CYTOKINETIC AND CYTOGENETIC CHANGES IN CULTURED HUMAN LEUCOCYTES AND HELA CELLS INDUCED BY BENZENE

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Action of benzene on human leucocytes and on HeLa cells were examined from cytokinetic and cytogenetic points of view. Dose-response type analysis revealed that decreased DNA synthesis of cultured human leucocytes and of HeLa cells were remarkable in dose level of 2.2×10^{-3} M of benzene. As far as chromosome aberrations of cultured human leucocytes were concerned, gaps and breaks were observed in dose levels of both 2.2×10^{-3} M and 1.1×10^{-3} M of benzene.

A number of studies has been accumulated on chromosome abnormalities of industrial workers exposed to ambient benzene^{1~4}). There are also experimental studies on induced chromosome abnormalities in bone marrow cells of mice⁵) and on incorporation of ³H-labelled thymidine into DNA of animals⁶) exposed to benzene. It is well known that ionizing radiation and some chemicals cause both chromosome abnormalities and inhibition of mitosis. In the previous study we reported decreased cell renewal rates in cultured human leukocytes⁷) and HeLa cells⁸). The present study aims to know about cytokinetic and cytogenetic changes in cultured human leucocytes and HeLa cells affected by benzene.

MATERIALS AND METHODS

1. Chromosome analysis

About 0.1 to 0.2 ml of human whole blood of each healthy donor (6 males were examined) was dropped into 5 ml of culture media which consisted of TC-199 Medium with 30% calf serum. The culture media contained 0.01 ml of PHA-P and 100 units/ml of penicillin, 0.1 mg/ml of streptomycin. Benzene was added to the culture media at dose levels of 2.2 and 1.1×10^{-3} M, 2.2×10^{-4} M, and 2.2×10^{-5} M at the beginning of the incubation and the culture bottles were sealed tightly. All samples were incubated at 37°C for 72 hr. Number of leucocytes were counted

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at the beginning of culture and at the end of incubation by the use of hemocytometer. Before harvesting, deacetyl-methylcolchicine (Colcemid-CIBA) was added after 70 hr of incubation to give a final concentration of $1\text{ }\mu\text{g/ml}$. Whole contents of culture media were centrifuged for 5 min at 800 to 1,000 r.p.m. The supernatant was replaced by about 5 ml of hypotonic saline (1:3 v/v physiological saline and Aqua dist.). After repeated centrifugation, the sedimented cells were fixed with fixative (1:3 v/v glacial acetic acid and methyl alcohol) and were kept in a freezer for a night. Mixed suspension of the cells were then spread over clean slide glasses, air-dried and stained with Giemsa solution. Photomicrographs were made for randomly selected cells in mitosis and the quality of each picture was assured by immediate comparison with the microscopic appearance of the cells photographed. Both numerical and structural analysis were performed of these pictures with reference back to the original slides as needed. Enlarged photomicrographs of the same cells were made for karyotype analysis when indicated. Besides ploidy analysis, chromosome and chromatid-type gaps and breaks were counted for each cell.

2. DNA synthesis

a) cell labeling. In case of human leukocytes, cell culture was carried out by the same way as in chromosome analysis. $1\text{ }\mu\text{c/ml}$ of ^3H -thymidine (SPA 5.0 Ci/mM) was added to the cultures 1 hr prior to the fixation. Cells were harvested at 24 hr, 48 hr, and 72 hr after the beginning of culture respectively. At each harvest, cells were treated with a hypotonic solution and fixed with freshly prepared fixative. After fixation the cells were spread over clean slide glasses. HeLa cells of wild type were plated on glass cover slip ($12\times 35\text{ mm}$) in 1 ml of Eagle's MEM medium (Nissui Seiyaku Co.) containing 10% calf serum with a final concentration of about 3×10^5 cells/ml, and incubated at 37°C for 3 days. Then the medium was removed and replaced by another medium containing 2.2 and $1.1\times 10^{-3}\text{M}$, $2.2\times 10^{-4}\text{M}$, $2.2\times 10^{-5}\text{M}$ of benzene. In this way the cells were exposed to benzene for 24 hr. Before harvesting, ^3H -labelled thymidine was added with a final concentration of $0.3\text{ }\mu\text{c/ml}$ (SPA 5.0 Ci/mM) for 20 min. After removal of the radioactive medium the cover slips on which cells were grown were washed twice with TC-Hanks solution (DIFCO) and fixed by methanol solution.

