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# Sister Chromatid Exchanges in Human Lymphocytes Treated with Combinations of Cytotoxic Drugs

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**Abstract**—*Lymphocytes from peripheral blood of either 'normal' donors or patients with cancer were stimulated to divide in culture medium containing 5-bromo-2'-deoxyuridine. During* 74 hr *of incubation, the cells were exposed to single agents or to permutations of two combinations of chemotherapeutic drugs, namely MOPP or cyclophosphamide and methotrexate. Only mustine and cyclophosphamide (activated and not activated) increased the frequency of sister chromatid exchanges (SCE). Neither vincristine, procarbazine or prednisolone with mustine, nor methotrexate with cyclophosphamide altered the number of SCEs expected from the use of mustine or cyclophosphamide alone. There was no difference in the response of cells from cancer patients and 'normal' subjects to the drugs. If the drugs of these two regimens do interact with one another to enhance the amount of subcellular damage, this is not manifested by changes in the number of SCEs in human lymphocytes* in vitro.

## INTRODUCTION

COMBINATIONS of cytotoxic drugs are frequently administered to patients with cancer, and the use of these drugs is increasing [1, 2].

Generally, the rationale for combining drugs is based on differences in the proportion and rate at which cells in the tumour are proliferating compared with the normal tissue [3]. In addition, agents may cooperate at a subcellular level [4, 5]. For instance, vincristine alters uptake and retention of cyclophosphamide and methotrexate in cells [6]. Only when the magnitude and mechanism of interaction of antineoplastic agents at a subcellular level is more explicitly understood can any scientific progress be made in improving combinations of agents and the doses required.

The aim of this study was to assess whether cytotoxic drugs interact to enhance subcellular damage *in vitro* compared to that obtained using single agents.

Two commonly used regimens were chosen. A combination of nitrogen mustard, vincristine, procarbazine and prednisolone (MOPP) has been used to successfully treat Hodgkin's disease [7]. Cyclophosphamide and methotrexate have been combined in the palliation of bronchogenic carcinoma [8, 9].

Peripheral blood lymphocytes are sensitive to cytotoxic agents [10] and this may be a limiting factor for the dose of chemotherapeutic drugs which can be given to patients. When lymphocytes are stimulated to divide *in vitro* [11], the effects of drugs can be measured.

Reciprocal exchanges between sister chromatids of a chromosome (SCE) [12, 13] are induced by a variety of chemicals and are generally a more sensitive indicator of small doses of these than chromosome or chromatid damage [14].

The efficacy of either MOPP or cyclophosphamide with methotrexate to produce SCEs in human lymphocytes has been compared with the effects of the component drugs given alone. Cyclophosphamide requires conversion into alkylating derivatives by mixed function oxidases in the liver in order to exert its pharmacologic effects [15]. Activation has been achieved *in vitro* using hepatic microsomes from rats [16] and the subsequent metabolites showing alkylating activity have been measured [17].

## MATERIALS AND METHODS

### *Subjects*

A brief description of the eight people whose blood was used has been tabulated (Table 1).

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*Table* 1

| Subject | Sex | *Laboratory workers** Approximate age (yr) | Smoking habits | Occupational exposure to ionising radiation |
|---|---|---|---|---|
| A | F | ≤20 | NS | No |
| B | F | 20–24 | NS | Yes |
| C | F | 25–30 | NS | Yes |
| D | M | 40–44 | S | Yes |

*Table* 1*b*

| Subject | Sex | *Patients with cancer†* Approximate age (yr) | Disease | Exposure to ionising radiation |
|---|---|---|---|---|
| 3/78 | M | ≤5 | Hodgkin's | |
| 24/78 | M | ≥60 | Carcinoma of bronchus | 24 Gy, 6 MV photons in 6 fractions, 2/week to left chest, 7 weeks before giving blood |
| 1/79 | M | ≥60 | Carcinoma of bronchus | |
| 7/79 | M | ≥60 | Carcinoma of bronchus | 30 Gy, 6 MV photons in 10 fractions, 5/week to chest, 5 weeks before giving blood |

*None of the individuals designated as exposed to ionising radiation had received a significant dose. None knowingly suffered from diseases caused by viruses when blood was taken.

†None of the individuals had received cytostatic drugs when they gave blood.

*Drugs and dosage*

Cyclophosphamide ('Endoxana', WB Pharmaceuticals Ltd., Bracknell, Berks), methotrexate solution (Lederle, Gosport), mustine hydrochloride (Boots Co. Ltd., Nottingham), vincristine sulphate ('Oncovin', Eli Lilly and Co. Ltd., Basingstoke) and tablets containing prednisolone (Merck Sharp and Dohme Ltd., Hoddesdon, Herts) or procarbazine hydrochloride ('Natulan', Roche Products Ltd., Welwyn Garden City, Herts) were diluted in sterile distilled water, generally by factors of ten, immediately prior to use. Prednisolone and procarbazine were also dissolved in acetone and diluted to 0.05% acetone or less in the culture medium. The doses refer only to the concentrations of drugs and do not include 'filler' material present in some preparations, which was added with the drugs to the culture medium.

