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Induction of Sister-chromatid Exchanges and Cell-cycle Delays in Human Lymphocytes by Vitamin A alone or in Combination with Melphalan and Caffeine

J. DOZI-VASSILIADES, A. MYRTSIOTIS, A. GRANITSAS and D. MOURELATOS
Department of General Biology, Faculty of Medicine, Aristotelian University, Thessaloniki 54006, *Greece*

Abstract—*In cultured human lymphocytes vitamin A was found to increase SCE rates, to reduce the mitotic index and to have no effect on cell kinetics. Vitamin A induces cytotoxic effects: (a) in combination with melphalan (MELPH), as can be deduced from the resulted synergism on induction of SCEs, the produced cell division delay and the suppressed mitotic index; (b) in combination with caffeine (CAF), producing synergism on induction of SCEs and suppressing the mitotic index; and (c) in combination with MELPH and CAF, producing cell-cycle delays and reducing the mitotic index.*

INTRODUCTION

ONE OF the well-documented effects of vitamin A is its ability in combination with CAF, to enhance the antitumour effect of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) in mice [1] and also to increase the antitumour effect of cyclophosphamide in murine L1210 leukaemia [2]. This prompted us to undertake the present study aimed at testing in human lymphocytes the effect of vitamin A alone or in combination with MELPH and CAF on SCEs, mitotic indices and cell kinetics. Chemically induced cytotoxicity in that it delays cell turnover times is clearly manifested as a change in the relative proportions of cells in their first, second and subsequent divisions [3]. Studies in search of relationship between SCE induction and other expressions of genotoxicity have found a positive relationship between SCEs and reduced cell survival and alteration in cell-cycle kinetics [3, 4]. These findings suggested that a common element, possibly a type of DNA damage, produced by certain agents was responsible for inducing SCE and reducing cell survival and cell growth [3, 4].

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MATERIAL AND METHODS

Heparinized blood samples were obtained and cultured from normal individuals, none of whom were known to be receiving drugs for medical or other reasons. Cultures were established by adding five drops of whole blood to 4 ml of chromosome medium 1A (Gibco) in universal containers and placed in a light-tight box and incubated at 37°C. Treatment with vitamin A (Sigma) at 4 μg/ml alone or in combination with MELPH (Sigma) and CAF (Sigma) at 180 μg/ml was given at the beginning of culture life. 5-Bromodeoxyuridine (Sigma) at 4 μg/ml was added 24 hr after initiation of culture. The experiment was repeated with six different blood donors with consistently similar results. Colchicine at 0.3 μg/ml was added for the last 2 hr of culture. The cultures were harvested after 72 hr incubation and chromosome preparations were made and stained by the FPG method [5]. The preparations were scored for cells in their first mitosis (both chromatids dark staining), second mitosis (one chromatid of each chromosome dark staining) and third and subsequent divisions (a portion of chromosomes with both chromatids light staining) and suitably spread second division cells were scored for SCEs. Mitotic

indices for 5000 activated lymphocytes were also determined in each type of culture in each donor. For the statistical evaluation of the experimental data the chi-square test was used for the mitotic index and for the cell-kinetic comparisons, whereas for the SCE frequencies Student's t test was performed to determine whether any values deviated significantly ($t \leqslant 0.01$) from the controls.

RESULTS

Vitamin A induces a statistically significant ($P < 0.01$) increase in SCE rates at a concentration of 4 μg/ml and reduces ($P < 0.001$) the mitotic index (Tables 1 and 3). The results in Table 1 indicate that when vitamin A was added in the cultures exposed to MELPH at a dose (150 ng/ml) sufficiently high to induce SCEs, the effect of vitamin A was synergistic, the SCE level achieved being consistently much higher than that expected by the simple addition of the effects of MELPH and vitamin A. The presence of vitamin A in cultures treated with MELPH induces cell division delays ($P < 0.001$) and suppresses significantly the mitotic index ($P < 0.001$) (Tables 2 and 3). Table 1 shows that vitamin A acts synergistically with CAF on induction of SCEs, while Table 2 demonstrates a suppression of the mitotic index ($P < 0.01$) in cultures treated with vitamin A in combination with CAF compared with cultures treated with CAF only. Table 1 shows the already reported synergistic effect of CAF with MELPH on SCEs which has been attributed to a possible interference of CAF with excision DNA repair [6].

