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Sister Chromatid Exchange in Chronic Ethylene Oxide-exposed Primates: Unexpected Effects of *in Vitro* Culture Duration, Incubation Temperature, and Serum Supplementation[1]

Karl T. Kelsey, John K. Wiencke, Ellen A. Eisen, Dennis W. Lynch, Trent R. Lewis, and John B. Little

Laboratory of Radiobiology, Department of Cancer Biology [K. T. K., J. B. L.] and Occupational Health Program, Department of Environmental Science and Physiology [K. T. K., E. A. E.], Harvard School of Public Health, Boston, Massachusetts 02115; Laboratory of Radiobiology and Environmental Health and Department of Epidemiology and International Health, University of California, San Francisco, San Francisco, California 94143 [J. K. W.]; and National Institute for Occupational Safety & Health, Division of Biomedical and Behavioral Sciences, Experimental Toxicology Branch, Cincinnati, Ohio 45226 [D. W. L., T. R. L.]

ABSTRACT

Ethylene oxide (EtO), a potent monofunctional DNA alkylating agent, has been shown to induce sister chromatid exchanges (SCE) in the peripheral blood lymphocytes of animals and workers exposed to it *in vivo*. We have previously reported that elevations of SCE persist for 6 years after cessation of EtO exposure in cynomolgus monkeys chronically exposed to EtO; the elevation in mean SCE was entirely attributable to a subpopulation of high SCE frequency cells (HFCs). We now report that the detection of persistent HFCs is dependent on the conditions of cell growth, and that EtO exposure increases the replication indices of lymphocytes from the exposed animals when these cells are examined at early cytogenetic harvest times. Culture of lymphocytes in differing serum supplements, changes in cytogenetic harvest times, and alterations in *in vitro* incubation temperature all markedly affected mean SCE frequency by influencing the detection of HFCs. The frequency of EtO-induced HFCs was independent of 5-bromodeoxyuridine concentration, used for differential staining of sister chromatids. These observations indicate that the detection of persistent alkylation-induced chromosomal changes, observed long after cessation of *in vivo* chronic exposure of these animals, is highly dependent upon factors affecting cell growth.

INTRODUCTION

EtO[2] is a highly reactive, monofunctional DNA alkylating agent that is commonly encountered in the workplace as an intermediate in the production of chemicals such as glycol ethers and polyester resins. EtO is also widely used as a fumigant for some food products and as a commercial sterilizing agent. Epidemiological studies of workers exposed to EtO have reported a significant excess of cancers, including leukemia and solid tumors (1–3). Cytogenetic studies of animals and human workers exposed to EtO have associated this exposure with the induction of elevated SCE frequencies in their peripheral blood lymphocytes (4–10). In some exposed workers the EtO-induced elevations in SCE have persisted for years after cessation of exposure (9).[3]

We have shown previously that EtO-induced SCE persist in peripheral blood lymphocytes for 6 years after the last EtO exposure of cynomolgus monkeys (11). The persistence of an elevated SCE frequency in the lymphocytes from these animals was entirely attributable to a subpopulation of cells with very high SCE frequencies. High SCE frequency cells (cells with more than 40 SCE/cell; HFCs) may be long-lived lymphocytes that inefficiently repair EtO-induced DNA damage (11).

In the present investigation we performed experiments to test whether HFCs, like other highly damaged cells acutely exposed to alkylating agents (12, 13), proliferate more slowly *in vitro* than unexposed cells and may thus be detected in greater numbers when cells are allowed to grow for additional times before cytogenetic analysis is performed. *In vitro* culture conditions which preferentially detect slowly proliferating HFCs could be used to improve the sensitivity of the SCE assay as an indicator of chromosomal damage. Cell cycle progression might also be a potential epidemiological confounder in chronic EtO exposure situations.

