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DEPARTMENT OF EXPERIMENTAL THERAPEUTICS

GENERAL SUMMARY

The principal objectives of cancer research in this department are to investigate the role of immunostimulant in immunotherapy or chemoimmunotherapy and to develop more selective and effective anticancer drugs to be submitted for clinical use.

The first of our research proposals is concerned with studies on immunotherapy of cancer. It is well known that BCG, *Corynebacterium parvum*, plant polysaccharides and others which are nonspecific immunostimulators have been widely used in the clinical and experimental treatment of cancer. However, immunotherapy is apparently capable of destroying only a limited tumor burden and must be combined with other therapeutic modalities to eradicate tumor cells. The chemoimmunotherapy is based on the concept that the combined use of an anticancer drug at lower dose and a nonspecific immunostimulator resulted in a reduction in such toxic side effects as leucopenia, thrombocytopenia, and depressed RES functions, and also provided a wider margin of safety. Meanwhile, it is known that OK-432 which is an unique anticancer preparation derived from group A streptococcus has not only direct oncocidal activity but also a host-mediated anticancer effect. Immunopotentiating effects of OK-432 can conveniently be classified as nonspecific in the sense that they do not require an immune response to tumor antigens, and as specific where there is adjuvant effect on tumor antigens.

Recently, it was demonstrated in this department that combination therapy with OK-432 and a suboptimal dose of 5-fluorouracil was synergistically effective in allogeneic and syngeneic tumor-host system. A number of drug combinations with OK-432 have been examined in experimental animals, and the background of therapeutic synergism —— a greater than additive anticancer effect without a corresponding increase in toxicity —— has been extensively studied.

On the other hand, current attempts at isolating chemically well-defined fractions from whole streptococcal cells have been given a priority, with the justification that they may lead to preparations that retain antitumor activity but have decreased or no toxicity. Up to the present no fraction has been obtained which is more effective in cytotoxic activity and in stimulation of the lymphoreticular system compared with those produced by OK-432. Although there are many complicated problems in the field of immunotherapy or chemoimmunotherapy, the evaluation of microbial products used in cancer immunotherapy should be carried out employing fully planned protocols with various tumor-host systems.

Another proposal for our research is the development of new anticancer

agents. Many substances derived from natural sources as well as synthetic compounds, have been submitted for the study in cancer research, and some of them have shown antitumor activity. Especially, a variety of metabolic products of streptomyces and fungi are currently in use as supportive measures for cancer treatment. Asterriquinone is a compound recently isolated from *Aspergillus terreus* IFO 6123, as one of its intracellular metabolic products. This compound which comprises a benzoquinone moiety and two indole rings was found to be moderately effective in suppressing the growth of transplantable murine tumors *in vivo* and in inhibiting the proliferation of HeLa cells in culture. Although the action mechanism of asterriquinone is not yet clear, it is likely that it acts on the membrane system of the tumor cell, based upon the data obtained in biochemical investigations and in morphological findings. Subsequently, it is thought that this compound or its analogs may be hopeful as a new type of candidate for an anticancer drug. Besides, it is of interest to isolate and characterize the major cytotoxic constituents of marine invertebrates, particularly the sea urchin.

Another approach to the development of anticancer drugs is to search for an useful candidate among chelating agents which are able to interact with vital components in tumor cells. As reported previously, 2-(2-hydroxyphenyliminomethyl) -4-*n*-hexylphenol (HP) and 2- (2-hydroxy-5-*n*-hexylphenyl) -8-quinolinol-4-carboxylic acid (HQII) were found to be effective on some transplantable rodent tumors. Since both compounds having metal-chelating ability inhibited selectively DNA biosynthesis in ascites hepatoma AH13 cells in culture, and interfered with the function of ribonucleotide reductase (iron-requiring enzyme), many chelating compounds were tested at random in this respect. Several compounds were active in suppressing the proliferation of the cultured cells, but did not exhibit antitumor activity *in vivo* very much. However, it is hoped that the approach employed in the present study will give a possible anticancer drug.

Furthermore, the primary purpose of cancer chemotherapy is in general to reduce the body burden of tumor cells. The concept of cell-cycle specific therapy of tumors following synchronization of tumor cells has recently found increasing interest. Therefore, it is important to establish a treatment schedule based on cell kinetics and pharmacodynamics in cancer chemotherapy.