b) Autoradiographic procedure. The cover slips containing fixed cells were dipped into liquid emulsion NR-M2 (SAKURA), and then stored at 4°C in the dark for about 2 weeks. They were developed by KONIDOL-X (SAKURA) and fixed by KONIFIX (SAKURA). Finally, the cells were stained with Geimsa solution.

Labeled cells were defined as cells with labeled nuclei, and labeled nuclei were defined as those had more than five silver grains in each nucleus, and regarded as in their course of DNA synthesis. Through observations of 300 to 500 cells about each donor or each concentration, percent of labeled cells were derived.

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Distribution of silver grains per each cell was calculated by means of scoring silver grains on 60 to 180 labeled nuclei.

RESULTS

1. Chromosomal changes

Numerical distribution on ploidy of chromosome and numbers of cells with gaps and breaks are shown in Table 1. This indicated that the higher the concentration of benzene was, the more the percent of both hypo- and hyper-ploidy was. Percent of cells with structural changes also increased according as benzene concentration increased. In control, there was 8.5% of cells with aberrations but at the dose level of $1.1 \times 10^{-3} \text{M}$ there was 21.8% of cells with gaps and breaks and at the $2.2 \times 10^{-3} \text{M}$ there was 15.3% of cells with gaps and breaks.

Fig. 1. Displaced chromosomal breaks of human leucocytes under the influence of $1.1 \times 10^{-3} \text{M}$ benzene. Giemsa air dry. $\times 3000$.

Table 1. Effect of benzene on chromosome aberrations of cultured human leucocytes.

Benzene concentration	Cells studied	Numerical cell distribution					Cell with aberration break and gap	
		<46		46	>46			
		No	%	No	No	%	No	%
Control	316	5	1.6	309	2	0.6	27	8.5
$2.2 \times 10^{-5} \text{M}$	129	5	4.6	122	2	1.5	12	9.3
$2.2 \times 10^{-4} \text{M}$	105	5	5.3	98	2	1.9	12	11.4
$1.1 \times 10^{-3} \text{M}$	147	6	3.6	137	4	2.7	32	21.8††
$2.2 \times 10^{-3} \text{M}$	111	6	5.4	102	3	2.7	17	15.3†

†† significant at $p < 0.01$

† significant at $p < 0.05$

2. *Percent of labeled cells*

Table 2 shows percent of labeled cells of cultured human leucocytes under the influence of $2.2 \times 10^{-3}\text{M}$ of benzene. The ratio of the percentages to that of the control were 0.40 after 48 hr and 0.53 after 72 hr of culture. Khi-square test revealed that there are significant differences between treated groups and control. [$\text{Pr}(\chi^2=10.821)=0.001$, $\chi_0^2=69.731 > 10.821$]

Table 3 shows percent of labeled HeLa cells. The ratios of the percentages of labeled cells to that of the control were higher in 10^{-5}M and 10^{-4}M level of concentration, but it became lower in 10^{-3}M level and there was also a significant difference between $2.2 \times 10^{-3}\text{M}$ group and the control. [$\text{Pr}(\chi^2=3.841)=0.05$, $\chi_0^2=4.261 > 3.841$]

Table 2. Percent ^3H -labeled cells of treating cultured leucocytes with $2.2 \times 10^{-3}\text{M}$ benzene for 24, 48 or 72 hr.

Experimental No.	24 hr		48 hr		72 hr	
	C	B	C	B	C	B
1	1.5	0.0	29.5	11.3	33.7	15.4
2	1.0	0.0	23.0	9.1	32.5	16.3
3	1.7	0.0	23.5	9.2	32.1	16.0
4	1.5	0.0	24.0	10.0	43.8	23.8
5	0.4	0.0	21.9	8.7	35.4	25.1
6	0.0	0.0	15.6	6.8	32.6	14.3
Average value	1.0	0.0	22.9	9.2	35.0	18.5
Ratio to the control			1	0.40	1	0.53

C: Control

B: Benzene added

Table 3. Percent ^3H -labeled cells of treating HeLa cells for 24 hr with benzene at $2.2 \times 10^{-5}\text{M}$, $2.2 \times 10^{-4}\text{M}$, $1.1 \times 10^{-3}\text{M}$ or $2.2 \times 10^{-3}\text{M}$.

