Eur J Cancer Clin Oncol, Vol. 18, No. 10, pp. 979–989, 1982.
Printed in Great Britain.

0277-5379/82/100979-11$03.00/0

Sister Chromatid Exchanges in Human Lymphocytes Exposed to Single Cytotoxic Drugs *in Vivo* or *in Vitro**

M. GILLIAN CLARE,† WILLIAM G. JONES and JANET H. TAYLOR
University Department of Radiotherapy, Regional Radiotheropy Centre, Cookridge Hospital, Leeds, LS16 6QB, U.K.

Abstract—*Venous blood was taken from apparently healthy volunteers and patients with cancer, the latter both before and at intervals after treatment with single cytotoxic drugs. Cells from untreated individuals were exposed to a range of concentrations of drugs in culture medium. Chlorambucil, treosulfan and cyclophosphamide (activated by hepatic microsomes) significantly increased the numbers of SCEs, both* in vitro *and in the lymphocytes of patients. While methotrexate and 5-fluorouracil had no effect, bleomycin slightly increased the number of SCEs, but only* in vitro. *Although the* in vitro *dose–effect relationship indicated which drugs would increase the frequency of SCE* in vivo, *the magnitude of the response tended to be overestimated. When patients were treated with drugs the frequency of SCE increased, then declined with time. Though this complicates the quantitative relationship betwen dose and damage, SCEs may be useful to monitor the effects of alkylating agents on normal tissues.*

INTRODUCTION

THE LOSS of reproductive integrity of cells in tissues which constantly replenish themselves limits the dose of most cytotoxic drugs given for the treatment of malignant diseases. To improve treatment, the effects of these agents on normal as well as tumour tissues must be qualitatively and quantitatively defined. Peripheral blood lymphocytes are counted routinely in patients receiving cytotoxic drugs and, if depressed below a critical level, the treatment is modified. Thus lymphocytes may be regarded as a 'limiting' normal tissue.

Chromosomal injury disrupts the function of cells and causes cell death [1, 2]. Examination of chromosomes in the lymphocytes of peripheral blood, stimulated to divide in short-term tissue culture [3], provides a convenient assay for sublethal intracellular damage. Staining techniques have been developed [4, 5] to show clearly reciprocal exchanges that occur between the chromatid arms of a chromosome, i.e. sister chromatid exchanges (SCEs). Most chemicals, particularly alkylating agents, which damage chromosomes also greatly increase the rate of SCE [6–8]. This provides a sensitive indicator of the quantity of agent which has reached the DNA.

The objective of this study was to evaluate whether SCE could be used to measure the effect of chemotherapy on a normal tissue. The ability of single, commonly used cytotoxic drugs to induce SCEs, either *in vitro* or in the leukocytes of cancer patients, has been investigated. One of these drugs was cyclophosphamide, which requires conversion by hepatic microsomes before it manifests biological activity [9]. Cells were exposed to cyclophosphamide in culture medium containing a preparation of enzymes from rat liver. The amount of chromosome damage was assessed after exposure of cells to each of the drugs in culture medium.

Treosulfan is used in the treatment of carcinoma of the ovary [10]. The relationship between the dose of this drug and the frequency of SCE was examined. The persistence of SCE

Accepted 25 May 1982.
*Supported by the Yorkshire Cancer Research Campaign.
†Requests for reprints should be addressed to G. Clare, currently at Shell Toxicology Laboratory (Tunstall), Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K.

in lymphocytes was measured after cessation of the treatment.

MATERIALS AND METHODS

Donors

Three laboratory workers, without known malignant or viral disease when blood was taken, volunteered as donors of blood. One (subject C) was a female non-smoker in the age group 25–30 years. The other two were male smokers, aged 40–45 years (subject D) and 30–35 years (subject E). Although workers C and D were designated as occupationally exposed to ionising radiation, no significant levels had been detected on their personal dose meters.

The patients with cancer varied as regards sex, age, disease and previous treatment, apart from seven women with carcinoma of the ovary (Table 1). The latter group received daily doses of treosulfan, during which time the white blood cell counts remained approximately constant. If the count was depressed the drug was temporarily discontinued until recovery was observed.

