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Cytogenetic Abnormalities of Hematopoietic Tissue in Retired Workers of the Ohkunojima Poison Gas Factory

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ABSTRACT

A high incidence of cancer of the respiratory tract has been reported among former workers in a poison gas manufacturing plant which operated on Ohkunojima from 1927 to 1945. This report provides evidence of a high incidence of chromosome abnormality and sister chromatid exchange (SCE) rate among the former workers, as well as cytogenetic changes in two patients among the former workers with chronic myelocytic leukemia (CML). A chromosome study of seven former workers with chronic bronchitis revealed a stable type of aberration, the average abnormality being 10.9 ± 4.4 percent, which is equivalent to those of atomic bomb survivors exposed at 1.2 km from the hypocenter. The SCE rate observed in 16 former workers ranged from 4.9 ± 2.1 to 17.8 ± 3.9, which was significantly higher than in the control group ($p<0.03$). One of the CML patients showed an extremely high percentage of missing Y chromosomes along with t(9;22) translocation which is a specific chromosome aberration for the disease. Furthermore, the patient had almost a 3 times higher SCE rate compared to the control group and a high incidence of chromosome abnormality (12.1 %) of the peripheral lymphocytes. These results suggest that the development of leukemia in this patient was strongly related to poison gas exposure.

Key words: *Mustard gas, Chromosome abnormality, Sister chromatid exchange, Chronic myelocytic leukemia*

Ohkunojima is a small island in the Seto Inland Sea of Japan, Hiroshima Prefecture, where, between 1929 and 1945, a special factory was established by the former Japanese army for the production of several types of poison gases, including Y perite or mustard gas, and Lewisite, which is chlorvinylarsine. Both are vesicant gases. Among these poison gases, the long acting vesicant, mustard gas, was produced in the highest quantities. In the factory, workers wore gas masks, rubber clothes, and took other measures for protection against poison gases. However, supplies were limited and, furthermore, the gas could penetrate through the protective clothing and masks and injure the skin of workers or cause acute irritation of the eyes and respiratory tract. The majority of former employees have complained of persistent productive coughs, bloody sputum, or intermittent fever. Studies of the former factory employees have revealed an excessive incidence of respiratory tract neoplasms among workers heavily exposed to mustard gas[12,16,17].

In the present study, cytogenetic abnormalities were investigated among workers with chronic bronchitis or other disorders and also in two patients with chronic myelocytic leukemia having a history of gas exposure. The former factory workers had a higher incidence of chromosome abnormalities and sister chromatid exchange rates compared to the control group. One of the two CML patients showed a high incidence of missing Y chromosomes in the bone marrow cells along with t(9;22) translocation, which is a specific chromosome abnormality for the disease and also a high incidence of SCE rate, suggesting a possibility of CML development in relation to exposure to the poison gas.

MATERIALS AND METHODS

Clinical samples:

Peripheral blood samples from 43 former workers with a history of poison gas exposure were collected for analysis of chromosome aberrations and sister chromatid exchange (SCE) rate at Ta-

danoumi Hospital, located near Ohkunojima. The former poison gas workers were divided into three groups according to their type of work in the factory[12]. Group A consisted of workers who had been engaged directly in the production of mustard gas and/or Lewisite. Group B consisted of workers who were engaged in the inspection of products and transport. Group C consisted of those who had been engaged in the production of other gases, or who had worked in medical and/or administrative jobs in that area. Bone marrow and peripheral blood samples from two CML patients were also obtained from Hiroshima University Hospital. The control group consisted of healthy individuals aged more than 50 years. The former workers at the factory, the CML patients and the control individuals were informed that blood or bone marrow samples would be taken for chromosome analysis and that their privacy would be protected.