	Rate of labeled cells (%)	Ratio to the control
Control	32.34	1
Benzene concentration		
$2.2 \times 10^{-5}\text{M}$	36.60	1.13
$2.2 \times 10^{-4}\text{M}$	33.00	0.99
$1.1 \times 10^{-3}\text{M}$	25.17	0.78
$2.2 \times 10^{-3}\text{M}$	23.59	0.73†

† significant at $p < 0.05$

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3. Number of silver grains

Table 4 shows average value of grain count of cultured human leucocytes under the influence of $2.2 \times 10^{-3}M$ of benzene. Test of significance by Marshall's S-test revealed a statistically significant difference between experimental groups and control. The ratio of each value to the control was 0.21 after 48 hr and 0.25 after 72 hr.

Table 5 shows average numbers of grain count in case of HeLa cells. Ratio of DNA synthesis to the control was 0.94 in $2.2 \times 10^{-5}M$ group, and 0.81 in $2.2 \times 10^{-3}M$ group in average respectively.

Table 6 shows frequency distribution of silver grains in case of HeLa cells. By means of Marshall's S-test, statistically significant differences were shown between $1.1 \times 10^{-3}M$ group to $2.2 \times 10^{-3}M$ group and control. The median in control was 36, however, it was 30.8 in $1.1 \times 10^{-3}M$, and it was 30.3 in $2.2 \times 10^{-3}M$. Both of them were lower than that of control.

Table 4. Silver grains of treating cultured human leucocytes with $2.2 \times 10^{-3}M$ benzene for 48 or 72 hr.

Experimental No.	48 hr		72 hr	
	C	B	C	B
1	38.7	7.7	41.9	10.9
2	36.1	7.2	40.7	10.0
3	37.4	8.2	39.8	11.1
4	36.8	8.0	38.3	9.3
5	35.3	7.9	40.4	10.3
6	35.9	7.4	40.9	9.6
Average value	36.7	7.7	40.3	10.2
Ratio to the control	1	0.21	1	0.25

C: Control

B: Benzene added

Table 5. Silver grains of treating HeLa cells for 24 hr with benzene at $2.2 \times 10^{-5}M$, $2.2 \times 10^{-4}M$, $1.1 \times 10^{-3}M$ or $2.2 \times 10^{-3}M$.

	Average number of grains	Ratio to the control
Control	41.30	1
Benzene concentration		
$2.2 \times 10^{-5}M$	38.96	0.94
$2.2 \times 10^{-4}M$	34.93	0.85
$1.1 \times 10^{-3}M$	34.23	0.83
$2.2 \times 10^{-3}M$	33.37	0.81

Table 6. Sample distribution and sample mean of silver grains of treating HeLa cells for 24 hr with benzene at $1.1 \times 10^{-3}M$ or $2.2 \times 10^{-3}M$.

Grains	Control	$1.1 \times 10^{-3}M$	$2.2 \times 10^{-3}M$
5 — 9	0	0	1
10 — 14	3	2	8
15 — 19	3	16	12
20 — 24	10	16	24
25 — 29	7	10	14
30 — 34	6	6	19
35 — 39	4	14	9
40 — 44	4	7	9
45 — 49	6	2	4
50 — 54	4	8	6
55 — 59	1	5	1
60 — 64	2	0	4
65 — 69	3	0	4
70 —	7	4	5
Total	60	90	120
Total grain count	2478	3080	4004
Average value per cell	41.30	34.23	33.37

DISCUSSION AND CONCLUSION

As far as our experimental results are concerned, influence of benzene on chromosome and on DNA synthesis of cultured cells were evident in comparatively higher dose levels. Moeschlin⁶⁾ found similar inhibition of DNA synthesis from a study on uptake of 3H -thymidine into DNA of bone marrow cells in rabbits exposed to benzene.

