Tumor Res. 2(1967) 193-210

MECHANISM OF NITROMIN RESISTANCY OF EHRLICH ASCITES TUMOR CELLS

Hideyuki Tsukada, Aiko Kaneko, and Toshio Murata

Cancer Research Institute and Department of Pathology

Sapporo Medical College

An increasing emphasis has been placed on the mechanism of drug resistancy of cancer cells, with an increasing demand of practical use of various cancer chemotherapeutica. A number of investigations, thus, hitherto have been concerned with the possible mechanism of natural and acquired resistance of various species of tumor cells to alkylating agents, and the following possibilities were pointed out in a review of Wheeler(21) in connection with alkylating agents. These are, (a) altered transport of the agent to the cells, (b) altered permeability of the cells to the agent, (c) deactivation of the agent, (d) decreased availability of critical target, (e) utilization of alternative metabolic pathways by the resistant cells, (f) increased production of essential materials to overcome the defficiency caused by the alkylation, and (g) failure to activate the agent.

Among these possibilities cited above, the present investigation was focused on the changes in the permeability of cell membranes, the changes in the content of non-protein sulfhydryl groups as one of the intracellular substances which may be involved in the mechanisms preventing the target metabolism from alkylation, and also the changes in the target metabolisms

of Ehrlich ascites tumor cells, caused by acquired resistance to an alkylating agent, nitrogen mustard N-oxide (Nitromin).

Materials and Methods

Male mice of JCL/ICR strain weighing between 21 and 23g were used. The animals were fed on a compressed diet (Oriental, No. NMF) and received drinking water *ad libitum*. The tumor used was Ehrlich ascites tumor of a hypotetraploid line, which was obtained from the Sloan-Kettering Institute, and has been kept in our laboratory through serial transfers in mice for the past 7 years (14). During this period, no alterations were found in their chromosomal morphology and biological features including virulence to mice (14, 15).

Schedule for preparation of resistant tumor line: The tumor ascites was drawn from mice on the 5th day after intraperitoneal inoculation with approximately 10^7 tumor cells. 1 ml of the ascites was incubated with 2 ml of PBS(3) containing 2.5 mg of neutralized Nitromin at room temperature for 30 min under a gentle stirring. After the incubation, the tumor cells were centrifuged at 1,000 r.p.m. for 3 min. The cells were suspended in PBS to give a final volume of 1 ml. 0.2 ml of the suspension thus prepared, was intraperitoneally inoculated into mice. After sufficient ascites was accumulated, i.e. 7 to 10 days, the ascites was drawn and the tumor cells were subjected to the same treatment with Nitromin as described above. These treatments were repeatedly made for each transplant generation for 30 generations, with Nitromin in a dosage of 2.5 mg for the initial 5 generations, 5.0 mg during the subsequent 5

generations, 7.5 mg in the next 5 generations, and 10 mg during the following 15 generations.

After the total of 30 generations with the tumor cells pre-treated *in vitro* with Nitromin had passed, 2 or 3 generations were furthermore added with the cells non-treated with Nitromin, before the cells were submitted to the examinations. On the 6th day after intraperitoneal inoculation with these tumor cells, the ascites was drawn, and the cells were washed 3-times with ice-cold PBS or Eagle's culture medium supplemented with 10% bovine serum, and then, the cells were suspended in respective medium in appropriate concentrations.

Degree of resistancy of tumor cells: Prolongation of survival time of mice inoculated intraperitoneally with tumor cells pre-treated with Nitromin *in vitro* was observed as a parameter for evaluating the degree of resistancy. 10^8 tumor cells and various doses of neutralized Nitromin in PBS of a total volume of 1.5 ml were incubated at room temperature for 30 min. The cells were centrifuged and suspended in 0.5 ml of PBS, and then, 0.1 ml of the suspension was inoculated into mice. The observation was made for 50 days.

Permeability of tumor cell membranes to Nigrosin: 10^8 tumor cells washed and suspended in 1 ml of PBS were incubated with 5 ml of PBS containing 20 mg of neutralized Nitromin or 0.3% (v/v) Tween 80, at 37°C. After various incubation periods, the mixture was rapidly cooled in ice, and then, was added with 6 ml of 0.5% Nigrosin-PBS. After being kept on ice for 5 min, the cells were microscopically examined with the aid of a hemocytometer.

