diameter) (NUNC) in medium containing 0.25% agar (Noble agar, DIFCO) and incubated for 12 days in an atmosphere of 7% CO_2 in air in the CO_2 incubator. Each dose was studied in triplicate dishes, and each experiment was repeated three times. The drug concentrations needed to decrease the survival to 50% and 10% were inhibition dose 50 (ID_{50}) and 10 (ID_{10}), respectively.

Enzyme assays. For the measurements of enzyme activities, the cells were spun down at 1000 rpm and 0° C and washed with Hanks' solution (repeated three times). The cells were then sonicated and centrifuged at 105,000 *g*. The activity of thymidine kinase, determined according to Klemperer and Haynes [25] and described previously [39], was measured in the cytosol. The reaction mixture of a 0.5 ml final volume contained 0.2 ml cytosol. Incubation was carried out at 37° C in the presence of 20 μ*M* thymidine, 10 m*M* TRIS-HCl buffer (pH 8.0), 2.5 m*M* $MgCl_2$, 5 m*M* ATP, and 3.7×10^7 Bq ^{14}C-thymidine (2.07×10^9 Bq/mmol). At different intervals of incubation period, aliquots were put on Whatman DE 81 discs. Thymidylate synthetase activity was estimated by the method of Roberts [36], with a slight modification (Hullán et al., to be published). The reaction mixture contained 4.5 nmol [^{3}H]-dUMP (22 kBq), 6 nmol tetrahydrofolate, 660 nmol formaldehyde, 1.5 μmol 2-mercaptoethanol, 7.5 μmol NaF, 0.25% bovine serum albumin, 7.5 μmol TRIS-HCl buffer (pH 7.5), and 0.03–0.09 ml cytosol. Radioactivity was measured in an Intertechnique SL 30 liquid scintillation spectrometer. The protein content of the cytosol was measured by the method of Hartree [19].

Chemicals. The hexitol compounds DAG, DBD, and DisuDAG were the products of the Chinoin Pharmaceutical Works, Budapest, Hungary. EMS, 5-BUdR, 6-TG, thymidine, hypoxanthine, and aminopterin originated from Sigma; PEG 6000 was obtained from Fluka.

Results

Chromosome studies

Cytogenetic properties of the P388 lines, sublines, and mutants were different (Table 1). The modal chromosome numbers of the P388/S and P388/ST were 39 and 78, respectively, and that of the P388/DAG line was 40. The modal number of TK$^-$ and HPRT$^-$ mutant lines was 38, and that of the hybrid was 76.

A large metacentric marker chromosome (M) was found in the P388/S line and two were found in the tetraploid line (Fig. 1 A, Table 1). A large submetacentric marker (SM_I) was present in the P388/DAG cells (Fig. 1 B, Table 1). Two new markers appeared in the HPRT$^-$ mutant cells: a submetacentric marker (SM_{II}) with a p:q ratio different from that of the SM_I marker, and a metacentric marker with a long centromeric region (MC) (Fig. 1 C). In the hybrid clone, all four markers of the parent mutant lines were observed (Fig. 1 D, Table 1).

Activity of TK and TS enzymes

The TK activity of the TK-deficient cells decreased to 1%, and the TS activity of the same cells increased to 182% compared with the TK and TS activities of the HPRT-deficient cells. The activities of the TK and TS enzymes in the hybrid cells were 13.5% and 80.6%, respectively, relative to those of the HPRT$^-$ cells (Table 2).

Drug sensitivity

The dose-survival curves of the sensitive P388/S and P388/ST, the DAG-resistant P388/DAG, and the hybrid cells obtained after 1 h DAG treatment were all of the sigmoid type, with a shoulder region (Fig. 2). P388/DAG

Table 1. Cytogenetic characteristics of P388 cell lines

	P388/S	P388/TS	P388/S TK$^-$	P388/DAG	P388/DAG HPRT$^-$	Hybrid
Modal chromosome number	39	78	38	40	38	76
Markers[a]:						
SM_I	–	–	–	93%	98%	97%
M	100%	100%	99%	–	–	100%
MC, SM_{II}	–	–	–	–	97%	96%

[a] Marker chromosomes: SM_I, submetacentric; M, metacentric; MC, metacentric, with a long centromeric region; SM_{II}, submetacentric, with p:q different from SM_I