Case history of the leukemia patients:

Case NT : On October 24, 1981, a 68-year old man was referred to our clinic from Tadanoumi Hospital with a 3-month history of progressive fatigue and leukocytosis for about one year. He had been engaged in the production of mustard gas at the factory (Group A) for 22 months and had been examined for his health condition once or twice a year for 13 years since 1969. His hemogram showed 48 $\times$ 10^9/L of white blood cells (with 11% basophils, 6% lymphocytes, 1% monocytes, 1% myeloblasts, 3% promyelocytes, 10% myelocytes, 7% metamyelocyte, 6% band form and 55% neutrophils), 11.9g/dl of hemoglobin and 206 $\times$ 10^9/L of platelets. Diagnosis of CML was made from the morphology of the bone marrow cells and the positivity of Philadelphia (Ph^1) chromosome. After busulfan therapy for three months, the leukocyte count was maintained at a 8–11 $\times$ 10^9/L level. He died of pneumonia on 15 Feb., 1983.

Case SN : On February 15, 1984, a 53-year old female was also referred to our clinic from Tadanoumi Hospital with a history of leukocytosis for two years. She had worked in the area as a Group B worker. Her hemogram showed 54.8 $\times$ 10^9/L of white blood cells (with 3% basophils, 3% eosinophils, 4% lymphocytes, 3% monocytes, 2% promyelocytes, 7% myelocytes, 5% metamyelocytes, 15% band form and 58% neutrophils), 12.8g/dl of hemoglobin and 93.7 $\times$ 10^9/L of platelets. Diagnosis of CML was made from the bone marrow findings and Ph^1 positivity in the bone marrow cells. She was treated with busulfan for nine years with frequent interruption of the treatment. She developed blast crisis and died of bleeding on April 28, 1993.

Chromosome analysis:

After drawing 10 ml of peripheral blood, lymphocytes in the buffy coat were washed once with saline and cultured for 48 hours with 7.9 ml of RPMI 1640 medium, 2.0 ml of fetal calf serum (FCS) and 0.1 ml of phytohemagglutinin (PHA, Welcome HA15). Two hours before cell harvest, they were treated with colchicine at a concentration of 0.02 mg/ml. Hypotonic treatment was performed with a mixture of 0.075 M KCl and 0.034 M sodium citrate at 4:1 after fixing with Carnoy's solution (a mixture of absolute methanol and glacial acetic acid at 3:1). Slides were prepared by the air-drying method. After the slides were well dried, G-banding with trypsin was performed. All the satisfactory metaphases were photographed and all cells with chromosome aberrations on the photograph were subjected to karyotyping. Detailed examination was made of the types of structural aberrations and breakpoints based on the human chromosome diagram according to ISCN (1985)[7].

Sister chromatid exchange rate:

After washing, the lymphocytes were cultured in RPMI 1640 medium with the same supplement as described above. PHA was added to the cells and were incubated at 37°C for 24 hours. After this period, BrdU, at a final concentration of 10 mM, was added to the cultures and the cells were incubated for an additional 48 hours. Following colchicine arrest, metaphase preparations were made. The air-dried slides were exposed to Hoechst 33258 (0.5 mg/ml) in phosphate buffer (pH 7.0), exposed to ultraviolet light, and stained with 4% Giemsa[11].

SCEs were scored in at least 20 metaphases in the second division from the peripheral lymphocyte preparation. Agreement of two investigators was obtained before each SCE was scored. All metaphases scored were subsequently photographed.

Chromosome analysis of leukemic marrow cells:

Mononuclear cells from bone marrow samples were purified from a Ficoll gradient and cultured at a concentration of 1 $\times$ 10^6 cells/ml in a medium containing 8 ml of RPMI 1640 and 2 ml of FCS. A total of 10^7 cells were cultured in a plastic flask for 24 hours. Cell harvest, hypotonic treatment and staining were the same as described above for the peripheral lymphocyte culture.