Cells were exposed to the same relative proportions of drugs as the total dose of each given throughout a 'course'. Patients were treated with courses at approximately monthly intervals, with some drugs prescribed more than once during the period. The largest amount of each drug in μg per ml which the cultures received was four or five times as high as that given on a mg per kg body weight basis during one course. The effects of a range of lower concentrations were also assessed. The drugs remained for the entire period of culture. If the largest quantities prevented cells reaching their second division in culture, only the effects of smaller doses are presented.

*Activation and measurement of cyclophosphamide*

Hepatic microsomes were prepared from a male hybrid Wistar rat, injected with a single dose of 100 mg/kg phenobarbitone on each of two consecutive days before sacrifice, to stimulate mixed-function oxidase activity in the liver [18]. The microsomes were suspended in 0.0067 M phosphate buffer, pH 7.0, such that 1.0 ml was equivalent to 1 g of original liver.

Tubes containing 1.0 ml suspension of microsomes, 1.0 ml of 0.0067 M phosphate buffer, pH 7.0, 1.0 ml solution of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 3.0 mM and 0.1 ml of either 0,

0.02, 0.2, 2.0, 20.0 or 200.0 $\mu$g cyclophosphamide in sterile distilled water were incubated at 37°C for 10 min. Aliquots of 0.2 ml were removed from each and added to the medium for the culture of lymphocytes.

The remainder was assessed for alkylating activity, using the method of Friedman and Boger [17]. The amount of activity was computed by referring to a calibration curve using nitrogen mustard and expressed as micromoles of alkylating products of cyclophosphamide, and will be referred to as 'activated' to distinguish it from the original substance.

*Culture and harvest of leukocytes*

The method, which was carried out aseptically, was modified from that described by Moorhead *et al.* [11]. Appropriate amounts of drug and 0.5 ml whole blood were added to glass Universal bottles. These contained 5.0 ml TC 199 at pH 7.2 (Flow Labs Ltd., Irvine), 0.25 ml donor calf serum (Gibco-Biocult Ltd., Paisley), 0.1 ml non-essential amino acids (Flow Labs Ltd.) 200 I.U. gentamycin sulphate (Warrick Pharmaceuticals Ltd., Bracknell), 0.1 ml PHA (reagent grade, Wellcome Reagents Ltd., London) and 10 $\mu$M 5-bromo-2'-deoxyuridine (Brd U, Sigma, London).

Cultures were incubated in total darkness for 71 hr at 37 ± 0.5°C, when vincristine sulphate (Eli Lilly and Co. Ltd.) was added to a final concentration of 0.1 $\mu$g/ml. After a further 3 hr of incubation the cells were harvested in a dark room illuminated through an Ilford S902 filter, using 0.075 M potassium chloride as the hypotonic solution. The suspensions of cells were fixed in freshly prepared methyl alcohol and glacial acetic acid (3:1). This mixture was changed three times. Preparations of cells on micro slides were air dried.

*Staining*

Using a method similar to that described by Perry and Wolff [13], the cells were stained for 15 min with Hoechst 33258 (Hoechst Chemicals Ltd., Hounslow) at a final concentration of 0.5 $\mu$g/ml in distilled water. After rinsing they were flooded with Sorenson's phosphate buffer, pH 6.8, and cover-slips floated on top. They were exposed to 365 nm u.v. light, 13 cm away (APW Allen, London) for 25 min. The slides were rinsed and stained for 6 min at 20°C with Giemsa (BDH, 0.68% w/v solution in methanol glycerol) diluted to 2% with phosphate buffer (pH 6.8).

*Counting SCE*

All slides were codified within any one experiment. Cells at metaphase in their second or third division which had clearly differentially stained chromosomes were chosen. For most samples more than 1000 chromosomes within 30 cells were scored. SCEs about the centromere were counted only when clearly distinguishable from chromosome twisting. The frequency of SCEs for the whole sample was expressed as follows:

$$\text{frequency of SCEs per chromosome} = \frac{\text{total number of SCEs}}{\text{total number of chromosomes}}.$$

## RESULTS

SCEs are more numerous in cells exposed to certain chemicals (Fig. 1).

The majority of cells were at their second division, but approximately 10–20% were at their third.