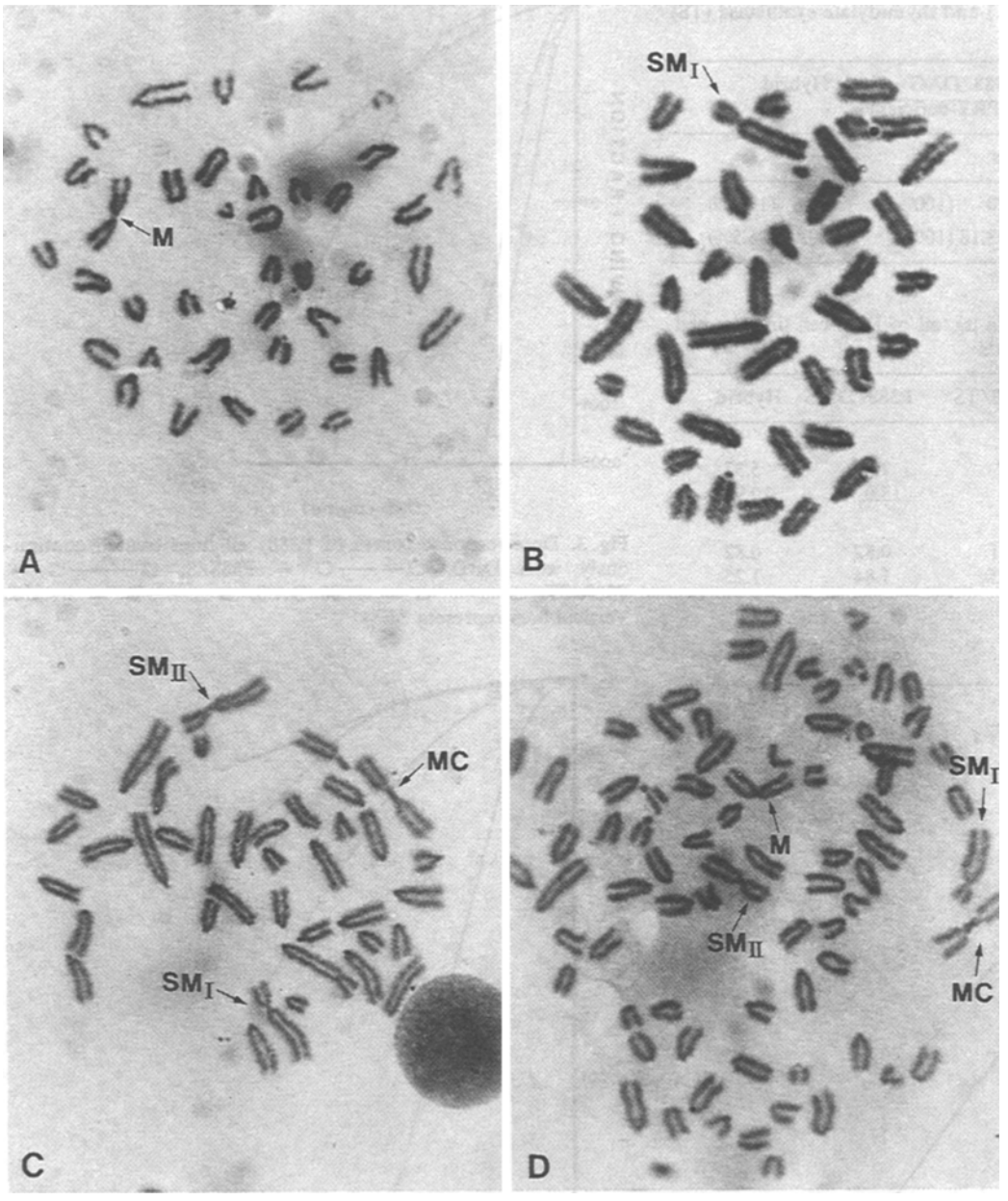

Fig. 1. (A) Karyotype of a P388/S cell. Note the large metacentric marker (*M*) (*arrow*). × 1,63. **(B)** Karyotype of a P388/DAG cell. SM_I is a submetacentric marker (*arrow*). × 1,63. **(C)** Marker chromosomes of P388/DAG/HPRT-deficient cell: two submetacentric (SM_I, SM_{II}) and a metacentric marker with a long centromeric region (*MC*) (*arrows*). × 1,000. **(D)** Karyotype of a hybrid cell. All four markers are present (*arrows*) (Giemsa staining). × 1,63

cells were 5–6 times less sensitive to DAG than the sensitive lines (Table 3). The hybrid cells were about 4 times more resistant than the P388/S and P388/ST cells. The difference was even larger at higher DAG doses. The dose-survival curves of P388/DAG and hybrid cells ran parallel (Fig. 2).