Contents of protein, glutathione, and sialic acid: On the tumor cells washed with and suspended in PBS, protein, glutathione, and sialic acid were determined according to the method of Lowry et al.(9), Grunet and Phillips (5), and Warren (19), respectively.

Incorporation of tritiated thymidine and uridine into perchloric acid-insolubles of tumor cells: Tumor cells washed with and suspended in Eagle's medium supplemented with bovine serum were used. 0.8 ml of the suspension containing 3.0×10^7 cells was added with 0.1 ml of PBS containing the compounds to be examined for their effects on the tumor cells. The mixture was incubated at 37°C for 5 min. Then, 0.1 ml of ^{3}H-thymidine (1μCi) or ^{3}H-uridine (2μCi) was added, and the incubation at 37°C was further continued for 30 min under a gentle stirring. At the end of the incubation, 8 ml of ice-cold 6% perchloric acid solution was added. After centrifugation, the sediments were washed 3-times with the acid solution. The sediments thus obtained, were suspended in 0.1 ml of 1N-NaOH solution, and then added with 0.6 ml of distilled water. 0.5 ml of the suspension, after an addition of 10 ml of dioxane-naphthalene scintillator (12), was assayed on its radioactivities using a Horiba liquid scintillation counter. 0.1 ml of the suspension was further subjected to determination of protein.

Incorporation of the labelled thymidine into the tumor cells was also examined autoradiographically. The cells incubated as described above, were spun down, and smear preparations were made. After drying, the preparations were fixed in absolute ethanol, washed with graded series of ethanol, and then further washed with distilled water. Autoradiography was made according to a dipping method with Sakura NR-M2 nuclear emulsion

Results

1. Acquisition of Nitromin-resistancy:

As shown in Table 1, mice bearing the original sensitive tumor cells were observed to survive for a period exceeding 50 days, when the cells were pre-treated with Nitromin in doses of more than 5 mg/1.5 ml before the inoculation. On the other hand, the animals inoculated with the tumor cells which had repeatedly been subjected to the "resistancy-treatment" died within a period comparable to those bearing the sensitive cells, even after the *in vitro* -treatment of the cells with 5 mg of Nitromin. 2 mice out of 5 inoculated with the cells which were previously subjected to the "resistancy-treatment" and were pre-treated with 7.5 mg of Nitromin died from ascites tumor on the 15th day. However, remaining 3 animals out of 5 produced solid tumor nodules subcutaneously at the site of the intra-peritoneal inoculation. These mice survived for considerably long

Table 1 Survival time of mice bearing Nitromin-sensitive and resistant Ehrlich ascites tumor cells.

Dose of Nitromin	Survival time of mice(days)	
	Original sensitive line	Resistant line
0	9, 11, 12, 14, 15	9, 9, 10, 11
2.5mg*	18, 18, 22, 46, survived**	
5.0mg	survived, survived, survived, survived, survived	9, 9, 11, 12, 14
7.5mg	survived, survived, survived, survived, survived	15, 15

Mice were inoculated intraperitoneally with $2x10^7$ tumor cells.

* Dose of Nitromin in 1.5 ml incubation medium.

** Observations were made for a period of 50 days.

periods and did not die from ascites tumor development.

2. <u>Changes of tumor cell membranes:</u>

Changes in the permeability of the cell membranes might be an appropriate parameter for evaluating those in their structural and functional integrity. As shown in Fig. 1, the permeability of the tumor cell membranes against Nigrosin was approximately the same in the resistant cells as in the original sensitive ones. However, when the cells were exposed to Nitromin or Tween 80, the permeability of the sensitive cells was increased at an increasingly rapid pace and was of a higher degree than that of the resistant cells.