MATERIALS AND METHODS

Animals. The study was initiated in 1979 with 36 adult male cynomolgus monkeys (*Macaca fascicularis*; Primate Imports Corp., Port Washington, NY), assigned randomly to two exposure groups and one control group of 12 animals per group (10). At the start of the study, the monkeys weighed 5.31 ± 0.82 kg. The animals have been continually housed in stainless steel cages with automatic watering and a 12-h on/12-h off (7:00 a.m./7:00 p.m.) lighting system. They have been fed a standard pellet diet (Purina Monkey Chow) supplemented with fresh fruit two to three times per week. These animals have not been subjected to any subsequent experimental manipulations and have not been exposed to any known mutagens or carcinogens since the cessation of EtO exposure in 1981.

Test Materials and Inhalation Exposures. Test materials and inhalation exposures have been previously described (10). Briefly, EtO (99.7% pure) was instilled under dynamic flow conditions in 4.5-m^3 stainless steel and glass inhalation chambers providing 12 to 15 air changes per hour, with controlled temperature (24 ± 3°C) and humidity (50 ± 10%). EtO was vaporized and metered into filtered air intake, and the EtO chamber concentrations were monitored two to four times per hour using infrared analysis. Charcoal tube samples of test atmospheres verified that chamber concentrations were within 10% of target value and that conditioned air chambers of controls were free of EtO.

Blood Collection. While each monkey was tranquilized by intramuscular injection of ketamine hydrochloride, the phlebotomy site was shaved and cleansed with isopropyl alcohol, and blood was withdrawn from the femoral vein in syringes. Blood was immediately placed in sterile vacutainers containing sodium heparin, mixed thoroughly and stored at room temperature until shipped. Vacutainer tubes were coded by NIOSH investigators at the time of venipuncture and shipped overnight via express package service for peripheral blood lymphocyte culture. In no case were transported samples subject to X-ray examination. Venous blood was drawn and coded by NIOSH investigators in late 1987 from all 22 animals for the initial study (Table 1). Phlebotomy was similarly performed over the ensuing several months for subsequent experiments on the exposed and control animals, as indicated.

Sister Chromatid Exchange Determination. The same experimental protocol previously described to study these animals (11) was used for the current study. To achieve differential staining of sister chromatids, 50 μM (or the dose reported) of BrdUrd was added to the culture

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[2] The abbreviations used are: EtO, ethylene oxide; SCE, sister chromatid exchange; HFC, high SCE frequency cells; BrdUrd, 5-bromodeoxyuridine; MMC, mitomycin C.

[3] C. Laurent, personal communication.

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medium 24 h after initiation of triplicate cultures. Cultures were incubated for 55, 72, or 96 h in complete darkness at 37°C in an atmosphere of 5% CO_2/95% air. Colcemid (0.1 μg/ml) was added to each culture 2 h before processing. For each animal, 50–100 metaphase spreads were examined to determine the mean SCE frequency. For each mean SCE frequency determination, only cells with 38 or more chromosomes (chromosomes 38–42) were scored. Replication indices were calculated by randomly examining 200 metaphases. The replication index was calculated as follows:

R.I. = (no. 1st divisions ×1 +

no. 2nd divisions ×2 + no. 3rd divisions ×3)/200

Statistical Analysis. The mean SCE/cell for individual animals, and for animals pooled by exposure groups, as well as the mean replication index were compared using a two-sided Student's *t* test. Cells with very high SCE frequencies (HFCs; mitoses with more than 40 SCE/cell) were also studied separately to facilitate the analysis of persistently elevated, EtO-induced SCE (see Ref. 11).

RESULTS

Animals. Two monkeys (one from the 50 ppm and one from the 100 ppm exposure group) died during the initial exposure. These deaths appeared unrelated to EtO exposure. Two monkeys per group were sacrificed at the termination of exposure. Since the cessation of exposure five more animals have died; two in the 100-ppm exposure group (51- and 57-month postexposure), one in the 50-ppm exposure group (46-month postexposure), and two controls (60- and 65-month postexposure). In each case, the deaths appeared to be unrelated to EtO exposure. One monkey in the 50-ppm group is diabetic and lymphocytes from this animal did not grow in response to mitogen stimulation in either 1981 or 1987.

