**109. Induction of Sister Chromatid Exchanges by
Cyclophosphamide in Human Lymphocytes
with Metabolic Activation**

By Kiyoshi INOUE,^{*)} Tadayoshi SHIBATA,^{*)} Shinichi MISAWA,^{**)}
Tatsuo ABE,^{**)} and Keiichi KAWAI^{***)}

(Communicated by Sajiyo MAKINO, M. J. A., Nov. 12, 1980)

Induction of sister chromatid exchanges (SCEs) has been useful as a more sensitive indicator for detecting mutagens and carcinogens than do the traditional chromosome-aberration formation (Beek and Obe, 1975; Kato, 1974; Latt, 1974; Perry and Evans, 1975; Solomon and Bobrow, 1975). Compounds that become effective by metabolic activation, however, have not been checked by conventional technique. To screen the mutagenicity of these compounds *in vitro*, Ames *et al.* (1973) adopted an activating system with a rat-liver microsome fraction in their bacterial assays. Similarly, in CHO cells Natarajan *et al.* (1976) demonstrated that dimethylnitrosamine and diethylnitrosamine induced chromosome aberrations and SCEs in the presence of a rat-liver microsome extract.

In this paper we attempted to show that cyclophosphamide (CPM) induced SCEs after *in vitro* activation with rat liver microsome in cultured human leukocytes.

Materials and methods. Liver homogenate fraction (S-9) was prepared from male Sprague-Dawley rats at 5 weeks of age which received KC-500 as a single i.p. injection of 500 mg/kg 5 days prior to their sacrifice. The liver was homogenized in the ice-cold 150 mM KCl solution. The homogenate was centrifuged at 4°C for 10 min at 9000 g. Supernatant (S-9) was stored at -80°C until used.

The S-9 mix consisted of 3 ml of S-9 fraction, 1 ml of 330 mM KCl, 0.1 ml of 500 mM MgCl₂, 1 ml of 50 mM glucose-6-phosphate, 1 ml of 40 mM NADP, and 3.9 ml of phosphate buffer (pH 7.4).

Freshly drawn heparinized human blood (0.5 ml), 0.1 ml of CPM and 0.5 ml of S-9 mix was mixed in a sterile plastic tube. After 0.5, 1 or 2 hr incubation at 37°C in a shaking water-bath, supernatant was poured off and leukocytes were washed twice with RPMI 1640

^{*)} Division of Food Sanitation, Osaka Prefectural Institute of Public Health, Osaka.

^{**)} Department of Medicine, Kyoto Prefectural University of Medicine.

^{***)} Department of Preventive Medicine, Kyoto Prefectural University of Medicine, Kyoto.

Table I. Sister chromatid exchanges induced in human leukocytes by various incubation times with cyclophosphamide (10^{-4} M) and S-9 mix

Treatment	Incubation time (min)	Number of cells scored	A1	A2	A3	Chromosome designation				F	GY	SCE/ chromosome	SCE/cell ±S.D.
BudR only	—	50	0.330	0.340	0.330	0.290	0.231	0.157	0.100	0.040	0.016	0.183	8.40 ± 3.29
CP 10 ⁻⁴ M alone	—	50	0.340	0.400	0.380	0.235	0.233	0.127	0.090	0.050	0.036	0.183	8.36 ± 3.53
S-9 mix only	30	50	0.370	0.330	0.320	0.285	0.203	0.143	0.077	0.065	0.052	0.175	8.06 ± 3.45
CP 10 ⁻⁴ M + S-9 mix	30	50	2.330	2.310	1.680	1.615	1.077	0.740	0.513	0.345	0.140	0.975	44.86 ± 10.92
S-9 mix only	45	50	0.350	0.440	0.180	0.250	0.213	0.140	0.073	0.045	0.028	0.170	7.80 ± 3.14
CP 10 ⁻⁴ M + S-9 mix	45	50	2.450	2.220	1.810	1.525	1.105	0.757	0.503	0.325	0.172	0.986	45.36 ± 9.60
S-9 mix only	60	50	0.450	0.350	0.290	0.330	0.221	0.137	0.097	0.060	0.032	0.187	8.62 ± 3.32
CP 10 ⁻⁴ M + S-9 mix	60	50	2.910	2.590	2.240	1.910	1.313	0.953	0.580	0.520	0.308	1.210	55.64 ± 11.48
S-9 mix only	120	50	0.420	0.420	0.220	0.360	0.221	0.130	0.080	0.030	0.020	0.182	8.36 ± 3.40
CP 10 ⁻⁴ M + S-9 mix	120	28	2.500	2.554	1.732	1.759	1.229	0.744	0.530	0.313	0.157	1.059	48.71 ± 15.09