The dose-response curves of the four cell lines were also sigmoid following continuous treatment with DBD (Fig. 3). The difference in sensitivity between the sensitive and DAG-resistant lines was more than fourfold. At higher doses of DBD, the DAG-resistant cells were more resistant to DBD than the hybrid cells (Table 3).

DisuDAG has previously been shown to have very low cytotoxicity [28, 32, 33]. Survival curves obtained with the P388 lines were also sigmoid, with a smaller shoulder for the P388/S sensitive cells and a wide one for the P388/ST tetraploid and the resistant cells (Fig. 4). The difference in sensitivity between the P388/S sensitive and the DAG-resistant cells was four- to fivefold. Surprisingly, the sensitivity of the P388/ST sensitive line was between those of the P388/S and the DAG-resistant P388/DAG lines (Fig. 4, Table 3).

Discussion

It has been shown that the resistance of the intraspecific hybrid P388 lymphoma cells to DAG is expressed in a quasi-dominant manner. Furthermore, P388/DAG cells

Table 2. Thymidine kinase (TK) and thymidylate synthetase (TS) activity in the cytosol

	P388/S TK-deficient	P388/DAG HPRT-deficient	Hybrid
	pmol/min per 10^7 cells		
TK	2.5 (1.14%)	220 (100%)	29.6 (13.5%)
TS	169.7 (182.1%)	93.18 (100%)	80.61 (86.5%)

Table 3. ID_{50} and ID_{10} values in μg/ml of P388 cell lines treated with cytotoxic hexitol compounds

Drug	P388/S	P388/TS	P388/DAG	Hybrid
DAG[a]:				
ID_{50}	1.37	1.0	7.75	5.37
ID_{10}	3.0	1.6	15.0	10.9
DBD[b]:				
ID_{50}	0.2	0.11	0.82	0.82
ID_{10}	0.35	0.25	1.84	1.25
DisuDAG[b]:				
ID_{50}	16.0	40.0	80.0	68.0
ID_{10}	28.0	70.0	200.0	110.0

[a] 1-h exposure
[b] Continuous treatment

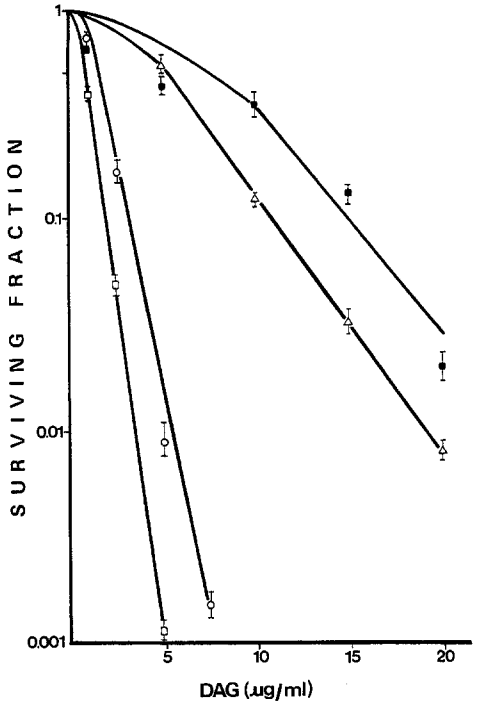

Fig. 2. Dose-response curves of P388 cell lines treated with DAG for 1 h. ○——○ = P388/S, □——□ = P388/ST, △——△ = P388/DAG, ■——■ = hybrid. Vertical bars represent SEM

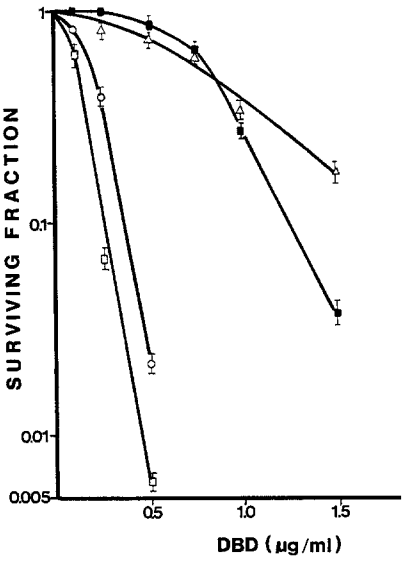

Fig. 3. Dose-response curves of P388 cell lines treated continuously with DBD. ○——○ = P388/S, □——□ = P388/ST, △——△ = P388/DAG, ■——■ = hybrid. Vertical bars represent SEM