Sister Chromatid Exchange Analysis. In initial experiments conducted at late (96-h) harvest times, we consistently observed significantly fewer HFCs as compared with earlier (72-h) harvest times in lymphocytes cultured from the highly EtO-exposed animals. Therefore, subsequent experiments were conducted to investigate the effect of harvest time on the detection of HFCs. Cells were harvested and slides stained for SCE at 55, 72, and 96 h after initiation of peripheral blood lymphocyte cultures. The 55- and 96-h time points were the earliest and latest feasible harvest times that would allow scoring more than 50-s division, differentially stained metaphases. The results of this analysis are shown in Table 1.

In each exposed animal (except number A39), the earlier harvest time (55 h) maximized the number of HFCs detected and was, therefore, associated with the highest mean SCE frequency. In animal number A39, the mean SCE frequency was maximal at 72 h. This increase in mean SCE level at the early harvest time in the EtO-exposed animals is clearly associated with the detection of a greater number of HFCs (Table 1). At the 96-h harvest time few of the HFCs were detectable. In one highly exposed animal (A53), 22% of all metaphases examined at 55 h had 40 or more SCE (HFCs). At the 72-h harvest time only 5% of the mitoses were HFCs and at 96 h there were no detectable HFCs after counting 100 cells. The mean SCE frequency for animal A53 correspondingly diminished from 21.47 SCE/cell at 55 h to 6.83 SCE/cell at 96 h.

The mean SCE frequencies in the HFCs and the non-HFCs (low SCE frequency cells, defined as cells with 40 or fewer SCE/cell) for all of the animals pooled by exposure group are shown in Table 2. As can be seen, the mean SCE frequency of low SCE frequency cells did not differ significantly from the control at any of the three harvest times (Table 2). Both the exposure-related increase in mean SCE frequency observed within each harvest time and the change in detection of HFCs over the three harvest times are entirely attributable to variation in the detection of EtO-induced HFCs. There was a slight decline in the mean SCE frequency in the control and treated animals at later harvest times (Table 2).

Since the detection of persistent, EtO-induced cytogenetic damage varied dramatically with harvest time, we examined the replication index for each culture to determine if the rate of lymphocyte growth might have been altered by exposure. The mean replication index at the 55-h harvest time in both groups of exposed animals was higher than that of the controls (Table 1). Although there was no significant difference when each exposed cohort is compared separately to controls, if the replication indices at 55 h from all of the EtO-exposed animals are compared to control, the replication index was significantly higher in the exposed animals ($P < 0.05$ by Student's *t* test). There was no appreciable correlation between an increase in replication index in individual exposed animals and the number of detectable HFCs (Table 1). In addition, there was no difference in replication index in the control and EtO-exposed animals at the 72-h harvest time.

Because cytogenetic harvest time dramatically affected the detection of HFCs, and because the replication indices in the exposed animals were higher than in controls, we tested the effect of other factors of *in vitro* lymphocyte culture that are known to alter cell growth and SCE frequency in peripheral blood lymphocytes. We cultured cells from a control and EtO-exposed animal at differing incubation temperatures. Peripheral blood was drawn from two additional animals in the 100 ppm exposure group and two animals in the control group. Incubation temperature systematically altered the detection of HFCs at the 55-h harvest time in the exposed animals (Table 3). Higher incubation temperatures increased the replication index and increased the detection of HFCs, although the optimal temperature for detection of HFCs varied between animals.