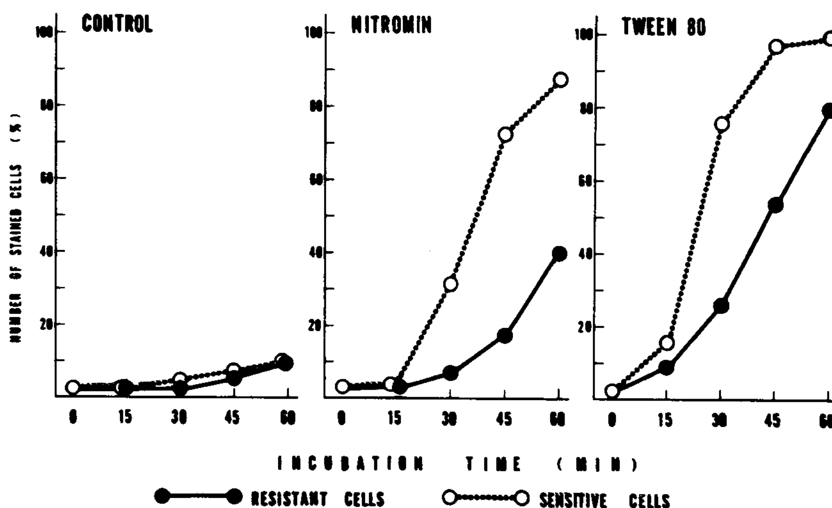


Fig. 1 Nigrosin-stainability of Ehrlich ascites tumor cells sensitive and resistant to Nitromin, in the presence of Nitromin and Tween 80.

3. Changes in the contents of glutathione and sialic acid of tumor cells:

As clearly noted in Table 2, the content of glutathione and sialic acid of the resistant cells was increased by approximately 240 and 170%, respectively, as compared with that of the sensitive counterpart. On the other hand, the protein content of the resistant cells was only slightly greater than that of the sensitive ones.

4. Changes in incorporation of tritiated precursors into the DNA and RNA of tumor cells:

As shown in Table 3, incorporation of ^{3}H-thymidine into DNA of the resistant cells was reduced to approximately a half of that of the sensitive cells, whereas incorporation of ^{3}H-uridine showed practically no difference between the two. When the cells were treated with Nitromin, incorporation of thymidine into the sensitive cells was more strongly inhibited than that into the resistant cell. With the same dose of Nitromin, uridine incorporation was less supressed than thymidine incorporation, and the supression of the former was found to be less in the resistant

Table 2 Contents of protein, sialic acid, and glutathione, of Ehrlich ascites tumor cells sensitive and resistant to Nitromin.

	Sensitive cells	Resistant cells
Protein, mg/10^9 cells	271 ± 22.9*(9**)	321 ± 25.0 (9)
Sialic acid, uM/10^9 cells	1.04 ± 0.140 (7)	1.77 ± 0.460 (13)
Glutathione, uM/10^9 cells	1.30 ± 0.480 (8)	3.13 ± 0.580 (9)

* Mean ± standard error.

** Number of examinations.

Table 3 Incorporation of ^{3}H-thymidine and ^{3}H-uridine *in vitro* into perchloric acid-insolubles of Ehrlich ascites tumor cells sensitive and resistant to Nitromin.

^{3}H-thymidine incorporation, c.p.m./mg protein		
	Sensitive cells	Resistant cells
Experiment 1.		
Non-treated	2530	1342
Nitromin(2.5mg)	90	180
Nitromin(2.5mg) + glutathione(4.0mg)	487	663
Experiment 2.		
Non-treated	2200	1354
Nitromin(2.5mg)	97	213
Nitromin(2.5mg) +glutathione(10.0mg)	744	1288

^{3}H-uridine incorporation, c.p.m./mg protein		
	Sensitive cells	Resistant cells
Experiment 1.		
Non-treated	2561	2850
Nitromin(2.5mg)	921	1323
Experiment 2.		
Non-treated	2630	2495
Nitromin(2.5mg)	1400	1597

cells as seen in the case of thymidine incorporation.

The addition of glutathione to the reaction mixture was observed to prevent the inhibitory action of Nitromin on thymidine incorporation into both sensitve and resistant cells. The inhibition in the resistant cells was almost abolished with 10 mg of glutathione.

