ENHANCEMENT OF PYRIMIDINE NUCLEOSIDE UPTAKE INTO K562 AND YAC-1 CELLS BY CADEGUOMYCIN

RONG TSUN WU†, TAKAYOSHI OKABE, SUN HEE KIM, HIDEO SUZUKI and NOBUO TANAKA*

Institute of Applied Microbiology, University of Tokyo, Tokyo 113, Japan

(Received for publication July 19, 1985)

Cadeguomycin markedly stimulated the uptake of thymidine, deoxycytidine and uridine into the acid-insoluble fraction of K562 human leukemic cells, but did not significantly affect adenosine incorporation. The enhancement of pyrimidine nucleoside uptake was 6~17 fold over the control. Aspartate incorporation into nucleic acid was not significantly blocked by the antibiotic, suggesting that the stimulation of pyrimidine nucleoside incorporation is not due to the inhibition of *de novo* pyrimidine nucleotide synthesis. Net DNA and RNA syntheses, observed by [^{32}P]phosphate uptake, were not significantly affected by cadeguomycin. The enzymatic activity of thymidine, deoxycytidine and uridine kinases was higher in cadeguomycin-treated cells than in untreated cells, suggesting that the enhancement of pyrimidine nucleoside uptake occurs in the phosphorylation process. The stimulatory activity of cadeguomycin of thymidine uptake was reversed by guanosine and deoxyguanosine, but not by adenosine and deoxyadenosine, suggesting that intracellular metabolism and/or action of cadeguomycin is related to that of guanosine and deoxyguanosine. The stimulation of pyrimidine nucleoside incorporation by cadeguomycin was also found with YAC-1 cells, but not with the other cell lines. The enhancement effect of the antibiotic seems to be not directly related to its cytotoxicity.

In the course of our screening for new antitumor antibiotics, we observed that a strain of *Streptomyces hygroscopicus* produces two substances; one inhibits thymidine uptake of K562 human leukemic cells and another enhances it. The inhibiting factor was identified with tubercidin, whereas the stimulating substance was found to be a new antibiotic which was named cadeguomycin, because the structure is 7-carboxy-7-deazaguanosine. The production, purification, structure assignment and biological activity were described in previous papers[1~3]. Since the antibiotic displays an unique property of enhancing thymidine uptake, we have studied the effect in detail and found that the stimulation is attributed to increased enzymatic activity of pyrimidine nucleoside kinases. The results are presented in this publication. A part of the preliminary results was summarized at an International Conference held in Tokyo, June, 1982[4].

Materials and Methods

Chemicals

[*Methyl*-^{3}H]thymidine (25 Ci/mmol), [5,6-^{3}H]uridine (44 Ci/mmol), [5-^{3}H]deoxycytidine (29 Ci/mmol), [2-^{3}H]adenosine (21 Ci/mmol), [5-^{3}H]guanosine (22 Ci/mmol) and [^{32}P]orthophosphate (1 mCi/ml) were products of Amersham International, England. L-[2,3-^{3}H]Aspartic acid (10 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

† The present address: National Yang-Ming Medical College, Taipei, R.O.C.

Cells

The K562 cell line was derived from human myelogenous leukemia[5] and YAC-1 from murine lymphoma induced by Molony leukemic virus[6]. L5178Y and L1210 were lymphoma cell lines of mice. A primary culture of Ehrlich murine carcinoma was also used. 3Y1 was a rat Fisher embryo cell line, W3Y a SV40-transformed 3Y1 cell line, DS-19-18 a Friend virus-induced mouse leukemia cell line, and X63.6.5.3 a murine myeloma cell line, deficient in hypoxanthine/guanine phosphoribosyltransferase[7]. All cell lines were grown in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum, benzylpenicillin (100 units/ml) and streptomycin (100 μg/ml), except L5178Y cells in which fetal calf serum was replaced by horse serum.

Radioisotope Incorporation

A microplate method was employed for the assay. Cells were suspended in the medium described above, and distributed into wells of microplates (Nunc, Denmark) in the presence or absence of cadeguomycin. Each well contained 10^4 cells/0.2 ml. The mixtures were incubated at 37°C for various periods in an atmosphere of 5% CO_2 and 95% air, and then radiolabeled precursors (0.2 μCi/well) were incorporated for 2 to 6 hours, as indicated. The cells were collected and washed in phosphate-buffered saline (PBS), and then in 5% cold TCA, using a multiple automated sample harvester (Abe Kagaku). 3Y1 cells were pretreated with 0.05% trypsin and 0.02% EDTA for 5 minutes before collection. The radioactivity was determined in a liquid scintillation counter (Beckman), using a toluene-based scintillator.