To further investigate the potential for *in vitro* culture variables that might alter the detection of HFCs, we cultured peripheral blood from one highly exposed and one control animal using as culture supplements different sources of commercially available fetal calf serum. As was previously noted (Tables 1 and 2), early harvest of cells grown in all sera maximized HFC detection. In one serum, peripheral blood lymphocytes grew very slowly, yielding no second division mitosis for SCE analysis at the 55-h harvest time. However, the 72-h harvest of lymphocytes grown in this serum supplement was associated with a dramatic increase in the detection of HFCs. This result is shown in Fig. 1. At 72 h, a mean of 31% of the cells scored in three separate experiments were HFCs, and the mean SCE frequency in these three experiments was 30.5 SCE/cell. All other serum supplements (four different supplements, culturing lymphocytes from the same highly exposed animal) resulted in a mean of 9.57 ± 1.2 (SD) SCE/cell. This increase above control in mean SCE frequency was completely attributable to an increase in the detection of HFCs, resulting in a bimodal SCE distribution (Fig. 1). The five serum supplements had no significant effect on the SCE frequency in the control animal.

To determine if the incorporation of BrdUrd into chromosomes of lymphocytes from EtO-exposed monkeys might be responsible for differences in the detection of HFCs, SCE frequencies were determined in experiments using different concentrations of BrdUrd. The BrdUrd dose-response for SCE

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Table 1 *Mean SCE frequency and replication index in peripheral blood lymphocytes from control and EtO-exposed monkeys at three harvest times*

Exposure group	Animal number	55-h harvest time		72-h harvest time		96-h harvest time	
		%HFC[a]	SCE/cell[b] ± SEM (R.I.)[c]	%HFC	SCE/cell ± SEM (R.I.)	%HFC	SCE/cell ± SEM
Control	A25	0	7.46 ± 0.37 (1.18)	0	6.42 ± 0.29 (2.01)	0	5.71 ± 0.24
	A28	0	8.00 ± 0.42 (1.53)	0	6.79 ± 0.34 (2.19)	0	6.03 ± 0.29
	A35	0	8.74 ± 0.42 (1.01)	0	9.28 ± 0.49 (1.76)	0	9.23 ± 0.40
	A42	0	5.17 ± 0.31 (1.14)[f]	0	5.20 ± 0.25 (1.62)	0	4.28 ± 0.19
	A58	0	9.14 ± 0.45 (1.32)	0	6.81 ± 0.30 (1.76)	0	5.98 ± 0.32
	A60	0	7.82 ± 0.50 (1.19)	0	5.50 ± 0.27 (2.04)	0	6.07 ± 0.34
	A81	0	8.55 ± 0.54 (1.21)	0	8.80 ± 0.44 (1.94)	0	7.35 ± 0.50
	A85	0	5.88 ± 0.33 (1.66)	0	5.58 ± 0.29 (2.11)	0	5.22 ± 0.24
	$N = 8$	0	7.60 ± 1.40 (1.28 ± 0.22)	0	6.79 ± 1.5 (1.93 ± 0.20)	0	6.23 ± 1.49
50 PPM	A26	6	11.7 ± 1.10 (1.44)	1	6.76 ± 0.65 (1.73)	0	5.01 ± 0.21
	A31	0	6.04 ± 0.43 (1.56)	0	6.91 ± 0.54 (1.60)	0	5.43 ± 0.24
	A33	5	11.17 ± 1.10 (1.28)	1	8.58 ± 0.74 (1.59)	2	6.44 ± 0.74
	A46	6.7	11.39 ± 1.57 (1.28)[g]	3	8.05 ± 0.76 (1.84)	1	7.17 ± 0.63
	A49	7	12.98 ± 1.15 (1.42)	2	7.20 ± 0.70 (2.12)	0	5.61 ± 0.30
	A50	3	10.03 ± 0.92 (1.47)	1	8.71 ± 0.83 (1.98)	0	7.40 ± 0.63
	A69	4	10.12 ± 1.19 (1.52)	1	7.53 ± 0.89 (2.05)	1	5.88 ± 0.44
	$N = 7$	4.5	10.40 ± 2.16 (1.42 ± 0.11)	1.3	7.68 ± 0.79 (1.84 ± 0.21)	0.6	6.13 ± 0.90
100 PPM	A29	12	17.62 ± 2.02 (1.60)	2	8.08 ± 0.96 (2.31)	3	9.06 ± 1.07
	A39	3	7.70 ± 1.05 (1.41)[d]	10	12.03 ± 1.79 (1.78)	2.2	7.22 ± 1.11[e]
	A53	22	21.47 ± 2.27 (1.31)	5	10.07 ± 1.30 (2.00)	0	6.83 ± 0.39
	A59	9	11.38 ± 1.66 (1.32)	3	7.08 ± 0.92 (1.94)	2	6.82 ± 0.91
	A61	9	13.36 ± 1.58 (1.79)	4	8.61 ± 1.07 (2.35)	3	7.34 ± 1.00
	A84	23	21.61 ± 2.13 (1.35)	8	11.83 ± 1.57 (1.95)	2	7.13 ± 0.61
	A242	6	10.44 ± 1.27 (1.50)	2	7.74 ± 0.93 (1.66)	3	7.87 ± 0.96
	$N = 7$	12	14.80 ± 5.51 (1.47 ± 0.18)	4.9	9.35 ± 2.00 (2.00 ± 0.25)	2.2	7.47 ± 0.79