The lesser intensity of thymidine incorporation into the resistant cells was also demonstrated in autoradiography. The number of cells heavily loaded with silver grains was significantly smaller in the resistant line

than in the sensitive ones. Furthermore, the percentage of the sensitive cells heavily loaded with silver grains was strikingly decreased in the presence of Nitromin, while on the other hand, the number of the resistant cells highly incorporated with the labelled thymidine was not significantly affected (Table 4, Figs. 2-5).

Table 4 Incorporation of ^3H-thymidine into Ehrlich ascites tumor cells sensitive and resistant to Nitromin.

Dose of Nitromin, mg	Number of grains on autoradiogram	Percentage of tumor cells	
		Sensitive cells	Resistant cells
0	0-5	20.0	34.6
	6-20	13.4	35.3
	21-50	18.8	25.6
	over 51	47.8	4.5
1	0-5	25.1	43.9
	6-20	26.8	36.0
	21-50	42.4	18.0
	over 51	5.7	2.1
2	0-5	39.0	52.7
	6-20	29.0	30.6
	21-50	29.2	14.1
	over 51	2.8	2.6

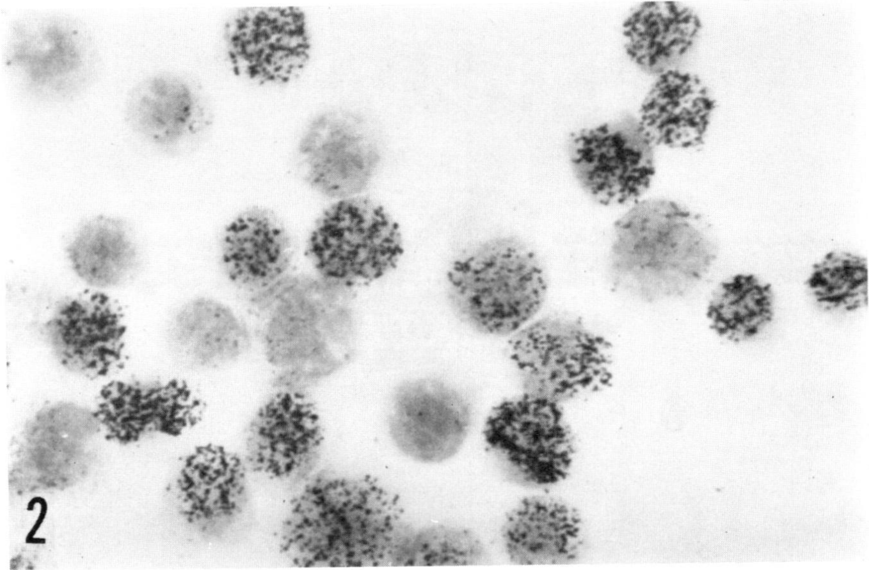
Discussion

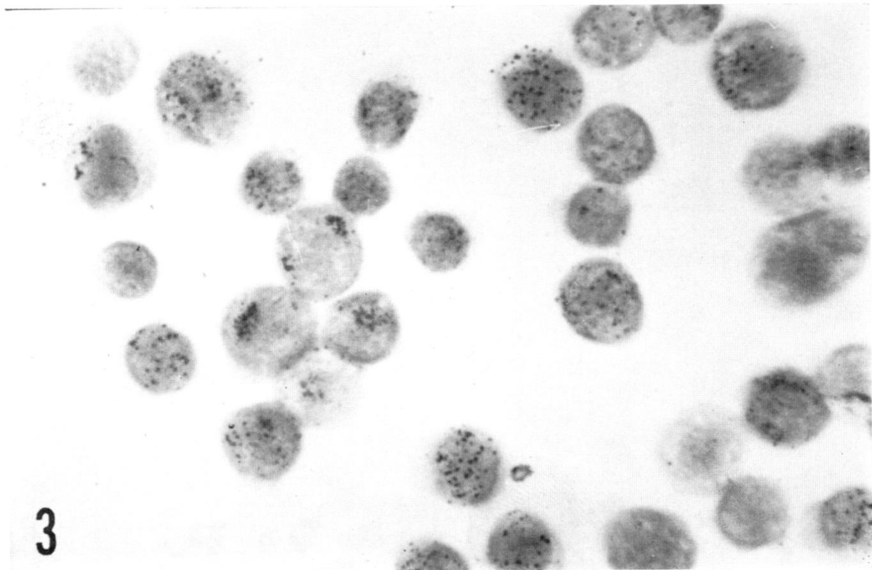
It seems appropriate to put an emphasis, first of all, on the changes in permeability of tumor cell membranes, since the initial site of the interaction between the cells and drug might be the cell membranes. It was reported that the permeability of the cell membranes of several species of