For examining the effects of purine nucleosides on the activity of cadeguomycin for [^{3}H]thymidine uptake, K562 cells were incubated with purine nucleosides of indicated concentrations and cadeguomycin (5 μg/ml) for 17 hours and then with [^{3}H]thymidine for 2 hours.

Stimulation Index

The ratio of [^{3}H]thymidine incorporation in the presence and absence of cadeguomycin was taken as the stimulation index.

[^{32}P]Orthophosphate Incorporation

K562 cells were suspended (2×10^5 cells/ml) in Dulbecco-Eagle medium supplemented with 10% heat-inactivated fetal calf serum, dialyzed 4 times against PBS during 16 hours, and were incubated with or without cadeguomycin at 37°C for 1 hour in 5% CO_2 and 95% air atmosphere. Then [^{32}P]orthophosphate was added to the cell suspension at a final concentration of 5 μCi/ml, and further incubated for 17 hours in the same conditions. The cells were washed twice with PBS and twice with 5% cold PCA by sedimentation. Nucleic acid fractions were extracted from K562 cells following the method of SCHMIDT and THANNHAUSER[8]. The DNA and RNA fractions were dissolved in BRAY's solution, and the radioactivity was determined in a liquid scintillation counter (Beckman).

Nucleoside Kinase Activity of Cell Extracts

The cell extracts were prepared as follows. After incubation with cadeguomycin (0, 1, 5 or 25 μg/ml) for 18 hours, K562 cells (*ca.* 7×10^5 cells/ml) were harvested by sedimentation, washed 4 times with cold PBS and suspended in 20 mM sodium phosphate, pH 7.0. The cell suspension was homogenized with a Dounce homogenizer (200 strokes) and centrifuged at $100{,}000\times g$ for 1 hour at 4°C. The supernatant was extensively dialyzed against 10 mM Tris-HCl, pH 8.0 to remove cadeguomycin and nucleosides. The dialysate was finally centrifuged at $10{,}000\times g$ for 20 minutes and the supernatant was stored at −80°C in a small aliquot until use. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.

The reaction mixture, in 0.1 ml, contained 50 mM Tris-HCl, pH 8.0, 5 mM ATP, 4 mM $MgCl_2$, enzyme or cell extract (45 μg of protein) and 1 μCi radiolabeled substrate. The concentrations of radiolabeled nucleosides were 4 μM of thymidine, 2.27 μM of uridine and 5 μM of deoxycytidine. The reaction mixture was incubated in an Eppendorf tube at 37°C for 30 or 60 minutes and placed in boiling water for 2 minutes to terminate the reaction. The mixture was briefly sedimented in an Eppendorf centrifuge and the supernatant (25 μl) was spotted on DEAE-cellulose disc paper (Whatman DE81, diameter 2 cm)[9,10]. The disc paper was washed in 1 mM ammonium formate for 10 minutes and then

in H_2O. The washing was repeated again. After washing with 95% ethanol, the disc was dried and the radioactivity remaining in the disc was counted in a toluene-based scintillator. The results were the average of triplicates.

For TLC analysis, the supernatant (10 μl) was spotted on Silica gel 60 F_{254} plastic sheet (Merck) and developed by a mixture of 1 M NH_4OH and isobutyric acid (3: 5). Cold thymidine, deoxycytidine and their mono-, di- and triphosphates were simultaneously developed as markers. The nucleosides and nucleotides were detected by UV light, and the radioactivity was determined as described above after the corresponding spots were cut off.

Cell Growth

The cells were cultured in the medium described above at 37°C for 72 hours in an atmosphere of 5% CO_2 and 95% air with or without cadeguomycin. The viable cell number was determined by trypan blue dye exclusion.