Drugs

Chlorambucil (CHL-Wellcome Foundations Ltd. Crewe, Cheshire), cyclophosphamide powder (CPM-'Endoxana', WB Pharmaceuticals Ltd. Bracknell, Berks), treosulfan tablets (TRS-Leo Labs Ltd. Aylesbury, Bucks), vials of methotrexate solution for intramuscular injection (MTX-Lederle Labs, Gosport, Hampshire), vials of 5 fluorouracil solution (5FU-Roche Products Ltd. Welwyn Garden City) and bleomycin powder (Lundbeck Ltd. Luton, Beds) were dissolved and diluted using sterile distilled water, generally by factors of ten-fold,

Table 1. Brief details of cancer patients studied

Donor	Sex	Age (yr)	Disease	Previous radiotherapy	Previous chemotherapy
22/78	M	66	Squamous carcinoma of right tonsillar fossa and tongue	None	None
23/78	M	39	Metastatic seminoma of right testis	1 year	3 years
2/79	M	57	Lymphocytic non-Hodgkin's lymphoma	None	None
19/79	F	44	Adenocarcinoma of left adrenal	With chemotherapy	None
38/79	F	23	Carcinoma of cervix, stage III	6 months	22 days
1/80	F	72	Pleomorphic adeno-carcinoma of ovary	8 weeks	None
4/80	M	57	Carcinoma of the bronchus	Immediately after 1st sample	None
20/79	F	61	Carcinoma of ovary	2 months	Cyclophosphamide daily for 4 weeks
23/79	F	53	Carcinoma of ovary	8 months	None
30/79	F	62	Carcinoma of ovary	None	None
31/79	F	62	Carcinoma of ovary	2 years	Treosulfan daily for 2 years
33/79	F	52	Carcinoma of ovary	9 months	Thiotepa 2 years, treosulfan 1 year 8 months
5/80	F	67	Carcinoma of ovary	None	Treosulfan 1 day

Details of surgery excluded. Approximate time between most recent treatment before blood sampling given for previous radiotherapy and chemotherapy. Radiotherapy consisted of low LET radiation, generally X-rays. The dose of approximately 40 Gy was usually split into 5 fractions per week for 4 weeks to the part of the body affected. Only when alkylating agents were used in previous chemotherapy is their name mentioned.

immediately prior to use. When the tablets were dissolved the solution was sterilised by passing it through a filter of pore size 0.23 μm.

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 cycle of chemotherapy. When present the drugs remained in the medium for the entire period of culture. If this prevented cells reaching their second division in culture only the effects of smaller doses are presented.

Activation and measurement of cyclophosphamide

The materials and methods for the activation and subsequent assessment of alkylating activity of cyclophosphamide have been described [11].

Briefly, various concentrations of cyclophosphamide were incubated at 37°C for 10 min with rat microsomes from 1 g of liver and reduced nicotinamide adenine dinucleotide phosphate at a final concentration of 1.0 mM.

Aliquots of 0.2 ml were removed from each tube and added to the medium for the culture of lymphocytes. The amount of alkylating activity in the remainder was estimated colorimetrically, calibrated against nitrogen mustard and expressed as micromoles of alkylating products of cyclophosphamide.

Culture and harvest of leukocytes

The procedures for obtaining samples of blood and the culturing, harvesting and staining of lymphocytes to demonstrate SCE were carried out as described previously [11]. Appropriate amounts of drug (if used) and 0.5 ml whole blood were added aseptically to 5.0 ml TC 199 at pH 7.2, 0.25 ml donor calf serum, 0.1 ml non-essential amino acids, 200 IU gentamycin sulphate, 0.1 ml PHA (reagent grade, Wellcome Reagents Ltd., London) and 10 μM 5-bromo-2'-deoxyuridine (BrdU, Sigma, London).

Cultures were incubated in total darkness for 71 hr at 37 ± 0.5°C, when 0.1 μg/ml vincristine sulphate was added. 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 microslides were air dried.

Staining

The cells were stained for 15 min with Hoechst 33258 at 0.5 μg/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). The slides were rinsed and stained for 6 min at 20°C with Giemsa (BDH, 0.68% solution in methanol glycerol) diluted to 2% with phosphate buffer (pH 6.8). The preparations of cells used to analyse chromosome damage were stained with Giemsa alone.

Recording chromosome damage or SCEs

All slides were codified within any one experiment. About 100 cells were selected from all cultures exposed to drugs *in vitro* and examined for any type of chromosome or chromatid damage. Chromosome breaks included acentric fragments of all sizes. Chromatid breaks included deletions and single fragments. Only data where chromosome damage is relatively more prevalent than SCE are presented.