[a] HFC, high frequency cell; metaphase with more than 40 SCE/cell.
[b] SCE/cell: mean SCE in mitotic figures with 40 or more chromosomes; based upon 100 cells per harvest time unless otherwise noted.
[c] R.I., replication index = (no. 1st division cells × 1 + no. 2nd division cells × 2 + no. 3rd division cells × 3)/200.
[d] 67 cells counted for SCE mean; [e] 90 cells counted for SCE mean; [f] 84 cells counted for SCE mean; [g] 59 cells counted for SCE mean.

Table 2 *Mean SCE/cell[a] ± SD in SCE frequency subpopulations of peripheral lymphocytes from control and EtO-exposed monkeys at three harvest times*

Exposure group	55 h		72 h		96 h	
	LFC[b]	HFC[c]	LFC	HFC	LFC	HFC
Control ($N = 8$)	7.6 ± 1.4	0	6.8 ± 1.5	0	6.2 ± 1.5	0
50 ppm ($N = 7$)	8.6 ± 1.5	43.5 ± 19.6	7.3 ± 0.8	41.0 ± 22.1	6.0 ± 0.9	42.8 ± 22.0
100 ppm ($N = 7$)	8.8 ± 2.0	58.8 ± 5.8	6.7 ± 0.2	59.4 ± 6.0	6.3 ± 0.6	50.4 ± 23.4

[a] 59 to 100 cells scored per animal, per harvest time.
[b] LFC, mean SCE/cell in mitotic figures with 40 or less SCE/cell.
[c] HFC, mean SCE/cell in mitotic figures with more than 40 SCE/cell.

Table 3 *Effect of incubation temperature on SCE after 55 h in peripheral lymphocytes from two control and two EtO-exposed monkeys*

Exposure group	Animal	Incubation temperature (°C)	%HFC[a]	SCE/cell ± SEM[b]	Replication index[c]
Control	A25	35	0	7.32 ± 0.59	1.30
		37	0	6.52 ± 0.34	1.27
		39	0	7.12 ± 0.44	1.63
	A42	35	0	3.94 ± 0.27	1.09
		37	0	4.81 ± 0.33	1.12
		39	0	4.38 ± 0.31	1.17
Exposed	A242	35	0	7.38 ± 0.57	1.11
		37	6	10.86 ± 1.60	1.16
		39	20	19.07 ± 3.08	1.29
	A84	35	0	8.11 ± 0.57	1.13
		37	16	18.72 ± 3.00	1.31
		39	12	13.30 ± 2.71	1.42