Results

The Effects on Nucleoside Incorporation in K562 Cells

Cadeguomycin was observed to enhance the incorporation of pyrimidine nucleosides into K562 cells. Preincubation of K562 cells with cadeguomycin (5 μg/ml) for 18 hours caused 17, 10 and 6 fold increases of uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]deoxycytidine, respectively, into acid-insoluble material (Fig. 1). The degree of stimulation was logarithmic with the concentration of the drug in a range of 0.2~5 μg/ml. A more significant enhancement was observed at an antibiotic concen-

Fig. 1. Stimulation by cadeguomycin of [^{3}H]thymidine, [^{3}H]deoxycytidine or [^{3}H]uridine incorporation in K562 cells after 18 hours preincubation.
○: [^{3}H]Thymidine, △: [^{3}H]deoxycytidine, ●: [^{3}H]uridine.

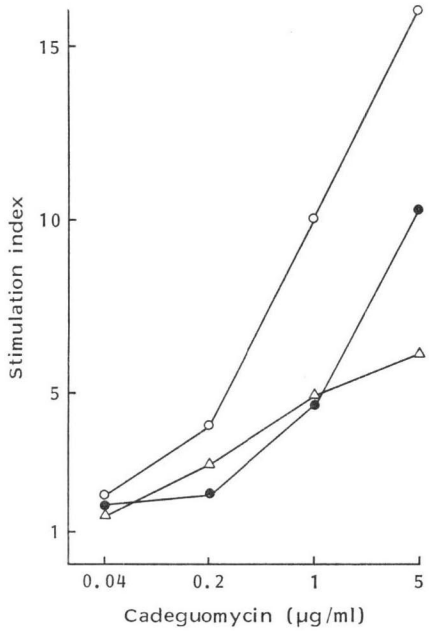


Table 1. Effects of cadeguomycin on [^{3}H]aspartic acid incorporation into DNA and RNA fractions of K562 leukemic cells.

Cadeguomycin (μg/ml)	Relative incorporation of [^{3}H]aspartic acid into	
	DNA fraction	RNA fraction
25	0.73	0.84
5	0.80	0.89
1	0.82	0.97
0.2	0.90	0.88
Control	1.00*	1.00*

* In the control, the uptake of [^{3}H]aspartic acid into the DNA fraction was 1,985±299 dpm/2×10^5 cells and into the RNA fraction 823±14.

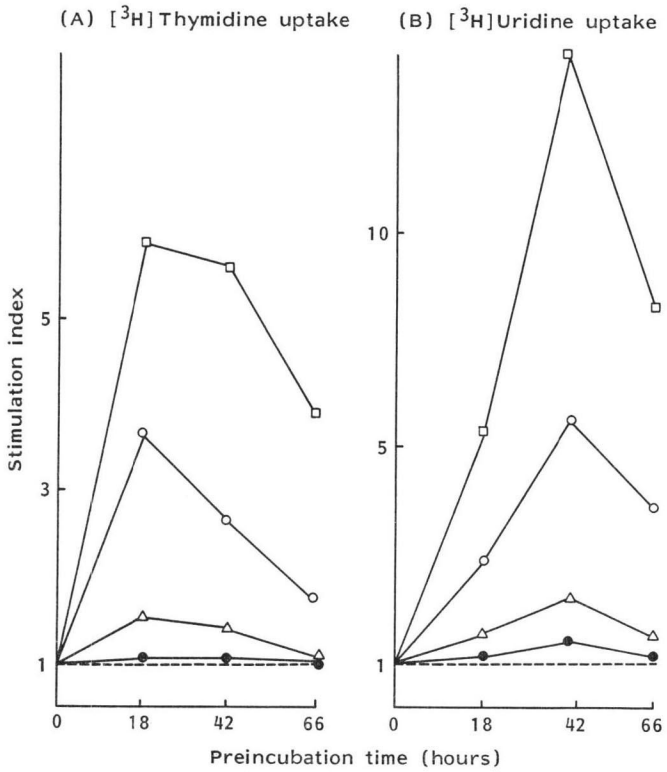
Table 2. Effects of cadeguomycin on [^{32}P]phosphate into DNA and RNA fractions of K562 leukemic cells.

Cadeguomycin (μg/ml)	[^{32}P]Phosphate incorporation into	
	DNA fraction	RNA fraction
25	0.85	0.89
5	0.86	1.03
1	0.96	1.00
0.2	0.93	1.04
Control	1.00*	1.00*

* In the control, the incorporation into DNA was 5,269±242 cpm/2×10^5 cells and into RNA 11,361±873.

Fig. 2. Stimulation by cadeguomycin of [^{3}H]thymidine or [^{3}H]uridine incorporation in K562 cells. Time dependency.