Cells at metaphase in their second or third division which had clearly differentially stained chromosomes were chosen to count SCEs. 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 results are presented as a frequency of SCE per chromosome (± 1 S.E.) for all the cells of that sample.

RESULTS

The average percentage of third division cells after exposure to cytotoxic drugs *in vitro* or *in vivo* was 24 and 11% respectively. There were about 1.3 times more SCEs in third than second division cells. Therefore the use of cells at their third division did not influence the results significantly.

On the basis of the amounts of drug (expressed in terms of their molecular weights), the order of effectiveness in inducing SCEs in normal lymphocytes *in vitro* was: activated cyclophosphamide > treosulfan > chlorambucil (Fig. 1). No drug significantly reduced the number of SCEs. Methotrexate had no effect on the number of lymphocytes which were transformed by the phytohaemagglutinin and caused neither chromosomal damage, nor SCE. No dose–response relationship was obtained for 5-fluorouracil as doses larger than about 0.1 μg/ml reduced the number of transformed lymphocytes and cells in their second division dramatically, but doses of up to 0.09 μg/ml did not significantly increase the frequency of SCE or cause chromosome or chromatid damage. Only bleomycin caused

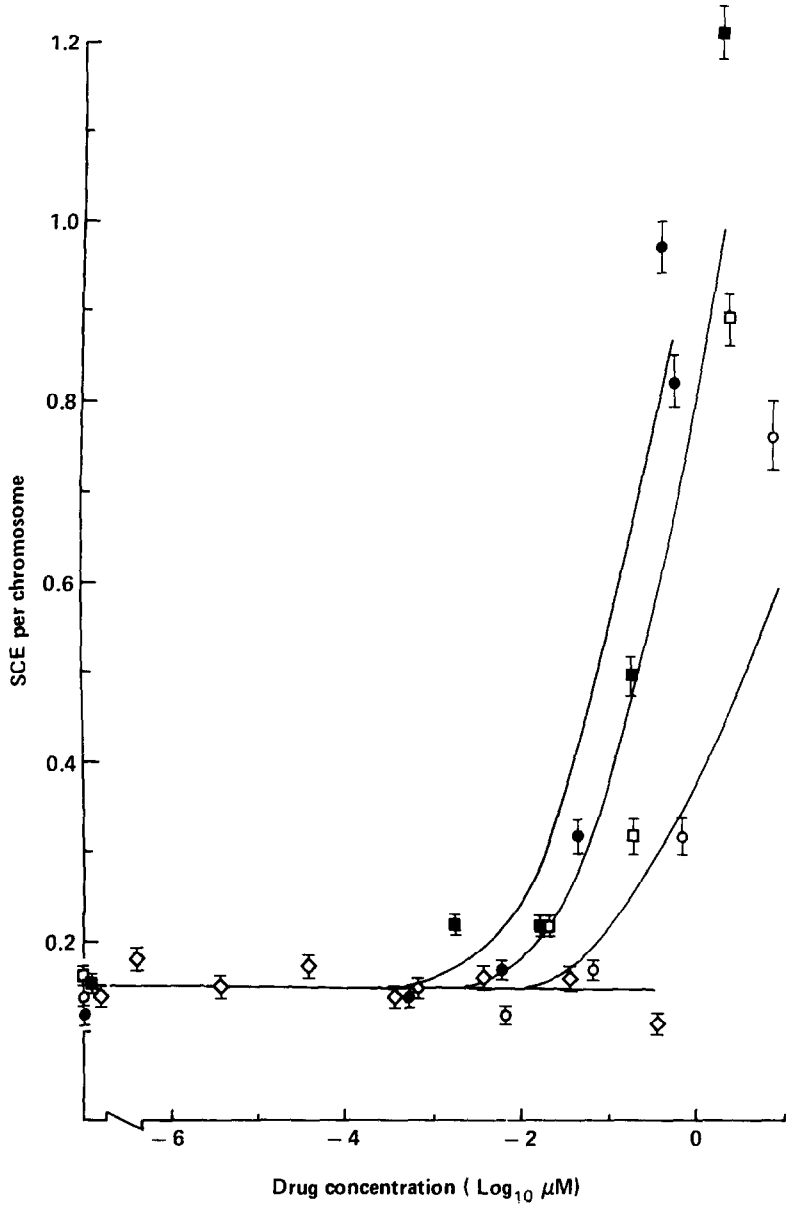

Fig. 1. The number of SCE per chromosome induced by doses of various chemotherapeutic agents in vitro. *Results* ± 1 *S.E. Key*: ○ *donor 2/79—chlorambucil*; ● *donor C—activated cyclophosphamide*; ◇ *donor D—methotrexate*; ■ *donor E—treosulfan*; □ *donor F—treosulfan. The total amounts of cyclophosphamide added to each culture were* 2.420 μg/ml, 0.242 μg/ml, 0.024 μg/ml, 0.002 μg/ml *and* 0.0002 μg/ml. *The percentage of activation was estimated to be* 5, 35, 60 *and* 65% *respectively.*