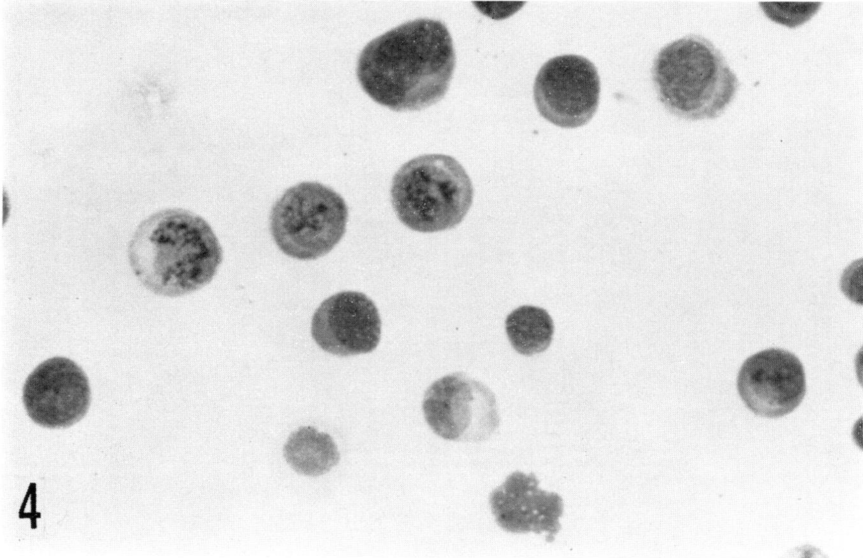
Figs. 2-5 Autoradiographs of Ehrlich ascites tumor cells after incubation with ^{3}H-thymidine. x 800

Fig. 2 Sensitive tumor cells in which a high incorporation is seen.

Fig. 3 Sensitive tumor cells treated with 1 mg of Nitromin. The incorporation is strongly inhibited.


2


3


4

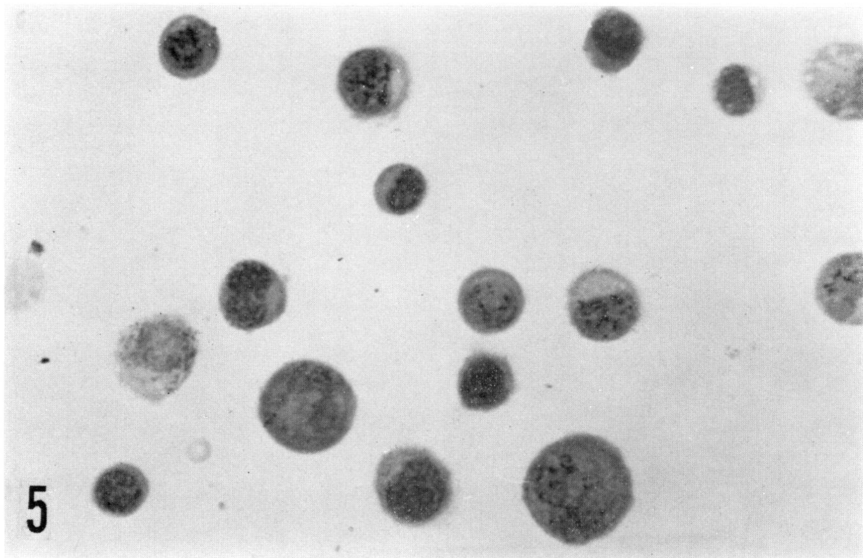

5

Fig. 4 Nitromin-resistant tumor cells in which a considerably low incorporation is noted.