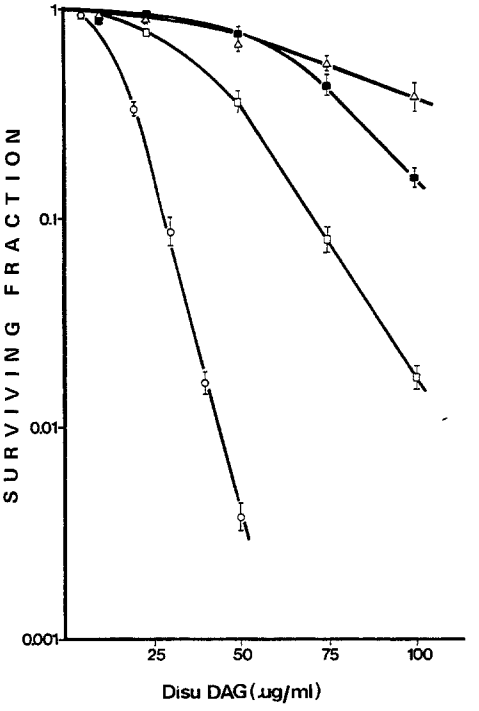

Fig. 4. Dose-response curves of P388 cell lines treated continuously with DisuDAG. ○——○ = P388/S, □——□ = P388/ST, △——△ = P388/DAG, ■——■ = hybrid. Vertical bars represent SEM

were cross-resistant to the hexitol derivatives DBD and DisuDAG, and this cross-resistance was dominantly inherited in the hybrid cells as well. Cross-resistance between hexitols was not surprising, because tumors resistant to alkylating agents have shown cross-resistance to other alkylating drugs [20]. This has also been observed under in vivo conditions [3, 16, 17]. The decreased sensitivity to DisuDAG of the tetraploid line, compared with that of the diploid one, cannot be explained by the bigger nuclear target size, because the sensitive diploid and tetraploid lines have shown equal sensitivity to UV light, to ethyl-methane sulfonate (EMS) [15], to colchicine [26], and to vinblastine [18]. In our experience, no difference in cell survival was observed when 1000 or 10,000 cells were treated with the

same dose (Pályi, unpublished data). We suppose that the cell membrane of the tetraploid line is less permeable to DisuDAG than that of the diploid line, as DisuDAG is a sterically larger molecule than the other two hexitols. In the majority of such studies, resistance to various cytostatic drugs and to X-rays was inherited dominantly, or codominantly in the hybrid cells [7, 13, 26, 35, 42]. Resistance was recessive only to ara-C in intraspecific hybrids of Chinese hamster cells made from crosses between ara-C- and vinblastine-resistant parental lines [18]. P388 cells seem to be generally more sensitive to drugs, at least to cytotoxic hexitols, than other cell types [32]. CHO cells were 6–15 times less sensitive than P388/S cells to DAG [2].

P388/DAG cells contained a marker chromosome, SM_I, which was lacking in the sensitive parental line. A submetacentric marker similar to SM_I was observed in 6% of the in vivo sensitive and 80% of the resistant cell population [3]. It was supposed that the resistant P388/DAG tumor had been derived by selection from the sensitive P388/S population [3].

The dominance of resistance to X-rays was fully expressed in intraspecific hybrids of sensitive and X-ray-resistant L5178Y mouse lymphoma cells [7]. It was shown that the increased DNA repair function of the X-ray-resistant parental cells was dominantly inherited in the hybrids [15]. Concerning the mechanism responsible for the resistance of the P388/DAG cells to DAG [6], it was shown that equal levels of DNA alkylation occurred in the sensitive and DAG-resistant P388 tumor cells when they were treated with bifunctional cytotoxic hexitols [23]. These observations suggest that possible differences between the two cell lines (in their cell membranes or in drug transport) did not play a role in resistance. The repair of DNA damage caused by the bifunctional alkylating agent DAG was increased in the resistant cells compared with that in the sensitive line [23].

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