relatively more chromosomal damage than SCE (Table 2), inducing principally breaks of chromatids and chromosomes.

Agents which did not increase the number of SCEs *in vitro* had no effect *in vivo*. Those agents which increased the number of SCEs *in vitro* also did so *in vivo* (Fig. 2).

A dose of 4.5 μg/ml of treosulfan increased the frequency of SCE in the lymphocytes of donor E by six or eight times compared with the control value *in vitro*, but only doubled the frequency of SCE in the lymphocytes of patient 1/80 *in vivo*. The largest amount of cyclophosphamide added to a culture was 2.42 μg/ml, of which 5% was activated. This increased the rate of SCE to seven times the control value. In contrast, patient 23/78 received 42.6 mg/kg of cyclophosphamide. The frequency of SCE was elevated by only 1.6 times, 21 days after the injection.

When patients with carcinoma of the ovary were treated continuously, without modification of the daily dose of treosulfan, the frequency of SCE was generally higher than it would have been without treatment (Fig. 3). There was a wide variation in response to similar total doses of drug. Small cumulative doses of more than 20 g produced a similar effect to 200 g of treosulfan.

There appeared to be a direct relationship

Table 2. Chromosomal damage and SCE after bleomycin in vitro

Bleomycin concentration (μg/ml)	No. of cells	Frequency per cell (± 1 S.E) Chromosome		Chromatid			No. of cells	SCE per chromosome ± 1 S.E.
		Exchanges	Breaks	Exchanges	Breaks	Gaps		
0	100	0	0	0	0	0.01 ± 0.01	30	0.06 ± 0.01
0.000002	100	0	0.02 ± 0.01	0	0	0	30	0.08 ± 0.01
0.00002	100	0	0.01 ± 0.01	0	0	0	30	0.10 ± 0.01
0.0002	100	0	0.03 ± 0.02	0	0	0	30	0.08 ± 0.01
0.018	100	0	0	0	0.01 ± 0.01	0.04 ± 0.02		—
0.18	100	0	0.22 ± 0.05	0.01 ± 0.01	0.09 ± 0.03	0.07 ± 0.03	30	0.10 ± 0.01
1.82	79	0.01 ± 0.01*	0.10 ± 0.03	0	0.04 ± 0.02	0.04 ± 0.02	30	0.11 ± 0.01

*Dicentric.

