STUDIES ON THE MECHANISM OF ACTION OF ALKYLATING AGENTS. I

Uptake and Subcellular Distributions of Cyclophosphamide and its Analogs in the Sensitive and Resistant Mouse Tumors

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INTRODUCTION

Cyclophosphamide is one of the most widely used alkylating agents, which has wide anticancer spectrum against human and experimental animal tumors. This agent is known to have little alkylating activity until it is activated by a hepatic microsomal enzyme.

Many reports are available as to the differences in drug uptake between sensitive and resistant tumors. Some reported that the difference was not as large as the difference in drug sensitivity, and others noticed that the extent of alkylation was closely correlated with sensitivity.

Shionogi adenocarcinoma 115 is a transplantable mouse tumor very sensitive to cyclophosphamide and to Ifosfamide, while it is insensitive to Trofosfamide. Sarcoma 37, on the contrary, is sensitive to Trofosfamide and is not sensitive to cyclophosphamide and to Ifosfamide. Initial concentrations of the agents in various organs and in the tumors have been compared and followed with the tumors up to 24 hours. Intracellular distribution of the drugs was then calculated and confirmed by means of the autoradiography, in order to determine whther the difference in sensitivity of these tumors can be related to these parameters.

MATERIALS AND METHODS

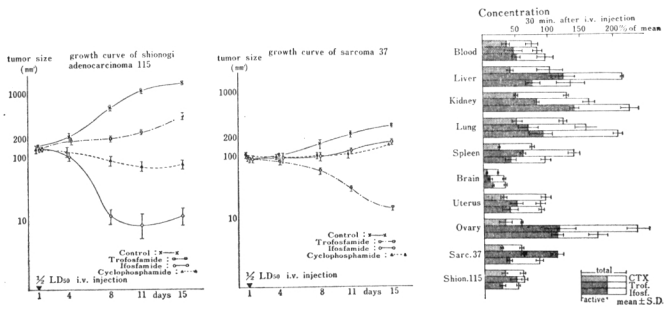
Alkylating agents

Cyclophosphamide and its analogs Trofosfamide and Ifosfamide were used. In some experiments tritium labeled Trofosfamide (sp. act. 447 μCi/mg) and Ifosfamide (sp. act. 446 μCi/mg) were used. These agents were obtained by the courtesy of Shionogi Pharmaceutical Company.

Experimental tumors

Two transplantable mouse tumors, Shionogi adenocarcinoma 115 and sarcoma 37, were used in this study. Mice were given various dose of alkylating agents on Day 10 of transplantation, when the tumors grew exponentially. These tumors had

Fig. 1 Growth curves of Shionogi adenocarcinoma 115 and sarcoma 37. Mice received single i.v. injection of the alkylating agents at a dosage of 1/2 LD_{50}, and the sizes of tumors were measured in square mm.



different sensitivity spectra to these alkylating agents used; namely, Shionogi adenocarcinoma 115 was highly sensitive to Ifosfamide and to the lesser degree to cyclophosphamide and insensitive to Trofosfamide, while sarcoma 37 was sensitive to Trofosfamide and insensitive to Ifosfamide and cyclophosphamide. Growth curves of the tumors are detailed in Fig. 1.

Chemical assay of the alkylating agents

Tumor bearing mice received single i.v. injection at a dose of 1/2 LD_{50} and were killed by decapitation after various time intervals. Organs or tumors were homogenized with glass homogenizer, and deproteinized with $HClO_4$. Samples were assayed by NBP method according to MORITA *et al*[17]. By this procedure values were expressed as those after hydrolysis and before hydrolysis, referred to total cyclophosphamide-like substance and its active metabolite(s), respectively.

Cell fractionation methods

Tumor cells and liver cells were fractionated by centrifugation according to the method by SCHNEIDER and HOGEBOOM[19]. Nuclear pellet was purified by the method of HIGASHI *et al*[9]., and was then dehistonized by the method described by SPELSBERG *et al*[21]. DNA was further purified by the method of STEELE[22]. In this experiment, tritium labeled Trofosfamide or Ifosfamide was given i.v. at a dose of 1 mg/kg and sacrificed after 30 minutes.