RESULTS

Chromosome aberrations:

Lymphocyte cultures were started in 13 former workers, of which 7 were successful for chromo-

Table 1. Chromosome aberrations in former workers at the poison gas factory

Case	Type of work	Duration of work (months)	Chromosome number		Total cell observed	Number of cells with aberration	Percent of abnormality	Karyotype
			46	47				
KM	A	24	50	1	51	7	13.7	del(9)(q31)del(1)(p31)del(5)(q31)/–6,+mar/ der(9)t(9;?)(p13;?)/del(22)(q13),–8,+mar/+8
OA	A	24	14		14	1	7.1	inv(10)(q11q24)
MM	A	18	24		24	1	4.2	t(12;21)(p12;p11)
AK	B	240	48	1	49	6	12.2	+13,–20/–8,+21/del(5)(q31)/ t(6;7)(p23;p15)/ t(9;11)(p13;p15)/+22,t(8;15)(p11;p11)
NT	B	72	12		12	1	8.3	del(3)(p25)
SY	B	60	37		37	6	16.2	t(1;17)(p22;p11)/del(3)(q27)/t(3;19)(p11;q11)/ t(3;22)(q27;q13)/del(6)(q23)/t(9;16)(p11;q11)
SZ	C	189	21		21	3	14.3	t(8;10)(q24;p15)/der(8)t(8;?)(q22;?)/der(11) t(11;?)(p15;?)
Total			206	2	208	25	10.9 ± 4.4	

Fig. 1. Chromosome abnormalities found in a worker who had worked in Group B for 20 years (case AK of Table 1), showing reciprocal translocation between chromosomes 8 and 15 and trisomy 22.

Fig. 2. Chromosome abnormality found in case SY(Table 1), representing t(9;16)(p11;q11).

some analysis. Five of these seven patients were also successfully examined for SCE. All of the former workers had suffered from chronic bronchitis. Three out of seven workers belonged to Group A, three to B and one to Group C according to their type of work in the factory or local area. Table 1 shows the type and duration of work at the factory, number of cells examined, chromosome aberration rate and abnormal karyotypes observed. All cases were found to have chromosome aberrations, ranging from 4.2 to 16.2. The average rate of abnormality was 10.9 ± 4.4 percent. Among 208 examined cells, 25 cells had chromosome abnormalities, including 6 numerical and 23 structural abnormalities. The types of structural abnormalities were 12 translocations, eight deletions, two markers and one inversion. Chromosome aberrations were different case by

case and no common abnormality or abnormal clone were observed. Aberration rate was not significantly different according to the type of work or working periods in the factory. Figures 1 and 2 represent the stable types of chromosome abnormalities found in former workers at the poison gas factory.

Table 2. SCE rate in former workers at the poison gas factory

Type	Case	Age at the time of examination	Type of work	Duration of works (month)	No. of cells examined	Average SCE ± SD	Range of SCE
Exopsed in poison gas	OK	88	B	249	27	17.8 ± 3.9	3 - 27
	SZ	80	C	189	26	12.1 ± 3.6	4 - 20
	IS	80	A	177	26	4.9 ± 2.1	1 - 10
	YT	76	C	84	13	6.7 ± 2.0	3 - 10
	NT	78	B	72	25	13.8 ± 3.3	0 - 16
	TR	73	B	70	33	8.7 ± 4.4	1 - 18
	OT	71	A	66	61	6.5 ± 3.1	1 - 14
	SY	67	B	60	9	9.7 ± 3.3	4 - 14
	MT	72	A	60	33	5.2 ± 2.4	1 - 9
	FI	66	A	56	22	5.7 ± 2.4	3 - 11
	HK	68	A	49	19	7.3 ± 4.6	1 - 18
	OT	74	A	36	30	8.3 ± 3.7	2 - 21
	KS	65	B	36	26	6.4 ± 3.8	2 - 15
	KM	68	A	24	32	9.0 ± 3.9	0 - 22
	OA	64	A	24	29	11.6 ± 2.9	2 - 17
	DK	66	A	13	14	5.6 ± 2.5	2 - 11
Control	1	56			25	4.2 ± 2.0	2 - 6
	2	60			23	4.7 ± 1.4	1 - 7
	3	72			20	5.3 ± 2.8	2 - 8
	4	51			27	4.7 ± 1.7	1 - 7
	5	68			26	4.9 ± 2.1	2 - 8

