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Sister-chromatid exchanges in human lymphocytes exposed to 1-*p*-(3-methyltriazeno)benzoic acid potassium salt

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Summary

The 1-*p*-(3-methyltriazeno) benzoic acid potassium salt (MTBA) is a triazeno analogue of dacarbazine, an antineoplastic agent capable of mediating the appearance of new antigenic specificities on cancer cells in mice, a phenomenon described as 'chemical xenogenization' (CX).

Recently we reported the clastogenic potential of MTBA on human lymphocytes. Since sister-chromatid exchange (SCE) assay is more sensitive than clastogenic tests, at least at low drug concentrations, we assessed SCE frequencies induced by MTBA on human lymphocytes stimulated by PHA. Drug treatment at 2–500 μg/ml was performed in vitro prior to or after PHA addition. SCE values increased significantly in a dose-dependent manner up to 200 μg/ml. However, SCE frequencies, as well as chromosome breaks, did not increase dramatically.

These data indicate that MTBA concentrations used for CX do not cause severe cytogenetic damage to immune cells at least in vitro.

The 1-*p*-(3-methyl triazeno) benzoic acid potassium salt (MTBA) has been found to be a suitable active substitute for its triazene analogue dacarbazine in inducing antigenic modifications of cancer cell membranes recognizable by the immune system (Nardelli et al., 1984a), a property defined as 'chemical xenogenization' (CX) (Bonmassar et al., 1972; Fioretti et al., 1981). Moreover, triazene compounds are alkylating agents (Preussmann and van Hodenberg, 1970; Skibba and Bryan, 1971) and therefore potentially clastogenic. In line with these observations we have recently described the clastogenic activity of MTBA in human lymphocytes (Vernole et al., 1987).

Sister-chromatid exchange (SCE) analysis is regarded as a sensitive test to detect exposure to mutagenic and/or cancerogenic substances (Littlefield, 1982) and also to evaluate patient sensitivity to chemotherapy (Mourelatos et al., 1983). Short-term cultures of human lymphocytes are

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widely used for these tests, both in vitro and in vivo (Lambert et al., 1982; Deen et al., 1986).

In the present report we describe the effects of MTBA on SCE frequency in human lymphocytes stimulated in vitro by phytohemagglutinin (PHA). It has been reported that SCE values can vary according to the time of addition of the substance (Morimoto et al., 1985). Therefore MTBA was added to lymphocytes at different times of culture, using the same concentrations of MTBA utilized in our previous clastogenic studies. SCE values increased after each treatment, according to individual sensitivity, time of addition and concentration of the drug.

Materials and methods

Human lymphocytes from heparinized blood of 8 normal donors were purified by sedimentation through a Ficoll–Hypaque gradient at $400 \times g$. Mononuclear cells (5×10^5 cells/ml) were cultivated in RPMI 1640, 20% v/v fetal calf serum, 1% w/v PHA-m and 10 μg/ml of 5-bromodeoxyuridine (5-BrdUrd). The cultures were always harvested at 72 h, after a 2-h incubation with 2 μg/ml of colchicine. MTBA was prepared as already described (Vernole et al., 1987). Before each treatment cells were centrifuged and resuspended at a concentration of 2×10^6/ml in RPMI 1640. Various concentrations of MTBA (2, 20, 200 or 500 μg/ml) were added to lymphocyte suspensions, according to the following schedule.

Treatment A. MTBA treatment occurred 2 h after PHA addition (3 samples: numbers 1, 2, 3).

Treatment B. MTBA treatment occurred after 24 h of culture (3 samples: numbers 4, 5, 6).

Treatment C. MTBA treatment occurred after 48 h of culture (3 samples: numbers 1, 2, 3).

Treatment D. MTBA treatment occurred immediately after cell separation, PHA was added at the end of the incubation with MTBA (2 samples).