There was a variation in the frequency of SCEs in untreated cells. For example, the value per chromosome from donor B changed from 0.07 to 0.11, when the two samples were separated by 16 months (Fig. 2). The incidence of SCEs in untreated samples was not influenced by age, sex, the disease of the cancer sufferers or smoking, possibly because of the small number of individuals studied. Thus the number of SCEs in the cells of subject 3/78, who had not previously been treated for Hodgkin's disease, fell within the 'normal' range (Table 2). The donors with cancer who had recently been irradiated had more SCEs in their cells than other individuals.

On five separate occasions, using blood taken principally from donors without clinically diagnosed cancer, both mustine alone and the MOPP combination caused a non-linear increase in the frequency of SCE with dose (Fig. 2). A small number of SCEs in untreated cells was reflected by a relatively smaller increase in the frequency of drug-induced SCE. A dose of 0.182 $\mu$g/ml ($-0.02\ \text{Log}_{10}\ \mu$M) mustine, which was the largest that permitted cells to survive to their second division for analysis, induced widely discrepant numbers of SCEs, even for donor B, whose blood was used twice for two experiments on the second occasion.

There was no apparent difference in response to mustine, whether used alone or as part of the MOPP regime.

Neither vincristine, prednisolone nor procarbazine diluted in water consistently affected the SCEs over the range of doses studied (Figs 2 and 3). When 0.045 $\mu$g/ml ($-0.62\ \text{Log}_{10}\ \mu$M) of mustine was added with three different

*Table 2*

| Number of individuals | Cancer patients | Previous radiotherapy | SCEs per chromosome (±1 S.D.) |
|---|---|---|---|
| 12 | No | No | 0.10 ± 0.03 |
| 19 | Yes | No | 0.13 ± 0.05 |
| 22 | Yes | Yes | 0.14 ± 0.03 |

Effect of disease and previous treatment on the frequency of SCE. 'Normal' individuals included samples taken at different times from the same person. Cancer patients suffered from diverse diseases, though none had received previous cytotoxic chemotherapy.

concentrations of any of the latter three agents, the levels of SCE could be ascribed to the effect of mustine alone (Figs 2 and 3).

The effects of large doses of mustine could not be measured, since cells accumulated at their first division. When exposed to a fixed amount of mustine and two different doses of vincristine, both of which would normally allow cells to divide twice, no cells at the second division were apparent after 74 hr in culture. Thus, these combinations of mustine and vincristine appeared to affect the kinetics of the lymphocytes more than the sum of their separate effects.

Prednisolone, at a dose of 4.56 $\mu$g/ml (1.02 $\text{Log}_{10}$ $\mu$M) in acetone and water, significantly raised the frequency of SCEs ($P < 0.05$), though doses of up to 45.6 $\mu$g/ml (2.02 $\text{Log}_{10}$ $\mu$M) failed to exert an effect when water was used as a diluent (Fig. 3c). Methotrexate had no effect (Table 3, Figs 4 and 5).

Cyclophosphamide induced a consistent dose-related increase in the frequency of SCEs, both in the cells from the three patients with lung cancer and in those of the other donors (Fig. 4). However, whilst SCEs were 2.6 times more frequent than in the control after a dose of 0.024 $\mu$g/ml ($-1.4$ $\text{Log}_{10}$ $\mu$M) activated cyclophosphamide, a similar dose of non-activated drug produced only a marginal positive change in two out of three experiments. Though the percentage of activation decreased as the concentration of cyclophosphamide increased, there was still most activated drug present in the culture containing the highest concentration of the original drug (Fig. 5).

Mustine was more effective than non-activated cyclophosphamide, but activated cyclophosphamide was most effective in inducing SCEs on a molar basis (Figs 2, 4 and 5).

*Table 3*

| Donor | Concentration ($\mu$g/ml) | SCE frequency (±1 S.E.) |
|---|---|---|
| C | 0 | 0.14 ± 0.01 |
| | $1.82 \times 10^{-6}$ | 0.18 ± 0.01 |
| | $1.82 \times 10^{-5}$ | 0.15 ± 0.01 |
| | $1.82 \times 10^{-4}$ | 0.17 ± 0.01 |
| | $1.82 \times 10^{-3}$ | 0.14 ± 0.01 |
| | $1.82 \times 10^{-2}$ | 0.16 ± 0.01 |
| | $1.82 \times 10^{-1}$ | 0.16 ± 0.02 |
| | 1.82 | 0.11 ± 0.01 |
| D | 0 | 0.07 ± 0.01 |
| | $1.82 \times 10^{-5}$ | 0.10 ± 0.01 |
| | $1.82 \times 10^{-4}$ | 0.09 ± 0.02 |
| | $1.82 \times 10^{-3}$ | 0.08 ± 0.01 |
| | $1.82 \times 10^{-2}$ | 0.17 ± 0.01 |

Effect of methotrexate on frequency of SCE in cells from donors C and D.