Sister chromatid exchange rate:

Cultures for SCE were carried out in 30 former workers, of which 16 cases were successful. Most of the cases suffered from chronic bronchitis or cardio vascular disorders. Table 2 shows the ages at the time of SCE examination, type and duration of work, number of cells examined, average SCE rate and its range in the order of working period. Nine former workers belonged to Group A and had worked at the factory for an average of 49.4 months. Five belonged to Group B and had a history of exposure for an average of 97.4 months. Two workers in Group C had worked for 189 and 84 months, respectively. Workers in the gas production area (Group A) had a shorter working period compared to Groups B and C. The SCE rate ranged from 4.9 ± 2.1 to 17.8 ± 3.9 in the exposed group. The mean SCE rate of the exposed group was significantly higher than that of the control group (average SCE rate 4.8) ($p < 0.03$). There was no clear relationship among type of works, smoking habit and SCEs rate. Fig. 3 shows an example of the distribution of cells with SCE in Case SZ who had worked at the gas factory for 189 months as a member of Group C. It reveals a high percentage of cells with more than 11 SCE.

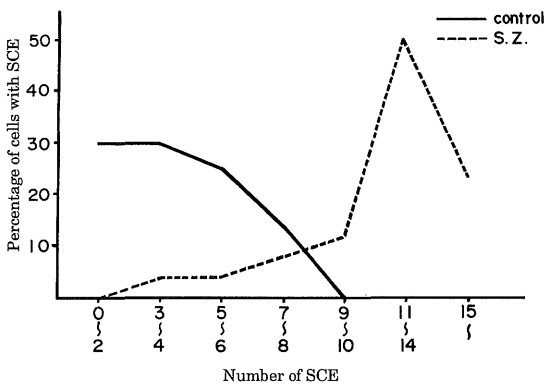

Fig. 3. Distribution of cells with SCE in case SZ (Table 2) who had worked at the gas factory for 189 months as a member of Group C.

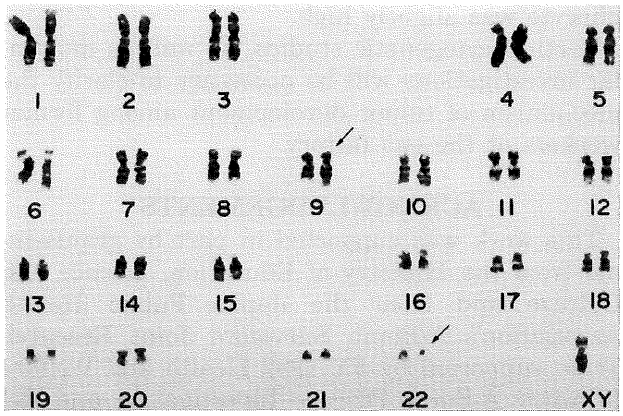

Fig. 4. Karyotype of bone marrow cell from case NT, representing reciprocal translocation of t(9;22)(q34;q11) and missing Y chromosome.

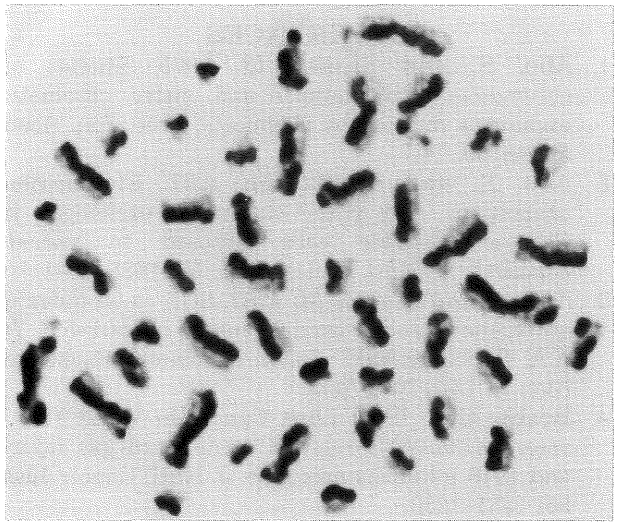

Fig. 5. Sister chromatid exchanges found in case NT, showing 17 exchanges in a metaphase.