Kissling and Speck⁹⁾ found chromosome aberrations in rabbits with benzene poisoning during a period of peripheral pancytopenia.

Recently, Vigliani¹⁰⁾, Selley¹¹⁾ and Forni^{12,13)} reported on patients with benzene induced aplastic anemia which showed chromosome aberrations and transitions into acute myeloblastic type of leukemia. Nowell and Hungerford¹⁴⁾ found Ph'-chromosome and showed the evaluation of the chromosomal finding in chronic myeloblastic leukemia.

According to Cleaver¹⁵⁾, decrease of silver grain count corresponds to the prolongation of S-phase of a cell cycle, and our study of benzene exposure S-phase of cultured human leucocytes and HeLa cells seemed to be prolonged. However, it is not known whether or not the delay of S-phase of affected cells is directly connecting to the mechanism of chromosome abnormalities.

Among many industrial solvents, benzene is said to be myelotoxic agent and

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pathogenic property of benzene is characteristic. In addition to common toxic action of organic solvents, it interferes cell differentiation in the haemopoietic system. Desaturation process of benzene in human body is already reported¹⁶⁾. A concentration of 20,000 ppm in air (65 mg/L at 20°C under 760 mmHg) is rapidly fatal and a concentration of 2,500 ppm causes serious disturbance on exposure for 60 min.¹⁷⁾ The relationship between DNA synthesis and chromosome breakage has been discussed by several workers about different chemicals.

In benzene poisoning, the presence of numerical and structural abnormalities of the chromosomes which were also observed in our study might be interpreted as consequence of an alteration of DNA which is realized before mitosis. In our experiment, the media contained 10^{-3} M to 10^{-5} M of benzene at the beginning of incubation. Similarity of dose-response relationship of mitotic inhibition and of chromosome change is also interesting. However, action of benzene to cells should be studied in comparison with those of ionizing radiation and other radiomimetic chemicals.

REFERENCES

- 1) Tough, I. M. and Court Brown, W. M. (1965). *Lancet*, **1**, 684.
- 2) Pollini, G. and Colombi, R. (1964). *Med. Lav.*, **55**, 241.
- 3) Forni, A., Pacifico, E. and Limonta, A. (1971). *Arch. Environ. Health*, **22**, 373.
- 4) Hartwich, Von G. and Schriker, K. Th. (1969). *Fortschr. Med.*, **13**, 563.
- 5) Koizumi, A. and Takada, M. (1966). *Jap. J. Ind. Health*, **8**, 203.
- 6) Moeschlin, S. and Speck, B. (1967). *Acta Haematol.*, **38**, 104.
- 7) Dobashi, Y., Tachibana, Y. and Koizumi, A. (1971). *Jap. J. Ind. Health*, **13**, 1, 56.
- 8) Dobashi, Y., Tachibana, Y. and Koizumi, A. (1971). *Jap. J. Ind. Health*, **13**, 3, 50.
- 9) Kissling, M. and Speck, B. (1971). *Helv. Med. Acta*, **36**, 59.
- 10) Vigliani, E. C. and Forni, A. (1969). *J. Occup. Med.*, **11**, 148.
- 11) Sellyei, M. and Kelemen, E. (1971). *Europ. J. Cancer*, **7**, 83.
- 12) Forni, A. and Luciano, M. (1967). *Europ. J. Cancer*, **3**, 251.
- 13) Forni, A. and Moreo, L. (1969). *Europ. J. Cancer*, **5**, 459.
- 14) Nowell, P. C. and Hungerford, D. A. (1960). *J. Natl. Cancer Inst.*, **25**, 1, 85.
- 15) Cleaver, J. E. (1967). *Thymidine Metabolism and Cell Kinetics*, p. 104, North Holland Research Monographs Frontiers of Biology, Amsterdam.
- 16) Sato, A. and Fujiwara, Y. (1972). *Jap. J. Ind. Health*, **14**, 3, 32.
- 17) ILO (1968). *Benzene: uses, toxic effects, substitutes*, p. 52. ILO, Geneva.