Cadeguomycin □: 1.0, ○: 0.2, △: 0.04, ●: 0.008 μg/ml, --- control.



tration of 25 μg/ml (data are not shown). Analysis by the method of SCHMIDT and THANNHAUSER[8] revealed that more than 95% of the acid-insoluble radioactivity was localized in the nucleic acid fractions (data are not shown). The stimulatory effect of cadeguomycin on thymidine and uridine incorporation continued for a fairly long period and found even 42 and 66 hours after introduction of the antibiotic to the cells (Fig. 2). The peak of stimulation by cadeguomycin of thymidine uptake was observed at 18 hours and that of uridine incorporation at 42 hours.

The incorporation of [^{3}H]adenosine into the acid-insoluble fraction of K562 cells was not significantly influenced by cadeguomycin (data are not shown).

The Effect on Pyrimidine Nucleotide Synthesis

Cadeguomycin failed to block [^{3}H]aspartate incorporation into nucleic acids, suggesting that the antibiotic does not significantly affect *de novo* pyrimidine nucleotide synthesis (Table 1).

The Effect on [^{32}P]Phosphate Incorporation into DNA and RNA Fractions of K562 Cells

Cadeguomycin did not enhance [^{32}P]orthophosphate incorporation into nucleic acid fractions of K562 cells (Table 2). The result suggests that the stimulation of pyrimidine nucleoside uptake does not result in enhancement of net nucleic acid syntheses.

Fig. 3. Enzymatic activity of thymidine, uridine and deoxycytidine kinases in cadeguomycin-treated K562 cells.

A: Thymidine kinase, B: uridine kinase, C: deoxycytidine kinase.

Cadeguomycin ○: 0, △: 5, ●: 25 μg/ml treated cell extract.

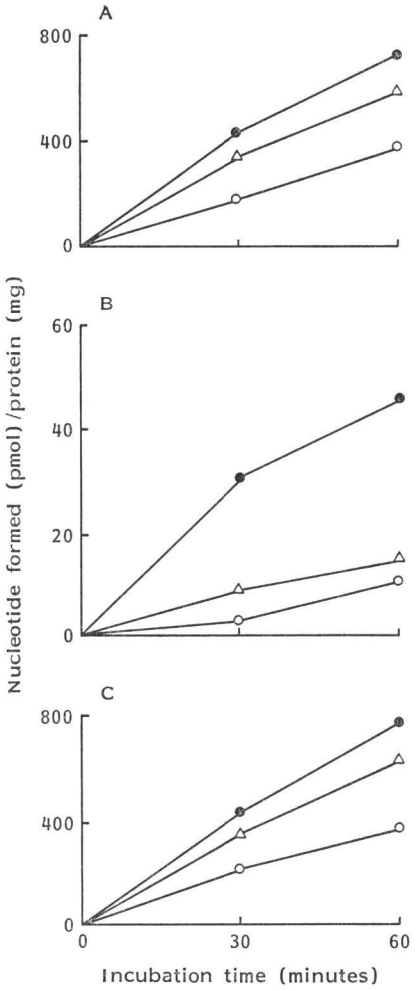


Table 3. Thymidine, deoxycytidine and their nucleotide kinase activities of extracts of K562 cells treated with cadeguomycin.

Cadeguomycin (μg/ml)	Nucleotide formed			
	dTMP	dTDP dTTP	dCMP	dCDP dCTP
5	6,910*	340	1,010	2,530
1	2,950	221	690	1,890
0	2,560	236	2,490	235

* The number represents pmol/mg protein/hour.
The enzymatic activity was determined by TLC analysis, using cell extracts.

Fig. 4. Effects of purine nucleosides on the stimulatory activity of cadeguomycin for [^{3}H]thymidine uptake in K562 cells.

Stimulation index without purine nucleoside was 9.0.

▲: Adenosine, △: deoxyadenosine, ●: guanosine, ○: deoxyguanosine.