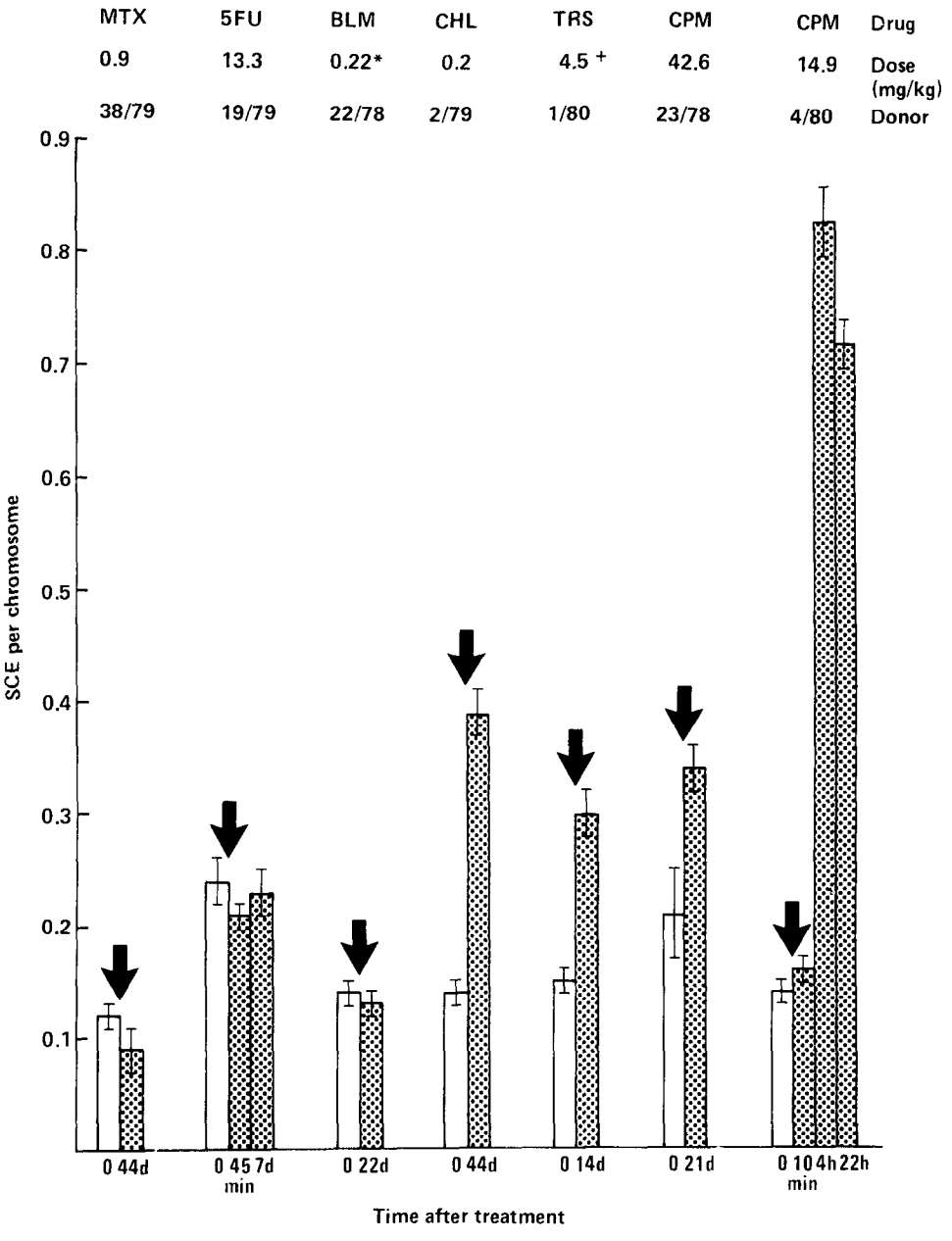

Fig. 2. SCE in lymphocytes of patients after treatment with single drugs. □ *Pretreatment, no previous chemotherapy;* ⇓ *drug given;* ■ *after treatment;* * 15 mg *on days* 0, 2 *and* 7 *after first blood sample taken;* +250 mg *daily for* 14 days.

between the numbers of SCEs and the daily dose of treosulfan (Fig. 4).

One donor (23/78) had previously received a total of 88.5 g at a rate of 500 mg/day. As soon as treatment was discontinued the frequency of SCE declined quickly at first and then more slowly, with a half-life of about 34 days. After about 11 weeks it reached a reproducible level consistent with a control value (Fig. 5).

DISCUSSION

The analysis of SCEs forms a sensitive, specific and accurate test which may indicate the recovery of lymphocytes from a chemical insult and the effective biological half-lives of alkylating agents *in vivo*.

Only those cytotoxic drugs which alkylate DNA (cyclophosphamide, chlorambucil and treosulfan) increased the frequency of SCE dramatically *in vitro*. Neither methotrexate nor 5-fluorouracil react directly with the DNA of cells [12, 13]. This appears to be a prerequisite for the formation of SCE [14]. Despite the contention that those chemicals which induce most damage to the DNA induce most SCEs [15], only the type of damage that also results in the reciprocal exchange between chromatids to produce quadriradial configurations at