Scintillation counting

Each sample containing tritium labeled agents was treated with Packard model 36 sample oxidizer, dissolved in Moncface 40 scintillation media and was counted with Packard model 3375 scintillation sepctrophotometer.

Determination of DNA and RNA

DNA was measured by the method of diphenylamine reaction by BURTON[1]. RNA was determined by the orcinol reaction described by KERR and SERAIDARIAN[13] with slight modification.

Autoradiographic technics

Intracellular localization of ^3H-Trofosfamide or -Ifosfamide was visualized using water soluble autoradiographic technic. The liver or tumor was excised 30 minutes after the i.v. injection of radioactive agents, and tissue samples were cut into approximately 5 mm cubes. These samples were divided into two groups according to their fixing technics. One was prepared to be freeze-dried and dehydrated in 10^{-4} Torr vacuum for 48 hours at −70°C, by the method of GERSH[6] with slight modification. The other group was fixed and dehydrated in acetone. Dehydrated tissues of both groups were embedded in Epon resin in 10^{-4} Torr vacuum by the method of LUFT[16]. Thin sectioned samples were prepared by dipping, and were exposed for 8 weeks at 4°C. Details of the technics were described elsewhere.

RESULTS

Figure 2 shows the concentration of cyclophosphamide, Trofosfamide and Ifosfamide in various organs, excised 30 minutes after the single i.v. injection at a dose of 1/2 LD_{50}. Concentrations of the agents were determined by NBP method[17]. A relatively high concentration was exhibited in the liver, kidneys and lungs. High uptake of Trofosfamide and Ifosfamide was noticed in the ovaries compared with that of cyclophosphamide, about twofold of the concentration in blood. Concentrations in the two tumor tissues examined were relatively low. Concentrations of active metabolites were about one half of total alkylating agents in all organs, and were not higher in the tumors than in other tissues examined.

Fig. 2 Concentrations of cyclophosphamide, Trofosfamide and Ifosfamide. Abscissa: drug concentrations expressed as a percentage of mean concentration (mean concentration = injected dose/body weight). Shaded values: active metabolite(s).

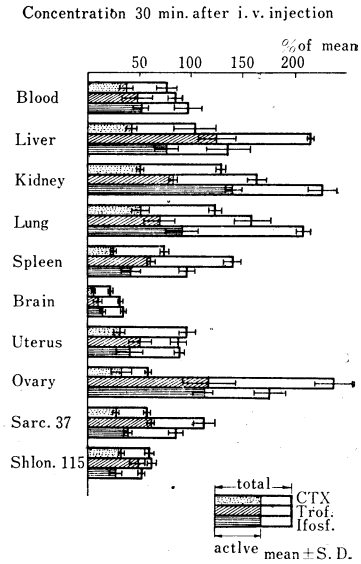


Fig. 3 Time course of drug concentration. Concentrations expressed as a percentage of mean concentration. Open symbols with interrupted lines : total alkylating agents. Solid symbols with solid lines : active metabolite(s).

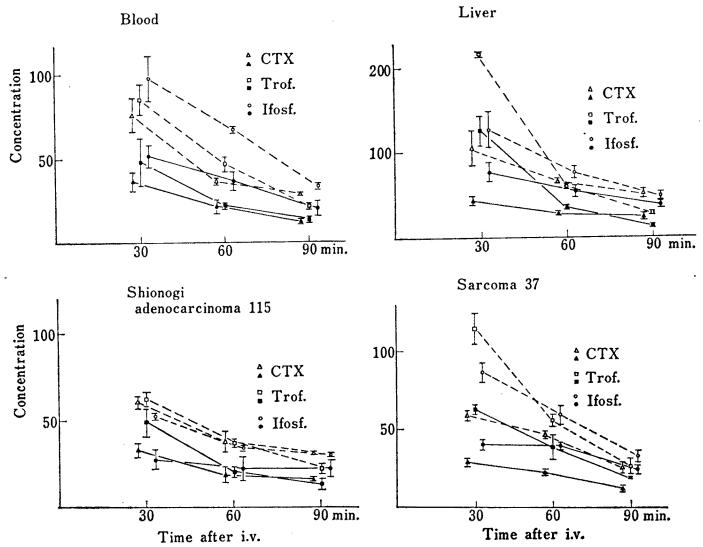
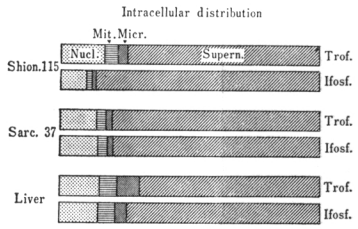