Fig. 5 Nitromin-resistant tumor cells treated with 1 mg of Nitromin. The incorporation is comparable to that in the non-treated cells.

rat ascites tumors which were naturally resistant to Nitromin was considerably lower than that of the sensitive cells; however, the cells having acquired resistance were found to show no appreciable decrease of the permeability to the agents (2, 7, 8, 10, 11, 23). These suggest that certain differences are present in the integrity of the cell membranes between the tumor cells having natural and acquired resistance to alkylating agents, notwithstanding that the acquired resistancy would be brought about by the selection of naturally resistant cells from a population in which resistant and sensitive cells were present concurrently.

The changes in the structure and function of the cell membranes of the sensitive cells and the cells having natural and acquired resistance have not fully been examined. Hirono et al. (6, 8) found that a stronger mutual adhesiveness of tumor cells was in parallel to a lesser permeability of the cell membranes in a series of rat ascites hepatoma having various degrees of natural resistance to Nitromin. In the present experiments, a solid tumor nodule was occasionally formed in the subcutaneous tissues of mice at the site through where intraperitoneal inoculation of the resistant cells was made. This phenomemon was not observed on the sensitive cells. This may suggest that the cells having acquired resistance to Nitromin also may have a stronger mutual adhesiveness than the sensitive cells, probably due to changes in the structure of the cell membranes.

The sensitivity of tumor cells to Nitromin was reported to be increased with Tween 80 on naturally resistant rat ascites tumor cells, while not to be increased on tumors with acquired resistance (23). On the other hand, in the present experiments, Nitromin as well as Tween 80 enhanced Nigrosin-uptake through the cell membranes in a lesser degree in the

resistant cells than in the sensitive counterparts. This suggests that, regardless of the specificity of Nitromin on the cell membrane structures, the membranes of the resistant cells may have a construction which are less permeable. In spite of the fact that DNA-biosynthesis in the resistant cells was found to be much lower than in the sensitive ones, mice inoculated with the resistant cells incubated in PBS did not survive longer than those bearing the sensitive cells. This may indicate that the resistant cells resist the incubation, while the sensitive cells may be destructed to some extent during the incubation due to higher sensitivity of the cell membranes to the incubation in the latter cells.

Sialic acid-containing glycoprotein is considered as a consistent component of tumor cell membranes (1, 18). Furthermore, it was reported that removal of sialic acid changed ATPase activity involved as well as relative orientation of proteins and phospholipids in the membranes (4). Since a considerable amount of sialic acid of tumor cells was reported to be located in the cell membranes (18), an increase in the acid content of the resistant cells might result in changes in their chemical integrity, and is considered to eventuate virtually in the changes in their functions related to the permeability. An increase in sialic acid content of the resistant cells may also contribute to an increase in r-glutamyl transpeptidase activity, which was reported as one of enzymes related to glutathione biosynthesis and bound to sialic acid (13).

A specific increase in the amount of non-protein sulfhydryl groups was reported in tumor cells with acquired resistance to alkylating agents. Hirono et al. (7, 8) emphasized the importance of the role of the increase in free sulfhydryl groups in the resistant cells, based on the likeliness

that the sulfhydryl is one of nucleophilic centers with which alkylating agents combine and thereby prevent other intracellular targets from the compounds. On the other hand, Ball et al. (2) stated that a slight increase in the sulfhydryl concentrations would not contribute to the removal of large amounts of the compounds. Notwithstanding these two divergent assumptions, it might be conceivable that the increase in free sulfhydryl groups might contribute to some extent to the mechanism of the resistancy.

DNA is said to be one of critical targets of alkylating agents in tumor cells. Wheeler (20) reported that, guanine, the chief target moiety of DNA to alkylating agents, was reduced in the resistant cells. Williams and Joranger (22) suggested the presence of altered pathways of DNA-biosynthesis in the resistant cells. Furthermore, it was assumed that the repairing process of DNA was accelerated or the presence of a shorter S-period in the cell life cycle was possibly responsible for one of mechanisms of the resistancy (2), since alkylating agents were found to be more toxic on the tumor cells in the S-period (17).