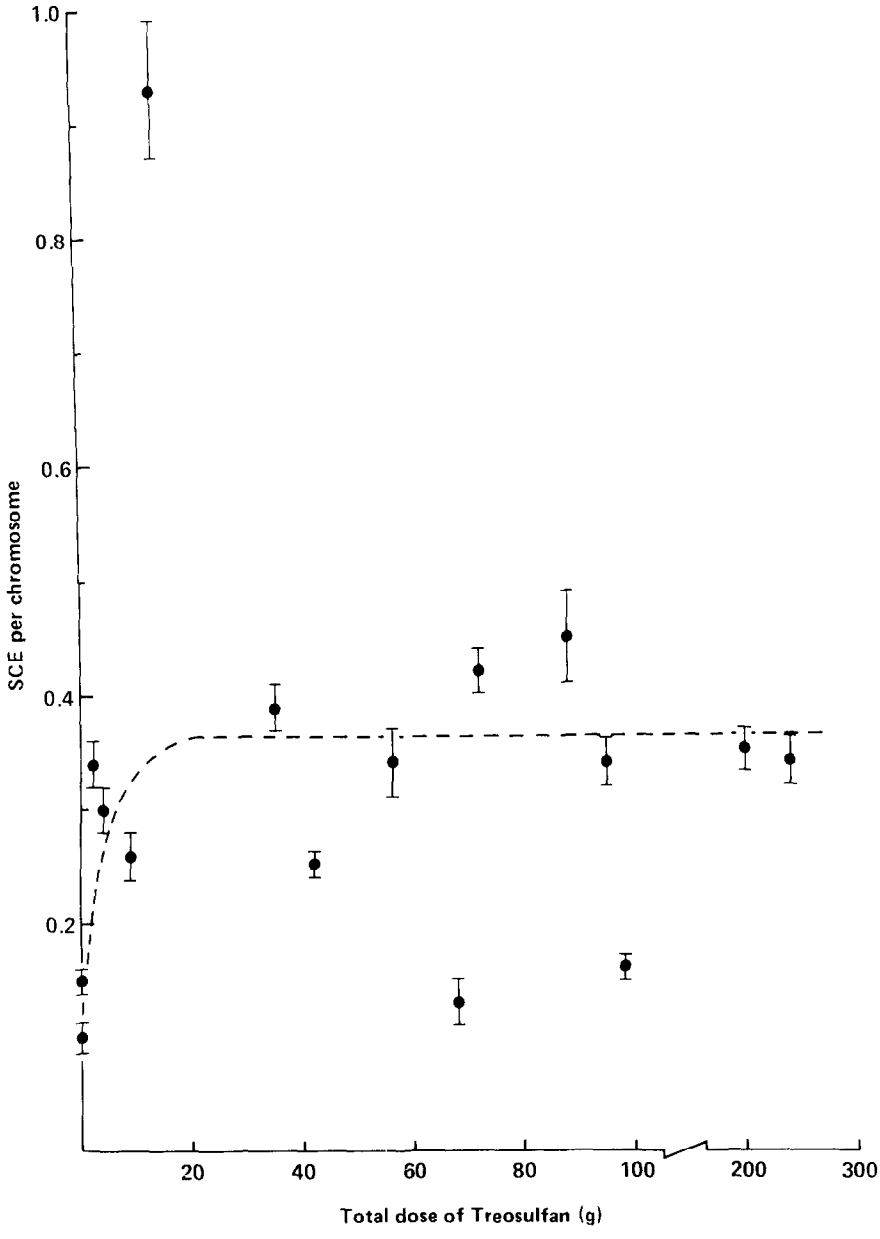

Fig. 3. Frequency of SCE in patients receiving daily doses of treosulfan, accumulated over different lengths of time.

metaphase is correlated with SCE [16]. Bleomycin cleaves DNA [17] and breaks chromosomes, but does not produce lesions which terminate in SCEs. These results emphasise the necessity of testing mutagenic agents with consideration of the special mechanism of their activity [18].

The analysis of SCEs in the peripheral blood lymphocytes of patients receiving methotrexate, 5-fluorouracil or bleomycin is an unsuitable method for detecting the damage induced by these drugs. Lambert *et al.* [19] also reported normal levels of SCE in the lymphocytes of patients exposed to these latter three agents.

Conversely, the use of SCEs to monitor the effects of chlorambucil, treosulfan and cyclophosphamide in patients appears promising.

Radiotherapy may raise the level, as in the control sample from donor 19/79. The type of malignant disease may be associated with a higher frequency of SCE than normal [20]. Nevertheless, the levels of SCE in cells are greatly elevated only by certain cytotoxic drugs. Thus the use of SCE provides a specific test, and one which is a more sensitive measure of the effects of alkylating agents than chromosome damage or overt toxicity, since the total white blood cell counts in women with carcinoma of the ovary remained approximately constant during a period when the levels of SCE were related to the daily doses of treosulfan.