Cells were incubated with MTBA for 1 h at 37°C in 5% CO_2 in air. The drug was then removed by washing with cold medium and the cells were cultured again in their conditioned medium until harvesting. Mitomycin C (MMC, 0.1 μg/ml) was added to cultures from 2 of the individuals studied with treatments A and C at 48 h of culture, to compare the effects of the 2 different drugs at similar molar concentrations.

Control cultures were treated as described, using medium alone.

Chromosome preparations were obtained by standard techniques (Hungerford, 1965) and treated according to Perry and Wolff (1974) to obtain sister-chromatid differential staining. Cells dividing for the first (M1), the second (M2), or more times in culture (M3 +) were recognized according to their differential staining pattern of sister chromatids (Tice et al., 1976). SCE were scored generally on 50 M2 cells, and on at least 20 M2 cells when cell growth was very poor. The distribution of M1, M2 and M3 + cells was assessed on 100 metaphases. 1000 cells were analyzed to calculate mitotic indices (MI). Student's t test was applied after transformation of all SCE values to their logs according to statistical considerations already described (Hirsch et al., 1984).

Results and discussion

The means for SCE and the relative standard errors (SE) for treatments A, B and C are shown in Table 1. A dose-dependent increase in SCE, statistically significant in most cases, was found up to a concentration of 200 μg/ml. At a concentration of 500 μg/ml of MTBA SCE values were lower than at 200 μg/ml in one sample only belonging to treatments A, C (i.e., sample 3), and B (sample 6). This is in line with our previous results on MTBA clastogenicity. A similar 'bend-down' of the dose–response curve was reported by other authors for treatments with MMC (Morimoto et al., 1985). It is possible that the majority of lymphocytes exposed to the highest concentration of the agent are too damaged to be able to reach division or otherwise their progression through the cell cycle is very much delayed.

Treatment D was performed using 20, 200 and 500 μg MTBA/ml, cultures were harvested, not only at 72 h but also at 88 h. Even at this later time, we could find M2 cells and evaluate SCE frequencies only at the lowest concentration of the drug. The respective mean SCE values for control cultures and those treated with 20 μg/ml were 5.6 and 7.1 (donor 1), 5.66 and 7.28 (donor 2), in both cases the differences over the control were significant ($p < 0.05$).

We also found that increased concentrations of MTBA resulted in a gradual increase of M1 cells

TABLE 1

INDUCTION OF SCE IN HUMAN LYMPHOCYTES BY MTBA

Treatment A: after 2 h of culture; treatment B: after 24 h of culture; treatment C: after 48 h of culture.

Treatment A						
Sample:	1		2		3	
MTBA (μg/ml)	mean	S.E.	mean	S.E.	mean	S.E.
0	6.35	0.57	5.90	0.65	7.48	0.39
2	8.90 *	0.51	8.65 *	0.92	10.40 *	0.56
20	10.10 *	0.51	9.90 *	0.81	10.32 *	0.51
200	10.70 *	0.61	10.05 *	0.80	11.80 *	0.45
500	12.30 *	0.82	11.05 *	0.98	9.08 *	0.69
Treatment B						
Sample:	4		5		6	
0	7.72	0.34	7.23	0.41	7.63	0.42
2	8.14	0.48	7.64	0.44	9.50 *	0.66
20	8.90 *	0.40	8.24	0.46	10.64 *	0.53
200	9.34 *	0.49	10.46 *	0.60	11.00 *	0.49
500	10.38 *	0.40	No cell growth		9.58 *	0.69
Treatment C						
Sample:	1		2		3	
0	5.95	0.31	6.40	0.59	6.64	0.47
2	not tested		10.25 *	0.73	10.44 *	0.49
20	8.32 *	0.52	14.00 *	1.23	12.96 *	0.59
200	11.25 *	1.54	15.65 *	0.99	13.88 *	0.61
500	8.97 *	0.57	15.30 *	0.94	12.92 *	0.96
MMC						
0.1 μg/ml	not tested		25.95 *	0.81	23.52 *	1.30

* $p < 0.01$ according to Student's t test.

and a decrease of M2 and M3 + cells (data not shown). MI were 4–5 times lower than control values only after treatment with 200–500 μg/ml of MTBA or with MMC, while no relevant difference was found at 2 and 20 μg MTBA/ml except in treatment D. This was in accordance with previous results showing that ^{3}H-thymidine uptake after G0 treatment was minimal at 200 and 500 μg/ml and also slightly depressed at 2 and 20 μg/ml (Vernole et al., 1987).