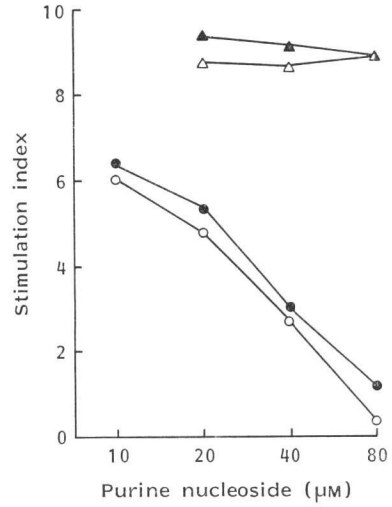
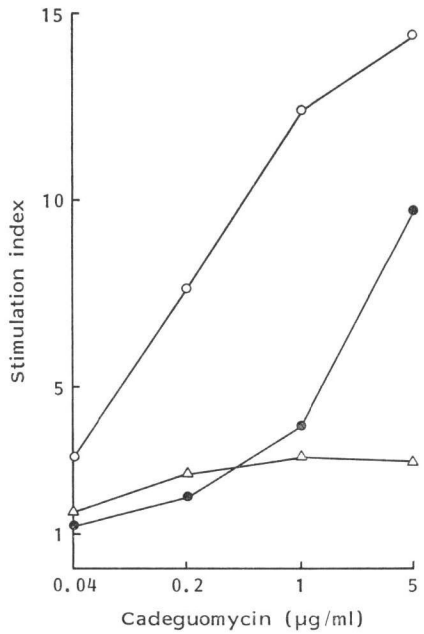


Fig. 5. Stimulation by cadeguomycin of [^{3}H]thymidine, [^{3}H]deoxycytidine or [^{3}H]uridine incorporation in YAC-1 cells after 18 hours preincubation.

○: [^{3}H]Thymidine, △: [^{3}H]deoxycytidine, ●: [^{3}H]uridine.



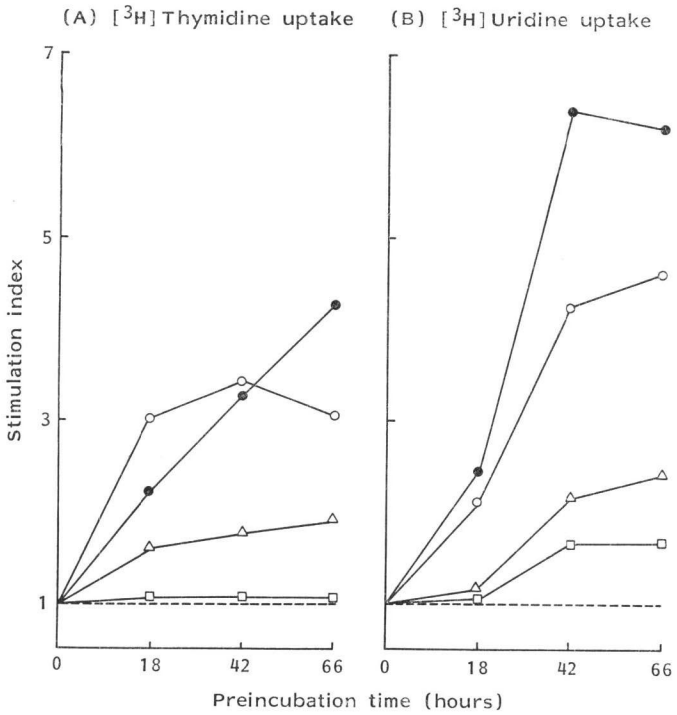
Enzymatic Activity of Thymidine, Uridine and Deoxycytidine Kinases in Cadeguomycin-treated K562 Cells

Since cadeguomycin-treated K562 cells showed higher incorporation of pyrimidine nucleosides than the untreated cells, pyrimidine nucleoside kinase activities in these cells were examined. As illustrated in Fig. 3, the activity of thymidine, uridine and deoxycytidine kinases increased when K562 cells were incubated with 5 or 25 μg of cadeguomycin per ml for 18 hours, and the enzyme levels depended upon the antibiotic concentrations. The enhancement of thymidine, deoxycytidine and their nucleotide kinases by cadeguomycin was also demonstrated by a TLC method (Table 3). The extracts of cells treated with 1 or 5 μg of cadeguomycin per ml showed increased activity of synthesizing dTMP, and dCDP and/or dCTP. On the contrary, the activity of adenosine kinase was not changed by the treatment with cadeguomycin (data are not shown).