Finally, studies on the new type of hemolytic toxin produced by hemolytic streptococci in the presence of oligoribonucleotide and colistin is also in progress in our laboratory. Since this hemotoxin was chromatographically detected as a segment which is different from the streptolysins, S and O, the accumulated data in this study may find a clue to the mechanism of production of streptococcal hemotoxins.

These works were supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

ABSTRACT

(47) Chemoimmunotherapy with OK-432 in experimental tumor systems

S. Koshimura, T. Ujiie, K. Ryoyama
T. Murayama, J. Inagaki and Hsu Shang-Lin

Many reports have already been presented about the antitumor activity of OK-432 for which the antitumor action is due to either the direct cytotoxic effect on tumor cells *in vitro* and *in vivo* or the potentiation of the reticuloendothelial system of the host[1]. Further, it is evident that OK-432 has brought about antitumor effects on various experimental tumors including spontaneously developed mammary carcinoma, chemically induced sarcoma and drug-resistant tumors, and the clinical effect of this preparation has also been appraised. Meanwhile, clinical usefulness has been prompted by the demonstration that OK-432 and other antitumor drugs acted synergistically against experimental tumors probably in conjunction with the immunologic defenses of the host[2,3]. The successful application of nonspecific immune stimulator, when used alone or combined with chemotherapy to augment host immunity, suggests that immunologic responses of the host may prove valuable parameters in the control of cancer. It is likely that the technique of administration regarding site, dosage, frequency and especially duration in chemoimmunotherapy is important for producing beneficial effects in cancer treatment.

In the present work, the effect and time of OK-432 administration combined with chemotherapy was investigated, employing some syngeneic mouse tumors, L1210 lymphoid leukemia, Lewis lung carcinoma and B16 melanotic melanoma. Table 1 presents the results indicating that when OK-432 was successively or intermittently given during the treatment with a suboptimal dose of cyclophosphamide against advanced Lewis lung carcinoma, the synergistic effect was clearly demonstrable in this chemo-

Table 1. Effect of treatment with cyclophosphamide(CPM) and/or OK-432 against Lewis lung carcinoma

Group	Treatment (ip)		Tumor weight(g) *1 on Day 20	T/C (%)	*t*-test
	CPM mg/kg	OK-432 KE/kg	Mean ± S. E.		
1	.	.	3.26 ± 0.41	.	.
2	.	100*2	2.59 ± 0.50	79.4	NS*4
3	.	50*3	2.01 ± 0.20	61.5	P<0.05
4	.	25*3	2.82 ± 0.37	86.5	NS
5	20*3	.	2.11 ± 0.51	64.7	NS
6	20*3	100*2	0.82 ± 0.30	25.1	P<0.005 P<0.005*5
7	20*3	25*3	0.57 ± 0.11	17.3	P<0.005 P<0.025*5

Animal: Male B6D2F$_1$ mice (6.5-week-old, 8 mice/group).
Inoculum: Lewis lung carcinoma cells 1.5 x 10^6/mouse, sc.

*1 Calculated by the formula of $\frac{L \times W^2}{2}$ in NCI protocols.

*2 On Days 4, 7, 10, 13. *3 On Days 4–13. *4 Not significant (P=0.05).
*5 Compared with group 5.

immunotherapy. Similar effectiveness was observed in the experiment in which the combined treatment with cyclophosphamide and OK-432 was adopted to the B6D2F1 mice subcutaneously implanted B16 melanoma, though survival time of the treated group animals was not so much longer than that of the control animals. A typical result obtained for combination therapy against L1210 leukemia is presented in Table 2. The antileukemic effect[4,5] elicited by combination therapy with adriamycin and cyclophosphamide was also enhanced by additional treatment with OK-432 after the chemotherapy, as shown in this table. In this case, a single injection of OK-432 on Day 5 gave a most pronounced result with over half long-term survivors. Synergistic effect was also produced by combination treatment with 5-fluorouracil, when OK-432 was administered only once on Day 4 after the chemotherapy[6]. Further, the mice surviving after the chemoimmunotherapy could reject the challenge of 10^3 L1210 cells but not P388 leukemia cells. On the contrary, the mice surviving through chemotherapy alone could hardly be rescued from leukemic death after reinoculation of L1210 cells. This adjuvant effect of OK-432 manifested by the chemoimmunotherapy was reversed by an immunosuppressant, such as azathiopurine or dexamethasone or by a macrophage inhibitor, such as trypan blue or κ -carrageenan.