Figure 3 shows the time course of the uptake of drugs in blood, liver and tumors. Tumor bearing mice were treated with single i. v. injection at a dose of 1/2 LD_{50}, and concentrations were also measured by NBP method[17]. Half life of the agents in blood was about 1 hour in all drugs, and the ratio of active metabolites increased to about 2/3 of total agents in 90 minutes. Initial concentrations of drugs in the liver were somewhat higher than in blood, but the half life of the agents was almost same as in blood, and the ratio of active metabolite(s) showed same increment. The survival curves of the agents in both tumors were essentially same as in blood, though the initial uptake of the agents was low.

Table 1 and figure 4 show the intracellular distribution of ^{3}H-Trofosfamide and ^{3}H-Ifosfamide in the tumor and liver cells. Mice received tritiated Trofosfamide or Ifosfamide from the tail veins at a dosage of 1 mg/kg. Cell fractionation was carried out according to the method described by SCHNEIDER and HOGEBOOM[19]. Samples were treated and counted as described in the materials and methods. Over 70% of the counts was in the supernatant fraction in all tissues. Only 10 to 17% of the counts was fixed in the nuclear fractions of the liver and the tumor cells. Uptake of Trofosfamide and Ifosfamide in the nuclear fractions of the liver and of sarcoma 37 was almost the same. In the nuclear fractions of Shionogi adenocarcinoma 115, Trofosfamide was fixed much abundantly than Ifosfamide, but not statistically significant. In the microsomal fractions, the radioactivities in the liver cells showed significantly higher than those in the tumor cells.

Nuclear pellet obtained by the method of SCHNEIDER and HOGEBOOM[19] was a crude one ; cytoplasmic remnants were attached to the nuclei especially in the case of tumor cells. Nuclei were further purified by citric acid procedure described by HIGASHI *et al*[9]. Pure nuclear pellet was dehistonized and purified stepwise, yielding DNA as described in materials and methods. Table 2 shows the results of the fixation of ^{3}H-Trofosfamide and ^{3}H-Ifosfamide. Values of the agents fixed to the nuclei varied moderately, but these values became much uniform after lysing to chromatin. Dehistonization of the tumor chromatin and the removal of the acidic protein yielded no noticeable reduction in the radioactivities. The fixation of the agents to the tumor cell DNA was about 0.3 mole per 10^8 daltons DNA, when the agents were injected to mice at a dosage of 1 mg/kg, while to the liver DNA fixation of the agents was about 0.5 mole. The fixation to the nuclear RNA of Shionogi adenocarcinoma 115 was almost the same as was to DNA when divided by unit weight of RNA or DNA. These values were slightly smaller in the

Fig. 4 and Table 1 Intracellular distributions of ^{3}H-Trofosfamide and ^{3}H-Ifosfamide, 30 minutes after the single i.v. injection at a dose of 1 mg/kg. Nucl. Fr.: nuclear fraction. Mit.: mitochodrial fraction. Micr.: microsomal fraction. Supern.: supernatant solution.