It might be worthy of note that incorporation of thymidine into the resistant cells was significantly low, although the DNA content of the cel was not altered (16). This may not be ascribed to the reduction of the permeability of the cell membranes to thymidine, since there was practical no difference in uridine incorporation between the sensitive and resistant cells. The activity of DNA-biosynthesis is considered to be specifically lowered in the resistant cells, while being less sensitive to the inhibiting action of alkylating agents. It seems likely that the altered DNA and likewise the altered pathways of DNA-biosynthesis might result in a reduction in a critical target of the resistant cells.

Summary

Several biochemical characteristics of Ehrlich ascites tumor cells resistant to Nitromin were examined with respect to their mechanism of resistancy.

1) The resistancy of the tumor cells to the compound was induced by repetition of the transplant generations with the cells pre-treated with the compound in vitro before inoculation into mice.

2) Permeability of the resistant cell membranes to Nigrosin was enhanced with Nitromin and Tween 80 in a lesser degree as compared with that of the sensitive cells.

3) Content of sialic acid and glutathione was greater in the resistant cells than in the sensitive cells.

4) Incorporation of tritiated thymidine into the resistant cells was approximately half of that into the sensitive cells, whereas incorporation of tritiated uridine was almost the same in both cell lines. The inhibition of DNA-biosynthesis caused by Nitromin was much stronger in the sensitive cells than in the resistant cells, and the inhibition was abolished by the presence of glutathione.

The possible role in the mechanisms of acquired resistancy, of the cell membrane permeability, the content of sialic acid and glutathione, and pattern of DNA-biosynthesis and its senistivity to the alkylating agent was discussed.

(Received Oct. 16, 1967)

References

1. Baba, T., Ishii, M., Arai, H., & Aoki, K.: Gann, 57, 265 (1966).

2. Ball, C. R., Conners, T. A., Double, J. A., Ujhzy, V., & Whisson, M. E.: Intern. J. Cancer, 1, 319 (1966).

3. Dulbecco, R. & Vogt, M.: J. Exp. Med., 99, 167 (1954).

4. Emmelot, P. & Bos, C. J.: Biochim. Biophys. Acta, 99, 578 (1965).

5. Grunet, R. R. & Phillips, P. H.: Arch. Biochem., 30, 217 (1951).

6. Hirono, I.: Gann, 51, 179 (1960).

7. Hirono, I.: Gann, 52, 39 (1961).

8. Hirono, I., Kachi, H., & Ohashi, A.: Gann, 53, 73 (1962).

9. Lowry, O. L., Rosenbrough, N. J., Farr, A. L., & Randall, R. J.: J. Biol. Chem., 193, 265 (1951).

10. Miura, Y., & Moriyama, A.: J. Biochem., 50, 362 (1961).

11. Miura, Y., Motose, K., & Moriyama, A.: J. Biochem., 49, 514 (1961).

12. Murata, T.: Sapporo Med. J., 32, 20 (1967).

13. Sjewczuk, A. & Connel, G. E.: Biochim. Biophys. Acta, 83, 218 (1964).

14. Tsukada, H.: Acta Path. Jap., 10, 599 (1960).

15. Tsukada, H., Fujiwara, S., Ezoe, M., & Fujiwara, F.: Gann, 54, 311 (1963).

16. Tsukada, H., Inoue, A., Onoé, T., Hirota, E., & Onodera, T.: Gann, 50 (Suppl.), 92 (1959).

17. Walker, I. G., & Helleiner, C. W.: Cancer Res., 23, 734 (1963).

18. Wallach, D. F. H., & Eylar, E. H.: Biochim. Biophys. Acta, 52, 594 (1961).

19. Warren, J.: J. Biol. Chem., 234, 1971 (1959).

20. Wheeler, G. P.: Cancer Res., 22, 651 (1962).

21. Wheeler, G. P.: Cancer Res., 23, 1334 (1963).

22. Williams, A. M., & Joranger, E.: Brit. J. Cancer, 15, 342 (1961).

23. Yamada, T., & Iwanami, Y.: Gann, 53, 73 (1962).