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5) Tobias, J. S., Parker, L. M., Tattersall, M. H. N. and Frei, E., III, Br. J. Cancer, 32, 199 (1975).
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Table 2. Combination therapy with adriamycin(ADR), cyclophosphamide(CPM) and OK-432 against mouse L1210 leukemia

ADR mg/kg, ip Day 1	CPM mg/kg, ip Day 1	OK-432 KE/kg, ip Day 5	Median survival days*	50-day survivors/No. of test mice
.	.	.	6.0	0/20
.	.	100	6.0	0/10
3.0	.	.	10.5	0/10
3.0	.	100	10.5	0/10
.	100	.	9.0	0/10
.	100	100	9.0	0/10
3.0	100	.	15.5	1/10
3.0	100	100	24.0	6/10

B6D2F1 male mice were given ip injection of 1×10^6 L1210 cells on Day 0.
* Excluded 50-day survivors.

(48) Effect of OK-432 on immunization with mitomycin-C-treated L1210 cells.

K. Ryoyama, T. Murayama and S. Koshimura

As reported previously, OK-432 produced an enhanced antileukemic effect against mouse L1210 leukemia when it was administered intraperitoneally to B6D2F1 mice in combination with 5-fluorouracil[1]. Since this increased therapeutic effect was not obtained in the mice previously loaded with X-ray irradiation or administration of immunosuppressant, it was thought that a synergistic effect against L1210 leukemia in combined treatment was produced in conjunction with the immunologic defences of the host.

Bartlett *et al.*[2] have defined immunotherapy as any manipulation that augments the ability of a tumor rejection immunity which is ultimately systemic and is specifically directed against antigens detectable on tumor cells. Several hypotheses have been presented on how nonspecific immunostimulators, such as BCG, or *Corynebacterium parvum* potentiate specific or nonspecific antitumor immunity.

From the standpoints described above further investigations on the problem of whether OK-432 augments the ability of specific antitumor immunity in the host animal which received mitomycin-C-treated L1210 cells (MMC-L1210) as a specific immunogen were carried out. The data obtained indicate that OK-432 increasingly potentiate the resistance against L1210 challenge to B6D2F1 mice when a specific immunogen was injected before OK-432 administration (Table 1), and this resistance may be specific to the tumor employed as immunogen, because the tested mice could not overcome the challenge of mouse P388 leukemia. BCG was also effective in this assay system when BCG was given simultaneously or 6 days prior to the injection of the tumor immunogen (Table 2).

On the other hand, it was interesting that spleen cells and serum from

Table 1. Effect of pretreatment with mitomycin-C-treated L1210 cells and OK-432 on subsequent challenge with viable L1210 cells

Pretreatment			Median survival days (range)	60-day survivors/No. of test mice
Mitomycin-C-treated L1210 cells (ip)	OK-432 (KE/mouse)			
None	None		11.0 (9– 12)	0/10
5×10^6	None		12.0 (10– 24)	0/10
5×10^6	5	ip	17.0 (10–>60)	2/10
5×10^6	2.5	ip	19.0 (10–>60)	1/10
5×10^6	1	ip	15.5 (11– 32)	0/10
5×10^6	1	iv	13.0 (9– 36)	0/10
5×10^6	1	ip[a)]	11.0 (9– 20)	0/10
1×10^8	2.5	ip	11.0 (5–>60)	1/10
1×10^7	2.5	ip	15.0 (9– 43)	0/10
1×10^6	2.5	ip	19.0 (9–>60)	3/10

L1210 challenge was done by ip inoculation of 1×10^4 cells 11 days after the pretreatment.
a) One KE of OK-432 was given four times on days 2, 5, 8 and 11 before the challenge.

the mice pretreated with OK-432 and MMC-L1210 immunogen might be cytotoxic for L1210 cells *in vitro* even though the mice showed a less increased resistance to challenge with viable leukemic cells. The results of these experiments also showed that cytotoxic activity of spleen cells induced by OK-432 was significantly greater than that induced by BCG when the tumor immunogen was injected. However, there were no demonstrable differences in ability potentiating cytotoxicity of spleen cells between OK-432 and BCG when they were administered without the tumor immunogen. These results suggest that both agents may differ from each other in biological properties as an immunopotentiator. Hawrylko and Mackaness[3] reported that BCG could induce systemic resistance when irradiated tumor cells were injected into the sites prepared by 10 day prior local injection of BCG but not when both of them were simultaneously administered.