We have previously suggested 2 hypotheses for the cell growth inhibition after MTBA treatment in G0, i.e., a cytostatic effect of the drug or a modification of PHA receptors on the cell membrane resulting in a less effective stimulation. This second hypothesis might be supported by our present results. In fact, if cells are already committed to proliferation by 2-h incubation with PHA, MTBA is not able to block cell growth.

Only few reports describe SCE differences after treatment at different times of cultures. Variations were reported for example for MMC and 4-nitroquinoline in human lymphocytes (Morimoto et al., 1985), and alkylating agents in CHO cells (Sono and Sakagushi, 1981).

Treatment 24 h before harvesting (treatment C) caused more SCE than treatment A performed on the same subject. The differences were significant, except at the concentration of 2 μg/ml (samples 2 and 3) and in case 3 also at 500 μg/ml. Cells in treatment C might not have had enough time to repair and recover before reaching mitosis. Alternatively the different sensitivity to SCE induction might be due to drug exposure of cells in different stages of the cell cycle, as already reported (Morimoto et al., 1985). Treatment A was performed on cells in G0–G1, while treatment C included cells in any phase of the cycle.

SCE values in the 2 samples treated with MMC were 25.9 and 21.15, compared respectively to 15.65 and 13.88, the highest values found after MTBA treatment. These data, like those reported in the literature for other bifunctional alkylating agents, are higher than those obtained after MTBA treatment even at high cytotoxic concentrations (500 μg/ml). The limited effects of MTBA in inducing SCE could be explained taking into account that triazene compounds are monofunctional agents, and so generally less powerful than the bifunctional ones in SCE induction. Moreover, their action at the DNA level is probably due also to other mechanisms different from alkylation (Matney et al., 1985; Vernole et al., 1987).

Previous studies showed that marked CX can be produced in vitro by MTBA concentrations of the order of 100 μg/ml in lymphoma cells (Nardelli et al., 1984b). It follows that MTBA concentrations able to induce CX would cause only moderate cytogenetic damage (i.e., less than that produced by 200 μg/ml, see Table 1) to human immune cells. Therefore the present findings could provide good support for the possible application of this new approach to cancer immunochemotherapy (Giampietri et al., 1981) to man.

Furthermore, the SCE assay might be useful to test individual sensitivity to the drug before starting in vivo treatments, since a positive correlation between in vitro and in vivo sensitivity has been reported (Tofilon et al., 1985; Deen et al., 1986).

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References

Bonmassar, E., A. Bonmassar, S. Vadlamudi and A. Goldin (1972) Changes of L1210 leukemia in mice treated with 5-(3,3-dimethyl-1-triazeno) imidazole-4-carboxamide, Cancer Res., 23, 1446–1452.

Deen, D.F., L.E. Kendall, L.J. Marton and P.J. Tofilon (1986) Prediction of human tumor cell chemosensitivity using the sister chromatid exchange assay, Cancer Res., 46, 1599–1602.

Fioretti, M.C., B. Nardelli, R. Bianchi, C. Nisi and G. Sava (1981) Antigenic changes of a murine lymphoma by in vivo treatment with triazeno derivatives, Cancer Immunol. Immunother., 11, 283–286.

Giampietri, A., A. Bonmassar, P. Puccetti, A. Circolo, A. Goldin and E. Bonmassar (1981) Drug-mediated increase of tumor immunogenicity in vivo for a new approach to experimental cancer immunotherapy, Cancer Res., 41, 681–687.