Intracellular distribution of ^{3}H-Trofosfamide

	Shionogi 115	Sarcoma 37	Liver
Nucl. fr.	17.13± 8.06	14.16±5.41	15.51±3.72
Mit. fr.	5.33± 0.47	3.37±0.46	6.81±1.51
Micr. fr.	3.07± 0.78	2.29±0.49	8.62±1.55
Supern.	74.47±11.12	80.18±6.37	69.06±5.68

%±S.D.

Intracellular distribution of ^{3}H-Ifosfamide

	Shionogi 115	Sarcoma 37	Liver
Nucl. fr.	10.29±0.82	14.59±0.14	15.48±2.95
Mit. fr.	2.04±0.45	4.07±0.50	6.44±0.82
Micr. fr.	1.05±0.12	1.92±0.07	4.29±0.78
Supern.	86.62±0.24	79.42±0.29	73.81±3.19

%±S.D.

Table 2 Fixation of ^{3}H-Trofosfamide and ^{3}H-Ifosfamide, 30 minutes after the i.v. injection. Nuclei were purified by the citric acid procedure.

Shionogi Adenocarcinoma 115		Pure nuclei (dmp/mg DNA)	Chromatin (dpm/mg DNA)	Dehistonized DNA pellet (dpm/mg DNA)	DNA (dpm/mg)	DNA (Mol/10^{9} dal)	RNA (dpm/mg RNA)
Trofosfamide	Tumor	2851±717	1601±115	1443±101	934± 54	0.281±0.02	1059±132
	Liver	4336±116	2461±277	2049±131	1604±204	0.482±0.06	993±255
Ifosfamide	Tumor	1926±353	1080±126	1032± 39	982± 23	0.383±0.01	1227± 46
	Liver	3390±428	2084±136	1914±301	1352±228	0.528±0.09	1564±107
Sarcoma 37		Pure nuclei (dpm/mg DNA)	Chromatin (dpm/mg DNA)	Dehistonized DNA pellet (dpm/mg DNA)	DNA (dpm/mg)	DNA (Mol/10^{9} dal)	DNA (dpm/mg RNA)
Trofosfamide	Tumor	1373±275	1100±106	1060±123	1111± 86	0.334±0.03	730± 65
	Liver	4942±602	2513±352	2410±137	1713± 65	0.515±0.02	1388±231
Ifosfamide	Tumor	2150±110	1110±103	1097±148	1008± 68	0.394±0.02	672±101
	Liver	3372±252	2721±293	2093±102	1391±143	0.543±0.05	775± 68

case of sarcoma 37.

Figures 5 and 6 show the autoradiograms of Shionogi adenocarcinoma 115, injected i.v. with 1 mCi/mouse of ^{3}H-Ifosfamide. Freeze-dried sample (Fig. 5) exhibited that grains were accumulated much abundantly in the cytoplasm than in the nucleus. In the acetone-fixed sample (Fig. 6) grains in the nucleus were almost same in number as those in the freeze-dried sample, but the grains in cytoplasm were almost completely disappeared. Grains in the nucleus seemed to be located on the chromosomes and on the nucleolus. Number of grains in the liver cells on the freeze-dried samples (not exhibited in the figures) was about three-fold of that of tumor cells, but the grains in the nuclei of both cells were about the same. There was no difference in the number of grains and in the mode of their distribution between Shionogi adenocarcinoma 115 and sarcoma 37. There was also no difference in the grain counts between two agents.

Fig. 5 Autoradiogram of Shionogi adenocarcinoma 115, injected with 1 mCi/mouse of ^{3}H-Ifosfamide. Excised tumor was freeze-dried and embedded directly in epoxy resin.