Other experiments carried out to examine the influence of combined treatment with chemotherapeutica and OK-432 on active immunity showed an interesting result. In the protocols, in which a large dose of cyclophosphamide (100 mg/kg) was given intraperitoneally to the mice previously treated with tumor immunogen on day −11 and OK-432 on day −6, the resistance of the hosts to the challenge of tumor cells seems to be dominated by administration time of cyclophosphamide. This may be concerned with modulation of the function of suppressor cells. In this respect, further studies on the differences in the mode of action between OK-432 and BCG, and on the factors to be influenced upon resistance to tumor challenge are currently underway.

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Table 2. Enhancement of antitumor immunity by OK-432 or BCG in relation to their administration time

Group	Pretreatment			Median survival days	60-day survivors
	Immunogen[a]	Potentiator[b]	Time		
1	−	None	.	10.0	0/26
2	+	OK-432	Day −17	20.0	2/16
3	+	OK-432	Day −11	19.0	2/16
4	+	OK-432	Day − 6	31.5[c]	4/16
5	+	BCG	Day −17	22.0[d]	3/16
6	+	BCG	Day −11	20.0	2/16
7	+	BCG	Day − 6	18.0	0/16

Challenge with L1210 was done by ip inoculation of 1×10^4 cells on day 0.
a) Mitomycin-C-treated L1210 cells (5×10^6) as immunogen were ip injected on day −11.
b) A dose of potentiator was 2.5 KE/mouse for OK-432 and 250 μg/mouse for BCG, respectively.
c) $P \leq 0.07$, compared with group 2; $P < 0.02$, compared with group 7.
d) $P \leq 0.05$, compared with group 7.

(49) Antitumor and other biological properties of sub-cellular fractions from group A streptococcus.

K. Ryoyama, T. Murayama, S. Koshimura and S. Sakai

It is known that a streptococcal preparation, OK-432, has both a direct action on malignant cells and an indirect effect through the general enhancement of the host immune responses. Therefore, many attempts have been made to isolate the active factor(s) from live group A streptococcus or OK-432; Koshimura *et al.* and Shoin have separately demonstrated the antitumor activity of cell-free extracts prepared from the coccus, and recently Koshimura *et al.* have reported the effectiveness in *in vitro* and *in vivo* of the protoplast membrane fraction freshly isolated from the coccus.

Rotta[1] showed that the cell walls of group A streptococcus induced non-specific resistance of mice to infection and that the activity of the fraction was dependent on its peptidoglycan but not on its protein and polysaccharide. Kotani *et al.*[2] also reported that the cell walls of group A streptococcus possessed the immunological adjuvant activities to induce delayed-type hypersensitivity and to stimulate antibody production in guinea pigs to ovalbumin when administered in the form of a water-in-oil emulsion. However, the biological active molecule in OK-432 has not yet been determined.

In this work, live bacterial cells harvested from a 15-hour culture of group A streptococcus were disrupted in a Ribi cell fractionator and fractionated by centrifugation into cell wall fraction, particle fraction and cytoplasmic soluble fraction. The protoplast membrane fraction was prepared by the method described previously. Each of the sub-cellular fractions thus obtained were tested for thier antitumor activities in following ways; 1) *in vivo* antitumor effect against Ehrlich ascites carcinoma, 2) growth inhibition of Yoshida sarcoma cells or ascites hepatoma AH13 cells in culture, 3) prophylactic activity in allogeneic and syngeneic systems and 4) inhibition of cellular uptake of isotope-labeled precursors in macromolecular biosyntheses.

Meanwhile, immune responses in the mice pretreated with each fraction were assayed in accordance with the following; 1) delayed-type hypersensitivity with picryl chloride, 2) antibody dependent cell-mediated cytotoxicity, 3) immunoglobulin subclasses and complement components in serum, 4) blastoid transformation of spleen cells *in vitro* and 5) adjuvant activity on acquired specific immunity.

As the results, none of the coccal cell fractions were more effective either in *in vitro* and *in vivo* cytotoxic potencies or in stimulation of lymphoreticular system, as compared with those produced by OK-432 administration. Further studies are needed on the problem of why full antitumor activity depends upon the integrity of the whole bacterial cell and why the oil-treated cell walls were effective.