Cytogenetic findings in leukemia patients:

At the time of diagnosis, Case NT was shown to have Ph^1 positive cells in the bone marrow, accompanied by 36.8% of cells with missing Y chromosomes in addition to Ph^1 chromosomes. Among 256 metaphases examined, 7 cells showed sporadic chromosome abnormalities along with Ph^1 or Ph^1 and missing Y chromosomes. Fig. 4 shows a karyotype with Ph^1 and missing Y chromosomes. PHA stimulated lymphocytes were also analyzed for chromosome abnormalities. In a total 124 cells examined, 87.9% of cells were normal, but the rest showed sporadic aberrations, most of which were of a stable type. The average SCE rate of the lymphocytes was 15.2/cell among 20 cells analyzed, a significantly higher SCE rate than in the control group. Figure 5 shows a metaphase of lymphocyte having 17 SCE. A chromosome study of the bone marrow cells from Case

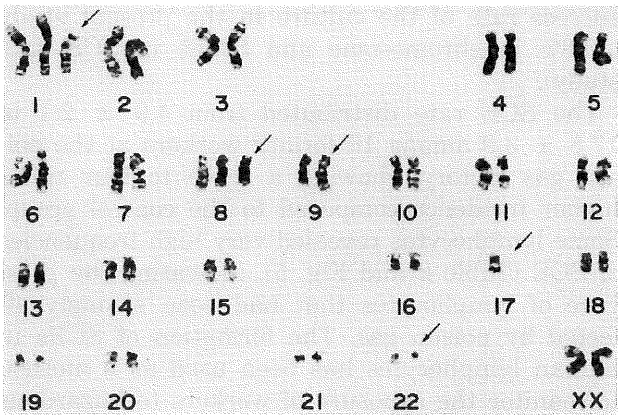

Fig. 6. Karyotype of bone marrow cell from case SN in accelerated phase of CML, representing 47, XX, +8,-17,t(9;22)(q34;q11),+der(1)t(1;17)(p13;q21).

SN with CML revealed Ph^1 positive cells in 100% at the time of diagnosis. After treatment with busulfan for eight years, she developed the accelerated phase and had additional chromosome abnormalities of +8,-17,+der(1)t(1;17)(p13;q21) (Fig. 6). The average SCE rate of lymphocyte was 8.0 ± 2.4 among 10 cells analyzed.

DISCUSSION

The present study demonstrates, for the first time, the high incidence of chromosome abnormalities and sister chromatid exchange in former workers at the poison gas factory. The chromosome aberration rates ranged from 4.2% to 16.2%, the average being 10.9%, which is equivalent to that of atomic bomb survivors exposed at 1.2 km from the hypocenter[8,15]. The types of chromosome aberrations were of the stable type as seen in atomic bomb survivors. However, the chromosome breakpoints in the poison gas workers seemed to be distributed in more peripheral regions of each chromosome, compared to those of atomic bomb survivors[5,9,15]. No specific chromosome abnormality nor clone formation was found in the seven former workers examined, whereas some clone formations were found among heavily exposed atomic bomb survivors[5,10]. Although the chromosome findings should have been confirmed by results from a larger number of the former workers, there were some difficulties in getting mitosis from these workers. Most of the former workers are more than 65 years old at present and have a low mitotic index of lymphocytes stimulated by PHA as an age effect. Furthermore, most of the former workers have suffered from chronic bronchitis and have been receiving steroid hormone or antipyretics which react as membrane stabilizers resulting in low sensitivity of the lymphocytes to PHA and in a low mitotic index. This was one of the reasons for the low

success rate of the culture in the present study (53.8% in chromosome and 53.3% in SCE rate study).