## DISCUSSION

The effect of cytotoxic drugs on cells from cancer patients and other donors, and then the induction of SCEs by combinations of these agents, will be considered.

Neither Hodgkin's disease not bronchiogenic carcinoma altered the sensitivity of the lymphocytes to the drugs as far as the number of SCEs displayed was concerned. Though recent irradiation increased the frequency of SCE slightly, the effect appeared to be small and temporary.

Though death of heavily damaged cells may have occurred, particularly after large doses of drugs, measurement of SCEs required that cells should have divided at least twice in culture, so only sublethal effects have been examined.

Even without mixing cyclophosphamide with hepatic microsomes, the drug caused SCEs to form in the lymphocytes. Either the cyclophosphamide spontaneously degraded into mutagenic derivatives when incubated at 37°C

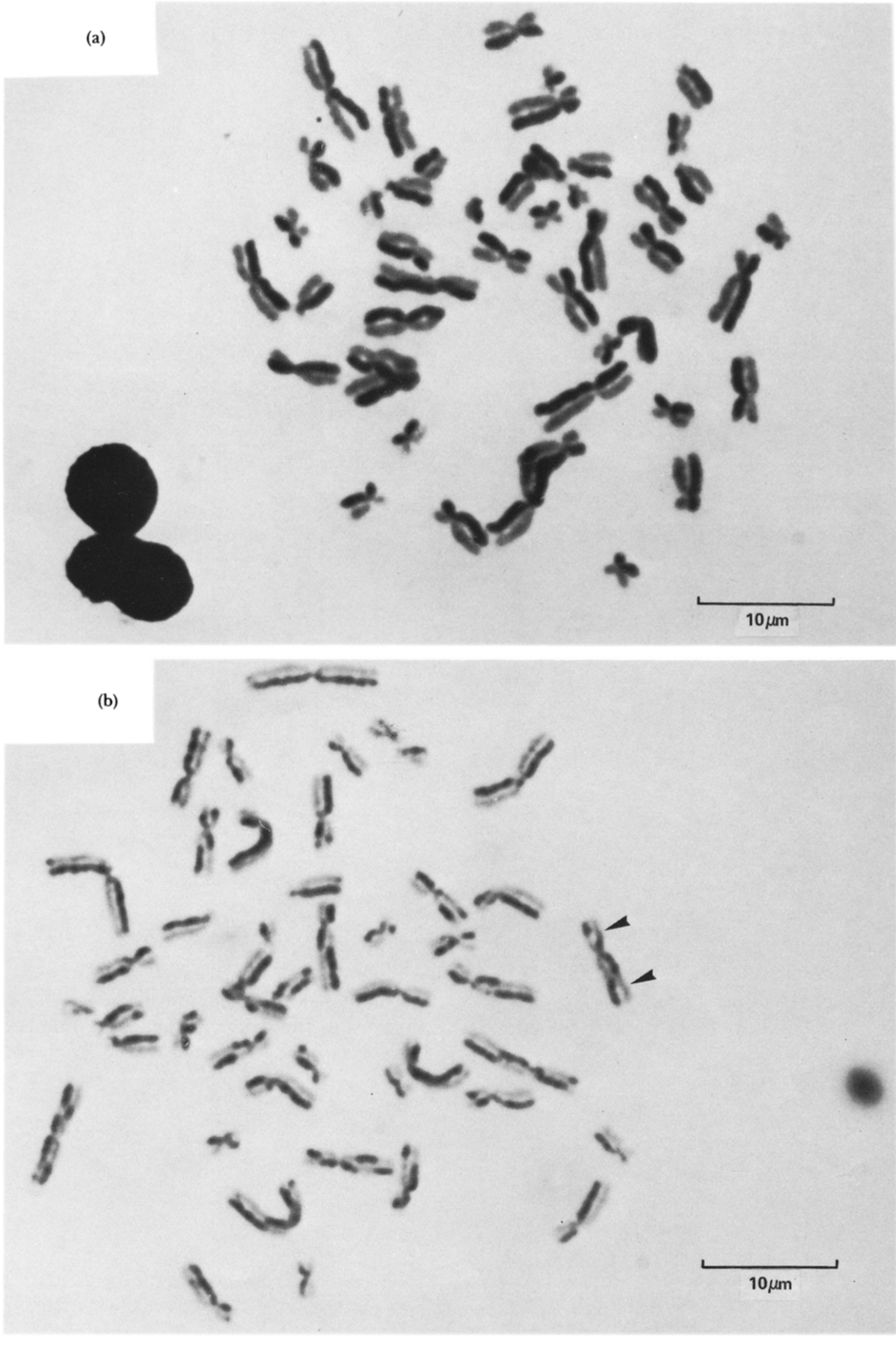


*Fig.* 1. *Untreated cell* (*a*) *with* 1 *SCE present, and cell containing about* 40 *SCE* (*b*) *after exposure to* 0.12 μg/ml *activated cyclophosphamide. Arrows indicate presence of SCE.*