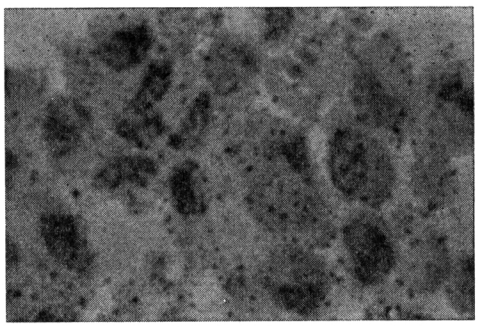
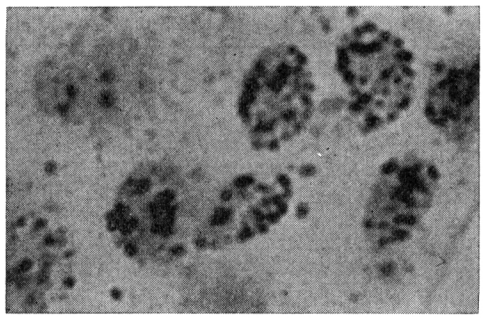


Fig. 6 Autoradiogram of the same tumor, fixed in acetone and then embedded in epoxy resin.



DISCUSSION

Cyclophosphamide itself is inactive as an alkylating agent and is not NBP-reactive. This agent is activated by a hepatic microsomal enzyme, yielded a single NBP-reactive metabolite, aldophosphamide[20]. Ifosfamide and Trofosfamide are thought to be activated in a similar manner[10]. By NBP method established by MORITA *et al*[17]., values of total cyclophosphamide-like substance and of its active metabolite(s) were separately determined; values before hydrolysis corresponded to active metabolites and values after hydrolysis to total alkylating agents. Ifosfamide and Trofosfamide were reported to be measured with the same method[10].

There are some papers in which cyclophosphamide levels were measured chemically using the NBP method. HIRATA *et al*[11]. reported that the half life of cyclophosphamide in blood was about 30 minutes, when administered i. v. to rabbits, and that its active metabolites were about one third at 30 minutes after the injection and one half at 90 minutes. On the other hand, MORITA *et al*[17]. reported that the half life of cyclophosphamide in rabbits was about 90 minutes, and that the active metabolites were about 50% at 1 hour and over 80 % after 3 hours. KUSAKABE[14] reported, in the experiment using dogs, that total cyclophosphamide-like substance was measurable until 18 hours and active metabolites until 12 hours. In our experiment with mice, half lives of three alkylating agents in blood were about 1 hour irrespective of agent, and the ratio of active metabolites was about 2/3 of total agents in 90 minutes. The survival of the agents in both tumors examined was almost the same as in blood and the liver, though the initial levels were somewhat lower than those in blood and the liver. There was no report available describing the uptake of the agents in tumors, using chemical assay methods. GRAUL *et al*[8]. reported using ^{3}H or ^{14}C labeled cyclophosphamide that the half life in Yoshida and Jensen's sarcomas was about 30 minutes and was almost same as in blood and the liver. In our experiment there was no correlation between drug sensitivity and the uptake of the agents or survival of the agents in the tumors.

In this paper, following experiments were carried out using isotopically labeled Trofosfamide or Ifosfamide. Here it comes into question whether or not the values obtained by labeled agents reflect the extent of alkylating activities, because these masked compounds are known to be activated and then degraded yielding inactive metabolites, though their radioactivities still remain. KUSAKABE[14] and MORITA *et al*[17]. reported in their short time experiments using ^{14}C-cyclophosphamide that the values calculated isotopically were in good accord with the values after hydrolysis measured chemically by NBP method. So we performed the subcellular studies using tritiated agents, assuming that the values calculated isotopically would be equivalent to those measured by NBP method.

Subcellular distributions of ^{3}H-Trofosfamide and -Ifosfamide were investigated using the procedure of SCHNEIDER and HODGEBOOM[19]. No noticeable difference was seen in the subcellular distributions between the liver cells and tumor cells, except that in the microsomal fraction the ^{3}H was taken up about 3-fold greater by the liver cells than by tumor cells. This may support the evidence that the activation of these masked compounds is car-