The SCE rate distributed from 4.9 ± 2.1 to 17.8 ± 3.9 among 16 former workers at the poison gas factory, showing a three to four times higher incidence compared to the control group. Some lymphocytes revealed very high frequencies of SCE (Table 2 and Fig. 5), indicating the presence of lymphocytes that had been strongly affected by poison gas. The formation of SCEs in human lymphocytes has been used as a method to monitor the exposure of workers to hazardous compounds[1,2,13,14]. Though a large number of environmental chemicals including mutagens and/or carcinogens have been studied, so far no report has been published on SCEs induced by mustard gas or Lewisite[3]. It is possible that the elevated SCE in the former workers at the poison gas factory in Ohkunojima island could be due to exposure to these poison gases, especially mustard gas.

A high incidence of cancer of the respiratory tract was reported among Allied troops exposed to mustard gas during the First World War[4] and also among former workers at the poison gas factory on Ohkunojima in Hiroshima[12,16,17]. Nishimoto[12] reported that the standardized mortality ratio was significantly elevated in Groups A and B who had inhaled mustard gas for a period of more than seven months, suggesting a possibility that cancer of the respiratory tract is induced by mustard gas.

Only two cases with chronic myelocytic leukemia have been found among the former workers since 1952. These two cases are reported here.

Case NT had Ph^1 positive cells which is specific for the disease, and also missing Y chromosomes in 36.8% of Ph^1 positive cells at the time of diagnosis. The high percentage of missing Y chromosomes shown in this case is unusual. A high percentage of missing Y chromosomes is normally found in aged persons with solid cancer or in immunologically deficient individuals. The former factory workers were found to have suppressed immunological competence[6,18,19,20]. Case NT had been engaged in the production of mustard gas for 22 months and seemed to have developed immunological deficiency. The high percentage of missing Y chromosomes may be related to his exposure to poison gas. Furthermore, he showed a high SCE rate of lymphocyte and a high incidence of abnormal chromosomes in the lymphocytes. From this evidence, it may be deduced that the development of CML in Case NT is related to poison gas exposure. On the other hand, Case SN showed standard chromosome abnormality for CML. Therefore, it is difficult to speculate that the cause of the disease was poison gas exposure in this case, though the average SCE rate of lymphocyte was slightly high.

Further cytogenetic studies, as well as molecular investigations will be necessary to clarify the mechanism of tumor development among former workers at the gas factory.

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REFERENCES

1. **Abe, S. and Sasaki, M.** 1975. Studies on chromosomal aberrations and sister chromatid exchanges induced by chemicals. Proc. Jpn. Acad. **53:** 46–49.
2. **Abe, S. and Sasaki, M.** 1977. Chromosome aberrations and sister chromatid exchanges in Chinese hamster cells exposed to various chemicals. J. Natl. Cancer Inst. **58:** 1635–1641.
3. **Abe, S. and Sasaki, M.** 1982. SCE as an index of mutagenesis and/or carcinogenesis. pp. 501–514. *In* A.A. Sandberg (ed.), Sister Chromatid Exchange. Alan R. Liss, New York.
4. **Beebe, G.W.** 1960. Lung Cancer in World War I veterans. Possible relation to mustard gas injury and 1918 influenza epidemic. J. Natl. Cancer Inst. **25:** 1231–1253.
5. **Honda, H., Kamada, N. and Bloom, A.D.** 1969. Chromosome aberrations and culture time. Cytogenetics **8:** 117–124.
6. **Hozawa, S., Ishioka, S., Yanagida, J., Takaishi, M., Matsuzaka, S., Ohsaki, M. and Yamakido, M.** 1989. Effects of periodic administration of Nocardia rubra cell-wall skeleton on immunoglobulin production and B-cell stimulatory factor activity in vitro in workers at a poison gas factory. Cancer Immunol. Immunother. **30:** 190–194.
7. **ISCN (1985).** 1985. An international system for human cytogenetic nomenclature. *In* D.G. Harnden and H.P. Klinger (eds.), published in collaboration with Cytogenet. Cell Genet. Karger, Basel.
8. **Kamada, N., Yuzaki, M., Tani, T., Ohkita, T., Okamoto, N. and Munaka, M.** 1980. Synthetic medical studies on atomic bomb survivors exposed in short distances. VIII. Estimation of radiation dose by means of chromosome analysis. Nagasaki J. Med. Ass. **55:** 722–725. (In Japanese)
9. **Kamada, N. and Tanaka, K.** 1993. Cytogenetic studies of hematological disorders in atomic bomb survivors. pp.455–474, *In* K. Ishihara and M.S. Sasaki (eds.), Radiation-Induced Chromosome Damage in Man. Alan R. Liss, New York.
10. **Kamada, N., Tanaka, K. and Hasegawa, A.** 1988. Chromosome aberrations and transforming