Effects of Purine Nucleosides on the Stimulatory Activity of Cadeguomycin

Guanosine and deoxyguanosine markedly reversed the enhancement of [^{3}H]thymidine uptake by cadeguomycin in K562 cells (Fig. 4). In contrast, the stimulation was not significantly affected by adenosine and deoxyadenosine. The results suggest that the intracellular metabolism of cadeguomycin, including phosphorylation, and the action of the drug and its metabolites are related to the metabolism of guanosine and deoxyguanosine, provided that the cellular uptake of nucleosides is carried out by a common transport system[11].

Fig. 6. Stimulation by cadeguomycin of [^{3}H]thymidine or [^{3}H]uridine incorporation in YAC-1 cells. Time dependency.
Cadeguomycin ●: 1.0, ○: 0.2, △: 0.04, □: 0.008 μg/ml, --- control.



The Effect on Pyrimidine Nucleoside Uptake in YAC-1 Cells

Cadeguomycin was also found to stimulate incorporation of pyrimidine nucleosides into YAC-1 cells. Preincubation of YAC-1 cells with cadeguomycin (5 μg/ml) induced 15, 10 and 2 fold increases of uptake of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]deoxycytidine, respectively (Fig. 5). The degree of enhancement depended upon the antibiotic concentrations. The stimulatory effect on thymidine and uridine uptake continued for a considerable period, and were significant even 42 and 66 hours after contact of cadeguomycin with the cells (Fig. 6). Thus, the effect of cadeguomycin on pyrimidine nucleoside uptake in YAC-1 cells seemed to be similar to that in K562 cells.

The Effects on Pyrimidine Nucleoside Incorporation in Other Kinds of Cells

Cadeguomycin did not show any stimulatory effects on [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]-deoxycytidine uptakes in L5178Y, L1210, 3Y1, W3Y, DS-19-18, X63.6.5.3 and Ehrlich carcinoma cells as well as human and mouse lymphocytes. Various extents of inhibition by the antibiotic were detected at high antibiotic concentrations. The effects on L1210 cells have been illustrated in the previous paper[4].

The Effect on Growth of Various Cells

At a high concentration, cadeguomycin showed cytotoxic effects on various cell lines: K562, YAC-1, L5178Y, L1210, 3Y1, DS-19-18 and X63.6.5.3. The antibiotic (25 μg/ml) produced *ca.* 50% growth inhibition in YAC-1 and L1210 cells, 30~40% inhibition in L5178Y, X63.6.5.3 and DS-19-18, and *ca.* 20% inhibition in K562 and 3Y1 cells.

Discussion

It has been revealed in the present experiments that cadeguomycin enhances pyrimidine nucleoside uptake into K562 and YAC-1 cells. Pyrimidine nucleoside and/or nucleotide kinases were increased by the treatment with the antibiotic and the increased enzymatic activity seems to result in stimulation of nucleoside incorporation. Cadeguomycin seems to induce the enzymes in K562 cells. The antibiotic did not directly affect pyrimidine nucleoside kinases (data are not shown), suggesting that it lacks in an allosteric effect or the phosphate is the active form. However, the mechanism of enhancement of the enzymatic activity remains to be determined.

The action of cadeguomycin is limited to K562 and YAC-1 cells. The narrow spectrum may be due to the level of the nucleotide pool in these cells. Alternatively, it may be attributed to the salvage pathway; cellular transport and metabolism. The antibiotic may be activated or phosphorylated, if the nucleotide is the active form, in these cells. However, the precise reason remains to be determined.

The K562 and YAC-1 cells are widely used as targets for their high and reproducible sensitivity to NK-mediated lysis[6,12,13]. However, the relationship of cadeguomycin action to NK sensitivity remains open to discussion.

Ross *et al.*[14] reported that deoxyguanosine inhibits growth of K562 cells at a high concentration of 100 μM and suggested that in addition to blocking ribonucleotide reductase, deoxyguanosine affects the salvage pathway. The reversal of cadeguomycin action by guanosine and deoxyguanosine suggest that a similar mechanism is involved in the enhancement by cadeguomycin of pyrimidine nucleoside uptake, although the precise mechanism remains to be determined.

Cadeguomycin prevents growth of various kinds of cells at a high concentration. Since the stimulation of pyrimidine nucleoside uptake is observed at a low antibiotic concentration and is limited

to K562 and YAC-1 cells, the enhancement of nucleoside uptake may be not related to the cytotoxicity.