The results obtained from cells exposed to the drug in culture medium qualitatively

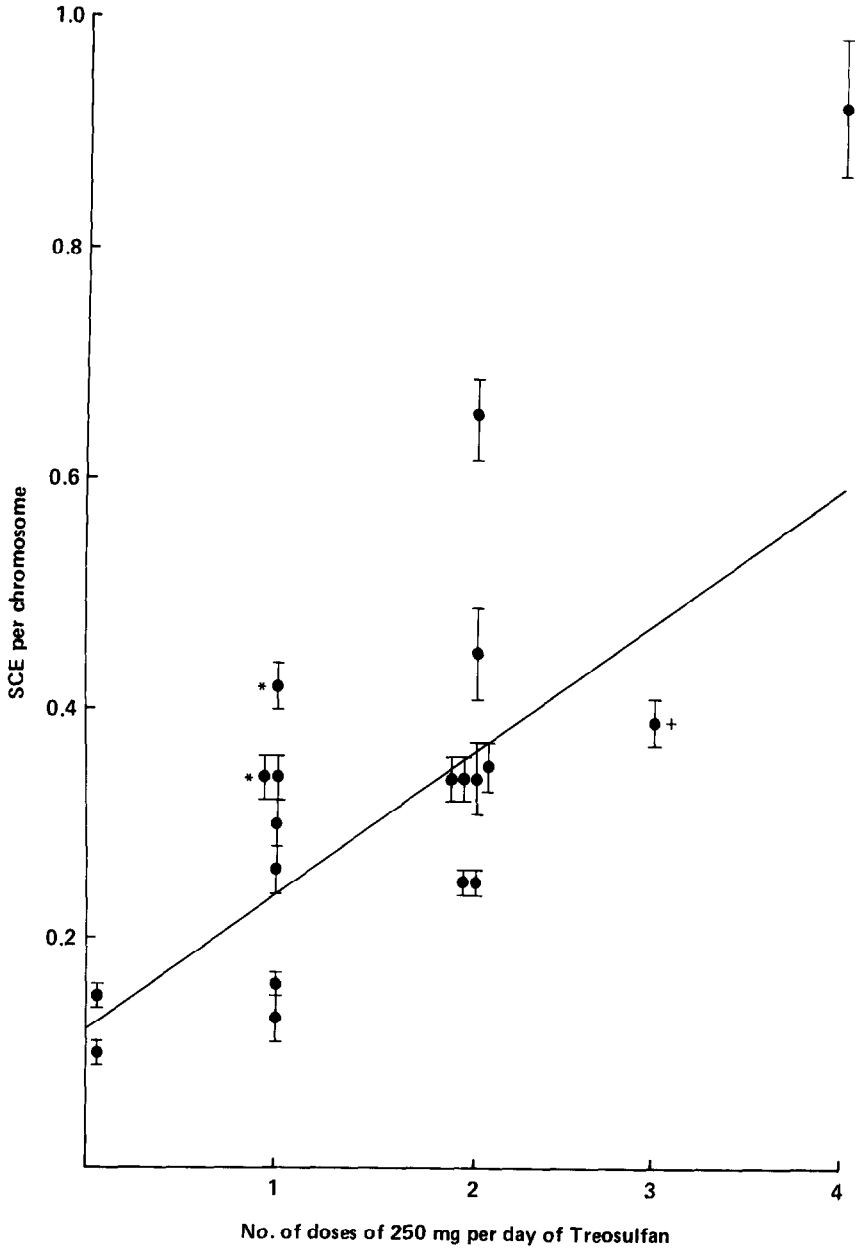

Fig. 4. Frequency of SCE in relation to the number of times a dose of 250 *mg of treosulfan was received each day by patients with carcinoma of the ovary.* * 125 mg *twice a day*; +250 mg *four times a day for* 14 days, *then twice a day for* 14 days.

reflected the effects of drugs in patients and probably indicate the maximum response that could be expected *in vivo*. The exposure of peripheral lymphocytes to a drug in a patient will depend upon the mode of administration, and its storage, metabolism and rate of excretion, which may occur within an hour or so of treatment [21]. Since each drug was present for the entire time of culture *in vitro*, unless the activity of the agent diminishes rapidly in solution the cells would be in contact with the agent for longer *in vitro* than *in vivo*.

There is a reduction in the number of SCEs with time after single doses of alkylating agents and a lack of correlation of the number of SCEs with the total dose of treosulfan accumulated over a long period. The transience of SCE has also been established by others [22, 23]. This decrease complicates the quantitative determination of dose using SCE.