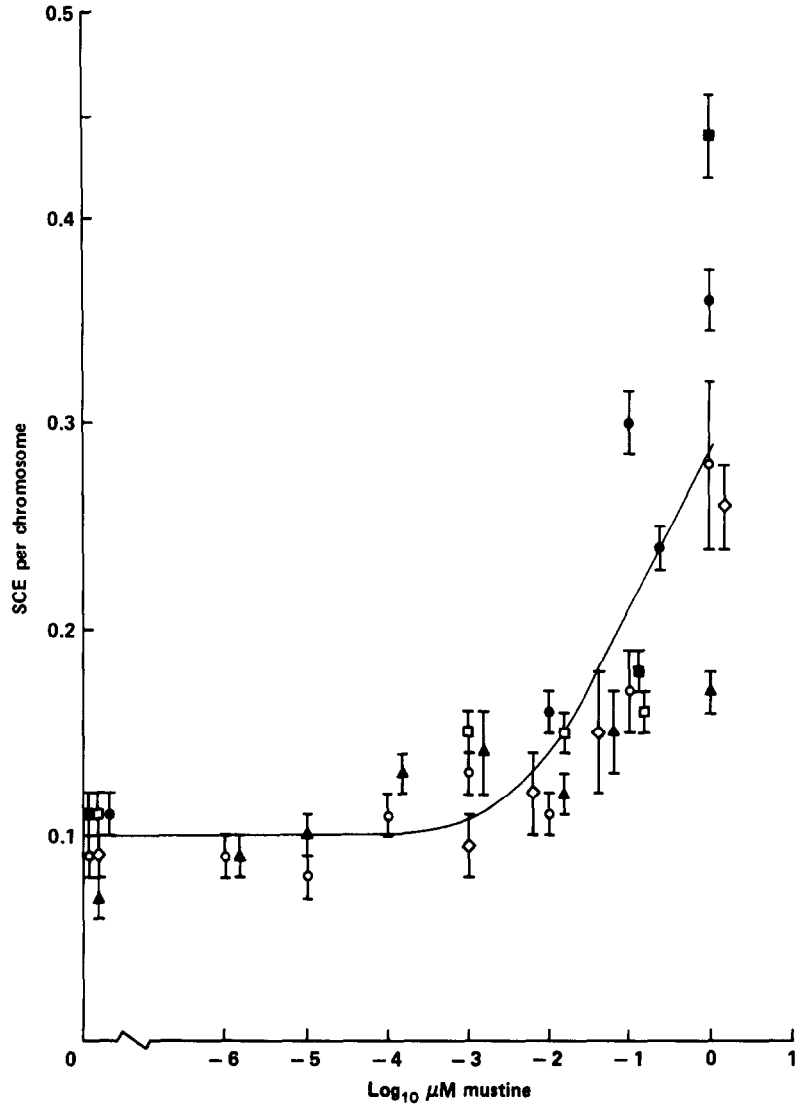


*Fig. 2.* ◇ *Cells from donor 3/78 exposed to MOPP;* ● *cells from donor B exposed to mustine only;* ○ *cells from donor D exposed to MOPP;* ■ *cells from donor A exposed to mustine only;* □ *cells from donor B exposed to MOPP;* ▲ *cells from donor B exposed to mustine only. Largest doses of MOPP used were approximately* 0.2 μg/ml *mustine,* 0.05 μg/ml *oncovin,* 3.0 μg/ml *procarbazine and* 0.75 μg/ml *prednisolone. The relative proportions of these drugs remained constant. Errors* ±1 *S.E. Line drawn through mustine data only.*

over three days [19] or the lymphocytes metabolised some of the drug [20]. One or all of the products of activation were probably responsible for inducing the majority of SCEs, since the frequency was more closely correlated with the activated dose than the total amount of cyclophosphamide.