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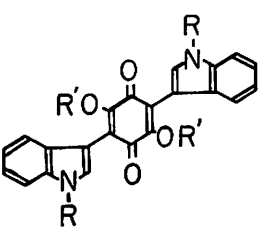
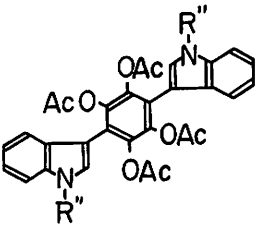
(50) Effect of asterriquinone and its related compounds on transplantable murine tumors.

S. Shimizu*, Y. Yamamoto* and S. Koshimura

Asterriquinone (ARQ, I), 2,5-bis[N-(1",1"-dimethyl-2"-propenyl)indol -3'-yl] -3,6-dihydroxy-1,4-benzoquinone, is a compound isolated by Yamamoto *et al.*[1] from *Aspergillus terreus* IFO 6123, as one of intracellular metabolic products. This compound comprises a benzoquinone moiety and two indol rings, which are derived from tryptophan in culture medium, as illustrated in following.





I, R=$C(CH_3)_2CH=CH_2$, R'=H IV, R"=$C(CH_3)_2CH=CH_2$

II, R= 〃 , R'=Me

III, R=$C(CH_3)_2CH_2CH_3$, R'=H

Recently, preliminary experiments carried out to examine the biological properties of ARQ showed that it inhibited the growth of Ehrlich ascites carcinoma *in vivo*[2]. Then, antitumor activity of ARQ and its derivatives, dimethyl ether (II), tetrahydro compound (III) and its tetraacetate (IV), were investigated in detail. The test systems employed were as follows:

The compounds to be tested were suspended in 0.25% carboxymethylcellulose (CMC) solution and intraperitoneally administered once daily to mice bearing Ehrlich carcinoma (EC) for 7 successive days, begining 24 hours after the implantation. In the ascites tumor system, in which 2×10^6 cells of EC were implanted intraperitoneally to ddY mice, evaluation of efficacy was determined on the basis of survival time and number of mice surviving over 50 days. In the solid tumor, in which 4×10^6 cells of EC were implanted subcutaneously in the left groin of the mice, antitumor activity was evaluated on Day 14 by the ratio of tumor weights of the treated and control animals

As shown in Table 1, remarkable increase in the life-time of the experimental animals was observed with dose response of ARQ, and all the survivors on Day 50 were autopsied without macroscopical tumor finding. Moreover, only a single intraperitoneal administration (100 mg/kg, Day 3) of ARQ was markedly effective in prolongation of survival time. On the other hand, ARQ suppressed the growth of tumor mass in the solid tumor system, as presented in Table 2. The effectiveness of ARQ was also demonstrated with broad antitumor spectra; i.e., ARQ was moderately effective

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against mouse P388 leukemia (T/C, 172%) and ascites hepatoma AH13 (T/C, 256%), and slightly against mouse L1210 leukemia (T/C, 147%) and Yoshida sarcoma (T/C, 162%), but not against ascites hepatoma AH109A (T/C, 121%). However, none of the ARQ derivatives, II, III and IV, were responsible to any transplantable mouse tumors nor EC cells.

From these results, it is suggested that two hydroxy groups freed from substituents, p-quinone ring and *tert*-pentenyl moieties in the ARQ molecule might be essential for exhibiting antitumor activity. Experiments made on the action mechanisms of ARQ revealed that this compound inhibited the proliferation of HeLa cells in culture even at a concentration of 10 μM (IC_{50}), and cellular uptake of [^{3}H]-thymidine by EC or HeLa cells was selectively inhibited *in vitro*. However, it is supposed that ARQ may act on the membrane system of tumor cells, based upon the fact that it caused lysis of tumor cells in buffer solution, and also upon the fact that increased morphological changes in cell surface were observed in specimens of the tumor cells exposed to ARQ *in vitro* and *in vivo*.

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Table 1. Antitumor effect of asterriquinone on Ehrlich ascites carcinoma in mice

Agent	Dose (mg/kg)	Treatment (ip)	50-day survivors/No. of test mice	Mean survival days	T/C (%)
0.25% CMC	.	Days 1-7	0/6	19.3	.
ARQ	40	do.	5/6	>47.3	>245
	20	do.	6/6	>50.0	>259
	10	do.	3/6	>43.2	>224
0.25% CMC	.	Day 3	0/6	18.2	.
ARQ	100	do.	5/6	>48.3	>265

Male albino mice (ddYS, 6 mice/group) were implanted intraperitoneally with 2×10^6 cells of Ehrlich carcinoma.