The presence of vitamin A in cultures treated with MELPH and CAF does not further elevate significantly the SCE rate but induces significant ($P < 0.01$) cell-cycle delays and reduction ($P < 0.001$) of the mitotic index (Tables 1–3).

DISCUSSION

Retinoids have already been reported to enhance SCEs in human fibroblasts [7, 8]. The SCE induction by vitamin A in human lymphocytes (Table 1) may be due to its ability to bind upon chromatin [9, 10]. The synergistic induction of SCEs by vitamin A in combination with MELPH (Table 1) may be attributed to the ability of vitamin A to produce sublethal injury to cellular membranes [11], so facilitating the action of MELPH upon DNA. Another possible explanation is that in human lymphocytes undertaking DNA repair after being damaged by MELPH, vitamin A may interfere with DNA repair and this might lead to an increase in the number of unrepaired lesions at the S phase. If damage to DNA is left unrepaired it can be linked causally to the formation of SCEs by a process that resides in semiconservative DNA replication [12]. Vitamin A has been reported to give 50% repair inhibition of DNA replication which normal human lymphocytes undertake after being damaged by u.v. irradiation [13]. CAF, which appears to have a high affinity for partially denatured DNA [14], and which is present throughout culture life, suppresses the mitotic index, induces SCEs and cell-cycle delays ($P < 0.001$), and acts synergistically with vitamin A on SCE

Table 1. Induction of sister-chromatid exchange in human lymphocytes treated with vitamin A alone or in combination with melphalan and caffeine

Agent and concentration	No. of cells	Mean SCE/cell ± S.E. (range)
Control (C)	198	9.9 ± 0.3 (3-19)
Vitamin A, 4 μg/ml (A)	187	11.7 ± 0.3* (5-23)
CAF, 180 μg/ml (CAF)	130	18.4 ± 0.5* (6-35)
Vit. A (4 μg/ml) + CAF (180 μg/ml)	105	23.0 ± 0.7 (10-55)
MELPH, 150 ng/ml (M)	157	25.5 ± 0.5 (12-40)
MELPH (150 ng/ml) + vit. A (4 μg/ml)	142	33.4 ± 0.6 (16-51)
MELPH (150 ng/ml) + CAF (180 μg/ml)	96	38.5 ± 1.4 (17-106)
MELPH (150 ng/ml) + CAF (180 μg/ml) + vit. A (4 μg/ml)	46	38.9 ± 0.8 (20-60)

*Significant ($P < 0.01$) by Student's t test (one-tailed).

Table 2. Mitotic indices in 72-hr human cultures treated with vitamin A alone or in combination with melphalan and caffeine

Treatment*	Mitotic indices (‰)	χ^2	*P*
C	10.7	—	—
A	7.87	12.8	<0.001
CAF	2.9	—	—
CAF + A	1.66	9.5	<0.01
M	8.9	—	—
M + A	5.03	31.9	<0.001
M + CAF	1.9	—	—
M + CAF + A	0.5	23.4	<0.001

30,000 cells were counted for each point.
*As in Table 1.

induction (Tables 1-3). This synergistic action may be attributed to the ability of vitamin A to induce sublethal injury to cellular membranes [11], facilitating the action of CAF upon DNA. The dose of CAF of 180 μg/ml would correspond to approximately 900 mg of CAF for an adult of 70 kg while the dose of vitamin A (4 μg/ml) would correspond to approximately 18 mg of vitamin A for an adult of 70 kg. Addition of vitamin A in cultures treated with MELPH and CAF does not significantly elevate further SCEs (Table 1). One possible explanation could be that there is a change in the duration of cell-cycle stages or that severely damaged metaphases do not reach mitosis. In the cell-cycle delays there are significant differences between MELPH plus CAF and vitamin A plus MELPH plus CAF treatments (Table 3). This cell-cycle delay was the greatest one; we found hardly any third division mitoses and the fraction of the first mitoses was greatly increased in the last treatment.