ried out by a microsomal enzyme in the liver but not in the tumor. Only 10 to 17% of ^{3}H was distributed in the nuclear fractions and over 2/3 of the radioactivities were in the supernatant solution. The resistant tumor contained as much ^{3}H as the sensitive tumor, and the distributions of the ^{3}H among the subcellular fractions were similar for two tumors. Similar results were obtained by WHEELER and ALEXANDER[25], who determined *in vivo* distributions of ^{14}C-cyclophosphamide among the subcellular fractions of the sensitive and resistant plasmacytoma, and reported that the extent and distributions of ^{14}C were similar. On the other hand, RUTMAN *et al*[18]. reported in their experiment using Lettré-Ehrlich ascites tumor cells that the sensitive cells took up 3~4× as much HN 2 into whole cells and into nuclei as did resistant cells. GOLDENBERG *et al*[7]. also reported that HN 2-sensitive lymphoblasts incorporated HN 2 1.7~3-fold as much as did resistant cells. These investigators suggested that the resistant cell lines derived their resistance, at least in part, from an effective dose reduction, and that the discrepancy between uptake and the 30-fold difference in drug resistance might be due to superior repair capacity of resistant cells.

Although the sucrose-calcium procedure provided satisfactory nuclei from normal rat liver, many transplantable tumors retain a substantial amount of cytoplasmic remnants when isolated by this method[9]. Nuclear pellet was further purified by citric acid procedure. Uptake of the drugs in the nucleus purified by this procedure is shown in the left hand column in Fig. 5. The liver cells took up 1.5~3.6× as much agents as did the tumor cells. The resistant tumors took up Trofosfamide or Ifosfamide into nuclei about 1.5-fold greater than did the sensitive tumors, but the significance was doubtful. Removal of the acidic protein(s) yielded 20~49% loss of the ^{3}H (2 nd column). Dehistonization from the chromatin showed no noticeable reduction in counts. A dose of 1 mg/kg of Trofosfamide or Ifosfamide could be expected to produce 0.28~0.39 alkylation per 10^{8} daltons DNA in the tumor cells and 0.48~0.54 alkylation in the liver cell DNA (5 th column). WHEELER and ALEXANDER[25] reported that a dose of 10 mg/kg of cyclophosphamide injected at 16 and 2 hours before sacrifice produced 2~3 alkylations per 10^{8} daltons DNA in the resistant plasmacytoma, whereas 0.8~2 alkylations in the sensitive tumor. In this paper alkylations of the nuclear RNA varied moderately but the extent of alkylation was almost same as of the DNA.

In our experiment the alkylation of the tumor cell DNA was similar irrespective of the agents or tumors, and the extent of alkylation of the DNA was not related with the drug sensitivity. Many reports are available concerning with the relationship between drug sensitivity and DNA alkylation. CONNORS and BALL[3] reported that the reaction of Melphalan with the DNA of sensitive and resistant Yoshida sarcomas was initially the same and that there was no factor in the resistant cell which had prevented the agent from reaching the DNA. RUTMAN group reported that the alkylation of DNA by HN 2 in the sensitive Lettré-Ehrlich cells was 2-fold greater than in the resistant cells[2)18)], but the sensitive and resistant cells did not differ significantly in the rate of removal of the alkylation[26]. GOLDENBERG *et al*[7]. reported that binding of HN 2 to DNA of the sensitive lymphoblastoma was 8-fold greater than of the resistant tumor. But these authors reported that the difference in the DNA alkylation of sensitive and resistant cells was inadequate to explain sufficiently the far greater difference in drug sensitivity. On the other hand, WHEELER and ALEXANDER[25] reported that the resistant plasmacytoma fixed about 2× as much cyclophosphamide to the DNA as did the sensitive tumor. CONNORS and DOUBLE[4] reported that in the resistant lymphoma cells the level of aniline mustard fixed to the DNA was more than twice as high as seen in the DNA of the sensitive tumor. Similar results were obtained by TRAMS *et al*[28]. All these investigators suggested that the extent of alkylation of the DNA was insufficient to explain the drug resistance, and that alkylation of the DNA might not be the mechanism of action. Possible mechanism of resistance was proposed by GOLDENBERG *et al*[7]. and CONNERS and BALL[3], who suggested that the resistant cells might have superior ability to repair their DNA damage.