Hirsch, B., M. McGue and J. Cervenka (1984) Characterization of the distribution of sister chromatid exchange frequencies: implications for research design, Hum. Genet., 65, 280–286.

Hungerford, D.A. (1965) Leukocytes cultured from small inocula of whole blood and preparation of metaphase chromosomes by treatment with hypotonic KCl, Stain Technol., 40, 333–336.

Lambert, B., A. Lindblad, K. Holmberg and D. Francesconi (1982) The use of sister chromatid exchange to monitor human populations for exposure to toxicologically harmful agents, in: S. Wolff (Ed.), Sister Chromatid Exchange, Wiley, New York, pp. 149–182.

Littlefield, L.G. (1982) Effects of DNA-damaging agents on SCE, in: A.A. Sandberg (Ed.), Sister Chromatid Exchange, Liss, New York, pp. 355–394.

Matney, T.S., T.V. Nguyen, T.H. Connor, W.J. Dane and J.C. Theiss (1985) Genotoxic classification of anticancer drugs, Teratogen. Carcinogen. Mutagen., 5, 319–328.

Morimoto, K., M. Sato-Mizuno and A. Koizumi (1985) Sister-chromatid exchanges and cell-cycle kinetics in human lymphocytes exposed to alkylating mutagens: apparent deformity in dose-response relationships, Mutation Res., 152, 187–196.

Mourelatos, D., J. Dozi-Vassiliades, V. Tsigalidou-Balla and A. Granitsas (1983) Enhancement by methylxanthines of SCE frequencies induced by cytostatics in normal and leukemic human lymphocytes, Mutation Res., 121, 147–152.

Nardelli, B., P. Puccetti, L. Romani, G. Sava, E. Bonmassar and M.C. Fioretti (1984a) Chemical xenogenization of murine lymphoma cells with triazene derivatives: immunotoxicological studies, Cancer Immunol. Immunother., 17, 213–217.

Nardelli, B., A.R. Contessa, L. Romani, G. Sava, C. Nisi and M.C. Fioretti (1984b) Immunogenic changes of murine lymphoma cells following in vitro treatment with aryl-triazene derivatives, Cancer Immunol. Immunother., 16, 157–167.

Perry, P., and S. Wolff (1974) New Giemsa method for the differential staining of sister chromatids, Nature (London), 251, 156–158.

Preussmann, R., and A. van Hodenberg (1970) Mechanism of carcinogenesis with 1-aryl-3,3-dialkyltriazenes. II. In vitro alkylation of guanosine, RNA and DNA with aryl-monoalkyltriazenes to form 7-alkylguanine, Biochem. Pharmacol., 19, 1505–1508.

Skibba, J.L., and G.T. Bryan (1971) Methylation of nucleic acid and urinary excretion of ^{14}C-labeled 7-methyl-guanine by rats and man after administration of 4(5)-(3,3-dimethyl-1-triazeno)-imidazole 5(4)carboxamide, Toxicol. Appl. Pharmacol., 18, 707–719.

Sono, A., and K. Sakagushi (1981) Inhibition of protein synthesis antagonizes induction of sister-chromatid exchanges by exogenous agents, Mutation Res., 80, 121–131.

Tice, R., E.L. Schneider and J.M. Rary (1976) The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics, Exp. Cell. Res., 102, 232–236.

Tofilon, P.J., I. Basic and L. Milas (1985) Prediction of in vivo tumor response to chemotherapeutic agents by the in vitro SCE assay, Cancer Res., 45, 2025–2030.

Vernole, P., D. Caporossi, B. Tedeschi, B. Porfirio, G. Melino, E. Bonmassar and B. Nicoletti (1987) Cytogenetic effects of 1-*p*-(3-methyl triazeno) benzoic acid potassium salt on human lymphocytes in vitro, Mutation Res., 189, 349–356.