Talbe 2. Antitumor effect of asterriquinone on solid tumor of Ehrlich carcinoma in mice

Agent	Dose (mg/kg)	Treatment (ip)	Tumor weight (g) Mean $\pm$ S.D.	T/C (%)
0.25% CMC	.	Days 1-7	1.24$\pm$0.19	.
ARQ	40	do.	0.43$\pm$0.10	34.7
	20	do.	0.71$\pm$0.16	57.3
	10	do.	0.80$\pm$0.17	64.5

Female albino mice (ddYS, 8 mice/group) were implanted subcutaneously with 4×10^6 cells of Ehrlich carcinoma.

(51) Chelating agents as the candidates for anticancer drugs[1]

T. Ujiie and S. Koshimura

Of many compounds synthesized in our laboratory, 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (HP) and its related compound, 2-(2-hydroxy-5-*n*-hexylphenyl)-8-quinolinol-4-carboxylic acid (HQ II), were found to be effective on some rodent tumors *in vitro* and *in vivo*.

Structure-activity relation studies indicated that hexyl, o, o'-dihydroxy, and azomethine groups were contributive to their biological activities[2].

Both compounds selectively inhibited DNA synthesis in AH13 cells in culture and blocked deoxyribonucleoside diphosphate formation catalysed by ribonucleoside diphosphate reductase (iron–requiring enzyme) *in vitro* and *in vivo*[3]. These actions are mostly restored by washing or addition of iron salts. Then, the expectation that among chelating agents which are able to interact with vital iron components in cells may have picked up the candidates for anticancer drugs prompted us to reassess chelating agents including HP and HQII in the following respects: prevention of cellular DNA synthesis, inhibitory effect on proliferation of cultured HeLa cells, anticancer activity (Ehrlich carcinoma, leukemias P388 and L1210), hydrophobicity, and iron chelate-forming ability.

Among the tested compounds, several agents including bathophenanthroline and 8-hydroxyquinoline were ten fold or more active than HP and HQII against the cultured cells, but none of them showed any recognizable anticancer activities *in vivo*.

Exceptionally, isoquinoline-l-carbaldehyde thiosemicarbazone (NSC-92188) and pyridine-2-carbaldehyde thiosemicarbazone (NSC-729) have iron chelate-forming ability, and their sites of action have been reported to be ribonucleotide reductases[4]. They were not only inhibitors against HeLa cells (ED_{50}: 0.44 and 2.1 μM) but also exhibited pronounced anticancer effects. While, a slight antileukemic effect on L1210 cells was observed in their hydrogenated derivatives, 1-(1-isoquinolylmethyl)- and 1-(2-pyridylmethyl)-thiosemicarbazides, of which chelating ability and hydrophobicity were altered from the thiosemicarbazones.

As bathophenanthroline is highly cytotoxic against the cultured cells to the same extent as the anticancerous thiosemicarbazones, it may not be unreasonable to assume that its chemical modulation concerned with hydrophobicity gives a HQII like but more active compound.

Further investigation in this line of work is now in progress.

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(52) Caffeine releases G_2 arrest caused by adriamycin[1].

T. Ujiie and T. Goshima

Studies were initiated to characterize the repair mechanism of cells damaged by an intercalating anticancer drug, adriamycin, whose mode of action and effect on cell cycle progression were also followed, using mainly HeLa cells in culture systems.

Adriamycin shows a strong lethal effect against proliferating HeLa cells in a dose-dependent manner. One hour exposure to 0.9 μM adriamycin reduced clonogenic cells to less than 1% of the untreated cells. Post-treatment with caffeine was found to enhance the cell-killing effect of this drug. The effect of caffeine was dose dependent. Caffeine causes transient retardation of growth rate of the untreated cells but has no lethal action. Curiously, it was observed that cells treated with caffeine after adriamycin exposure appeared to be normal size in contrast to enlargement of the cells which were exposed to adriamycin followed by incubation in the absence of caffeine.

Further studies by stathmokinetic and cytofluorographic methods to elucidate the morphological changes gave the following results: Adriamycin leads to accumulation of cells in the G_2 phase at 0.9 μM and caffeine (2 mM) appears not to be effective against cell cycle traverse of untreated cells, while release of the G_2 blockade caused by adriamycin occurs by caffeine treatment after exposure to the drug.