Gibas and Limon [15], as well as Ishii and Bender [16], found significantly lower yields of SCEs induced in PHA-activated lymphocytes by two 9-aminoacridine derivatives and/or MMC in early-, as compared with late-, proliferating lymphocytes. Also, the greatest reduction in mitotic index appeared in the treatment of vitamin A plus MELPH plus CAF. The results in Table 3 reveal that vitamin A in combination with MELPH and CAF acts synergistically on cell-division delay, the cell-division delay achieved being higher than that expected by the simple addition of the effects on cell-division delay of vitamin A and MELPH plus CAF.

The synergistic cytotoxic effects by vitamin A and MELPH or by vitamin A in combination with MELPH and CAF may be of use in the treatment of human cancer. Cancer therapy in many instances is designed to produce severe damage to the neoplastic cell. However, if an agent penetrates a cell and induces damage its biological effect may be minimized by the cell's capacity for repair [17]. The ability to repair various types of damage to DNA is probably a general property of living cells. This is of considerable interest to the problem of cancer because it provides a mechanism by which the rate

Table 3. Frequency of cells in first, second or subsequent (3rd+) division in 72-hr human cultures treated with vitamin A alone or in combination with melphalan and caffeine

Treatment*	No. of cells in cell cycles (%) 1st	χ^2	*P*	2nd	χ^2	*P*	3rd+	χ^2	*P*
C	582 (50.12)	—	—	456 (39.27)	—	—	123 (10.59)	—	—
CAF	710 (74.5)	129.9	<0.001	226 (23.71)	57.3	<0.001	17 (1.78)	64.3	<0.001
A	613 (50.24)			479 (39.18)			129 (10.57)		
CAF + A	569 (77.1)			162 (21.95)			7 (0.94)		
M	658 (56.67)	—	—	387 (33.33)	—	—	116 (9.99)	—	—
M + A	623 (65.99)	18.7	<0.001	293 (31.0)	1.1	>0.05	28 (2.96)	39.2	<0.001
M + CAF	578 (80.27)	—	—	136 (18.88)	—	—	6 (0.83)	—	—
M + CAF + A	396 (86.4)	7.0	<0.01	58 (12.66)	7.4	<0.01	4 (0.9)	0.06	>0.8

*As in Table 1.

of potential genetic damage induced by various antitumour agents may be modified [17]. Although the relationship between SCEs and DNA repair mechanisms is still speculative, SCEs are being increasingly used as highly sensitive indicators of chromosome damage and/or subsequent repair [4, 12, 18, 19]. Vitamin A has already been reported to enhance the antitumour effect of cyclophosphamide in murine L1210 leukaemia [2]. If vitamin A and CAF do interfere with DNA repair in man, as seems likely, it should be possible to increase the effectiveness of antitumour agents by inhibiting or deranging the DNA repair system. The partial substitution of antitumour therapeutic regimens by reduced therapeutic regimens and a non-toxic concentration of vitamin A and CAF may also be considered in view of the side-effects which may arise from antitumour therapy. A considerable enhancement of the antitumour effect of BCNU by vitamin A and CAF has been reported for the mouse [1].

Therapeutic effects with this treatment regimen represent an improvement over those reported previously for vitamin A and BCNU alone. Enhancement to a similar or greater extent than was seen previously was obtained with lower doses of both vitamin A and BCNU [1]. Our hypothesis, that the combination of vitamin A, MELPH and CAF may be used in treatment of cancer, might be validated only with future experiments using a lower dose of these drugs to study the synergistic effects of this combination upon human tumour cells.