[a] HFC, high SCE frequency cell: cells with more than 40 SCE.
[b] 50 cells per point scored for SCE mean.
[c] 200 cells counted for determination of replication index.

was examined with differing *in vitro* growth conditions for lymphocytes from one highly exposed (100 ppm) monkey. Extreme conditions (varying doses of BrdUrd added to cultures containing different serum supplements) were used to maximize the detection of any effect of BrdUrd incorporation on detection of HFCs. No significant variation in the detection of HFCs was apparent, under either of the growth conditions tested, over a 10-fold range of concentrations of BrdUrd in the culture medium (Table 4).

DISCUSSION

Previous cytogenetic studies of acute exposures to alkylating agents have indicated that mutagen exposures slow the progression of cells through the cell cycle and that highly damaged cells may progress more slowly than less damaged cells. Consequently, highly damaged cells should be detected in greater numbers when cytogenetic analysis is delayed (12–14). We found that cells with high frequencies of SCE from animals chronically exposed to EtO were less frequently detected after longer cell culture times. Detection of HFCs was maximal at 55 h and by 96 h, in most exposed animals, few HFCs were observed. Thus, it is likely that the mechanisms responsible for our observations in chronically exposed animals differ from those involved in acute treatments of cells with alkylating agents.

Incubation temperature has been reported to slightly alter SCE frequencies and affect lymphocyte growth kinetics, with cells having higher replication indices at higher temperatures (15–17). We found that incubation temperature does not significantly affect baseline SCE in unexposed monkeys when lymphocytes are harvested at an early time (55 h). However, the replication index of monkey lymphocytes increased with temperature (Table 4), consistent with the results of other investigators studying human lymphocytes (15–17). In the EtO-exposed animals, the replication index and the detection of HFCs increased at higher temperatures.

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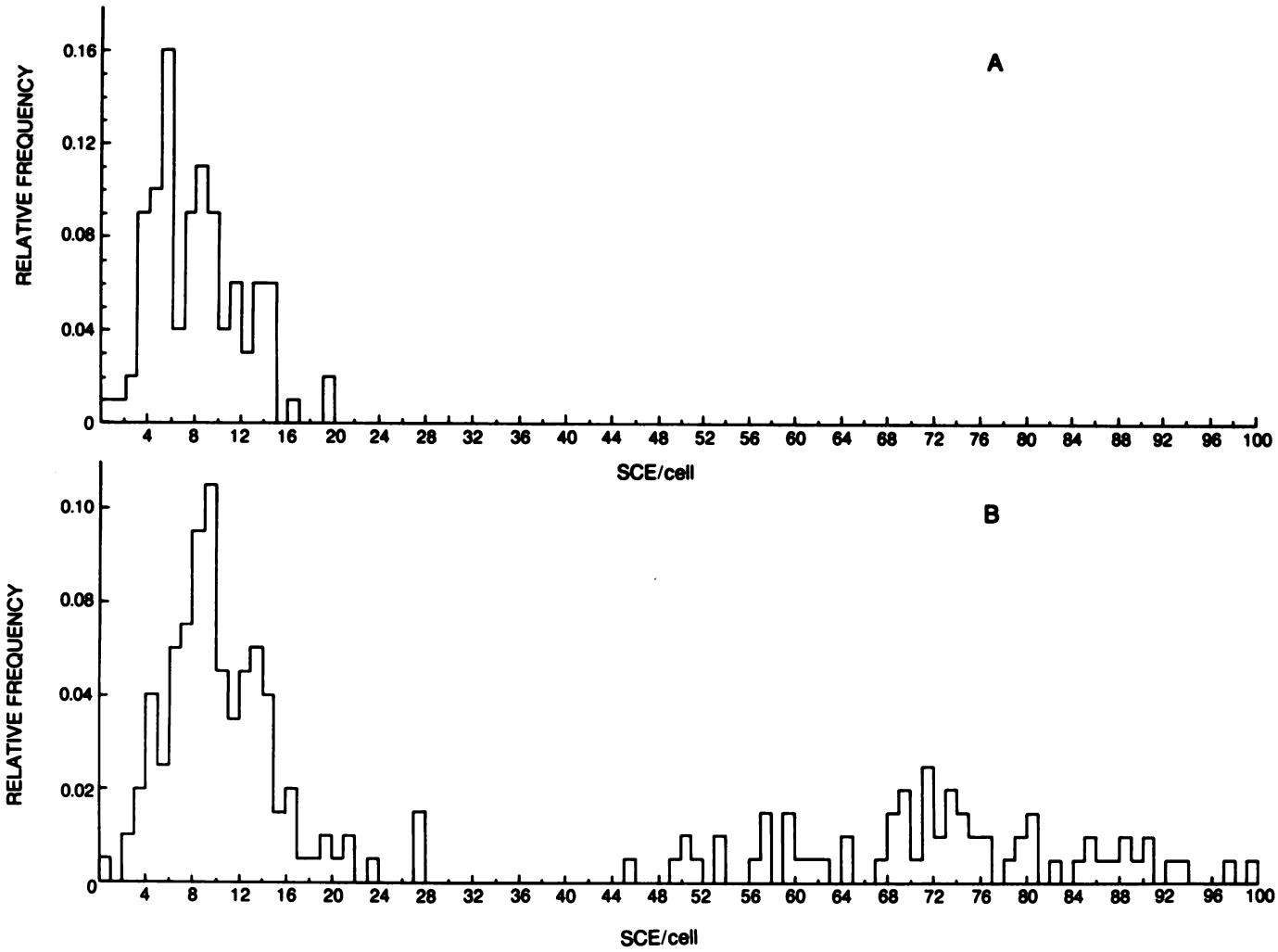