As a DNA synthesis inhibitor, hydroxyurea, did not affect the "caffeine effect", showing that caffeine acts on G_2 cells which have been blocked further progression to mitosis. It is unlikely that caffeine affects S phase cells to accelerate the progression rate. The caffeine (1, 3, 7-trimethylxanthine) could not be substituted for other cyclic AMP-like agents including dibutyryl cyclic AMP, butyrate, and papaverine, except by theophylline (1, 3-dimethylxanthine). Since the "caffeine effect" even appeared to be reversed by dibutyryl cyclic AMP, another function of caffeine may be suggested. It has been reported that mammalian cells are arrested at a number of distinct stages in the G_2 phase by many drugs, including adriamycin, daunomycin, actinomycin D, neocarzinostatin, and nitrosoureas. As in the case of adriamycin, whether or not the G_2 arrest caused by these drugs is released by caffeine treatment was followed by the stathmokinetic method. The results showed that among the tested compounds, G_2 blockade occurring by exposure to 1-(4-amino-2-methylpyrimidine-5-yl)methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride was prominently released by caffeine treatment. But, the effects of actinomycin D and neocarzinostatin were hardly reversed by caffeine at all.

The "caffeine effect" was observed in KB and CHO cells as well as in HeLa cells. However, mouse L and L1210 leukemia cells could not respond to caffeine treatment although they were arrested by adriamycin within the G_2 phase. There may be a possibility that adriamycin and/or

caffeine act(s) on target point(s) in a different fashion from HeLa cells.

Recently, Tobey presented the hypothesis that a surveillance mechanism operates throughout the G_2 phase to eliminate from the proliferative mode cells with altered DNA[2]. It may be thought that caffeine acts on the surveillance mechanism operating at multiple stages within the G_2 phase to suppress it. As a result, cells arrested in the G_2 stage by adriamycin are thought to permit traversing cell cycles even though abortive reproduction happens. Since the G_2 blockade caused by actinomycin D or neocarzinostatin is not reversed by caffeine treatment, it seems that any specific stages in the G_2 phase might be susceptable to caffeine.

In conclusion, the G_2 blockade in the cell cycle progression caused by adriamycin exposure is released by post-treatment with caffeine or theophylline. Cyclic AMP-like activity of caffeine may not be concerned with this phenomenon. Moreover, as the cell-killing effect of adriamycin is reinforced by post-incubation with caffeine and is dependent on its doses, a post-replication repair system may be operative in adriamycin-damaged cells. However, the question of whether or not there is any relationship between the release effect and the potentiation of cell-killing action of adriamycin, both caused by caffeine, is not yet clear.

1) Ujiie, T. and Goshima, T., Proc. Jpn. Cancer Assocc., 37th Ann. Meet. (Tokyo), 162 (1978).
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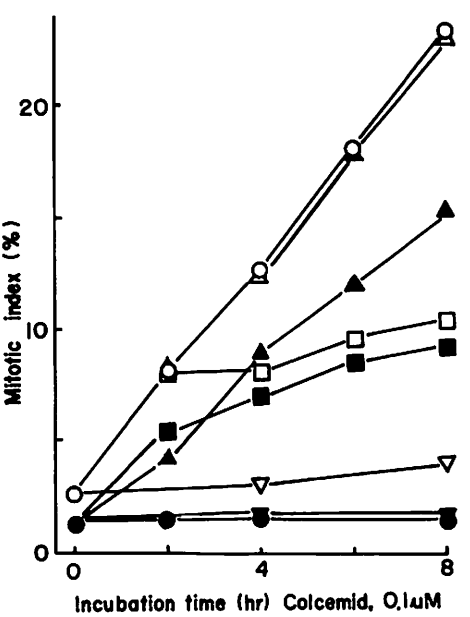

Figure. Effect of caffeine on $G_2 \rightarrow M$ traverse of adriamycin-treated HeLa cells—Stathmokinetic analysis.

Adriamycin (0.9 μM) exposure (1 hr) (black)
○, ●, without caffeine
△, ▲, caffeine 2 mM
□, ■, caffeine 2 mM + hydroxyurea 10 mM
▽, ▼, caffeine 2 mM + cycloheximide 0.4 mM

(53) Studies on the streptococcal hemolysin formed in the presence of colistin.