The largest dose of cyclophosphamide probably saturated the microsomes and NADPH, since initially only 5% was converted to metabolites. However, this dose increased the frequency of SCEs 7.4 times above the control value. By comparison, using rodent cell lines, with periods of one hour or less incubation of the cells, 280 μg/ml cyclophosphamide and S9 mix increased the rate of SCE 4.5 times [21]. A dose of 10 μg/ml cyclophosphamide caused a multiplication of about five times in the frequency of SCEs[22]. Chinese hamster V79 cells in diffusion chambers were exposed to cyclophosphamide injected into the host mice at 2.5 μg per g body weight. The frequency of SCEs was increased 2.4-fold [23]. Thus the cyclophosphamide appeared to be more effective in inducing SCEs in this study than other workers have experienced. The different response may either reflect differences in the sensitivity of metabolic capacity of the types of cells used, death and removal of damaged cells, or the effectiveness of activation. For instance, the proportion of microsomes transferred to the culture medium in this study may have continued the process of metabolism, especially if the original con-

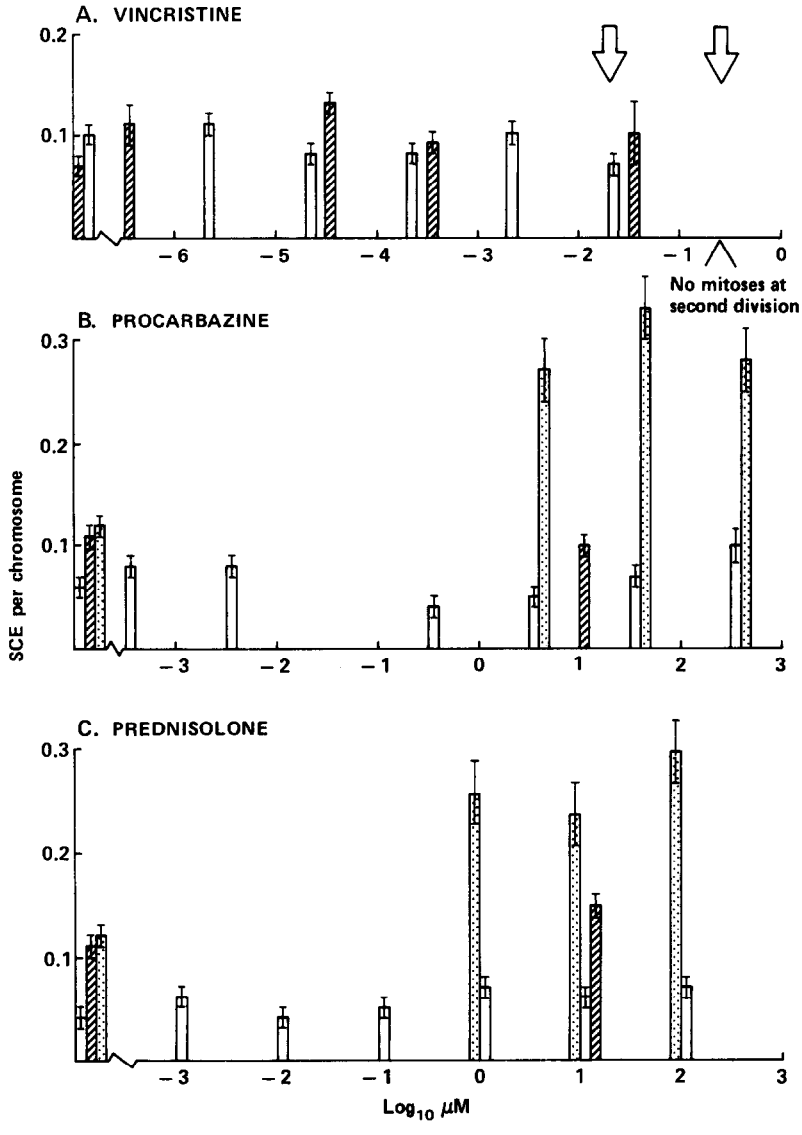


*Fig. 3.* (*A*) ▨ *Effects of vincristine on cells from donor D*; □ *effects of vincristine on cells from donor C*; ⇩ *effects of various doses of vincristine and* 0.045 μg/ml *mustine on cells from donor A. No mitoses at second division.* (*B*) ▨ *Effects of procarbazine dissolved in acetone* (0.02%) *on cells from donor D*; □*effects of procarbazine on cells from donor C*; ▩ *effects at various doses of procarbazine and* 0.045 μg/ml *mustine on cells from donor A.* (*C*) ▨ *Effects of prednisolone dissolved in acetone* (0.02%) *on cells from donor D*; □ *effects of prednisolone on cells from donor C*; ▩ *effects of various doses of prednisolone and* 0.045 μg/ml *mustine on cells from donor B. All results* ± 1 *S.E.*

version took place at a limited speed. If so, the magnitude of effect would be greater than that expected *in vivo*, particularly since detoxification and removal of the drug would also occur *in vivo*. All drugs were left in the medium for the entire culture period to enhance the chances of interaction between the drugs.