Fig. 1. *A*, distribution of SCE/cell (100 cells) for one control animal (animal A25) and harvested at 72 h; *B*, distribution of SCE/cell (200 cells) for one ethylene oxide exposed animal (animal A84) harvested at 72 h. Cells from the exposed animal were cultured in a serum supplement which was experimentally found to enhance the detection of high SCE frequency cells when cytogenetic harvest was done at 72 h.

Table 4 *Effects of different BrdUrd concentrations on SCE frequency and cell cycle kinetics in peripheral blood lymphocytes from an ethylene oxide-exposed and control monkey*

BrdUrd concentration (μM)	SCE/cell[a] Overall mean	LFC mean	HFC mean	Cell[b] replication index	%HFC[c]
Exposed, animal A84					
Serum D (72-h harvest)					
10	23.2	9.3	54.9	1.91	30.7
50	35.1	11.3	73.0	1.90	38.7
100	31.2	15.5	64.6	1.84	32
Serum B (72-h harvest)					
25	11.5	7.2	78.3	1.35	6.6
50	10.0	8.1	81.8	1.36	2.6
100	11.2	10.2	63.0	1.21	2.6
200	11.1	11.1		1.16	
Control, Animal A25					
Serum B (72-h harvest)					
25	4.7	4.7		1.51	0
50	7.0	7.0		1.53	0
100	7.3	7.3		1.36	0
200	9.5	9.5		1.19	0

[a] Mean SCE/cell overall, for the low SCE frequency population (LFC), and for the high SCE frequency population (HFC; cells with more than 40 SCE); a total of 75 cells were scored per point.
[b] 200 cells per point were scored for cell replication index.
[c] %HFC, percentage of cells with greater than 40 SCE/cell.