Table II. The frequencies of sister chromatid exchanges and the concentration of cyclophosphamide in the presence and absence of S-9 mix

Concentration of cyclophosphamide	S-9 mix	Treatment time (hr)	No. of cells scored	A1	A2	A3	Chromosome designation				F	GY	SCE/ chromosome	SCE/cell $\pm$ S.D.
0	+	1	25	0.340	0.320	0.320	0.280	0.197	0.140	0.040	0.050	0.016	0.161	7.40 $\pm$ 2.35
10 ⁻⁸ M	+	1	25	0.500	0.380	0.360	0.220	0.275	0.093	0.087	0.033	0.024	0.191	8.80 $\pm$ 3.22
10 ⁻⁷ M	+	1	25	0.420	0.430	0.440	0.370	0.261	0.067	0.073	0.030	0.016	0.197	9.08 $\pm$ 3.66
10 ⁻⁶ M	+	1	25	0.460	0.620	0.360	0.350	0.253	0.073	0.030	0.030	0.032	0.199	9.16 $\pm$ 4.27
10 ⁻⁵ M	+	1	25	0.620	0.620	0.520	0.380	0.285	0.127	0.140	0.040	0.016	0.243	11.16 $\pm$ 4.12*
10 ⁻⁴ M	+	1	25	2.280	2.200	2.180	1.770	1.237	0.767	0.460	0.380	0.184	1.030	48.76 $\pm$ 10.76*
0	—	24	25	0.280	0.280	0.300	0.230	0.213	0.113	0.107	0.020	0.016	0.159	7.32 $\pm$ 3.28
10 ⁻⁶ M	—	24	25	0.320	0.320	0.440	0.170	0.192	0.153	0.073	0.030	0.008	0.157	7.24 $\pm$ 2.98
10 ⁻⁵ M	—	24	25	0.320	0.380	0.300	0.310	0.253	0.113	0.080	0.050	0.008	0.184	8.44 $\pm$ 3.53
10 ⁻⁴ M	—	24	25	0.300	0.240	0.500	0.270	0.227	0.153	0.040	0.080	0.024	0.177	8.16 $\pm$ 3.94

* $P < 0.001$.

medium supplemented with 15% fetal calf serum. Blood cultures were set up using the same medium, with phytohemagglutinin P, and 5-bromodeoxyuridine (BrdU) was added as to give a final concentration of 5 $\mu\text{g}/\text{ml}$. These cultures were kept in the dark at 37°C for 80 hr. Colchicine (0.2 $\mu\text{g}/\text{ml}$) was added for the last 2 hr of cultivation. Cells were treated with 0.075 M KCl, fixed in methanol: acetic acid (3:1), and chromosome preparations were made by the conventional air drying method. For SCE analysis slides were stained in the aqueous solution of Hoechst 33258, mounted in McIlvaine's disodium phosphate/citric acid buffer pH 8.0, and exposed to a fluorescent lamp for 1 hr on a hot plate at 55°C. The slides were then stained in Giemsa (2% in phosphate buffer, pH 7.0) for 6 min. Twenty-five or 50 cells were scored for SCEs in each treatment.