Various doses of vincristine, procarbazine, prednisolone and methotrexate, the largest of which were four or five times as high as those given to patients on a mg per kg basis during one course of chemotherapy, did not significantly affect the frequency of SCEs. Since prednisolone and procarbazine mixtures were prepared from tablets, the lack of effect may be ascribed to the use of an inappropriate solvent. However, procarbazine hydrochloride is very soluble in water and prednisolone is soluble to about 770 μg/ml, so only the 'filler' material was likely to have been affected by the use of acetone. More work is necessary to confirm whether prednisolone dissolved in acetone would consistently increase the frequency of SCEs, since a single result does not preclude a chance effect. However, all of these drugs have elicited a response in other biological systems, examining various endpoints [24–28].

Cross-linkage of DNA appears to be a prerequisite for the formation of SCEs [29, 30]. Thus the inactivity of vincristine, prednisolone and methotrexate may be accounted for by

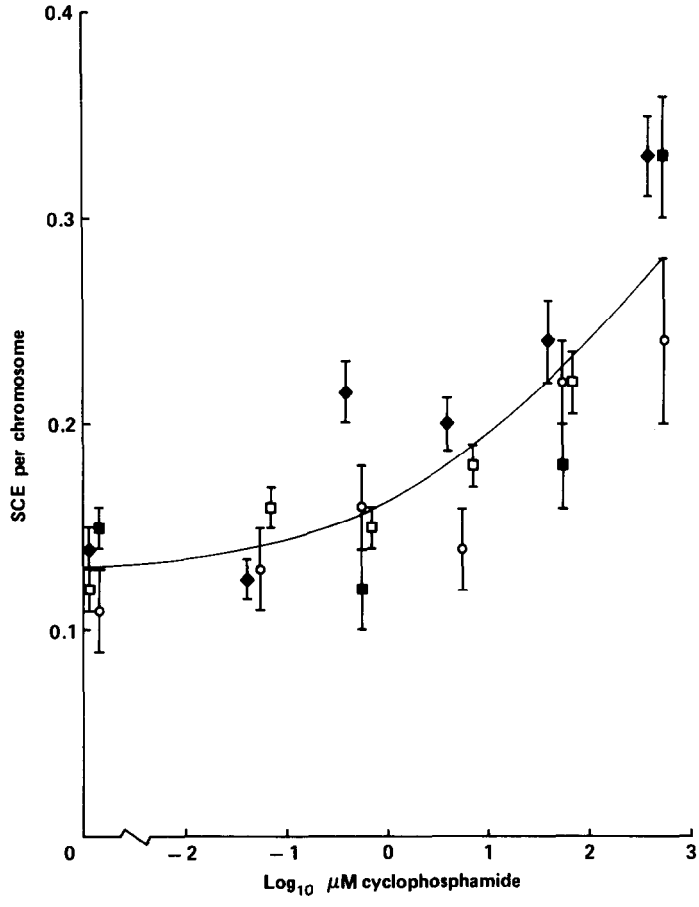


*Fig. 4.* □ *Blood from donor C exposed to inactivated cyclophosphamide*; ○ *blood from donor* 1/79 *exposed to inactivated cyclophosphamide*; ■ *blood from donor* 7/79 *exposed to inactivated cyclophosphamide and methotrexate*; ◆ *blood from donor* 24/78 *exposed to inactivated cyclophosphamide and methotrexate. Largest doses of combination of inactivated cyclophosphamide and methotrexate received were* 14.5 μg/ml *and* 2.7 μg/ml *respectively. All errors* ± 1 *S.E. Line drawn through all data.*

their inability to react directly with, and distort, DNA.

Procarbazine, a methyl hydrazine derivative, is metabolically converted into a mutagen in mammals, where it is a potent inducer of micronuclei in the bone marrow of mice [31]. Procarbazine also induces chromatid exchanges and breaks in hypotetraploid Ehrlich and diploid P388 ascites tumours in mice [32], and SCEs in the bone marrow of Chinese hamsters [33]. An increase in the frequency of SCEs in human lymphocytes *in vitro* could therefore probably be achieved by exposing cells to the drug in the presence of a microsomal activation system.

Mustine did not interact with other drugs in the MOPP regime, nor did cyclophosphamide with methotrexate, in inducing SCEs. If this effect is specific to normal rather than malignant cells, multiple agent chemotherapy could confer an advantage, particularly as damage to the DNA of normal cells by the treatment may induce new malignancies [34]. The absence of any interaction of the drugs in inducing SCE does not preclude cooperation to enhance subcellular effects other than their reaction with DNA.

Vincristine and mustine together appeared to prevent cells from dividing at doses where the single drugs would have failed to do so. There remains a tentative suggestion that these drugs act synergistically in a manner unconnected with the formation of SCEs. For example, Ezdinli and co-workers [35] demonstrated that multiple drug therapy decreased the percentage of rosette-forming cells in the peripheral blood more profoundly than did single agents.