Serum supplements used in the cultivation of lymphocytes for cytogenetic studies have complex effects on SCE. Baseline SCE and the lymphocyte cell cycle in unexposed individuals are minimally affected by the type of serum or plasma used (14, 18–20). In contrast, serum has marked effects on SCE induced *in vitro* by benzo(*a*)pyrene (19). Even more dramatic are the effects that serum can have on *in vivo* SCE analysis. For example, in one study of EtO-exposed sterilizer operators, a significant increase in SCE frequencies was observed when lymphocytes were grown in fetal calf serum, but not when the cells were cultured in human plasma (20). Our observations on the effects of different serum supplements on the detection of HFCs in peripheral blood from chronically EtO-exposed primates also shows that this factor can quite significantly affect SCE frequencies.

Previous work with mutant cell lines and with lymphocytes from individuals with the genetic disease Bloom's Syndrome has shown that the dose of BrdUrd affects the detection of high SCE frequency cells (21, 22). We found, however, that the detection HFCs which were induced by chronic EtO exposure in monkeys were not dependent on BrdUrd dose. This indicates that the subpopulation of EtO-induced HFCs are likely not produced by a lesion similar to that which causes the high SCEs observed in cells from individuals with Bloom's Syndrome or cells derived from mutant lines sensitive to BrdUrd-induced SCE.

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We also observed that persistent EtO-induced SCE in lymphocytes, occurring after chronic inhalation exposure, was associated with an increased replication index in cells from the exposed animals. The detection of the persistently damaged cells is maximized at early cytogenetic harvest times. However, it remains unclear whether the persistently damaged cells themselves are responsible for the observed increase in replication index. The magnitude of the difference in replication index between control and exposed groups, as well as the absence of any difference at 72 h, is consistent with the effect being attributable to the HFC subpopulation. However, the animals with the most HFCs were not consistently those with the highest replication indices, arguing that the damaged cells are not responsible for this effect.

One possible explanation for our observations is that the HFCs are either a subpopulation of cells particularly susceptible to EtO-induced damage, or are deficient in the repair of EtO-induced lesions and that this subpopulation has a rapid entrance into the cell cycle or a shorter cell cycle (or both of these). It has been reported in humans that lymphocyte subpopulations have different cycling times and different SCE baselines (23–25).

Our observation that the detection of EtO-induced HFCs depends on harvest time and cell growth conditions, suggests alternative explanations for several observations of other investigators who have studied EtO-exposed human workers with persistently elevated SCE in their lymphocytes. Nichols *et al.* (26) showed that *in vitro* MMC treatment of lymphocytes from EtO-exposed workers with a persistently high frequency of SCE results in a decrease in their overall SCE mean. This may be due to hypersusceptibility of the high SCE frequency cells to MMC-induced damage. Increased cellular sensitivity to MMC might arise as a result of altered repair of a variety of lesions caused by prior EtO exposure. Pero *et al.* (27) found diminished unscheduled DNA repair synthesis in human lymphocytes after EtO exposure. However, the decrease in SCE frequency in these individuals may be due to MMC-induced alterations in lymphocyte growth (cell-cycle delay) and a subsequently decreased detection of HFCs.

The variations in HFC detection related to lymphocyte cell cycle progression we observed may also provide an explanation for the very significant temporal variation in SCE mean in workers with very high SCE frequencies produced by EtO exposure (ranging from 18.8 to 43.6 and from 19.3 to 36 of SCE/cell in two different workers), noted by Stolley *et al.* (9) over the 41 months of their study. This variability had no clear pattern of increase or decrease over time. Our data suggest that this variability may be related to changes in cell growth and detection of HFCs over the 41 months of study.

Our observations also have important implications for the use of SCE in epidemiological studies. For example, the use of earlier *in vitro* lymphocyte harvest times may more sensitively detect the effects of chronic *in vivo* exposure to DNA alkylating agents on chromosomes. In addition, other factors that influence lymphocyte kinetics (*e.g.*, immune status, concurrent disease and human exposure patterns) might also be important considerations in study design. Our findings also indicate that the interpretation of increased SCE frequencies in peripheral blood lymphocytes after *in vivo* exposure to DNA alkylating agents may be significantly more complex than has been previously appreciated.

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