Results. The effect of the incubation time on the frequency of SCEs induced by CPM (final concentration 10^{-4}) in the presence of S-9 mix is presented in Table I. The S-9 mix alone did not increase SCE frequencies (7.80–8.62/cell) in various incubation times. The rat liver microsome extracts converted CPM into an active form that induce SCEs. Incubation of leukocytes for 60 min in the presence of S-9 mix caused a maximum increase of SCEs induced by CPM. At 120 min incubation, the lethality of S-9 mixture was high, and the

Fig. 1. Sister chromatid exchanges found in a human lymphocyte treated for 1 hr with CPM (10^{-4} M.) in the presence of S-9 mix.

Fig. 2. a: Effect of incubation time on the incidence of SCEs induced by cyclophosphamide (10^{-4} M.) in the presence of S-9 mix. b: The frequencies of SCEs induced by cyclophosphamide after treatment (for 60 min) with S-9 mix, and after treatment (for 24 hr) without S-9 mix.

number of scorable cells was low.

Human leukocytes were separately incubated with CPM at final concentrations of 10^{-8} M., 10^{-7} M., 10^{-6} M., 10^{-5} M. or 10^{-4} M. for 24 hr served controls. As shown in Fig. 1 and Table II, it was clear that there was a significant increase in frequency of SCE by CPM at the higher concentration of 10^{-5} M. and 10^{-4} M. when S-9 mix was added. Treatment with CPM alone showed no significant increase of the SCE-frequency. The results obtained are summarized in Fig. 2.

Discussion. SCEs have been known to be rather effective indicator of chromosome damages. Perry and Evans (1975) found that 12 of 14 known directly acting mutagens increased the frequency of SCEs to a great extent. However, the another class of chemicals such as CPM was not effective as a chromosome damaging agent until it was first metabolized in the liver and converted into an active alkylating intermediate(s). Natarajan *et al.* (1976) demonstrated that the liver microsomal activation used in bacterial tests *in vitro* were successfully used in cytological investigations on mammalian cells, and showed that DMN and DEN induced chromosomal aberrations and SCEs in CHO cells in the presence of S-9 mix. Similarly, Stetka and Wolff (1976) demonstrated that CPM produced a significant increase in SCE-frequency of CHO cells after incubation with rat-liver S-9 mix.

However, for evaluating mutagenic hazards to man, the develop-

ment of human culture system using metabolic activation may be of special interest. Bimboes and Greim (1976) reported that DMN induced chromosome aberrations in human leukocytes after *in vitro* activation with mouse liver microsomes. In their experiments, leukocytes were treated with DMN and S-9 mix for 45 min, because incubation exceeding 60 min increased cytotoxicity to the lymphocytes. In our system, the incubation time was fixed for 60 min, because 60 min incubation showed higher frequencies of SCEs than other incubation time did.

Summary. Human leukocytes were treated with CPM for the establishment of the *in vitro* activating system to detect inactive mutagenic chemicals in the presence of S-9 mix. CPM caused a maximum increase in SCE-frequency after 60 min incubation. Further, the frequency of SCEs was markedly increased by additional doses of CPM. It thus appears that chemicals requiring metabolic activation induce SCEs in the human leukocyte test system.

Acknowledgement. Our cordial thanks are due to Emeritus Professor Sajiro Makino, M. J. A., for revising the manuscript with invaluable advice.

References

- Ames, B. N., W. E. Durston, E. Yamazaki, and F. D. Lee (1973): Proc. Natl. Acad. Sci. USA, **70**, 2281-2285.
- Beek, B., and G. Obe (1975): Humangenetik, **29**, 127-134.
- Bimboes, D., and H. Greim (1976): Mut. Res., **35**, 155-160.
- Kato, H. (1974): Nature, **251**, 70-71.
- Latt, S. A. (1974): Proc. Natl. Acad. Sci. USA, **71**, 3162-3166.
- Natarajan, A. T., A. D. Bates, P. P. W. van Buul, M. Meijers, and N. de Vogel (1976): Mut. Res., **37**, 83-90.
- Perry, P., and H. J. Evans (1975): Nature, **258**, 121-125.
- Solomon, E., and M. Bobrow (1975): Mut. Res., **30**, 273-278.
- Stetka, D. G., and S. Wolff (1976): Mut. Res., **41**, 343-350.