The induction of SCE correlates more closely with genetic damage such as mutation rather than non-specific cytotoxicity [28, 36]. Whilst SCEs and chromosome aberrations may be alternative responses to damage [37], premutational lesions may be qualitatively distinct from prelethal lesions [38]. This suggests that SCEs are not directly associated with events

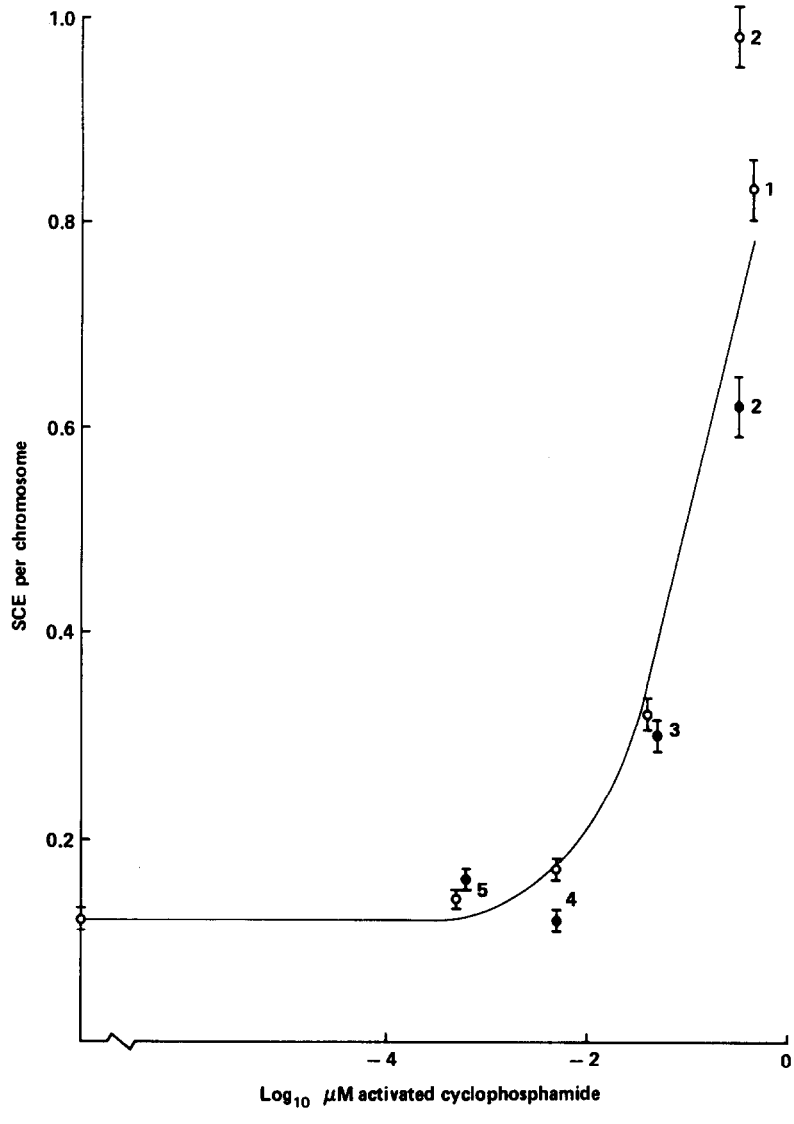


*Fig. 5.* ○ *Effects of activated cyclophosphamide on cells from donor C;* ● *effects of activated cyclophosphamide and methotrexate on cells from donor C. Control sample contained the activation mixture, diluted to about* 1 *in* 23 *parts. The amounts of cyclophosphamide added, the percentage of activation and the quantity of activated cyclophosphamide calculated to be present in the medium in* $Log_{10}$ μM *are as follows*: (1) 2.42 μg/ml, 5%, $-0.36$ $log_{10}$ μM; (2) 0.242 μg/ml, 35%, $-0.52$ $log_{10}$ μM; (3) 0.024 μg/ml), 50%, $-1.36$ $log_{10}$ μM; (4) 0.002 μg/ml, 60%, (*estimated* $-2.28$ $Log_{10}$ μM; (5) 0.0002 μg/ml, 65%, (*estimated*) $-3.25$ $log_{10}$ μM. *Methotrexate was added to cultures* (2)–(5), *from* 0.364 μg/ml *in culture* (2) *diluted by a factor of ten for each culture to* (5). *Line drawn through all data.*

causing cell death. However, since an agent often produces a spectrum of intracellular damage [39], the presence of SCEs could indicate the manner in which other forms of sublethal injury are accumulating.

It would be of interest to test the effect of combining cyclophosphamide and mustine to establish whether agents with similar modes of action would induce SCEs additively or synergistically. If they failed to interact, the widely held view that combining agents enhances their efficacy at a subcellular level may need to be more critically appraised.

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