REFERENCES

1. Cohen MH. Enhancement of the antitumor effect of BCNU by vitamin A and caffeine. *JNCI* 1972, **48**, 927-932.
2. Cohen MH, Carbone PP. Enhancement of the antitumor effects of BCNU and cyclophosphamide by vitamin A. *JNCI* 1972, **48**, 921-926.
3. Morimoto K. Induction of SCEs and cell division delays in human lymphocytes by microsomal activation of benzene. *Cancer Res* 1983, **43**, 1330-1334.
4. Morris SM, Heflich RH. A comparison of the toxic and SCE-inducing effects of inhibitors of ADP-ribosyl transferase in Chinese hamster ovary cells. *Mutat Res* 1984, **126**, 63-71.
5. Perry P, Wolff S. New Giemsa method for the differential staining of sister chromatids. *Nature (Lond)* 1974, **251**, 156-158.
6. Mourelatos D, Dozi-Vassiliades J, Granitsas A. Antitumour alkylating agents act synergistically with methylxanthines on induction of SCEs in human lymphocytes. *Mutat Res* 1982, **104**, 243-247.
7. Juhl HJ, Shuper CC, Bertram CR *et al.* Retinoids induce SCEs in human diploid fibroblasts. *Mutat Res* 1978, **58**, 317-320.
8. Tetzner C, Juhl HJ, Rudiger HW. SCE induction by metabolically activated retinoids in human diploid fibroblast cultures. *Mutat Res* 1980, **79**, 163-167.
9. Liau G, Ong DE, Chytil F. Interaction of the retinol/cellular retinol-binding protein complex with isolated nuclei and nuclear components. *J Cell Biol* 1981, **91**, 63-68.
10. Omori M, Chytil F. Mechanism of vitamin A action. Gene expression in retinol-deficient rats. *J Biol Chem* 1982, **257**, 14370-14374.
11. Meeks RG, Zaxarevits D, Chen RF. Membrane effect on retinoids: possible correlation with toxicity. *Arch Bioch Biophys* 1981, **207**, 141-147.
12. Wolff S, Rodin B, Cleaver JE. SCEs induced by mutagenic carcinogens in normal and Xeroderma Pigmentosum cells. *Nature (Lond)* 1977, **265**, 341-349.
13. Gaudin D, Gregg RS, Yieldin KL. DNA repair inhibition: a possible mechanism of action of co-carcinogens. *Biochem Biophys Res Commun* 1971, **45**, 630-636.
14. Domon M, Burton B, Porte A, Rauth AM. The interaction of caffeine with UV-light-irradiated DNA. *Int J Radiat Biol* 1970, **17**, 395-399.
15. Gibas Z, Limon J. The introduction of SCEs by 9-aminoacridine derivatives. *Mutat Res* 1979, **67**, 93-96.
16. Ishii Y, Bender MA. Factors influencing the frequency of MMC-induced SCEs in 5-bromodeoxyuridine-substituted human lymphocytes in culture. *Mutat Res* 1978, **51**, 411-418.
17. Byfield JE, Murnane J, Ward JF, Calabro-Jones P, Lynch M, Kulhanian F. Mice, man, mustards and methylated xanthines: the potential role of caffeine and related drugs in the sensitization of human tumours to alkylating agents. *Br J Cancer* 1981, **43**, 669-683.
18. Faed MJW, Mourelatos D. Enhancement by caffeine of SCEs in lymphocytes from normal subjects after treatment by mutagens. *Mutat Res* 1978, **49**, 437-440.
19. Ishii Y, Bender M. Caffeine inhibition of pre-replication repair of MMC-induced DNA damage in human lymphocytes. *Mutat Res* 1978, **51**, 419-425.