Cadeguomycin displays a synergistic activity with cytosine arabinoside (ara-C) by enhancing enzymatic activity of deoxycytidine and/or its nucleotide kinases, resulting in a high intracellular content of ara-CTP[3,15]. It is of interest that the extracts of K562 cells treated with cadeguomycin showed increased activity of synthesizing dCDP and dCTP (Table 3). It suggests that the levels of dCMP and/or dCDP kinases are also enhanced and therefore the formation of ara-CTP is accerelated by the antibiotic.

Cadeguomycin stimulates thymidine incorporation into K562 cells even at low concentrations of 0.2 and 1 μg/ml (Fig. 1), but thymidine kinase activity is only marginally increased at 1 μg/ml (Table 3). The discrepancy seems to be due to the different cell densities used.

Acknowledgments

This work was partly supported by a Grant-in-Aid for cancer research from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. H. UMEZAWA, Institute of Microbial Chemistry, Tokyo, for his kind advice and cooperation. They are greatly indebted to Meiji Seika Kaisha, Ltd. for the preparation of cadeguomycin.

References

1) TANAKA, N.; R. T. WU, T. OKABE, H. YAMASHITA, A. SHIMAZU & T. NISHIMURA: Cadeguomycin, a novel nucleoside analog antibiotic. I. The producing organism, production and isolation of cadeguomycin. J. Antibiotics 35: 272~278, 1982
2) WU, R. T.; T. OKABE, M. NAMIKOSHI, S. OKUDA, T. NISHIMURA & N. TANAKA: Cadeguomycin, a novel nucleoside analog antibiotic. II. Improved purification, physicochemical properties and structure assignment. J. Antibiotics 35: 279~284, 1982
3) YUAN, B. D.; R. T. WU, I. SATO, T. OKABE, H. SUZUKI, T. NISHIMURA & N. TANAKA: Biological activity of cadeguomycin. Inhibition of tumor growth and metastasis, immunostimulation, and potentiation of 1-β-D-arbinofuranosylcytosine. J. Antibiotics 38: 642~648, 1985
4) TANAKA, N.: Recent studies on mechanism of action of three antitumor antibiotics. *In* Trends in Antibiotic Research. Genetics, Biosyntheses, Actions & New Substances. *Ed.*, H. UMEZAWA *et al.*, pp. 79~88, Japan Antibiotics Res. Assoc., Tokyo, 1982
5) LOZZIO, C. B. & B. B. LOZZIO: Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. Blood 45: 32~34, 1975
6) KIESSLING, R.; E. KLEIN, H. PROSS & H. WIGZELL: Natural killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Molony leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. 5: 112~117, 1975
7) KEARNEY, J. F.; A. RADBRUCH, B. LIESEGANG & K. RAJEWSKY: A new mouse myeloma line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J. Immunol. 123: 1548~1550, 1979
8) SCHMIDT, G. & S. J. THANNHAUSER: A method for the determination of deoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. 161: 83~89, 1945
9) FURLONG, N. B.: A rapid assay for nucleotide kinases using C^{14}- or H^3-labeled nucleotides. Anal. Biochem. 5: 515~522, 1963
10) LEUNG, W. C.; D. R. DUBBS, D. TRKULA & S. KIT: Mitochondrial and herpesvirus-specific deoxypyrimidine kinases. J. Virol. 16: 486~497, 1975
11) WILEY, J. S.; S. P. JONES, W. H. SAWYER & A. R. P. PATERSON: Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. J. Clin. Invest. 69: 479~489, 1982
12) ORTALDO, J. R.; R. K. OLDHAM, G. C. CANNON & R. B. HERBERMAN: Specificity of natural cytotoxic reactivity of normal human lymphocytes against a myeloid leukemia cell line. J. Natl. Cancer Inst. 59: 77~83, 1977
13) HERBERMAN, R. B.; M. E. NUNN & D. H. LAVRIN: Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. I. Distribution of reactivity and specificity. Int. J. Cancer 16: 216~229, 1975
14) ROSS, D. D.; S. A. AKMAN, A. W. SCHRECKER & N. R. BACHUR: Effects of deoxynucleosides on cultured human leukemia cell growth and deoxynucleotide pools. Cancer Res. 41: 4493~4498, 1981
15) SUZUKI, H.; R. T. WU, S. H. KIM, T. OKABE & N. TANAKA: Potentiation by cadeguomycin of 1-β-D-arabinofuranosylcytosine cytotoxicity for K562 human leukemic cells. (in preparation)