Nuclear RNA of the sensitive and resistant tumors was alkylated to the same extent in our experiment. GOLDENBERG *et al*[7]. reported that the binding of HN 2 to RNA of sensitive lymphoblastoma was significantly higher than that observed in the resistant cells. Whether or not the RNA could

be the primary target of alkylation by which the alkylating agents exert their cytotoxic effects is another question. WHEELER[24] reviewed literatures and reported that alkylation of the RNA also could affect the synthesis of proteins and enzymes, and the alkylation of the proteins could alter enzyme activity. However, he concluded that the DNA was most sensitive to alkylation and was the primary site of alkylation. LAWLEY[15] reported that DNA and RNA were alkylated to the same extent, but that it was unlikely that either RNA or protein was sufficiently damaged, since their sizes as molecular tragets were much smaller than those of DNA.

In our autoradiographic study it was shown that grains of Trofosfamide or Ifosfamide were fixed firmly to the tumor cell nuclei in the acetone-fixed samples, whereas grains in the cytoplasm almost thoroughly disappeared. This would suggest that the agents in the nuclei were bound firmly to the nucleic acids, whereas in the cytoplasm binding to protein or RNA was loose, and that the loosely bound agents were solved out into acetone. These data would give support to the opinion that the DNA is the prime target of alkylation. There are some autoradiographic studies on the distribution of cyclophosphamide[5][12], but these works were insufficient in resolution, and no comment was proposed on the intracellular distribution.

The present study was designed to detect parameters which could explain the differences in sensitivities of tumors to alkylating agents. However, there was no difference in the uptake of the alkylating agents between the sensitive and resistant tumors. There were no differences in their subcellular distributions. There were also no noticeable differences in the extent of alkylation of the DNA and RNA. Parallel experiments investigated in our laboratory on the alkylation and interstrand cross-linking of the DNA and on the repair of the alkylated DNA gave us more detailed suggestions on the difference in drug sensitivity. The results will be reported in the forthcoming papers.

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REFERENCES

1) BURTON, K.: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62 : 315～323, 1956

2) CHUN, E. H. L.; L. GONZALES, F. S. LEWIS, J. JONES & R. J. RUTMAN : Differences in the *in vivo* alkylation and cross-linking of nitrogen mustard-sensitive and -resistant lines of Lettré-Ehrlich ascites tumors. Cancer Res. 29 : 1184～1194, 1969

3) CONNORS, T. A. & C. R. BALL : Possible mechanisms of acquired resistance to alkylating agents. pp. 13～21, in Gann monograph 2, eds. GOLDIN, A. *et al.*, Maruzen, Tokyo, 1966

4) CONNORS, T. A. & J. A. DOUBLE : Alkylation of the DNA of sensitive and resistant tumors by nitrogen mustard derivative. Int. J. Cancer 5 : 375～383, 1970

5) GERHARDS, H. J. & E. H. GRAUL : Autoradiographische Untersuchungen über die Verteilung von ^{3}H-Cyclophosphamid in der Ratte. Arzneimittelforschung 20 : 601～607, 1970

6) GERSH, I.: The Altman technique for fixation by drying while freezing. Anat. Rec. 53 : 309～337, 1932

7) GOLDENBERG, G. J.; C. L. VENSTONE, L. G. ISRAELS, D. ILSE & I. BIHLER : Evidence for a transport carrier of nitrogen mustard in nitrogen mustard-sensitive and -resistant L 5178 Y lymphoblasts. Cancer Res. 30 : 2285～2291, 1970

8) GRAUL, E. H.; E. SCHAUMLÖFFEL, H. HUNDESHAGEN, H. WILMANNS & G. SIMON : Metabolism of radioactive cyclophosphamide. Animal tests and clinical studies. Cancer 20 : 896～899, 1976

9) HIGASHI, K.; S. N. NARAYANAN, H. R. ADAMS & H. BUSCH : Utilization of the citric acid procedure and zonal ultracentrifugation for mass isolation of nuclear RNA from Walker 256 carcinoma. Cancer Res. 26 Part 1 : 1582～1590, 1966