gene in leukemic and non-leukemic patients with a history of atomic bomb exposure. pp.125-134. *In* R.W. Miller (ed.), Unusual Occurrences as Clues to Cancer Etiology, Japan Sci. Soc. Press, Tokyo.

11. **Murthy, P.B., Kamada, N. and Kuramoto, A.** 1985. Increased sister chromatid exchange frequency in bone marrow cells of myelodysplastic syndromes. Cancer Genet. Cytogenet. **15:** 151-158.
12. **Nishimoto, Y., Yamakido, M., Ishioka, S., Shigenobu, T. and Yukutake, M.** 1988. Epidemiological studies of lung cancer in Japanese Mustard Gas Workers. pp.95-101. *In* R.W. Miller (ed.), Unusual Occurrences as Clues to Cancer Etiology, Japan Sci. Soc. Press, Tokyo.
13. **Perry, P. and Evans, H.J.** 1975. Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature **258:** 121-125.
14. **Popescu, N.C., Turnbull, D. and DiPaolo, J.A.** 1977. Sister chromatid exchange and chromosome aberration analysis with the use of several carcinogens and noncarcinogens. J. Natl. Cancer Inst. **59:** 289-293.
15. **Tanaka, K., Kamada, N., Ohkita, T. and Kuramoto, A.** 1983. Nonrandom distribution of chromosome breaks in lymphocytes of atomic bomb survivors. J. Radiat. Res. **24:** 291-304.
16. **Wada, S., Miyanishi, M., Nishimoto, Y., Kanbe, S. and Miller, R.W.** 1968. Mustard gas as a cause of respiratory neoplasia in man. Lancet **June 1:** 1161-1163.
17. **Wada, S., Yamada, A., Nishimoto, Y., Tokuoka, S., Miyanishi, M., Katsuta, S. and Umisa, H.** 1963. Neoplasms of the respiratory tract among poison gas workers. J. Hiroshima Med. Ass. **16:** 728-745. (In Japanese)
18. **Yamakido, M., Yanagida, J., Ishioka, S., Matsuzaka, S., Hozawa, S., Takaishi, M., Inamizu, T., Akiyama, M. and Nishimoto, Y.** 1986. Immune functions of former poison gas workers. I. Mitogenic response of lymphocytes and serum factors. Hiroshima J. Med. Sci. **35:** 117-126.
19. **Yamakido, M., Yanagida, J., Ishioka, S., Matsuzaka, S., Hozawa, S., Takaishi, M., Inamizu, T., Akiyama, M. and Nishimoto, Y.** 1986. Immune functions of former poison gas workers. II. Lymphocyte subsets and interleukin-2 production. Hiroshima J. Med. Sci. **35:** 127-134.
20. **Yamakido, M., Matsuzaka, S., Yanagida, Y., Hozawa, S., Yukitake, M. and Nishimoto, Y.** 1984. Effect of N-CWS injection against Influenza virus infection in the retired workers of the Okunojima poison gas factory. Hiroshima J. Med. Sci. **33:** 547-551.