M. Fujita and S. Koshimura

Group A hemolytic streptococci have been shown by Todd[1] to produce two extracellular hemolysins, which were designated as the oxygen-labile hemolysin "Streptolysin O" (SLO) and the oxygen-stable "Streptolysin S" (SLS).

Since the discovery of Okamoto[2] that the formation of SLS by hemolytic streptococci was greatly enhanced by the addition of yeast ribonucleate to the culture medium, much work has been done on this hemolysin by many researchers[3].

Recently, it was found by Fujita[4] that when resting cells of hemolytic streptococci, Su-strain, were incubated with oligoribonucleotide and cysteine in Bernheimer's basal medium in the presence of colistin, there occurred a marked increase of hemolytic activity ($2-5 \times 10^6$ HU/ml) in the medium, as compared with that ($2-8 \times 10^4$ HU/ml) in a colistin-deficient medium. Such increased production of hemolysin was not obtained by the addition of either colistin alone or oligoribonucleotide alone to the coccal suspension. A hemolytic moiety formed by the streptococci in the presence of colistin and oligoribonucleotide was temporarily designated as colistin-induced hemolysin (CIH). The ability of the cocci to form CIH was sustained in the cells grown at late exponential phase in the culture medium containing 0.01% sodium thioglycollate.

Further, both Su and Blackmore strains—the former produces SLS and SLO, and the latter SLS only — were demonstrated to be useful in acquiring high potent hemolytic activity, while the C203U strain, which produces only SLO was not useful in this respect[5]. The crude preparation of CIH was fractionated on DEAE-cellulose column, resulting in isolation of two peaks (F-I and F-II) with hemolytic activity. Considering the responsiveness of two hemolytic fractions to various inhibitors and activators, F-II is likely to correspond to SLS, while F-I may, at least, be a segment different from the streptolysins, S and O.

In order to define more clearly the nature and properties of CIH, the crude toxin materials were further purified by using ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE-Sephadex column chromatography and CM-Sephadex column chromatography. This toxin, whose molecular weight was determined as about 61,000 by gel filtration, seems to be a typical protein, considering the fact that its ultraviolet absorption spectrum was minimum at 253 nm and maximum at 278 nm, and that trypsin destroyed the hemolytic activity of the purified toxin, as well as SLO, while did not SLS. The specific activity of the final preparation was 3.8×10^6 HU/mg and one HU equivalent to 0.26 ng of protein. As reported before[4,5], this toxin was not activated by any of several SH-compounds, but hemolysis by CIH was inhibited by minute amounts of cholesterol.

As shown in Table 1, the biological and chemical properties of CIH preparation showed considerable resemblance to those described for SLO, without activation by thiol substances. Bernheimer summarized as shown

Table 1. Comparison of some biological properties of streptolysin O(SLO), streptolysin S(SLS) and CIH

Nature	SLO	SLS	CIH
1. Activation by SH-compounds	+	–	–
2. Antigenicity	+	–	.
3. Inducing substances	Unknown	RNA Serum Tween	Colistin plus Oligoribonucleotide
4. Inactivation by trypsin	+	–	+
5. Inactivation by chymotrypsin	+	+	+
6. Inhibition of hemolysis by cholesterol	+	–	+
7. Inhibition of hemolysis by trypan blue and lecithine	–	+	+
8. Induction period of hemolysis	short	long	short

below[3,6] that SLO is the prototype of a series of extracellular substances that are synthesized by a considerable number of bacterial species; for example, pneumolysin, tetanolysin, θ-toxin, cereolysin and others. The members of this group possess a number of common features: (a) All are products of gram-positive bacteria belonging to the genera, *Streptococcus, Listeria, Clostridium,* and *Bacillus.* (b) The biological activity of all of them is abolished by oxygen, but their inactivation is reversible by SH-compounds. (c) Most and presumably all of them are irreversibly inactivated by low concentrations of cholesterol. (d) They are immunologically related to one another. CIH is an extracellular substance obtained from *Streptococcus haemolyticus,* and is irreversibly inactivated by low concentrations of cholesterol, While its activity is not potentiated by SH-compounds. Therefore, it seems that CIH may be closely related to oxygen-labile hemolysin. The supposition may be emphasized that this hemotoxin is a protein, and also the kinetics of hemolysis by this toxin is like that of SLO[5].

Further studies on the chemical, biological and immunological natures of this hemotoxin are now in progress.

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