10) HILL, D. L.; W. R. LASTER, Jr., M. C. KIRK, S. E. DAREER & R. F. STRUCK : Metabolism of Iphosphamide [2-(2-chloroethylamino)-3-(2-chloroethyl) tetrahydro-2 H-1, 3, 2-oxazaphosphorine 2-oxide] and production of a toxic Iphosphamide metabolite. Cancer Res. 33 : 1016～1022, 1973

11) HIRATA, M.; H. KAGAWA & M. BABA : Studies on cyclophosphamide. Part I. Chemical determination and degradation kinetics in aqueous media. Annual Report of Shionogi Res. Lab. 17 : 11～17, 1967

12) KALLENBACH, H. H.; R. SCHUSTER & A. GREGL: Autoradiographische Untersuchungen über die Verteilung von C-14-Lost markiertem Cyclophosphamid in bestrahlten und unbestrahlten

Impftumoren (Yoshida-Sarkom). A. Krebsforsch. 66 : 218～224, 1964

13) KERR, S. E. & K. SERAIDARIAN : The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J. Biol. Chem. 159: 211～225, 1945

14) KUSAKABE, H.: On the activation of cyclophosphamide *in vivo*. Med. J. Osaka Univ. 19 : 279～292, 1967 (in Japanese)

15) LAWLEY, P. D.: Effects of some chemical mutagens and carcinogens on nucleic acids. Progr. Nucl. Acid Res. 5 : 89～1311, 1966

16) LUFT, J. H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9 : 409～414, 1961

17) MORITA, M.; Y. TOCHINO, T. IWATA & T. MINESHITA : Studies on cyclophosphamide. Part II. The separative determination of cyclophosphamide and nor-mustard in biological materials. Annual Report of Shionogi Res. Lab. 17 : 114～121, 1967

18) RUTMAN, R. J.; E. H. CHUN & F. A. LEWIS : Permeability differences as a source of resistance to alkylating agents in Ehrlich tumor cells. Biochem. Biophys. Res. Commun. 32 : 650～657, 1968

19) SCHNEIDER, W. C. & G. H. HOGEBOOM : Intracellular distribution of enzymes. V. Further studies on the distribution of cytochrome C in rat liver homogenates. J. Biol. Chem. 183: 123～128, 1950

20) SLADEK, N. E.: Bioassay and relative cytotoxic potency of cyclophosphamide metabolites generated *in vitro* and *in vivo*. Cancer Res. 33 : 1150～1158, 1973

21) SPERSBERG, T. C.; A. W. STEGGLES, F. CHYTIL & B. W. O'MALLEY: Progesterone-binding component of chick oviduct. V. Exchange of progesterone-binding capacity from target to nontarget tissue chromatins. J. Biol. Chem. 247 : 1368～1374, 1972

22) STEEL, W. J.: Localization of deoxyribonucleic acid complementary to ribosomal ribonucleic acid and preribosomal ribonucleic acid in the nucleolus of rat liver. J. Biol. Chem. 243 : 3333～3341, 1968

23) TRAMS, E. G.; M. V. NADKARNI & P. K. SMITH: On the mechanism of action of the alkylating agents. I. Interaction of alkylating agents with nucleic acids. Cancer Res. 21 : 560～566, 1961

24) WHEELER, G. P.: Studies related to the mechanisms of action of cytotoxic alkylating agents : A review. Cancer Res. 22 : 651～688, 1962

25) WHEELER, G. P. & J. A. ALEXANDER : Studies with mustards. V. *In vivo* fixation of C^{14} of labeled alkylating agents by bilaterally grown sensitive and resistant tumors. Cancer Res. 24 : 1331～1337, 1964

26) YIN, L.; E. H. L. CHUN & R. J. RUTMAN : A comparison of the effects of alkylation on the DNA of sensitive and resistant Lettré-Ehrlich cells following *in vivo* exposure to nitrogen mustard. Biochim. Biophys. Acta 324 : 472～481, 1973