SCEs may only be induced in cells which are in direct contact with the drug during division. However, it is unlikely that sufficient chlorambucil, treosulfan and cyclophosphamide remained in the blood of subjects 2/79, 1/80 and 23/78 respectively at least two weeks after therapy to account for the elevated levels of SCE. Also, when cells have been exposed *in vivo* to either cyclophosphamide or mitomycin C, those cells which have been washed after

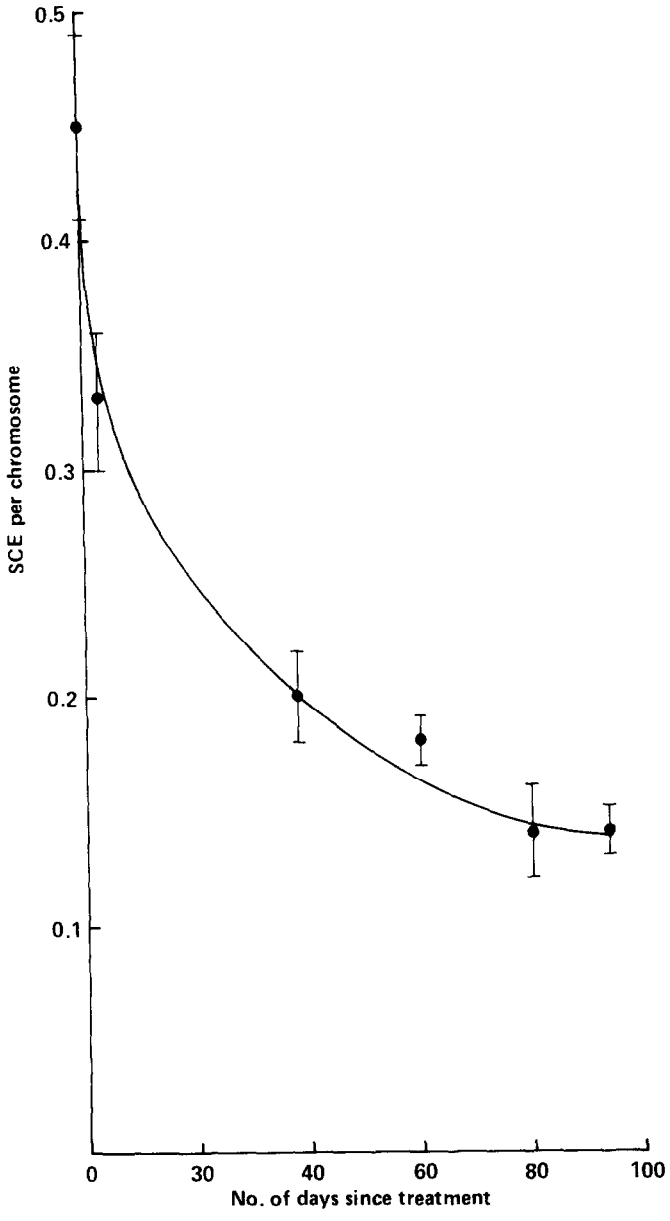

Fig. 5. Decrease in frequency of SCE in lymphocytes of patient 23/79 with increasing time after treosulfan was terminated.

venepuncture before culture contain as many SCEs as unwashed cells [24, 25]. This suggests that lymphocytes can accumulate damage and retain it in a form where it can be expressed after synthesising DNA.

The damage induced by certain drugs may be rapidly repaired and therefore short-lived [23]. Alternatively, since lymphocytes move in and out of the circulation [26], damaged or old lymphocytes may be removed and replaced by cells not previously in contact with as large a concentration of drug. Several subpopulations of peripheral blood lymphocytes probably exist, with different half-lives. which are tentatively assessed as being three years [27]. Unless a subpopulation of short-lived lymphocytes has been selected, the decrease in the number of SCEs after treatment with treosulfan was stopped cannot be solely explained by cell death.

Though treosulfan induces acute lymphocytic leukaemia [10] and SCE in groups of women with ovarian carcinoma, the frequency of these events is not correlated with the total cumulative dosage. Thus SCEs may be useful for monitoring those susceptible to carcinogens [28].

Acknowledgements—The authors are indebted to Prof. C. A. F. Joslin and Dr. B. Dixon for their help and encouragement, and to Prof. C. A. F. Joslin, Dr. S. Corbett and Dr. Barnard for providing samples of blood from their patients. We also acknowledge the skilful technical assistance provided by Mrs. E. Blain, and thank Shell Research Limited for preparing the manuscript.

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